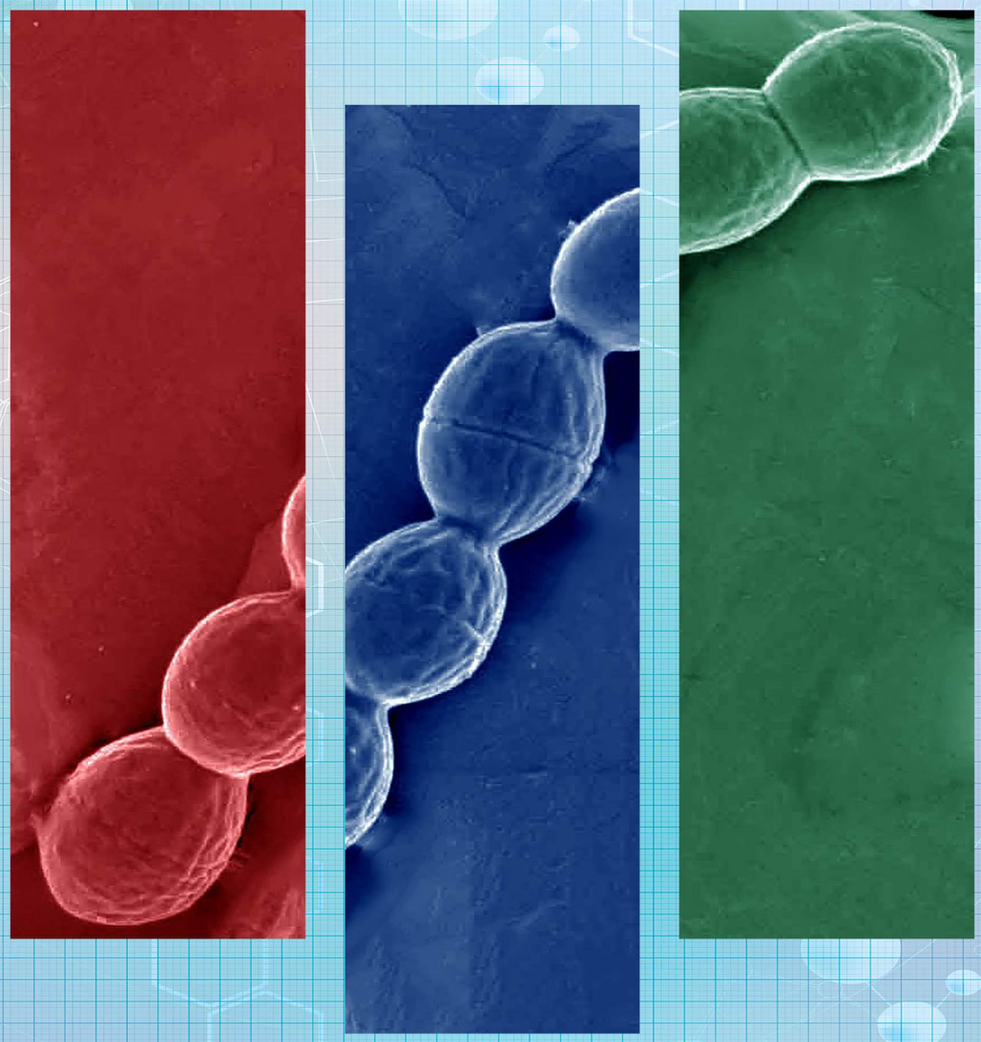
Physiological and Molecular Adaptations of *Lactococcus lactis* to Near-Zero Growth Conditions



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Physiological and Molecular Adaptations of *Lactococcus lactis* to Near-Zero Growth Conditions

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Onur Ercan

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Chapter 1

General Introduction and Thesis Outline

The biotechnology of industrial fermentation and microbial cell-factories

Industrial fermentation biotechnology employs microbes to convert raw materials to a range of fermented products and thereby represents an important economic process. Most of the industrial fermentation processes are derived from naturally occurring and/or artisanal production of foods that have been made compatible with large scale industrial processes, and include a variety of fermented foods and beverage products, like bread, beer, cheese, and wine. Many of these processes have their origin in classical fermentation processes like the production of fermented dairy products (*e.g.* yoghurt) and alcoholic beverages (*e.g.* wine), which can be traced back to Sumerians and Babylonians in the 7th millennium BC, and to Egyptians in the 4th millennium BC (38). Besides such traditional microbial applications, biotechnological applications of microbes have expanded over the last century or so, to their use in the production of fine chemicals, enzymes, pharmaceuticals and food ingredients.

An important milestone in the history of fermentation processes, was the discovery of tiny 'animalcules' around 1680, by Antonie van Leeuwenhoek, who provided the first descriptions of what we now refer to as microorganisms (13). Until the middle of 18th century, several scientists (in)directly contributed to important advances in understanding fermentation, verifying that microbes were involved in the fermentation process and identifying end products such as alcohol and acetic acid, although these observations remained unconnected and a clearly definition of the main driver of fermentation processes was still to be discovered (13). In 1857, Louis Pasteur demonstrated that fermentation is accomplished as a consequence of the activity of microbes (39), and with this breakthrough discovery the era of the microbiology of industrial fermentation was launched. During the late 19th and by the middle 20th century, improved techniques for the production of pure cultures of yeast and lactic acid bacteria (LAB) led to the development of starter cultures and mass production of the fermentation 'workhorses' (yeast, molds, and bacteria), providing more reliable fermentation processes and allowing the massive expansion of the fermented food and beverage industry. Nowadays, biotechnological applications of microorganisms not only includes their use in the production of food-stuffs, but also has expanded to their use in production of a range of microbial products including antibiotics and other pharmaceuticals, enzymes, peptides and specific metabolites that function as biochemicals in mass-production of bio-fuels that aim to

replace petro-chemical products and/or serve as high value fine-chemicals in food or pharma industries, but can also serve as molecular building blocks for the production of complex chemicals like bioplastics (48).

Industrial microbes are, therefore, often referred to as 'cell factories' that convert low-cost renewable resources to added-value products in processes that in many cases include the formation of biomass through microbial growth. The self-replicating nature of microbes is an important trait that distinguishes microbial production from chemical production. In some applications, the microbial biomass can actually be considered as the product of interest, like the production of baker's yeast, lactic acid bacteria starter cultures or probiotics. However, several other industrial fermentation processes do not aim to generate microbial biomass, but rather strive for maximal formation of microbial metabolites using the microbes as cell-factories. In these latter applications, the formation of microbial biomass is actually considered as a loss of resource, since biomass formation competes for substrate with the formation of product. In such applications (*e.g*. production of bulk chemicals, enzymes, fuels, flavor, texture, and health metabolites) the self-replicating characteristic of cellular biocatalyst can negatively impact the rate of product yield, which makes microbial biomass an undesired by-product that reduces economical profit in this type of industrial application (8). Therefore, depending on the application the industrial us of (fermentative) microbes has a great interest in the ability to control the self-replicating characteristics of the microbes they work with, either to maximize biomass yield on a particular substrate where the microbes themselves are the product, or in the case of cell factories to minimize biomass formation and maximize the uncoupling of product formation and growth.

Lactic acid bacteria

LAB have occupied a central role in various industrial processes. For example, they are used in production of biomass, (*e.g.* starter cultures or probiotics), which is critical in the food fermentation industry for the production of fermented consumer products that are dairy-based like yoghurt, cheese, sour cream, and buttermilk, or employ alternative food raw materials like vegetables, fruits, or meat. In these products LAB contribute to the aroma profile, enhance texture, and improve shelf life and microbial safety of the end product (35). Moreover, LAB are also employed in the production of bulk ingredients (*e.g.* lactic acid),

fine-chemicals and ingredients (*e.g.* riboflavin, folate, bacteriocin, exopolysaccharides), food additives (*e.g.* acetic acid).

LAB constitute a group of low G + C Gram-positive non-sporulating bacteria, that commonly inhabit natural ecosystems that are anaerobic or micro-aerobic. They are functionally grouped on basis of their ability to produce lactic acid through mixed-acid and/or homolactic fermentation metabolism. LAB form a heterogeneous group of bacteria that live in a variety of environments, including the human and animal body, and plant materials. These non-pathogenic and benign bacteria are used in diverse industrial food and feed fermentations that employ 6 major genera: *Lactobacillus* (milk, meat, vegetables), *Lactococcus* (milk), *Leuconostoc* (milk, vegetables), *Oenococcus* (wine), *Pediococcus* (milk, meat, vegetables), and *Streptococcus* (milk) (32). As a key-component of numerous fermentation processes, especially in starter cultures, their robustness of performance during fermentation is of critical importance for successful product formation. Since many industrial processes include exposure to several stress conditions, such as heating, drying, freezing, and thawing, and/or starvation the performance of industrial LAB is challenged by these conditions, underpinning the importance of robustness (60). Consequently, selection of LAB that perform well in fermentation and tolerate the industrial fermentation and processing conditions is an important topic to sustain, and where possible increase the production yield in the fermentation-related industries.

The selection approaches to identify novel starter cultures and strains as well as understanding their (industrial-) stress robustness has been rapidly improving and expanding since the initiation of the LAB genomics era that was initiated with the publication of the complete genome sequence of *L. lactis* IL1403 in 2001 (9). In the years that followed, genome sequences of representative species and strains of the genera *Lactobacillus*, *Streptococcus*, *Lactococcus*, *Oenococcus*, *Leuconostoc*, and *Pediococcus* were determined and published (32). The emergence of publically available complete genome sequences of LAB has accelerated by the recent advances in high-throughput next-generation sequencing technologies, which has enabled comparison of LAB strains of a species, revealing their evolutionary relatedness and sequence and function conservation, horizontal gene transfer, and expansion of selected gene families within the group of LAB, but also disclosing aspects

like genome decay, and metabolic simplification (36). The current genome sequencing projects of multiple strains of LAB species will allow advanced comparative genomic analyses, but will also provide novel approaches towards extended functional genomics approaches, including transcriptomic, proteomic, and metabolic approaches, and will tremendously expand our basic genetic and physiological knowledge of the LAB. Such knowledge can drive novel research approaches to improve industrial fermentation processes and/or enhance the fermentation capacity and robustness of microbes, ultimately aiming to provide the consumer with fermented products of increased quality in terms of taste and texture, and/or health-promotion, and microbial safety.

Lactococcus lactis

Lactococcus lactis is a mesophilic, coccoid-shaped bacterium with a relatively small genome (2.5 Mbp) (43). Like many other LAB, it lacks the genetic capacities that enable cell-motility and sporulation. The *Lactococcus lactis* species encompasses a diverse group of subspecies and strains, that include *L. lactis* ssp. *lactis*, ssp. *cremoris*, and ssp. *hordniae* as the main subspecies based on their phenotypic characteristics (Fig. 1). The ssp. *lactis* is able to grow at 40°C or in the presence of 4% NaCl, can utilize arginine as an energy source, and lacks glutamate decarboxylase activity. These phenotypic characteristics are the predominant discriminating phenotypic traits that distinguish spp. *lactis* from ssp. *cremoris* (30, 40). Moreover, the ssp. *lactis* strains are generally also more robust than the ssp. *cremoris* strains in terms of acid, bile salt, and freezing stress resistance (31). Confusingly, the genotypic characterization of strains of these subspecies is sometimes not entirely congruent with their distinction on basis of phenotypic traits (30). For example, the fully sequenced *L. lactis* MG1363 (59) clusters among the ssp. *cremoris* genotype, but the strain's phenotype includes some typical ssp. *lactis* traits. Due to these discrepancies between the genotype and phenotype classifications of some strains, the nomenclature of *L. lactis* has been revised several times.

Strains of the species *L. lactis* are frequently encountered in milk fermentations and are predominant components in many starter cultures applied in food fermentation processes, *e.g.* in the manufacture of cheese, sour cream, and various other fermented milk products. Starter cultures of *L. lactis* play a key role in preserving the food-raw material by acidification and also contribute to the formation of flavor and texture of cheese that is determining the

product's sensory qualities (47). In addition to these industrial (and artisanal) food fermentation applications, this species is also frequently encountered in other habitats, including (decaying) plant materials, and several fish and meat fermentations. The limited polymer degradation capacity of plant-derived lactococci is in agreement with the notion that in this habitat, they are commonly found in co-occurrence with yeasts and fungi that are capable of liberating nutrients from the plant polymers (43).

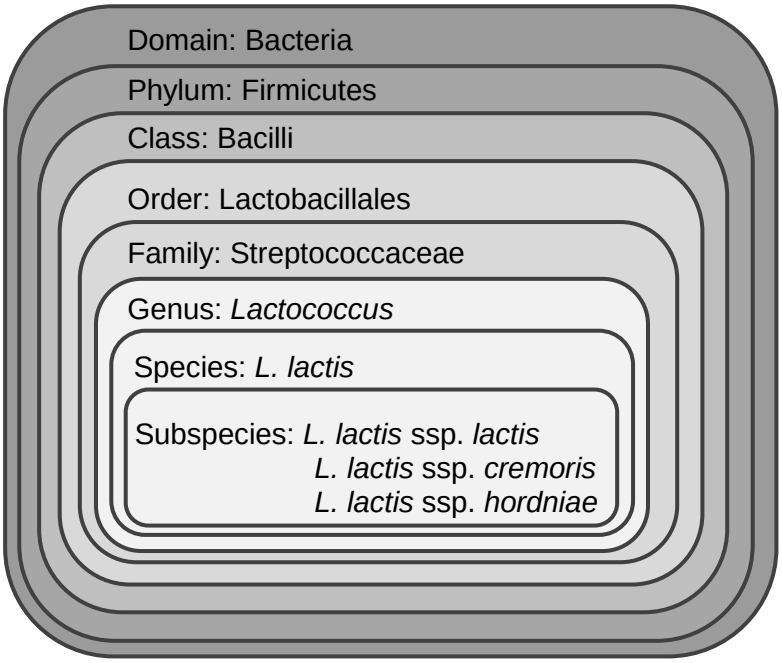

Figure 1: Taxonomy of *Lactococcus lactis*.

Dairy lactococci have been suggested to have evolved from plant isolates (29, 44, 55). In dairy lactococci the utilization of extracellular proteins, *e.g.* milk casein, is facilitated by a cell-wall-bound extracellular protease that converts casein into peptides, which are subsequently transported into the cell by the related peptide-transport systems (34). The protein-rich milk environment is in agreement with the observation that strains isolated from dairy niches are commonly characterized by a high number of amino acid auxotrophies (20, 24) and other characteristics that reflect their dependency on milk proteins as a source of amino acids like a large repertoire of peptide and amino acid import systems, as well as a panel of intracellular peptidases (37, 45). Notably, the genes coding for the production of the extracellular protease are often plasmid-located in lactococci, and this plasmid has been reported to be rapidly lost during propagations in milk, which is an apparent paradox when

considering the role of the protease in digesting milk proteins (3). However, a recent modeling study allowed the quantitative description of culture systems composed of protease producing cells and their cheater (protease negative) derivatives, allowing to explain several of these counterintuitive observations from a community perspective (3).

Plant versus dairy isolates of *L. lactis*

The strain used in this thesis, *L. lactis* KF147, was originally isolated from mung bean sprouts. The complete genome sequence of *Lactococcus lactis* ssp. *lactis* KF147 was determined and was predicted to contain 2578 protein-encoding genes (of which 59 are pseudogenes) (43). The genome encodes all enzymes required for the glycolysis and pyruvate dissipation pathways, which is congruent with its facultative heterofermentative phenotype. *L. lactis* KF147, like many dairy lactococci can convert the available carbohydrate source into formate, acetate, ethanol, and 2, 3-butanediol as end-products, but under most conditions executes a typical homolactic metabolism. The genome sequence of this plant derived *L. lactis* strain reflects many adaptations to the plant-associated habitat, which are in particular apparent from the repertoire of enzymes and pathways predicted to be involved in growth on carbohydrates derived from plant cell wall polysaccharides, including arabinose, galactose, xylose, mannose, and galacturonate. The gene sets predicted to enable the utilization of plant polymer derived carbohydrates in *L. lactis* KF147 are proposed to reflect the adaptation of this strain to growth on plant cell wall substrates (44). An additional adaptation to the plant environment recognized in strain KF147 is the lack of a strong metabolic focus on harvesting amino acids from environmental proteins through proteolytic degradation, which is also reflected by a relatively low number of amino acid auxotrophies relative to typical dairy-isolates of the same species (43, 44). These proposed niche-specific characteristics were clearly supported by the genome-wide adaptations detected in *L. lactis* KF147 derivatives that were adapted to efficient growth in milk using experimental evolution approaches. These studies revealed that adaptive mutations included the activation of functions related to peptide import, in combination with mutations leading to repression of *de novo* amino acid synthesis, and pathways related to the utilization of (plant-derived) alternative carbon substrates (2).

Functional genomics in *L. lactis*

Recent comparative genomics studies revealed that non-dairy isolates of *L. lactis* carry many accessory gene sets, which generally encode proteins involved in the interaction with the ecosystem they inhabit, additional biochemical pathways, or are related to specific phages or mobile genetic elements that are not found in dairy isolates (36, 44). Non-dairy *L. lactis* strains have gained an important position as model organisms for the studies targeting the natural diversity of this species in both ecological studies and industrial fermentation applications, with the inclusion of 'genomics-based' technologies, including complete genome sequencing, functional genomics and evolutionary approaches.

Functional genomics tools are also often used to investigate adaptive responses and regulatory networks that deal with relative short-term changes of *e.g*. gene expression levels in changing environments. Comparison of genome-wide transcriptome profiles of microbes in response to diverse environmental conditions can reveal general stress response features and can support the identification of cellular biomarkers for stress-adaptive behavior. Notably, functional genomics approaches have started to elucidate the gene expression repertoire of *L. lactis* during its growth in milk (19), or during the cheese production process (17, 51). Moreover, a R-IVET based (7) genetic screening strategy has enabled the genome-wide identification of genes activated in *L. lactis* during extended cheese ripening (4). These studies are starting to unravel the environmental adaptations of *L. lactis* to the industrial conditions encountered during typical food fermentation processes. The importance of understanding the gene expression repertoires of *L. lactis* under industrial conditions becomes clearly apparent from studies revealing the prominent environmental control and strain-specific regulatory diversity of the expression of enzymes relevant for flavor formation (5). As a consequence, *L. lactis* (and other LAB) starter culture strain selection procedures require *in situ* screening procedures that mimic the conditions of the eventual application as closely as possible, which has led to the development of 96-well based miniature cheese production systems (6). In addition to determination of the gene-expression repertoires under specific (industrial) conditions, the unraveling of the involvement of transcriptional regulators and their target regulons that drive the observed gene expression adaptations in fluctuating environmental conditions, is crucial to understand bacterial behavior under changing environmental conditions, and to eventually control and improve bacterial performance in fermentation

applications. Transcriptional regulators play a key role in controlling the expression of a number of cellular processes, including metabolism and stress response, and their relative high abundance in the genome sequence illustrates the capability of *L. lactis* to adapt and survive in a diversity of different environmental conditions.

Survival strategies of microbes under environmental stress conditions

During industrial fermentation processes, lactococci are exposed to extreme fluctuations in their environmental conditions, leading to several stress conditions, such as heat, freezing, thawing, drying, low pH, high osmotic pressure, and/or nutrient starvation. For example, throughout the process of cheese manufacturing, cells encounter large changes of temperature, sudden osmotic stresses or oxygen exposure, as well as restricted substrate availability for extended periods, and thus experience a variety of stress and nutrient starvation conditions (46).

The most common and detrimental impact of stress is the deformation and damage of macromolecules, including the composition and integrity of the cytoplasmic membrane, function and folding of proteins, and the functional properties of the nucleic acids, which may be lethal for microorganisms (11, 33). Thus, to survive and remain metabolically active under erratically fluctuating or limited fluctuating environmental conditions, microorganisms must induce several maintenance and repair mechanisms, such as metabolite export systems, detoxification enzymes and macromolecule-repair systems. Adaptations to environmental changes frequently include physiological adjustment strategies, such as the re-allocation of resources, including the re-orientation of energy fluxes from growth to survival mechanisms (11, 41).

When microbes encounter a certain stress condition for longer period of time (limited fluctuations), they can adjust their genetic repertoire by evolutionary processes to increase their survival fitness and proliferative capacity in such ecosystems. Intrinsic stress-resistance encompasses evolutionary adaptation that has been proposed to follow a generic growth versus survival trade-off strategy, which allows microorganisms to become resistant to stress through evolutionary adaptation, rather than flexible activation of specific stress responses (50). However, this constant resistance strategy involves trade-offs that limit microbial metabolic function and capacity to respond to enriched conditions (41). For example, Gram-

positive bacteria, which have thick peptidoglycan cell wall, are more intrinsically resistant to drying and rehydration stress as compared to Gram-negative bacteria, which have a thin cell wall but are protected by an outer membrane. However, the production of thick cell walls creates a significant metabolic burden in terms of substrate and metabolic energy (41, 42). Moreover, evolutionary adaptations may include genetic alterations leading to the constitutive expression of stress tolerance related functions, but also encompass changes in the genetic repertoire of microbes by horizontal gene transfer by which stress tolerance functions may be acquired by microbes from neighboring cells within same habitat (25). Thereby evolutionary processes enable microbes to genetically adapt to new environments to ensure improved survival and growth under newly encountered conditions. Although this evolutionary adjustment has a longer time span, adaptation process through acquisition of exogenous genetic material is accelerated in several bacteria, e.g. *Bacillus subtilis*, *Streptococcus pneumonia*, by activating their natural competence machinery (27). Thus, some microbes are able to increase their evolutionary adaptation rate to increase their chances of survival and adequate fitness's in a new ecosystem by recruiting and harnessing functions imported from neighboring cells.

In erratically fluctuating environments, gene-regulatory adaptation enables microorganisms to activate a specific set of response mechanisms to prevent or counteract the damage induced by stress conditions. Thereby the enduring energy burden related to maintaining the expression of the stress response genes is minimized, and energy expenditure related to expression of these mechanisms is restricted to conditions where these functions are required (41). Although these adaptation processes are energetically expensive for microorganisms, their complete development may be hampered by restricted nutrient and energy availability, posing the risk of being insufficiently or too slowly launched to counteract the stress conditions experienced. Nevertheless, the microbial resistance to stresses commonly involves the stress-induced launching of genetic capacities that aim to withstand stress-induced damage (58). Moreover, the adjustment of the expression of stress associated functions may also occur in absence of specific stress conditions. For example, compared to fast or logarithmically growing cells, slow-growing cells or those obtained from the stationary phase of growth differentially express overlapping pathways that elevate their resistance to various stresses, *e.g*. heat, acid, osmotic, oxidative stresses (11, 33).

In (non-sporulating) bacteria, stress response mechanisms depend on the strictly regulated expression of multiple genes, which controls a diversity of cellular processes, including functions like cell division, DNA metabolism, general housekeeping, membrane integrity, transport, etc., and that act in concert to increase bacterial stress resistance (54). The coordination of stress response gene expression is achieved by (partially overlapping) regulatory networks that allow the bacteria to effectively respond to several environmental changes (54). A canonical cellular stress response to prevent stress caused loss of function due to protein denaturation is the production of chaperons and/or the production of a protein degradation machinery to remove denaturing and aggregating proteins. Chaperon proteins are involved in maturation of newly synthesized proteins and in (re-) folding of denaturing proteins (49). Five molecular chaperons, which are DnaK/HSP70, DnaJ/HSP40, GrpE, and HSP60, are part of a so-called minimal stress proteome in several microorganisms, which underpins the strong evolutionary conservation of this cellular function and the significance of chaperons to withstand stress (33). In addition, if denaturing proteins fail to be refolded with the help of chaperones, they may be removed through a dedicated stress response proteolytic system. The ClpP protease plays a key role in degradation of damaged proteins, and its activity is facilitated by the Clp-ATPases chaperones that are involved in recognition of abnormally folded proteins in many microbes (28, 49). Similarly, cold-shock proteins (CSPs) have roles as RNA and/or DNA stabilizing chaperons to enhance or maintain the efficiency of translation or transcription and/or replication, respectively (54). Moreover, in several bacteria, the Uvr system which is part of the acid tolerance response, involving systems that maintain internal pH homeostasis as well as the repair of chromosomal DNA upon damage by for example acidic pH conditions (26).

Stress responses in *L. lactis*

When *L. lactis* strains are exposed to heat stress condition, two canonical chaperone complexes composed of DnaK-GrpE-DnaJ and GroES-GroEL, which are associated to the class I stress response, are strongly induced (49). The genes involved in class I stress response, in lactococci, are regulated by the repressor HrcA that binds to the palindromic operator sequence CIRCE (controlling inverted repeat of chaperone expression) positioned in the promoter regions of the *hrcA-dnaK-grpE-dnaJ* and *groEL-groES* operons (49, 54). In

addition, the expression of class III heat shock genes are controlled by the repressor CtsR, which binds to a direct repeat called the CtsR-box that is located in the promoter regions of the *clpB, clpC, clpE, clpP* genes (28).

Under aerobic conditions, *L. lactis* can use oxygen through the coupled NADH oxidase/NADH peroxidase system and thereby modulate the intracellular redox balance ($NADH/NAD^+$ ratio), which the homolactic mixed-acid fermentation switch (54). However, the exposure to molecular oxygen in this species leads also to the formation of reactive oxygen species *i.e.* hydroxyl radicals ($OH^\bullet$), superoxide (O_2^-), and hydrogen peroxide (H_2O_2), that are toxic due to their damaging effects on virtually all cellular components (*e.g.* proteins, nucleic acids, cellular membrane) and thereby eventually causing cell death (2, 54). In order to remove these highly reactive molecules from the cell, lactococci possess a flavoprotein NADH oxidase (*noxCDE*) that converts O_2 directly to water without producing H_2O_2 as an intermediate and a NADH oxidase/NADH peroxidase system, through which O_2 oxidizes NADH into NAD^+ by NADH oxidase, and the H_2O_2 produced in this reaction is converted to H_2O by NADH peroxidase. Besides, to eliminate O_2^-, *L. lactis* expresses a superoxide dismutase (*sod*A) that converts O_2^- to the less-damaging H_2O_2 (23).

Nutrient restriction and starvation stress elicits various responses in lactococci, which have been studied mainly focusing on limitation of carbon and nitrogen sources. Nitrogen (*i.e.* amino acids) starvation mainly constrains protein synthesis capacity. In *L. lactis*, the transcriptional regulator protein CodY that binds to a consensus CodY-box, plays a central role in controlling cellular responses to nitrogen availability. It regulates the expression of pathways involved in import and degradation of oligopeptides, as well as import of di/tripeptides and amino acids (*e.g.* Val, Leu, Ile, His, Arg, Glu, and Asn) in response to the availability of amino acids or peptides (12, 21). During nitrogen starvation, CodY-associated repression of peptide and amino acid transporter mechanisms is relieved to sustain an adequate, growth supporting nitrogen metabolism in lactococci (21). On the other hand, carbon (*i.e.* carbohydrate) starvation in *L. lactis* elicits the depletion of cellular energy, and as a consequence can indirectly induce an increased resistance to many stress conditions, including heat, oxidative, ethanol, acid and osmotic stress, and allows the improvement of microbial robustness during poor nutrinet conditions (54). Apparently, the organism is able to

elevate the expression of its generic stress responses under especially carbohydrate stravation conditions, enabling its enhanced survival under these challenging conditions. Notably, carbohydrate utilization is strictly regulated in many bacteria, including *L. lactis* by catabolite control. When bacteria encounter a mixture of carbon sources in their environment, they tend to selectively utilize the carbon source that allows maximal efficiency for growth. To this end, sophisticated regulatory circuits have evolved to sense the availability of carbon sources in the environment and adjust the catabolic capacities of the cell to preferentially use the preferred carbohydrate rather than use all carbohydrates simultaneously. This regulatory circuit has been named carbon catabolite control or carbon catabolite repression (CCR) (53). In low-GC Gram-positive bacteria, including *L. lactis*, global CCR is achieved by the transcriptional control of catabolic operons by the global regulator catabolite control protein A (CcpA), of which the activity is modulated in response to the availability of preferred carbon sources (*i.e.* glucose). Moreover, upon CCR relief, the emergence of intracellular availability of specific carbohydrates can lead to the formation of the carbohydrate specific inducers that trigger the expression of the corresponding catabolic pathways, and thereby this specific induction contributes a more specific level of gene expression control to the overall CCR regulation (53). Under conditions of starvation CCR is relieved to induce the expression of the genetic repertoire involved in alternative nutrient acquisition, coordinating the switching from preferred to less preferred carbohydrate-nutrients.

Starvation, stationary phase, and 'zero-growth'

Microbial adaptations to environmental conditions that allow only low specific growth rates, has commonly been studied using chemostat cultivation with low dilution rates, to mimic a lower specific growth rates (22). In a steady-state chemostat culture, the specific growth rate is fixed by controlling the supply rate of a single growth-limiting nutrient, and is equal to the dilution rate D, which equals the medium flow rate divided by the working volume in the fermenter. Moreover, to keep the culture volume constant, spent medium and biomass are continuously withdrawn from the fermentation vessel at a rate that equals the influx of fresh medium. Cultivation parameters, such as temperature, pH, oxygen level, and stirring can be controlled. However, chemostat cultivation is not very appropriate for the study of extremely low specific growth rates, because the extremely low dilution rates ($< 0.05\ h^{-1}$) result in the

biofilm formation and eventually stimulate the overcrowding of the culture by evolutionary adapted subpopulations (15, 18).

Another model to study slow- or non-growing microbes is the stationary phase of batch culture. In laboratory studies lactococci are grown in batches of synthetic and defined media or rich complex media with a single or multiple carbon and energy sources (*e.g*. lactose, glucose, etc.). Under anaerobic batch-culture conditions, lactococci typically go through five growth phases (Fig. 2) (10).

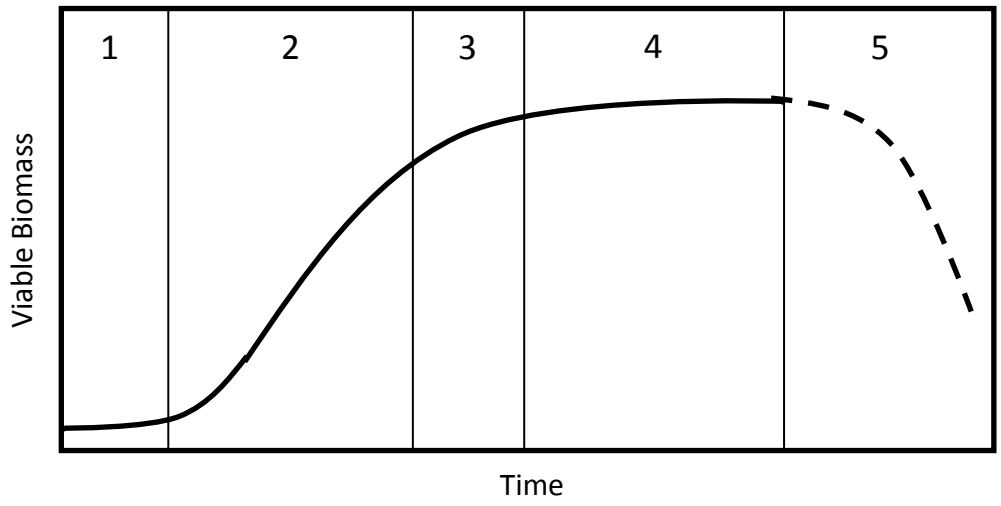

Figure 2: A typical growth profile of *L. lactis* in a batch cultivation with five different phases: 1) lag-phase, 2) exponential phase, 3) late-exponential phase, 4) stationary phase, 5) death phase.

The first phase is the lag phase, in which activation of synthesis of enzymes that are required for the new set of conditions takes place and the specific growth rate slowly accelerates. The second phase is the exponential growth phase where the cells grow at their maximum specific growth rate and display the highest specific organic acid formation rate. The third phase, or the late-exponential phase, can be seen as a transition period between the logarithmic- and stationary phase of growth. During this transition, growth decelerates as a consequence of increasing environmental acidity and lactate concentrations, and/or nutrient starvation, depending on the media used. In the fourth phase the medium can no longer sustain growth, lactate concentration has increased to high levels, and as a consequence pH of the culture dramatically decreased, leading the culture to enter the so-called stationary phase of growth, where the specific growth rate further declines to ultimately become zero. The fifth phase

(autolytic or death phase), is characterized by nutrient deprivation and low pH leading the cells to deteriorate and lose viability, which in *L. lactis* involves also an active autolytic process driven by the peptidoglycan hydrolase AcmA as well as cell-wall associated proteolytic functions (14). In contrast to chemostat cultures, the environmental conditions experience by the microbes in a batch culture situation are inconsistent due to the increasing concentrations of metabolic end-products and eventually cell debris from the dead cells. Therefore, in such cultivation systems it is difficult to disentangle the adaptive responses elicited by nutrient depletion from those elicited by metabolite accumulation and the exposure to cell debris.

The definition of 'zero-growth' used in this thesis differs from stationary-phase cells. The aim of the project was to reproducibly obtain and study a situation in which non-growing biomass remains metabolically active and did not deteriorate as a consequence of nutrient depletion and starvation.

Retentostat cultivation

Retentostat cultivation has been developed to examine physiological and molecular adaptations of microorganisms to near-zero growth rates (57). In essence, retentostat cultivation is a modification of chemostat cultivation in which the complete biomass is maintained in the fermenter by removing the spent-medium effluent through a retention filter unit, at a rate that equals the fixed medium supply rate. Prolonged retentostat cultivation leads to growth rates that approximate zero while the rate of energy transduction (through substrate consumption and conversion) is anticipated to approximate the maintenance energy requirements, *i.e*. the amounts of energy demanded for maintenance-related processes such as cellular homeostasis, rearrangement of metabolic pathways, induction of defense and repair systems (Fig. 3). Retentostat cultivation involves a gradual transition from a growing to a near-zero growth state under culture conditions that are kept constant, which sustains high culture-viability and when maintained long-enough leads to near-zero growth rates. Thereby retentostat cultivation provides an attractive model system to study near-zero growth rates in microorganisms that has prominent advantages (high viability, constant conditions, no cell turn-over, metabolic energy flux towards maintenance, etc.) compared to batch and chemostat approaches.

Retentostat cultivation has been reported for various microorganisms, including *Escherichia coli* (16), *Bacillus polymyxa* (1), *Paracoccus denitrificans*, *Bacillus licheniformis* (57), *Nitrosomonas europaea* and *Nitrobacter winogradskyi* (52). In these microbial physiology studies, the microbes proceeded through an exponential growth phase into an unbalanced linear growth phase, eventually leading to conditions that displayed low-specific growth rates, but never succeeded to reach near-zero growth conditions. Moreover, these studies did not include molecular analyses of the adaptations of these microorganisms to the slow-growth conditions.

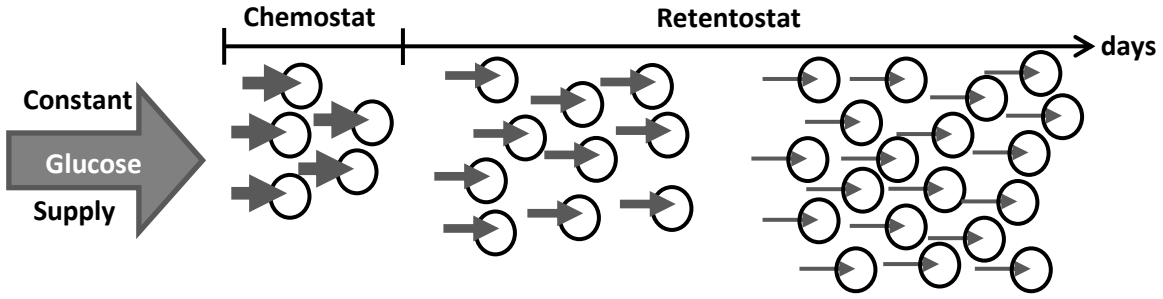

Figure 3: Scheme of glucose consumption rate per cell during chemostat and retentostat cultivation.

Microbiology by numbers: mathematical modelling

Mathematical modeling has been used for decades to understand the mechanisms and dynamics of experimental findings. Not only mathematical models have been used to verify hypotheses made from experimental data, but also designing and testing these models has allowed for experimentally feasible and testable experimental predictions. With advances in molecular biology, genetic manipulation, and the accessibility of complete genome sequences, new models have been developing that integrate dynamics of sets of biochemical interactions. Several modelling methods have been used to successfully address specific questions about the system of interest, and have been frequently applied to model microbial quality and safety of food products, which is determined by the measurement of the levels of pathogenic and spoilage microbes during the product's life cycle. The adaptation of microbial population and the quality features are affected by the local environmental conditions (56). Thus, the mathematical modelling of the development of microbes is a crucial step in quantitatively describing the influence of processing conditions on food quality and safety.

Predictive microbiology deals with the development of accurate and versatile mathematical models that enable the description of microbial adaptation in an environment as a function of time as well as the chosen environmental conditions (56). The modelling process aims to increase the value of such description and capture existing microbiological knowledge about the microbial physiology or behavior into mathematical models (56). In addition to classical microbial growth models, the last decades have also seen the development of several models that quantitatively describe microbial inactivation. These kinetic models describe microbial survival as a function of time, which is important for the prediction of food safety and quality during the shelf life of the product.

Outline of this thesis

This research describes the quantitative physiological and molecular adaptations of *Lactococcus lactis* KF147 to a near-zero growth state induced by carbon-limited retentostat cultivation. Zero-growth is here defined as non-growing, but metabolically active, and where product formation capability is still maintained in the microbial culture. To achieve this aim, the experiment was initiated using a steady-state chemostat at a dilution rate of 0.025 h^{-1}, in which the retentostat-cultivation regime was initiated by installing the biomass retention filter unit in the medium effluent line. Retentostat cultivation was subsequently continued at a dilution rate of 0.025 h^{-1} for extended periods of time.

Chapter 2 describes how retentostat cultivation enables uncoupling of growth and non-growth related processes in *L. lactis*, allowing the detailed quantitative analysis of the physiological adaptations of this bacterium to near-zero specific growth rates. The set-up allowed the estimation of accurate substrate- and energy-related maintenance coefficients and biomass yields under non-growing conditions, which were in good agreement with those calculated by extrapolation from chemostat cultivations at high dilution rates. Furthermore, biomass accumulation at near-zero growth rate could be accurately predicted on basis of maintenance coefficients calculated from the retentostat cultures.

Chapter 3 describes the genome-wide transcriptional responses of *L. lactis* at extended retentostat cultivation. In this chapter, transcriptome and metabolome analyses were integrated to understand the molecular adaptation of *L. lactis* to near-zero specific growth rate, and expand the studies in chapter 2 towards gene regulations patterns that play a

profound role in zero-growth adaptation. The transcriptional patterns in combination with the metabolome data underpinned the strong intertwinement of carbon- and nitrogen-metabolism in *L. lactis* KF147 and pinpointed a possible role of the global Nitrogen-metabolism regulator CodY in regulation of the transcriptional adaptations observed.

Chapter 4 describes the enhanced robustness to several stress conditions of *L. lactis* after its adaptation to extremely low-specific growth rate by carbon-limited retentostat cultivation. The experimental survival data were fitted using the Weibull microbial inactivation model to estimate the kinetic parameters. In this chapter correlations were modelled that quantitatively and accurately describe the relationships between growth-rate, stress-robustness, and stress-gene expression levels, revealing correlation coefficients for each of the varieties involved. In addition, this work illustrated that stress-gene expression values and/or specific growth rate may serve as a biomarker for the prediction of overall robustness of a (retentostat) culture of *L. lactis* KF147.

Chapter 5 evaluates the distinction between the transcriptome responses to extended carbon-limited growth and severe starvation conditions, where the latter condition was elicited by switching off the medium supply of the retentostat cultures described in chapter 1. In addition, this study evaluates the recovery capacity of strictly starved *L. lactis* cultures by re-initiation of the medium supply after 24 hours of starvation. The starvation phase elicited a strong stringency response that is proposed to involve a regulatory role of CodY, while this same regulator may also orchestrate the parallel induction of expression of several other genetic capacities, including the competence operons.

Chapter 6 highlights the comparison of the physiological and molecular adaptations of industrially important microorganisms towards carbon-limited retentostat conditions. The comparison addresses adaptations of physiological characteristics, such as specific minimal growth-rates achieved, cell-morphology, maintenance energy demand, but also addresses the near-zero growth transcriptional responses of these phylogenetically distant industrial workhorses, which include *Lactobacillus plantarum*, *Lactococcus lactis*, *Bacillus subtilis*, from the bacterial kingdom, and *Saccharomyces cerevisiae* and *Aspergillus niger* from the eukarya.

Chapter 7 summarizes the main results obtained in this thesis, and gives some concluding remarks and future perspectives.

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Chapter 2

Quantitative physiology of *Lactococcus lactis* at exteme low-growth rates

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Abstract

This paper describes the metabolic adaptation of *Lactococcus lactis* during the transition from a growing to a non-growing state using retentostat cultivation. Under retentostat cultivation, the specific growth rate decreased from 0.025 h^{-1} to 0.0001 h^{-1} in 42 days, while doubling time increased to more than 260 days. Viability of the overall culture was maintained above 90% but included approximately 20% damaged cells, which had lost their colony forming capacity on solid media. Although culture biomass and viability had reached a steady-state after 14 days of retentostat cultivation, the morphology of the cells changed from coccus-to-rod shape at later stages of retentostat cultivation, by which the cell's surface to volume ratio was estimated to increase 2.4-fold. Furthermore, the metabolic patterns switched between homolactic and mixed-acid fermentation during the retentostat cultivation. Retentostat cultivation enabled the calculation of accurate substrate- and energy-related maintenance coefficients and biomass yields under non-growing conditions, which were in good agreement with those calculated by extrapolation from chemostat cultivations at high dilution rates. In this study, we illustrate how retentostat cultivation allows decoupling of growth and non-growth associated processes in *L. lactis*, enabling the analysis of quantitative physiological responses of this bacterium to near zero-specific growth rates.

Keywords: *Lactococcus lactis*, zero-growth, quantitative physiology, maintenance energy, modeling

Introduction

Due to variable-nutrient accessibility in natural environments, microorganisms live a feast or famine existence, with famine as the prevalent state (23). The famine condition is different from starvation, because concentrations of (fermentable) substrates are usually not absolutely zero in natural habitats (9). In famine conditions, microorganisms either scavenge the environment for available substrates or induce adaptive responses to facilitate high-affinity nutrient acquisition (15). Also under industrial fermentation conditions, microorganisms may experience long periods of extremely low nutrient availability. For example, lactic acid bacteria (LAB) have strongly restricted access to nutrients during the ripening process of cheese (43) and dry sausage productions (21). Nevertheless, some LAB can survive in this process during months of ripening and may still contribute to flavor formation in the product matrix (8, 21). Another example is that in plain yogurt that contain no added sugar, in which LAB can sustain viability for a month during refrigerated storage (1).

On basis of variations in nutrient availability, microorganisms direct metabolic energy to either growth- or non-growth (maintenance) related processes. When environmental conditions are favorable and nutrient supplies are abundant, microorganisms direct nutrient and energy resources primarily to growth-related cellular processes (32). Once nutrient and energy supplies are limited, microorganisms predominantly employ metabolic energy for maintenance-related processes, involving shifts in metabolic pathways, including changes in energy storage and utilization pathways, but also the activation of defense mechanisms, and altered ion turn-over (52). This metabolic energy distribution between growth and maintenance was defined by Pirt, who described the linear correlation between specific substrate consumption rate and specific growth rate and deduced the energy flux required for maintenance-related processes (34). Maintenance energy is considered to be constant for a microorganism under specific cultivation conditions. Therefore, when energy sources are limited in the environment, reduced amounts of energy becomes available for growth-associated processes, resulting in slow- or zero-growth of microorganism (14).

Zero-growth is defined as a metabolically active, non-growing state of a microorganism in which product-formation capability is maintained. Zero-growth is fundamentally different from starvation, which involves deterioration of physiological processes and loss of metabolic

activity (24). The physiology of slow-growing and nutrient-starved bacteria has been most commonly studied in batch cultures moving from the exponential growth to the stationary phase, finally leading to the induction of starvation conditions (38). Poolman and co-workers proposed an alternative experimental set-up to study carbon-starved bacteria in which the medium supply in a steady-state chemostat cultivation is switched off (37). However, both set-ups have two main characteristics that are not commonly encountered in natural ecosystems; (i) they proceed through a sudden transition from steady-state to death phase and (ii) they include unsteady culture condition due to the accumulation of dead cells and end-products after inducing cell-death through a process of autolysis (10, 14, 16). Another approach to study microbial physiology at slow-growth is by chemostat cultivation, in which the specific growth rate can be readily manipulated by changing the dilution rate while maintaining constant environmental conditions (10). However, chemostats cannot be performed at extremely low dilution rates due to homogeneity problems of the culture. To mimic zero-growth cultures under controlled conditions, retentostat cultivation or recycling fermentor systems have been designed (19). Retentostat cultivation is a modification of continuous cultivation in which the growth limiting carbon source is fed at a constant rate, while biomass is retained in the bioreactor by a retention filter-probe in the effluent line. Extended retentostat cultivation leads to growth rates that approximate zero while the rate of energy transduction (through substrate consumption and conversion) equals the maintenance energy requirement (5, 56). Retentostat cultivation involves a gradual induction and progressive transition from a growing to a non-growing state under environmental conditions that remain constant, sustaining high viability of cells and approaching zero-growth rates, illustrating the strength of this approach.

Retentostat cultivation studies have been reported for a variety of microorganisms, including *Escherichia coli* (6)*, Paracoccus denitrificans*, *Bacillus licheniformis* (56), *Bacillus polymyxa* (2), *Nitrosomonas europaea* and *Nitrobacter winogradskyi* (46). In these studies, biomass accumulation of microorganisms at low specific growth rates could not be predicted by the basic extrapolation of results obtained at high specific growth rates. Following exponential growth phase, the microorganisms proceeded through an unbalanced linear growth phase, in which they continued to grow at low specific growth rates (55). In the latter growth phase, metabolism was redirected to alternative substrates for growth, including the induction of

global adaptive responses, such as the stringent and general stress responses (44, 47). This stringent response switches off growth-related processes and leads to elevation of intracellular phosphate and guanine nucleotide, ppGpp and pppGpp, which trigger general defense mechanisms in microorganisms (44). In addition to these general defense mechanisms, subpopulations with distinct physiological states (*e.g*. dormant cells or spores) and genetic variations were also reported to emerge under slow-growth conditions (6, 10, 47). Finally, it was concluded that Pirt's equation does not describe the growth of prokaryotes at slow-growth rates (2, 6, 46, 55). However, in a recently published quantitative physiological analysis of *Saccharomyces cerevisiae* (5) and *Lactobacillus plantarum* (14) under retentostat conditions, the maintenance energy and substrate requirements of microorganisms were calculated based on either substrate-limited chemostats at high specific growth rates or retentostat cultivations at low specific growth rates. In these two retentostat studies, Pirt's equation fitted well with the results, which is in clear contrast to the retentostat experiments with other microorganisms mentioned above.

Lactococcus lactis is used in food fermentation for the production of cheese, sour cream, and other fermented milk products. In addition, this species is also frequently encountered in other ecosystems, in particular in decaying plant material, in which nutrients may become more accessible locally (42, 51). *L. lactis* strain KF147 is an isolate from mung bean sprouts, and its genome sequence reflects many adaptations to the plant environment, especially related to growth on plant-cell wall carbohydrates. Because the plant environment provides lower protein concentrations as compared to the dairy environment, plant-isolated lactococcal strains are not strongly tuned towards harvesting amino acids through proteolytic degradation of environmental proteins and have a lower number of amino acid auxotrophies as compared to dairy derived strains (41, 42). This difference was also clearly apparent in the accumulating mutations and molecular adaptations identified in *L. lactis* KF147 when it was adapted to efficient growth in milk using experimental evolution procedures, which revealed mutations leading to strong activation of peptide import capacities and repression of *de novo* amino acid biosynthesis in the milk-adapted derivatives of strain KF147 (3).

The aim of our study was to investigate metabolic adaptations and quantitative physiology of the plant-isolate *Lactococcus lactis* KF14 at extreme low-growth rates. To this end, *L. lactis*

KF147 was cultivated under carbon-limited retentostat conditions. Throughout retentostat cultivations, the metabolic behavior of *L. lactis* remarkably fluctuated between homolactic and mixed-acid fermentation patterns. Nevertheless, biomass accumulation at near-zero specific growth rates could be accurately predicted based on a maintenance coefficient calculated from the retentostat cultures.

Materials and Methods

Bacterial isolates and media. *Lactococcus lactis* subsp. *lactis* strain KF147 originates from mung bean sprouts, and its genome sequence has been determined (42). Pre-cultures for chemostat and retentostat cultivations were inoculated in 50 ml M17 (48) broth supplemented with 0.5% glucose (w/v) and grown overnight at 30°C. Overnight cultures were harvested by centrifugation (6,000 g, 10 min., 4°C) and washed twice with physiological salt solution (0.9% NaCl in water). Subsequently, the culture was inoculated into chemically defined medium (CDM), supplemented with 25mM of glucose (14). To minimize variation in medium composition during long-term cultivation, 120-liter batches of medium were prepared, filter sterilized, and used for both the chemostat and retentostat cultivations.

Chemostat cultivation. Duplicate chemostat cultures were performed at dilution rates of 0.025, 0.05 and 0.1 h^{-1} in 1.5-l fermentors (Applikon Biotechnology, The Netherlands) with a stirrer speed of 100 rpm. Chemostat cultures with 1.5-l working volume were performed at 30°C under anaerobic conditions with a nitrogen flow at 15 ml/min at a stirring speed of 100 rpm while pH was controlled at 5.5 with 5 M NaOH. Once the culture density had reached an optical density at 600 nm (OD_{600}) of 0.8, the feed pumps were switched on and the fermentors were operated in chemostat mode at the dilution rates mentioned above. To ensure a constant working volume, a conductivity contact was positioned at the surface of the culture that triggered a peristaltic pump, which removed the filtrate at a rate equivalent to the dilution rate. Steady-state was obtained after six volume changes in which values of OD_{600} and cell dry weight (CDW) remained constant for at least two volume changes.

Retentostat cultivation. Retentostat set-up was assembled with a 1.5-l fermentor (Applikon Biotechnology, Schiedam, The Netherlands) and an autoclavable polyethersulfone cross-flow filter (Spectrum Laboratories, CA, USA). The filter was connected to the fermentor with an outer loop. Additionally, an extra outer loop was also provided as a spare filter. If the main

filter had been clogged during retentostat cultivation, the spare filter would have been used while replacing the clogged filter.

Two independent retentostat experiments with 1.5-l working volume were performed under the same conditions, initiating from chemostat cultivation at dilution rates of 0.025 h^{-1}. After steady-state had been achieved with six volume changes in chemostat mode, the fermentors were switched to retentostat mode by removing the effluent through the cross-flow filter. Retentostat mode was continued to perform at a dilution rate of 0.025 h^{-1} and sparged with nitrogen at the same rate as the chemostat cultures to maintain anaerobic conditions. As removal of samples could interrupt biomass accumulation, sample volume and sampling frequency were minimized.

Biomass, substrate and metabolites determination. During fermentations, culture samples were withdrawn at regular intervals to measure CDW, glucose and organic acid concentrations. To measure the culture density, 0.2 ml of culture samples were taken and the OD_{600} was measured with semi-micro cuvettes (10 x 10 x 45 mm) in a UV/VIS spectrophotometer. For CDW determination, 5 ml of culture was passed through pre-weighted membrane filters with a pore size 0.45µm (Merck Millipore, Darmstadt, Germany) using a vacuum filtration unit (Sartorius stedim biotech, Gottingen, Germany). Subsequently, membrane filters were dried at 55°C, and the biomass collected on the membranes were determined in g/ml. Concentrations of residual glucose and organic acids in the culture supernatant were determined by high performance liquid chromatography (HPLC). To this end, cells were removed from 2 ml of culture samples by centrifugation (20,000 g for 2 min. at 4°C), the obtained supernatant was filter sterilized and stored at -20°C until HPLC analysis. After the supernatants had been separated by HPLC using a Rezex ROA-organic acid column (300 x 7.8 mm) (Phenomenex Inc., CA, USA), with sulphuric acid (5 mM; 0.6 ml min^{-1}) as mobile phase at 60°C, lactate, acetate, formate and ethanol were detected by refractive index detector (Shimadzu Scientific Instruments, MD, USA) (22).

Cell viability and culturable cell estimation. The viability of cells in the culture was assayed by LIVE/DEAD *BacLight*™ Bacterial Viability and Counting Kit (L34856) (Molecular Probes Europe, Leiden, The Netherlands). According to the manufacturer's instructions, the analysis was performed using the BD FACSAria II flow cytometer (BD

Biosciences, CA, USA). The kit contains two fluorescent dyes, which bind to DNA: Red fluorescent propidium iodide (PI) and green fluorescent SYTO9. While SYTO9 can cross intact cell membranes, PI can across only permeabilized cell membranes. In addition, viability was assessed by quantification of colony forming units (CFUs) on GM17 agar plates, using serial dilutions of the cultures (10-fold dilution in M17 broth) in triplicate (40).

Cell morphology analyses by microscopy. Cells morphology was analyzed by phase contrast microscopy, using a Leitz Dialux® 20 microscope (Leica Microsystems, Wetzlar, Germany) at a magnification of 1,000-fold. Images were collected and analyzed with a Cohu High Performance CCD Camera (Cohu, Inc., San Diego, USA) using the Leica Q Fluoro V1.0 software.

Calculation of energy parameter. Two main energy parameters, maintenance energy coefficient (m_{ATP}) and maximum specific growth yield (Y_{ATP}), were calculated using the Herbert-Pirt (35) equation (equation 1). While this equation contains m_{ATP} and Y_{ATP}, it describes the specific ATP production rate (q_{ATP}).

$$q_{ATP} = \frac{\mu}{Y_{ATP}} + m_{ATP} \quad (1)$$

The Herbert-Pirt equation was modified to describe the specific substrate consumption rate (q_s), including substrate-related maintenance coefficient (m_s) and maximum specific growth yield (Y_{sx}^{max}) (equation 2).

$$q_s = \frac{\mu}{Y_{sx}^{\max}} + m_s \quad (2)$$

In addition to calculations of energy parameters, biomass accumulation during retentostat cultivations was fitted to the van Verseveld (56) equation (equation 3). In this equation, C_x indicates the biomass concentration ($g_{DW}\ L^{-1}$), $C_{x,0}$ is the initial concentration ($g_{DW}\ L^{-1}$), D is the dilution rate (h^{-1}), $C_{s,in}$ is the substrate concentration in the medium (25 mM glucose and 1.85 mM citrate), C_s is the residual substrate concentration in the effluent (no glucose or citrate left), m_s is the substrate-related maintenance coefficient ($mmol_{carbon}\ g_{DW}^{-1}\ h^{-1}$) and Y_{sx}^{max} is the substrate-related maximum specific growth yield ($g_{DW}\ mmol_{carbon}^{-1}$).

$$C_x(t) = \left(C_{x,0} - \frac{D.(C_{s,in} - C_s)}{m_s} \right).e^{-m_s.Y_{sx}^{\max}.t} + \frac{D.(C_{s,in} - C_s)}{m_s} \quad (3)$$

The specific growth rate was calculated as the first order derivative of the fitted equation divided by the calculated biomass concentration at the corresponding time (equation 4).

$$\mu(t) = Y_{sx}^{\max}.\left(D.(C_{s,in} - Cs) - C_{x,0}.m_s\right).e^{-m_S.Y_{sx}^{\max}.t} \cdot \frac{1}{C_{x,t}} \quad (4)$$

After determining the metabolically active biomass of the cultures, biomass accumulations and specific growth rates were calculated with equations 3, and 4, respectively, in two independent retentostat cultivations.

Results

Biomass accumulation, viability and morphology in retentostat cultures. *Lactococcus lactis* KF147 was grown under anaerobic retentostat conditions in a chemically defined medium (CDM) with glucose and citrate as the growth limiting substrates. Two independent retentostat cultivations were performed for 42 days to investigate the strain's physiological adaptation to near-zero growth conditions. During cultivation of *L. lactis* KF147 under retentostat conditions, biomass accumulation, cell morphology, and culture viability were measured at days 0, 2, 7, 14, 21, 28, 35, and 42.

In two independent fermentors, biomass accumulation sharply increased during the first 7 days of retentostat cultivation, followed by a slow increase of biomass from day 7 to 14 (Fig. 1A). During the latter cultivation period, the specific growth rate significantly decreased from 0.025 h^{-1} (chemostat growth and initial retentostat growth rate) to 0.004 h^{-1} after 7 days, and 0.0003 h^{-1} after 14 days (Fig. 1B). Finally, while biomass approached a near-zero growth steady-state after approximately 14 days in retentostat cultivation (Fig. 1A), the specific growth rate reached its minimum of 0.0001 h^{-1} at the end of the cultivation (after 42 days) (Fig. 1B). This final growth rate correlates with a culture doubling time of more than 260 days (Fig. 1B). In addition to experimental measurements, biomass accumulations and specific growth rates under retentostat conditions were predicted with the equation 3 and equation 4 (see Materials and Methods), respectively. The calculated biomass accumulation and specific

growth rate matched very well with the experimentally determined biomass accumulation and specific growth rate in both cultures (Fig. 1A & 1B).

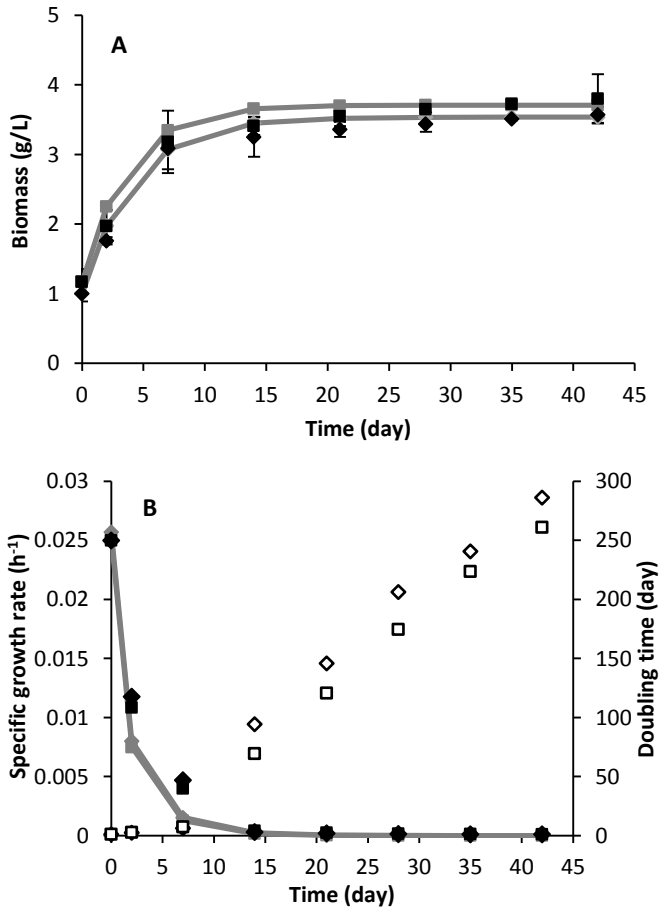

Figure 1: Growth of *L. lactis* KF147 under retentostat conditions. Data from retentostat cultivation 1 and 2 are represented as diamonds and squares, respectively. A steady-state anaerobic chemostat culture was switched to retentostat mode at time zero. Data points represent average ± mean deviation of triplicate samples.
(A) Measured biomass concentration (g/L). The biomass calculated from the fitted van Verseveld equation for retentostat 1 and 2 (grey lines with diamonds and squares, respectively) are shown.
(B) Specific growth rate (h^{-1}) (closed diamonds and squares) and doubling time (day) (open diamonds and squares) based on cell dry weight. In addition, corresponding calculated specific growth rates for retentostat 1 and 2 (grey lines with diamonds and squares, respectively) are shown.

The culture viability was assayed using LIVE/DEAD staining combined with fluorescent activated cell sorting (FACS) analysis (Fig. 2A) and plate counting assay (Fig. 2B). Viability assessment of *L. lactis* KF147 cultured extensively under retentostat conditions (after 35 and 42 days of retentostat cultivation) could be divided into three subpopulations on basis of their SYTO9 and propidium iodide (PI) staining characteristics (Fig. S1). The population with strong red and weak green fluorescence intensity was considered to represent dead cells, of which the membranes had become permeable to PI, whereas the population with strong green and weak red fluorescence intensity was considered as live cells with intact membranes. The third population with increased red and green fluorescence intensity (indicated by blue color in the cytogram) was classified as damaged cells, which is considered as intermediate state that allows reduced amount of PI to penetrate as compared to the dead cells. Using these

criteria, approximately 5% of the cell population was considered to be dead throughout the retentostat cultivation, while live cell population represented 93% ± 1% of the total population at early stages of retentostat cultivation (days 0 and 2). The live cell population declined during extended retentostat cultivation (*i.e*. after 35 and 42 days) to 76% ± 4% (Fig. 2A). Correspondingly, the damaged cell population increased from 1.2% ± 1% (days 0 and 2) to 21% ± 5% (days 35 and 42) of the total population during retentostat cultivation (Fig. 2A). Colony forming unit (CFU) enumeration by plate counting established significantly increasing CFU counts during the first 14 days of cultivation. Following this initial increase, CFU counts gradually decreased during extended retentostat cultivation up to 35 days of cultivation, when a steady- state in terms of -CFU number was reached that was maintained during the last week of retentostat cultivation (Fig. 2B). The CFU enumeration appeared to match with the proportion of live cells detected by FACS analysis, suggesting that the damaged cells could no longer form a colony.

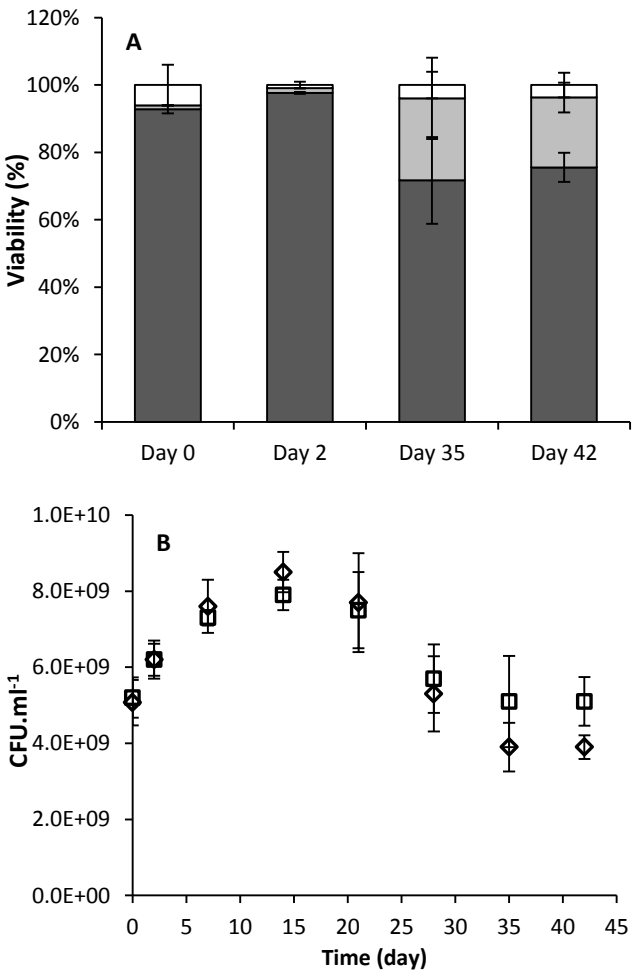

Figure 2: Viable and culturable *L. lactis* KF147 under retentostat conditions.
(A) Percentages of biomass viability assayed by LIVE/DEAD *BacLight* kit using FACS in retentostat culture. This assay includes percentages of live (dark gray), damaged (light gray), and dead (white) cells during retentostat cultivations. Data points represent average ± mean deviation of measurements of two independent cultures.
(B) Culturable cell estimation by plate count ($cfu.ml^{-1}$) from retentostat 1(diamonds) and 2 (squares) on solid medium. A steady-state anaerobic chemostat culture was switched to retentostat mode at time zero. Data points represent average ± mean deviation of triplicate samples.

Cell morphology of the bacteria growing under retentostat conditions was examined using phase contrast microscopy at days 0, 2, 7, 14, 21, 28, 35, and 42 (Fig. S4). At days 0 and 2, individual cells were relatively small and had the typical 'cocoid' shape. However, after 21 days of retentostat cultivation, the cells became slightly more rod-shaped and increased in size. These morphological transitions became even more apparent after 35 and 42 days of retentostat cultivation (Fig. S4). To quantify these morphological transitions from coccus-to-rod shape and corresponding size-increase, intensity of forward scattering light-area (FSC-A), which relatively correlates with cell surface area, was extracted from flow cytometry analysis of retentostat culture samples at days 0, 2, 35 and 42 (Fig. 3). For the distribution of live cell population, the FSC-A median value shifted from 1031 (in chemostat mode) to 2197 after 35 days, and reaching a maximal value of 2436 at the end of the retentostat cultivation (Fig. 3). These measurements imply that the cell surface to volume ratio increased 2.4-fold during extended retentostat cultivation as compared to chemostat cultivation.

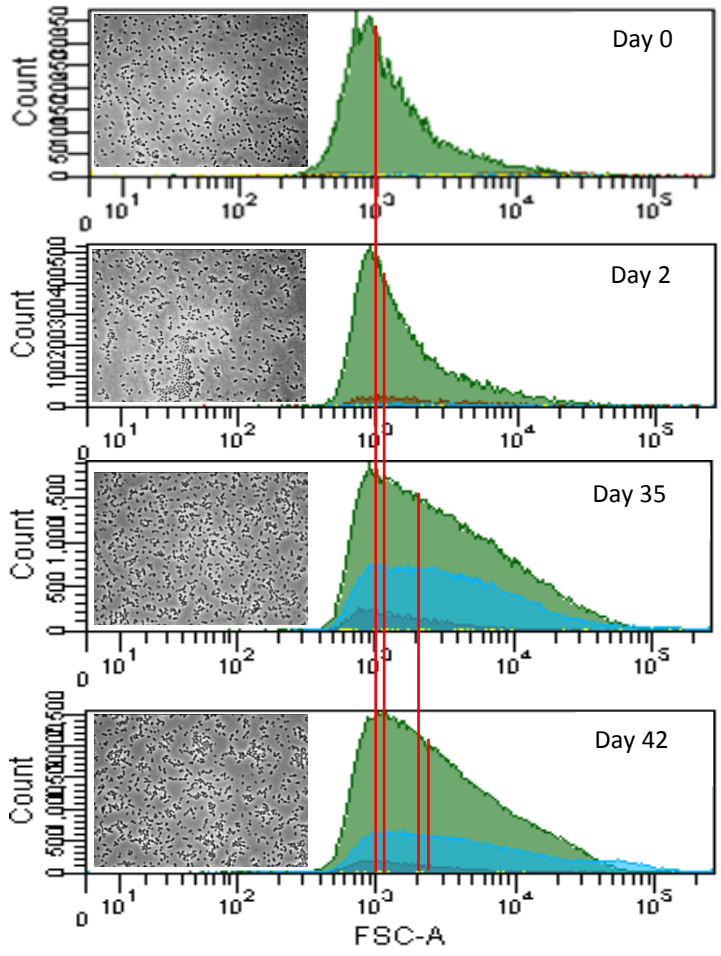

Figure 3: Morphological changes of *L. lactis* KF147 during retentostat cultivation 1 at days 0, 2, 35, and 42. Bright field images of cells and forward light scatter-area (FSC-A) graphs of FACS (live cells, green; damaged cells, blue) are presented to illustrate the morphological changes of cells during retentostat cultivation. Red lines indicate median values of each FSC-A graphs.

Metabolic profiling during retentostat cultivation. The specific production rates for fermentation metabolites were measured for lactate, formate, acetate and ethanol, which were the major end-products of the cultivation (Fig. 4). Due to low dilution rate of the cultivation, the culture displayed typical mixed-acid fermentation behavior with 5% of carbon flux directed towards lactate (the specific rate of lactate production of 0.097 $mmol_{lactate}\ g_{DW}^{-1}\ h^{-1}$) and 95% of the flux towards formate, acetate and ethanol during chemostat growth (day 0). During retentostat mode of growth (after day 0), the specific production rates of formate, acetate and ethanol gradually declined until day 21, while the specific rate of lactate production slightly decreased from 0.097 to 0.038 $mmol_{lactate}\ g_{DW}^{-1}\ h^{-1}$ in 7 days (Fig. 4). After 7 days, the specific rate of lactate production increased, and then, reached a maximal production rate of 0.211 $mmol_{lactate}\ g_{DW}^{-1}\ h^{-1}$ on day 21 (Fig. 4). After 21 days, the carbon flux distribution re-oriented and was characterized by approximately 40% of carbon flux towards formate, acetate and ethanol branches of pyruvate dissipation and 60% of flux towards lactate formation. A similar flux distribution was observed after 28 days of retentostat cultivation.

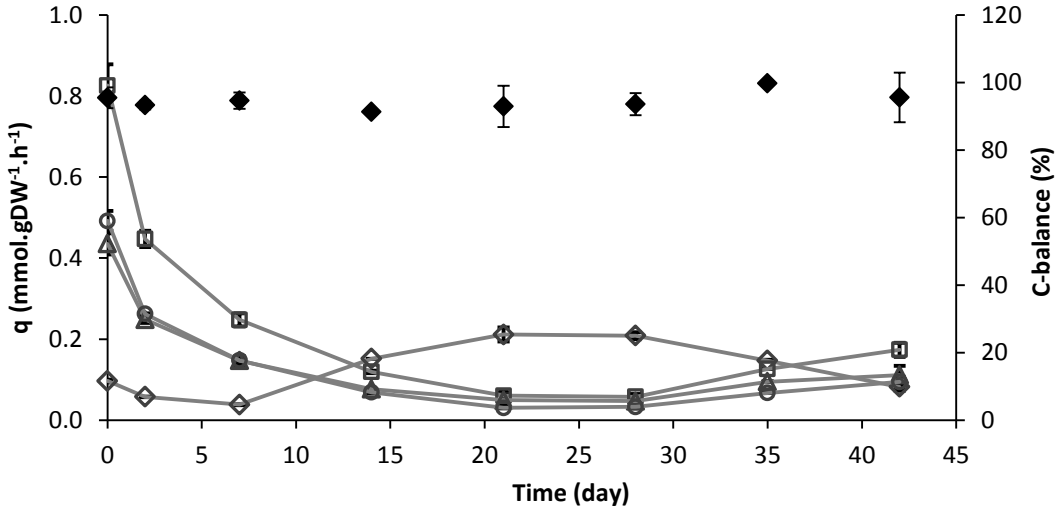

Figure 4: Metabolic profile and carbon balance (closed diamonds) of *L. lactis* KF147 in retentostat culture 1. Specific rate of lactate (open diamonds), formate (squares), acetate (triangles) and ethanol (circles) productions ($mmol.g_{DW}^{-1}.h^{-1}$). A steady-state anaerobic chemostat culture was switched to retentostat mode at time zero. Data points represent average ± mean deviation of triplicate samples.

However, further extension of the retentostat cultivation led to a gradual return towards the typical mixed-acid fermentation behavior that was also observed during the early stages of retentostat cultivation. At the end of retentostat cultivation, carbon flux distribution observed

with 82% of flux towards formate, acetate and ethanol, while 18% of flux towards lactate production with the specific rate of lactate production of 0.082 $mmol_{lactate}$ g_{DW}^{-1} h^{-1}. During these metabolic re-orientations, all the glucose and citrate in the medium were completely consumed and carbon balances calculated on the basis of the major end-products were consistently above 90% (Fig. 4). Notably, both independent retentostat cultures displayed similar fermentation patterns over time, including highly consistent specific rates of metabolite production, and carbon recovery.

Maintenance energy requirement in chemostat and retentostat cultures. Traditionally, energy-related maintenance coefficient (m_{ATP}) and maximum specific growth yield (Y_{ATP}) have been predicted by extrapolation of measurements obtained from chemostat cultures at high specific growth rates (μ of ≥ 0.05 h^{-1}). In such chemostat cultures, a linear correlation is observed between μ and specific ATP production rate (q_{ATP}). This correlation is constant with a growth rate-independent maintenance energy requirement (5, 35) that can be deduced by extrapolation of the growth rate to zero. In this study, we estimated the maintenance energy requirements of *L. lactis* KF147 under carbon-limited chemostat cultivations at high specific growth rates (0.025 to 0.1 h^{-1}), and determined the actual maintenance energy requirements in cultures that were approximating zero-growth in retentostat cultivations (0.0001 h^{-1}). To calculate maintenance energy requirements in retentostat cultivations, q_{ATP}, which was calculated from the lactate and acetate fluxes (1 and 2 ATP produced, respectively: $q_{ATP} = q_{lactate} + 2q_{acetate}$) (31), versus μ was plotted (Fig. 5A). Due to the linear relationship between q_{ATP} and μ on the plot, the slope represents $1/Y_{ATP}$ and the intercept with the y-axis represents m_{ATP}. The calculated m_{ATP} values from two retentostat cultivations were 0.29 and 0.32 $mmol_{ATP}$ g_{DW}^{-1} h^{-1}, while the calculated Y_{ATP} values were 0.04 and 0.05 g_{DW} $mmol_{carbon}^{-1}$, respectively.

In addition to the energy-related maintenance coefficient and maximum specific growth yield, the substrate-related maintenance coefficient (m_s) and maximum specific growth yield (Y_{sx}^{max}) of *L. lactis* KF147 were calculated under retentostat conditions. To calculate m_s and Y_{sx}^{max}, the specific substrate (glucose and citrate) consumption rates (q_s) were plotted versus the growth rate (μ) (Fig. 5B). By analogy to the Pirt equation for maintenance energy requirement, the slope represents $1/Y_{sx}^{max}$ and the interception with the y-axis represents the

m_s. The calculated values m_s values from two retentostat cultivations were 1.14 and 1.09 $mmol_{carbon}\ g_{DW}^{-1}\ h^{-1}$, while the calculated Y_{sx}^{max} values were 8.9 and 10.7 $mg_{DW}\ mmol_{carbon}^{-1}$ in both retentostat cultivations. In conclusion, the two independent retentostat cultures at extremely low specific growth rates displayed highly similar growth kinetics, and physiological parameters, including their substrate consumption and energy production rates.

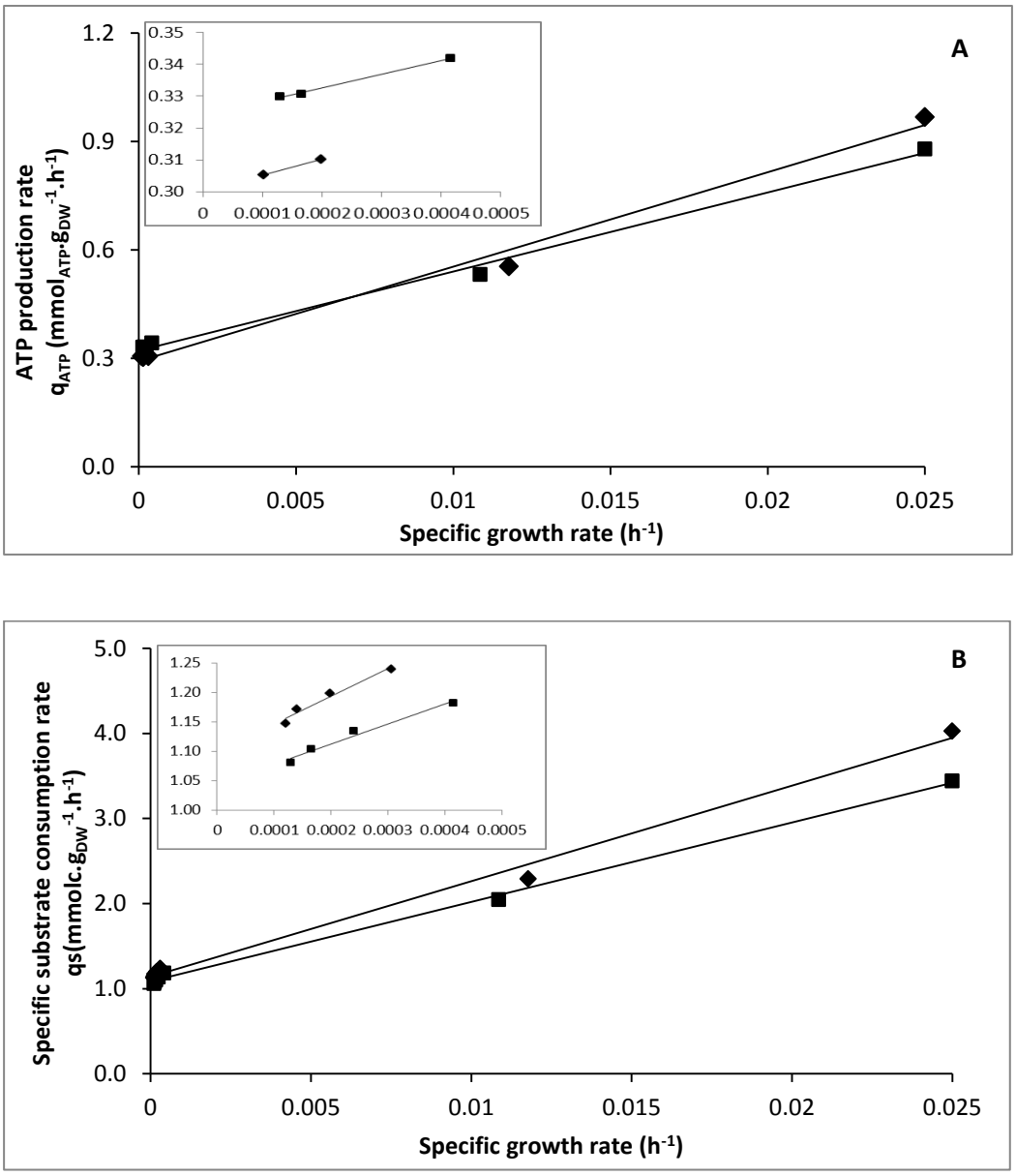

Figure 5: Determination of energy- and substrate-related coefficients. Data from retentostat fermentations 1 and 2 are indicated by diamonds and squares, respectively. Axes titles in the insets are the same as the main figures. (A) Plot of the specific ATP production rate qATP ($mmol_{ATP}^{-1}.g_{DW}^{-1}.h^{-1}$) against μ ($h^{-1}$). (B) Plot of the specific substrate consumption rate qs ($mmolc.g_{DW}^{-1}.h^{-1}$) against μ (h^{-1}).

Table 1: Substrate-related maintenance coefficient and maximum specific growth yield for several *Lactococcus lactis* strains grown under carbon limited chemostat cultures.

Organism	**Maintenance coefficient** (m_s) $mmol_{carbon}\ g_{DW}^{-1}\ h^{-1}$	**Max. specific growth yield** (Y_{sx}^{max}) $mg_{DW}\ mmol_{carbon}^{-1}$
L. lactis subsp. *cremoris* Wg2 (at pH 5.7) (47)	4.92	4.27
L. lactis subsp. *cremoris* Wg2 (at pH 6.4) (47)	6.48	4.60
L. lactis subsp. *cremoris* Wg2 (at pH 7) (47)	4.20	5.10
L. lactis subsp. *cremoris* Wg2 (33)	6.96	4.18
L. lactis ATCC 11454 (29)	4.37	5.70
L. lactis KF147 (in this study)	1.11	9.80

The substrate-related maintenance energy coefficient m_s and maximum specific growth yield Y_{sx}^{max} calculated from retentostat cultivations appeared to be significantly different from those that were previously reported for other *L. lactis* strains in chemostat cultures (Table 1). Therefore, we decided to predict m_s and Y_{sx}^{max} for *L.* lactis KF147 by extrapolation of measurements obtained from chemostat cultivations, analogous to what was previously done for the other *L. lactis* strains. To this end, a set of reference chemostat cultivations was performed at different dilution rates (0.025 to $0.1h^{-1}$), and these were used to predict m_s and Y_{sx}^{max} of *L. lactis* KF147 in anaerobic, carbon-limited cultures. Analogous to previous studies (Table 1), the data obtained from these chemostat cultures displayed a linear correlation between specific substrate consumption rate (q_s) and μ (Fig. S3). The maintenance energy coefficient m_s and maximum specific growth yield Y_{sx}^{max} were calculated by extrapolation of the chemostat cultures towards zero-growth rate. The obtained values of m_s and Y_{sx}^{max} deduced from the chemostat extrapolation approach were in complete agreement between those obtained from the retentostat cultivation, indicating that the maintenance energy calculated for the *L. lactis* KF147 strain is considerably lower than those calculated for the other (dairy-derived) strains of this species.

The extended retentostat cultivation leads to growth rates that approximate zero while the rate of energy generation by substrate consumption and conversion equals the maintenance energy requirements. This implies a quantitative conversion of all carbon sources that are consumed to lactate, acetate, formate, and ethanol. In the retentostat cultivations, the specific rates of

ATP production, and carbon sources consumption decreased and asymptotically approached the value of m_{ATP} and m_s, respectively (Fig. S2A & S2B). Furthermore, biomass accumulation and specific growth rate could accurately be predicted on basis of the calculated m_s and Y_{sx}^{max} in these retentostat cultures. Consequently, biomass-model prediction confirmed measured cell dry weight from the retentostat experiments (Fig. 1A).

The distribution of energy and substrate consumptions between growth- and maintenance-related processes was calculated during the transition from the initial chemostat (retentostat cultivations (Fig. 6A & 6B). The calculated values for the maintenance energy coefficient m_{ATP} and the biomass yield Y_{ATP} indicated that the amount of energy used for maintenance-related processes increased from 33.9% ± 4.3% to 97.3% ± 0.1% of the total ATP generated within 14 days of retentostat cultivation (Fig. 6A).

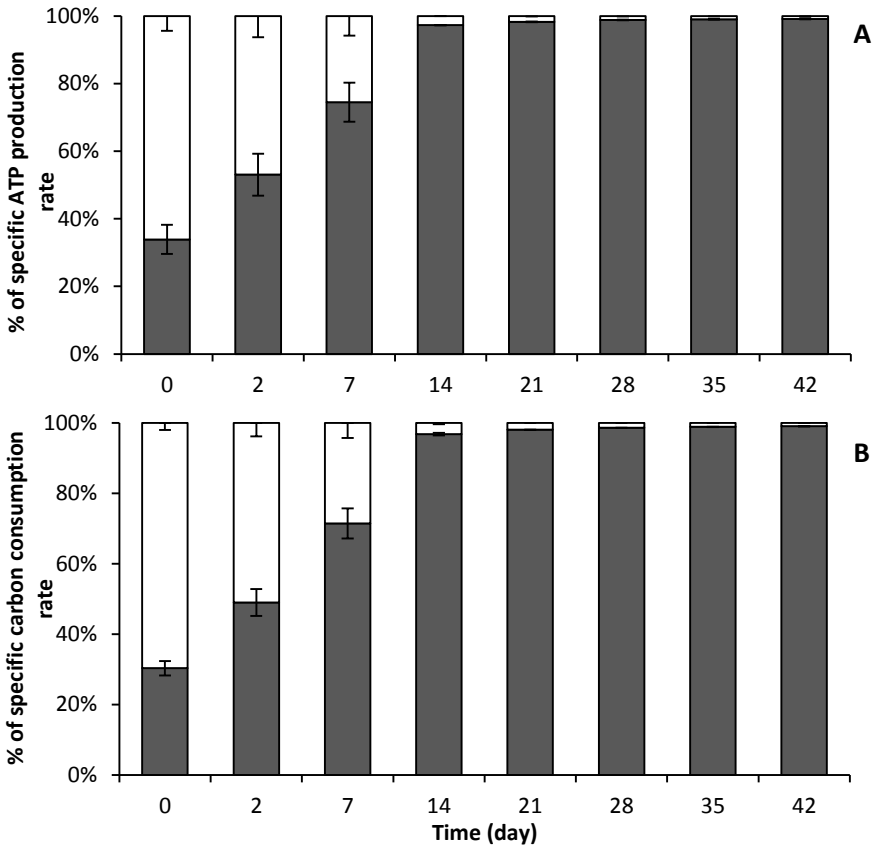

Figure 6: Percentage distribution of energy (A) and substrate (B) costs between maintenance (gray) and growth (white) processes. Data points represent average ± mean deviation of measurements of two independent cultures.

At the end of retentostat cultivation, *L. lactis* used 99.2% ± 0.1% of the total ATP for maintenance-related processes. Similarly, the calculated maintenance-related substrate

coefficient m_s and the biomass yield Y_{sx}^{max} indicated that the relative amount of carbon used for maintenance-related processes increased from 30.3% ± 2.0% to 96.8% ± 3.2% during the first 14 days of retentostat cultivation (Fig. 6B), and increased to 99% ± 0.1% towards the end of the retentostat cultivation. These measurements of energy and substrate cost distributions between maintenance- and growth-related processes confirmed that the retentostat cultures reached a typical near-zero growth state after 14 days.

Discussion

Adaptation of *L. lactis* to extremely low-growth rates induced by extreme limitation of carbon and energy sources using retentostat cultivation was analyzed by investigation of biomass accumulation, culture viability and metabolite production. In addition, the energy requirements and distribution between growth and maintenance related processes of *L. lactis* KF147 were compared between extremely slow-growth and chemostat conditions. Finally, cell dry weight accumulation under retentostat conditions were confirmed using biomass model-predictions. This study revealed remarkable metabolic shifts between mixed-acid and homolactic fermentation behavior of the *L. lacits* KF147 culture during retentostat cultivation. This observation is in clear contrast with previous studies of retentostat cultivation with lactic acid bacteria that revealed a stable metabolic profile with lactate as the major end-product (14, 20, 28, 58). In the current study, *L. lactis* reached a state of 'near-zero growth' after approximately 14 days of retentostat cultivation, coinciding with a shift from mixed-acid to homolactic fermentation behavior. Notably, metabolic behavior returned towards mixed-acid fermentation metabolism after prolonged retentostat cultivation (after 28 days), which is energetically more favorable compared to homolactic fermentation (7). Despite these drifts of metabolic behavior, biomass, extracellular protein content and total ATP yield were quite stable.

In homolactic acid fermentation, pyruvate is metabolized into lactate via lactate dehydrogenase (LDH), which regenerates NAD^+ that is then available to drive glycolytic conversion of the available carbohydrate (glucose and citrate in the current set-up). However, mixed-acid fermentation by *L. lactis* includes the dissipation of pyruvate into acetyl-CoA by either pyruvate formate lyase (PFL) (anaerobic conditions) or pyruvate dehydrogenase (PDH) (aerobic conditions) to produce formate or CO_2, respectively. Acetyl-CoA is subsequently

converted to acetate by phosphotransacetylase (PTA) and acetate kinase (ACK), producing additional ATP as compared to lactate production, or to ethanol by alcohol dehydrogenase (ADH) which includes the regeneration of NAD^+ (7, 12). Homolactic fermentation of *L. lactis* is observed when cells are rapidly growing for example in conditions of carbon excess, while mixed-acid fermentation metabolism is observed under anaerobic conditions when specific growth rate is low and/or under conditions of carbon limitation (49), although growth on media with excess of specific carbon sources (*e.g.* galactose) has also been associated with mixed-acid fermentation behavior (50). Moreover, the availability of molecular oxygen exerts a strong regulatory effect on the fermentative metabolism of *L. lactis*. Oxygen availability elicits the switching from homolactic to mixed-acid fermentation, which is controlled by activation of the NADH-oxidase enzyme, that dissipates the intracellular NADH pool, leading to repression of NADH dependent metabolic reactions like LDH and stimulating NAD^+ dependent reactions such as PDH (27). The metabolic shift from homolactic to mixed-acid fermentative metabolism in *L. lactis* involves a reorientation of pyruvate dissipation towards increased conversion by PFL or PDH at the expense of conversion by LDH. This metabolic shift is proposed to be at least in part controlled by allosteric control of intermediary metabolites. Homolactic fermentation correlates with elevated concentrations of intracellular glyceraldehyde-3-phosphate (GAP) and dihydroxyacetone-phophate that inhibit PFL activity, as well as fructose-1, 6-biphosphate (FBP) that is a known allosteric activator of LDH (12, 50). Alternatively, it has been suggested that the metabolic shift from homolactic to mixed-acid fermentation depends on glycolytic flux rates, where high flux results in homolactic fermentation and decreased flux-rates direct the pyruvate dissipation capacity towards mixed-acid production (12). This explanation is based on the potent inhibitory capacity of intracellular inorganic phosphate on pyruvate kinase and LDH in *L. lactis*, which implies that slow glycolytic flux results in starvation-simulating conditions and accumulation of inorganic phosphate (P_i) as opposed to binding of phosphate in the form of ATP during high glycolytic flux conditions. Along these lines, Lohmeier-Vogel and co-workers have shown that glucose metabolizing cells have lower levels of Pi as compared to maltose metabolizing cells, which is explained by a slower import rate for maltose by an ATP-dependent permease compared to the phosphotranferase system involved in glucose import (26).

Even though environmental regulation or control mechanisms of homolactic and mixed-acid fermentation behavior in *L. lactis* are known, the question why *L. lactis* exerts an energy-inefficient metabolism (homolactic fermentation) under certain conditions, while it can also exert an energy-efficient metabolism (mixed-acid fermentation) under other conditions, remains unanswered. Several hypotheses have been proposed to explain this frequently observed counterintuitive and energy-inefficient homolactic behavior. According to the chemical warfare hypothesis, high lactate or ethanol concentration are aimed to inhibit the growth of less resistance organisms in the environment that may compete for the same energy sources. This implies that lactate is produced as a joint effort of the lactococcal population to inhibit or kill the competing microbes in the environment, and to this end the individual cells sacrifice some energetic gain that could be obtained from mixed-acid fermentation (36). However, this concept would imply that "cheater" mutants would arise that are lactate tolerant, but would be energetically favored by executing mixed-acid rather than homolactic fermentation. This would increase their relative fitness while they would still benefit from the homolactic "warfare" executed by the other members of the community. Eventually, in a pure culture the "cheater" mutants could completely take over the population (30). Molenaar and colleagues proposed another hypothesis in which the homolactic and mixed-acid fermentation metabolism shifts are the resultant of a tradeoff between the metabolic or energetic efficiency of pathways and the costs invested in producing enzymes for the pathways that is varying with substrate concentrations and/or growth rate (30). The tradeoff hypothesis implies that metabolism tends towards catalytically efficient metabolism under conditions with higher substrate concentrations, which would allow for growth rate maximization. On the other hand, at low substrate concentrations, a mixture of metabolically and catalytically efficient pathways would lead to a higher growth rate than that of one of the pathways alone. Under those conditions, where available substrate is expensive, microorganisms would invest to gather substrate via a catalytically efficient pathway while gaining more energy via a metabolically efficient pathway. Consequently, microorganisms could optimize cellular economy by mixing both pathways at low substrate concentration. In *L. lactis*, the metabolically and energetically efficient pathway leading to acetate, formate, and ethanol, requires more enzymes and is catalytically less effective (low k_{cat}) as the catalytically efficient but metabolically inefficient pathway that leads to lactate formation (high k_{cat}) (30). In our

study, considering the warfare hypothesis, when the population reached steady-state at high density culture and extremely limited substrate availability, some lactococcal cells could switch to homolactic fermentation to inhibit the growth of other lactococcal cells by increased lactate concentration. Thus, this subpopulation could decrease the number of cells in the culture and access to more substrate. However, lactate tolerant "cheater" mutants (another subpopulation) could increase and execute mixed-acid fermentation, which would raise their relative fitness. Consequently, if the cultivation had run for longer periods of time, lactate producing, and lactate tolerant subpopulations could have coexisted and oscillated to take over the entire population in the high density culture. However, predictions of the frequency of "cheater" phenotype emergence on basis of the reported mutation frequency of *L. lactis* ($1.34x10^{-7}$ mutations/generation; 3) and the number of cells in the retentostat culture ($8.5x10^{9}$) implies that roughly 500 mutations may have occurred, which indicates that the option to explain the observed metabolic shift by "mixed-acid cheater" emergence is highly unlikely. Alternatively, considering the tradeoff hypothesis, the explanation for the shift between mixed-acid and homolactic fermentative metabolisms at extremely low substrate concentration may be found in optimizing cellular economy and increasing the growth rate by employing both types of metabolic pathways. During the earlier stages of retentostat cultivation, homolactic fermentation, *i.e.* catalytically efficiency, could be elevated to gather more substrate, while mixed-acid fermentation, *i.e.* energetically efficient, metabolism is sustained in parallel. Once the cells acquired enough substrate for maintenance-associated processes, metabolism could shift to predominant mixed-acid fermentation at the expense of homolactic fermentation to generate more ATP from the acquired substrate. This fluctuation would then be predicted to continue during prolonged cultivation.

Importantly, the measurements obtained in the retentostat cultivation enabled the accurate prediction of biomass accumulation and specific growth rate in the retentostat on basis of the calculated maintenance coefficient. Additionally, carbon metabolism in *L. lactis* may not have evolved for zero-growth under carbon limitation, because maintenance coefficients calculated from retentostat physiology at extremely low growth rates (0.0001 h^{-1}) and from extrapolated chemostat cultures at high dilution rates (0.025 to 0.1 h^{-1}) are virtually identical. Moreover, specific carbon consumption and ATP production rates, which are constant during the extended retentostat cultivation equal to culture's maintenance carbon and energy

requirements. The maintenance energy coefficients calculated from chemostat and retentostat cultures indicate that *L. lactis* KF147 has a substantially lower maintenance energy demand as compared to several other microorganisms (2, 5, 6, 14, 46, 56). This is especially remarkable when we compare maintenance energy coefficients of the KF147 strain with those determined for other strains of *L. lactis* (29, 33, 47). The plant-isolated *L. lactis* KF147 strain requires approximately 5-fold less substrate-related maintenance energy relative to the dairy-isolated *L. lactis* subsp. *cremoris* Wg2 and *L. lactis* subsp. *lactis* ATCC 11454 strains. Because plant-associated environments are characterized by highly variable chemical and physical conditions, and can commonly be classified as poor conditions for microbial growth due to low pH, high osmolarity and/or low nutrient concentrations, we hypothesize that plant-isolated lactic acid bacteria have adapted to these harsh environmental conditions by minimizing their maintenance energy demand. In contrast, dairy-isolated strains derive from a relatively nutrient-rich environment, such as milk, where amino acids from degraded caseins are readily available. Thereby, dairy-isolated strains may have evolved to increased carbon source and energy requirements for maintenance relative to their plant-isolate counterparts. This contrast of nutrient-richness in benign versus harsh environments of dairy and plant habitats is also reflected in the more profound metabolic and physiological diversity encountered in plant-isolated lactococci as compared to dairy-isolated strains (39, 54). For example, plant-isolated strains commonly produce higher levels of antimicrobial compounds, can ferment a wider range of different carbohydrates, display lower acidification rates on lactose containing media, and require less externally supplied amino acids repertoires for growth as compared to dairy-isolated strains (41, 43).

Among the most visible adaptive strategies of bacteria is their capacity to change cell morphology upon changing environmental conditions. In non-sporulating bacteria, nutrient starvation has been shown to lead to growth arrest, which is associated with transitions of cell morphology (53). As an example, starvation induced growth arrest leads to cell size reduction in bacteria like *Escherichia coli* (25), as well as some enterococci (13) and lactococci (17). Under the retentostat conditions, the morphology of *L. lactis* KF147 changed from the typical coccus- to a more rod-like shape, which coincided with increased cell surface/volume ratios. A possible explanation for the observed morphological transition may be related to reduced

activity of the cell division machinery due to the lack of energy, whereby the cells tend to become extended, rather than form septa and divide.

Retentostat conditions allowed the viability to be sustained above 90%, as determined by the LIVE/DEAD *BacLight* kit. However, 21% from 93% of viable cells were qualified as damaged population at the end of retentostat cultivations by the staining assays and appeared to have lost the capacity to form a colony on solid media. In the corresponding cytograms (Fig. S1), the damaged population was observed as a cloud of particles that seem to be more closely related to the cloud of the live cells, whereas the dead cells were clearly separated in a distinct population. This implies that these damaged cells are apparently only partially permeable, and their membrane characteristics are distinct from dead cells. On the other hand, absence of the damaged cell population in CFU counting may suggest that the damaged cells are in some form of dormant state that has been described before for lactococci that were maintained under severe starvation conditions (11, 45, 57). The damaged cell population is highly unlikely to serve as nutrient resource for cell turn-over in the retentostat population, since the cell debris that may eventually be released from damaged or dying lactococcal cells does not provide readily fermentable substrates that sustain the growth of *L. lactis*. In our opinion, this excludes the possibility that the retentostat culture is subject to population turn-over during extended retentostat cultivation.

Near-zero growth conditions cannot be achieved in extremely low dilution-rate chemostats or batch cultures, and retentostat cultivation offers a unique set-up allowing not only to reach extremely low growth rates while maintaining high cell-viability, but also enabling accurate calculations of maintenance energy requirements. Moreover, the set-up allowed the accurate modeling of the interrelationship between energy, biomass formation and growth.

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Supplementary information for chapter 2

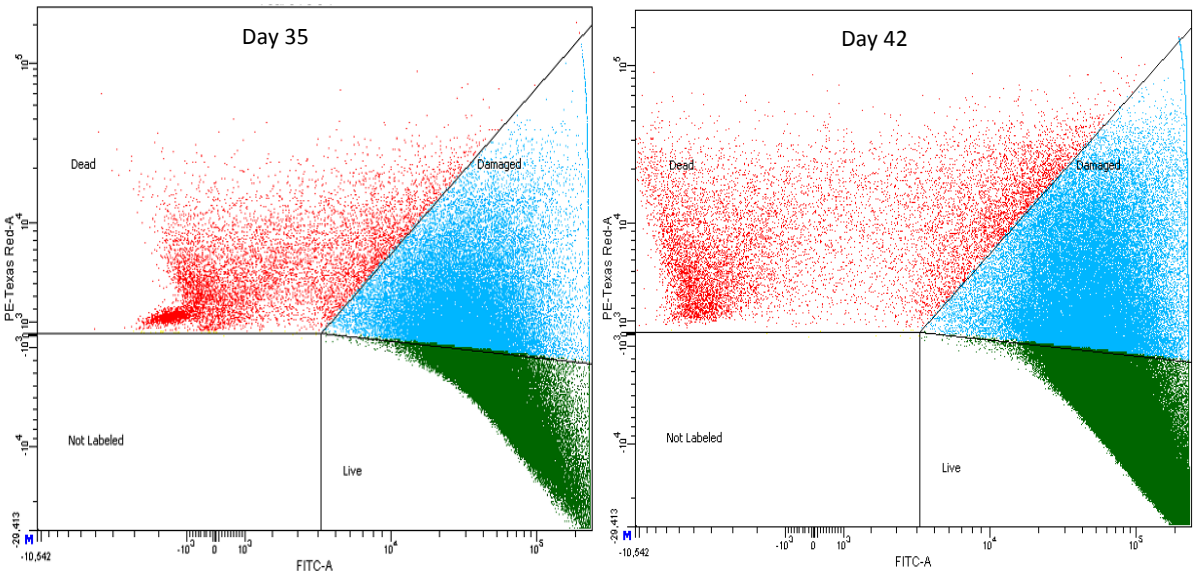

Figure S1: Flow cytometric analysis of *L. lactis* KF147 in samples taken on days 35 and 42 under retentostat cultivation. Bacterial cells were stained with a mixture of SYTO9 and PI, and then analyzed at red fluorescence (PE-Texas Red-A) versus green fluorescence (FITC-A) on a flow cytometer.

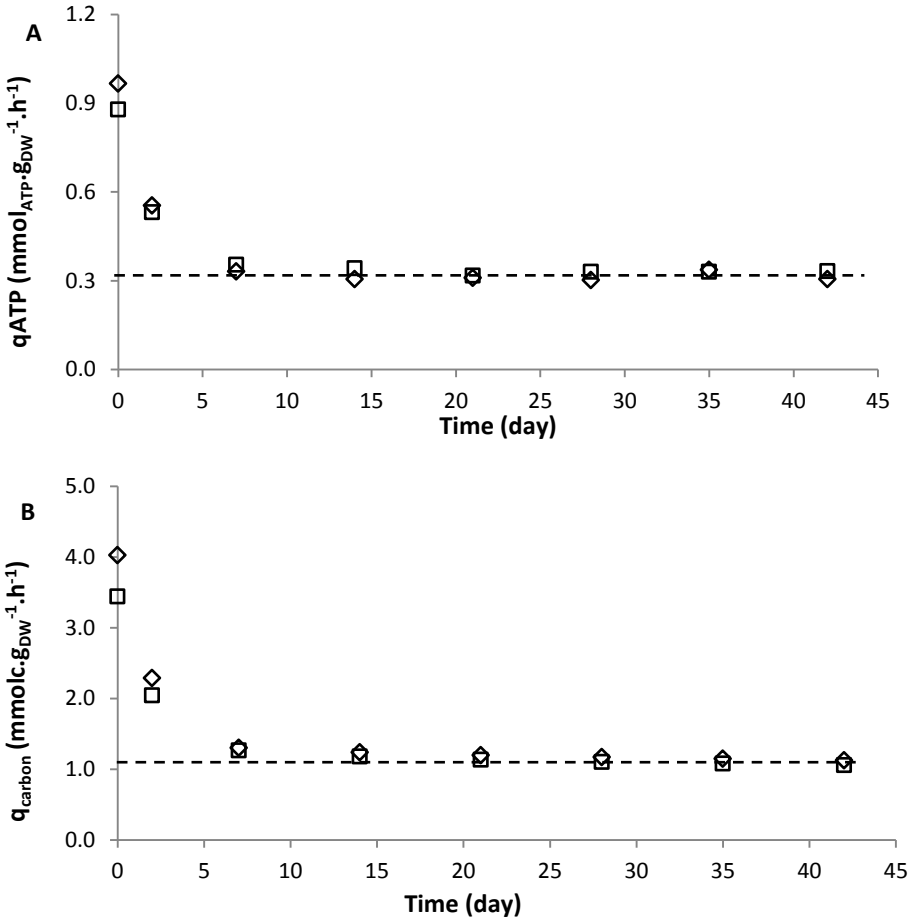

Figure S2: Kinetics of specific ATP production and carbon consumption rates in retentostat cultures of *L. lactis* KF147. A steady-state anaerobic chemostat culture was switched to retentostat mode at time zero. (A) Specific ATP production rate (q_{ATP}) and (B) specific carbon consumption rate (q_{carbon}). The dashed lines indicate the average of energy (m_{ATP})- and substrate-dependent maintenance coefficient (m_s), respectively, calculated from retentostat 1 (diamonds) and retentostat 2 (squares) cultivations.

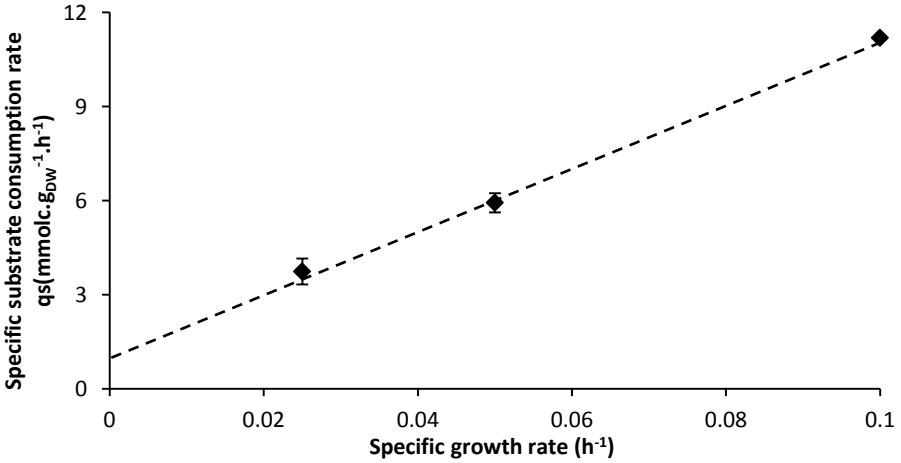

Figure S3: Plot of the specific substrate consumption rate qs against μ in anaerobic, carbon-limited chemostat cultures of *L. lactis* KF147. Each data point represents the average of data from three different chemostat cultures.

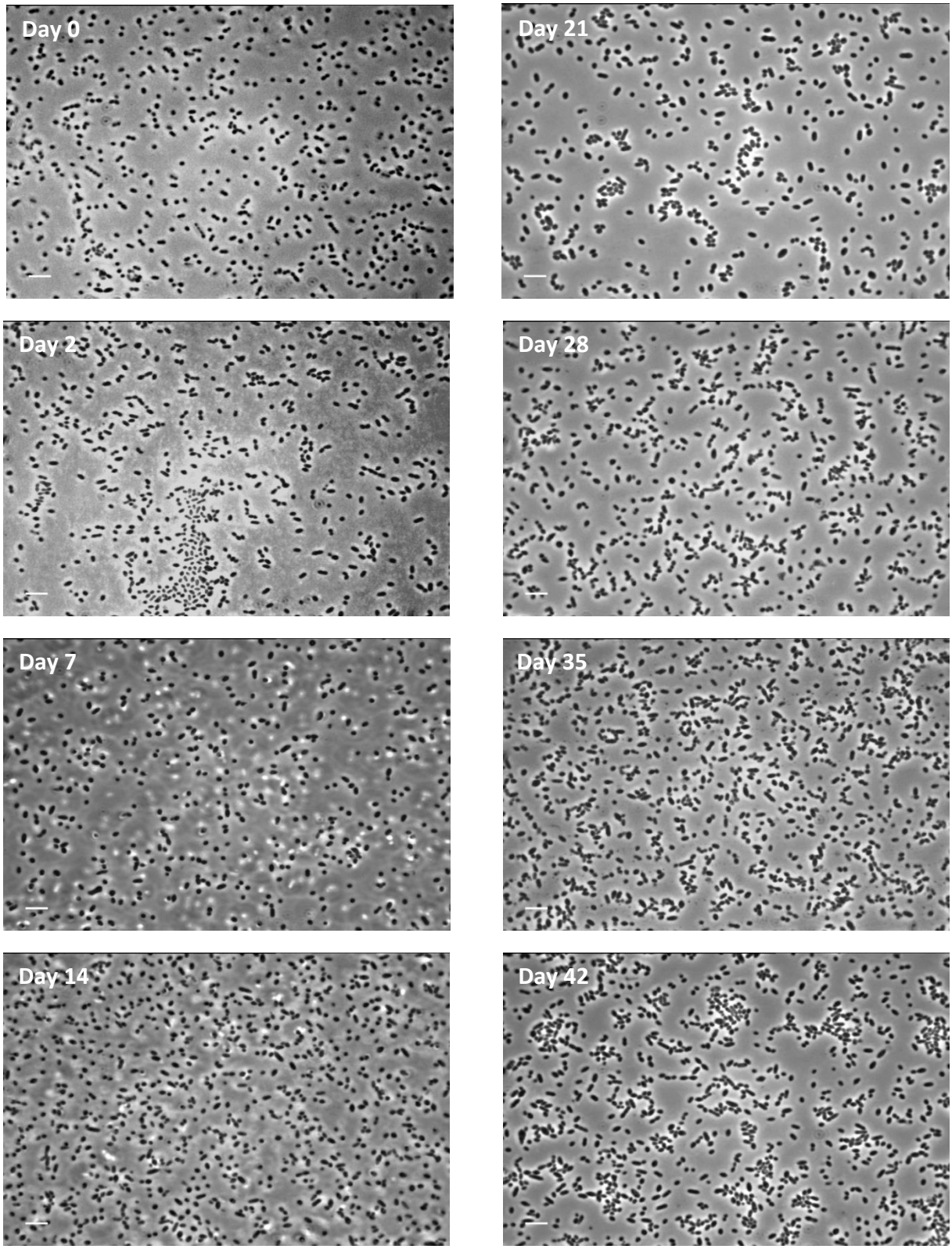

Figure S4: Bright field images of *L. lactis* KF 147 cells from retentostat 1 in the beginning of the retentostat, days 2, 7, 14, 21, 28, 35, and 42. Scale bar 1 cm.

Chapter 3

Molecular and metabolic adaptations of *Lactococcus lactis* at near-zero growth rates

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Submitted for publication

Abstract

This paper describes the molecular and metabolic adaptations of *Lactococcus lactis* during the transition from a growing to a near-zero growth state using carbon-limited retentostat cultivation. Transcriptomic analyses revealed that metabolic patterns shifted between homolactic and mixed-acid fermentation during retentostat cultivation, which appeared to be controlled at the transcription level of the corresponding pyruvate-dissipation enzyme pathway encoding genes. During retentostat cultivation, cells continued to consume several amino acids, but also produced specific amino acids, which may derive from the conversion of glycolytic intermediates. We identify a novel motif containing CTGWCAG, in the upstream regions of several genes related to amino acid synthesis, which we propose to be the target site for the *Lactococcus lactis* KF147 CodY. Finally, under extremely low carbon availability, carbon catabolite repression was progressively relieved and alternative catabolic functions were found to be highly expressed, which was confirmed by enhanced initial acidification rates on various sugars in cells obtained from near-zero growth cultures. The present integrated transcriptome and metabolome study provides molecular understanding of the adaptation of *Lactococcus lactis* to low-growth rate conditions, and expands our earlier analysis of the quantitative physiology of this bacterium at near-zero growth rates towards gene regulations involved in zero-growth adaptation.

Keywords: *Lactococcus lactis*, zero-growth, transcriptome, amino acids, CodY-binding site, alternative carbon scavenging

Introduction

Fundamental knowledge of microbial physiology and cellular regulation is obtained mainly from studies of microorganisms in batch cultures. However, the pace of life and its associated physiological phases in batch cultivation differ strongly from what is found in natural environments (24). During the early phase of batch cultivations, all nutrients, including carbon and energy sources are usually present in excess, and specific growth rate of the microorganism equals the maximum specific growth rate (4). Thereby, our understanding of microbial energy metabolism originates mostly from microbial population studies performed under laboratory conditions that include rapid growth, high metabolic activity, and high cell density. However, natural microbial communities generally live in relative famine conditions with low specific growth and metabolic rates due to severely limited nutrient and energy source availability (27). Analogously, under specific industrial fermentation conditions, microorganisms may experience strongly restricted access to nutrients for longer periods of time. For example, lactic acid bacteria (LAB) experience long periods of extremely low nutrient availability during the maturing process of dry sausage (25) and cheese (41) productions. Despite these harsh conditions, several LAB succeed to survive in these processes during months of maturation and may thereby continue to contribute to flavor and aroma formation in the product matrix (10, 15, 25).

L. lactis is used in food fermentation processes for instance for the production of cheese, sour cream, and other fermented milk products. This bacterium converts available carbon source into lactic acid which results in acidification of the food raw-material. Moreover, *L. lactis* is also commonly encountered in diverse natural environments, especially in decaying plant materials, in which nutrients may become more available (39). The strain used in this study, *L. lactis* KF147, was isolated from mung bean sprouts, and its genome sequence reflects many adaptations to the plant-associated habitat, which are in particular apparent from the repertoire of enzymes and pathways predicted to be involved in utilization of plant cell wall polysaccharides.

To study physiological and genome-wide adaptations of microorganisms to near-zero growth rates, retentostat cultivation or recycling fermenter set-ups have been designed (23). Retentostat cultivation is an adaptation of chemostat cultivation in which growth-limiting

substrate is supplied at a fixed dilution rate, while the complete biomass is retained in the bioreactor by removing the spent medium effluent through an external cross-flow filter. Prolonged retentostat cultivation leads to growth rates that approximate zero while the rate of energy transduction (through substrate consumption and conversion) equals the maintenance energy requirements (*e.g*. osmoregulation, turnover of damaged cellular components) (14, 47). Therefore, retentostat cultivation comprises a gradual transition from a growing to a near-zero growth state under stable environmental conditions and sustaining high cell-viability.

Although retentostat cultivations have been performed to study the fundamental physiology of some microorganisms, including *Escherichia coli* (7), *Bacillus polymyxa* (2), *Paracoccus denitrificans*, *Bacillus licheniformis* (47), *Nitrosomonas europaea* and *Nitrobacter winogradskyi* (43) at low-growth rates, they were not consistently complemented with genome-wide molecular analysis. Exceptions are the retentostat studies performed with *Lactobacillus plantarum* (22), *Aspergillus niger* (26), and *Saccharomyces cerevisiae* (4, 5) that included the analysis of metabolic and transcriptome responses.

Previously, we have described how retentostat cultivation allows decoupling of growth and non-growth-related processes in *L. lactis* KF147, allowing the investigation of the quantitative physiology of *L. lactis* at extremely low-growth rates, where the culture approached a specific growth rate of 0.0001 h^{-1}, corresponding to a doubling time of more than 260 days (14). This study allowed the accurate calculation of maintenance energy requirements and other quantitative physiology parameters of the strain subjected to retentostat cultivation (14). Furthermore, maintenance-related substrate coefficient (m_s) and the biomass yield (Y_{sx}^{max}) pointed out that the amount of carbon used for maintenance-associated processes increased from approximately 30% to 97% during the first 14 days of retentostat cultivation, and increased even further to 99% towards the end of the retentostat cultivation (14). The measurement of substrate cost distribution between maintenance- and growth-associated processes confirmed that the retentostat cultures reached a typical near-zero growth state after 14 days. In addition, the retentostat culture of *L. lactis* showed significant metabolic shifts within central carbon metabolism, switching between mixed-acid and homolactic fermentation behavior after prolonged retentostat cultivation (14).

In the present study, we complement the previous quantitative physiological study with an in-depth molecular level analysis of *L. lactis* KF147 under these retentostat cultivation conditions, including metabolome and transcriptome analyses, to decipher the molecular adaptation underlying the previously reported physiological observations. The genome-wide transcriptome analysis was in good agreement with previously observed oscillation of the culture between lactic and mixed-acid fermentation at extremely low-growth rates. Moreover, integrated metabolome and transcriptome analyses were employed to create a global view of the interconnected carbon- and nitrogen-metabolism under these near-zero growth conditions. Notably, these conditions also elicited a progressive relief of carbon catabolite repression, which prepared the strain to effectively scavenge alternative carbon sources when they become available. The transcriptome adaptations are discussed in the context of regulatory circuits that are proposed in their control, including a possible role for the central carbon- and nitrogen-metabolism control proteins CcpA and CodY, respectively.

Materials and Methods

Bacterial isolates, media and cultivation conditions. *Lactococcus lactis* subsp. *lactis* strain KF147 originates from mung bean sprouts, and its genome sequence was determined (39). Pre-cultures for retentostat cultivations (14) were inoculated in 50 ml M17 broth (44) complemented with 0.5% glucose (w/v) and grown overnight at 30°C. Overnight cultures were harvested by centrifugation (6,000 g, 10 min., 4°C) and washed twice with physiological salt solution (0.9% NaCl in water). Next, the culture was inoculated into chemically defined medium (CDM) containing 0.5% glucose (w/v) for chemostat cultivation. After steady-state had been achieved with six volume changes in chemostat, the fermenters were switched to retentostat mode by withdrawing the effluent through the cross-filter, and pH was controlled at 5.5 with 5 M NaOH in chemostat and retentostat cultivation (14). To keep the medium composition constant during long-term cultivation, 120-liter batches of medium were prepared, filter sterilized, and used during retentostat cultivations (14).

Two independent, carbon source-limited retentostat cultivations were performed under anaerobic conditions, initiating from chemostat cultivation at dilution rates of 0.025 h^{-1} as previously described (14). Retentostat set-up was assembled with a 1.5-l fermentor (Applikon Biotechnology, Schiedam, The Netherlands) and an autoclavable polyethersulfone cross-flow

filter (Spectrum Laboratories, CA, USA). As removal of samples could interrupt biomass accumulation, sample volume and sampling frequency were minimized.

Biomass and amino acids determination. During fermentations, culture samples were withdrawn at regular intervals to measure cell dry weight (CDW) and amino acid concentrations. For CDW determination, 5 ml of culture was passed through pre-weighted membrane filters with a pore size 0.45µm (Merck Millipore, Darmstadt, Germany) using a vacuum filtration unit (Sartorius stedim biotech, Gottingen, Germany). Subsequently, membrane filters were dried at 55°C for 24 hours, and the biomass collected on the membranes was determined in g/ml.

Concentration of amino acids in the culture supernatant and in the medium feed were measured by EZ:fast free amino acid analysis kit (KG0-7165) (Phenomenex, CA, USA). According to the manufacturer's instructions, the analysis was performed using a gas chromatography (GC) (Thermo Scientific, MA, USA) with a flame ionization detector (FID) (Thermo Scientific, MA, USA).

Acidification activity. Acidification profiles were determined in 200 µl PBS buffer (initial pH 6) with 0.5% of a chosen carbon source (glucose, ribose, mannitol, sucrose, fructose, and raffinose) (v/v) at 30°C, inoculated with 5 x 10^8 cfu/ml of stock cultures of retentostat cultures collected at days 14, 21, 35, and 42, using 96-well microplate HydroPlate® HP96U (PreSens, Regensburg, Germany). These plates encompass an optical pH sensor on the bottom of each well, which can be read-out through the bottom of the plate using a fluorescence reader. According to the manufacturer's instructions, pH values of cell suspensions in PBS buffers with the chosen carbon source were measured every 10 minutes for 10 hours with a microplate fluorescence reader (Tecan Safire II, Grödig, Austria). The maximum acidification rate (V_{max}) value that has an arbitrary and pH-based unit (pH Ux$10^{-3}$$min^{-1}$), was calculated on basis of the slope of the pH versus time plot using at least 8 subsequent time-points (regression coefficient > 0.99).

RNA isolation and transcriptome analysis. Total *L. lactis* RNA was isolated from two independent retentostat cultures harvested at days 0, 2, 7, 14, 21, 28, 35, and 42. RNA extraction, reverse transcription, labeling, hybridization, and data analysis were done as

previously described (34). Briefly, following methanol quenching, RNA was phenol-chloroform extracted and purified using the High Pure RNA isolation kit (Roche Diagnostics, Mannheim, Germany). The quality of the obtained RNA was verified with 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) using Agilent RNA 6000 Nano kit (Agilent Technologies, Santa Clara, CA), and samples with a 23S/16S RNA ratio equal to or higher than 1.6 were taken for cDNA synthesis, which was synthesized using the Superscript TMIII reverse transcriptase (RT) enzyme (Invitrogen, Carlsbad, CA). cDNA labeling was performed as previously described (34), using the CyScribe GFX purification kit (GE Healthcare, Buckinghamshire, United Kingdom), and labeled differentially using Cy-3 or Cy-5 labels (Amersham; CyDye postlabeling reactive dye pack; GE Healthcare, Buckinghamshire, United Kingdom). After a second purification with the CyScribe GFX purification kit (GE Healthcare, Buckinghamshire, United Kingdom), *L. lactis* KF1471 cDNA was hybridized to oligonucleotide DNA microarrays for this strain (Agilent Technologies, Santa Clara, CA). The probes for the microarray were designed using OligoWiz (48). Hybridization and scanning procedures were performed as previously described (34). As fluorescence intensities can differ between individual array slides, slide scanning was carried out at several photo multiplier tube values, and the optimal scan of each individual microarray was selected on the basis of signal distribution (combination of a low number of saturated spots and a low number of low signal spots). The data were normalized using the Lowess and inter-slide scaling normalization as available in MicroPrep (46). Median intensities of different probes per gene are given as absolute gene expression intensities. The microarray hybridization scheme for the transcriptome analyses at retentostat cultivations consisted of a compound loop design with 26 arrays (Fig. S8). Microarray data and the experimental procedure have been submitted to the NCBI Gene Expression Omnibus (GEO) under accession number GPL17806 and GSE51494 (http://www.ncbi.nlm.nih.gov), respectively.

The gene expression intensities were compared and clustered using Short Time-series Expression Minor (STEM) (version 1.3.6, http://www.cs.cmu.edu/~jernst/stem/) (16). The STEM clustering algorithm enriched with Gene Ontology (GO) terms was applied with Bonferroni correction method, a maximum number of model profiles of 50, a maximum unit change in model profile between time points of 2. To analyze the glycolysis and BCAA related genes expression results, heat maps of gene expression levels were constructed for the

transcript profiles using the MultiExperiment Viewer (MeV) (http://www.tm4.org/mev/) (37). The correlation of the transcriptome data at each time-point in duplicate retentostat cultivations was calculated by Pearson correlation coefficient and showed in hierarchical clustering linkage using the MeV tool.

DNA motif mining. Gene expression ratio data from the significant model profiles of the STEM clustering during retentostat cultivation was used as a data source to mine for transcription factor binding sites (TFBSs) in the genome of *L. lactis* KF147. The PePPeR database was used as a source of literature based regulon clusters (11). Binding sites searches were performed for sequences within 300 bp upstream of the translational start site of each gene. To identify upstream binding sites the algorithm for fitting a mixture model by expectation maximization (MEME) was performed (3), using the parameters mod anr (unlimited number of motifs per upstream sequence), revcomp (allowing motifs to be present on both "+" and "-" strand), allowing for maximally 3 motifs to be found and without any restriction on the total number of motifs to be found.

Results

Physiology of *L. lactis* KF147 at near-zero growth rates. In our earlier study, metabolic adaptations and physiology of *L. lactis* KF147 at extreme low-growth rates were studied in two parallel, anaerobic, carbon-limited retentostat cultivations (sustained for 42 days) (14). During retentostat cultivation biomass accumulated, reaching a plateau level after approximately 14 days and ultimately growth rates declined from 0.025 h^{-1} (chemostat conditions) to approximately 0.0001 h^{-1} after 42 days of retentostat cultivation (Fig. S1; 14). Despite the imposed extremely low-growth rate, the culture viability was sustained above 90% during prolonged retentostat cultivation (14). To expand these earlier observations, in the current study, the genome-wide transcriptome and targeted metabolome patterns were determined to investigate the molecular adaptations of *L. lactis* KF147 under near-zero growth conditions.

Transcriptome data analysis. To examine genome-level adaptation of *L. lactis* KF147 to near- zero growth conditions, time-resolved transcriptome analysis was performed on the two independent retentostat cultures. Samples were taken before starting the retentostat cultivation regime (t = 0 day; chemostat conditions at D = 0.025 h^{-1}), and 2, 7, 14, 21, 28, 35, and 42 days

after initiating the retentostat regime. Hierarchical clustering and Pearson correlation analysis illustrated that the transcriptome profiles taken from the two replicated fermentations were highly similar for the different sampling time-points (Pearson correlation > 0.92), illustrating that the results obtained are highly reproducible in biologically independent repetitions (Fig. 1). In addition, the cluster analysis separated clearly the first growth-related major cluster, including samples taken on days 0, 2, and 7, from the second major cluster that includes samples taken on days 14, 21, 28, 35, and 42, and reflects the near-zero growth conditions (Fig. 1). These two main clusters underpin the separation of the transcriptome signatures related to growth and near-zero growth associated processes.

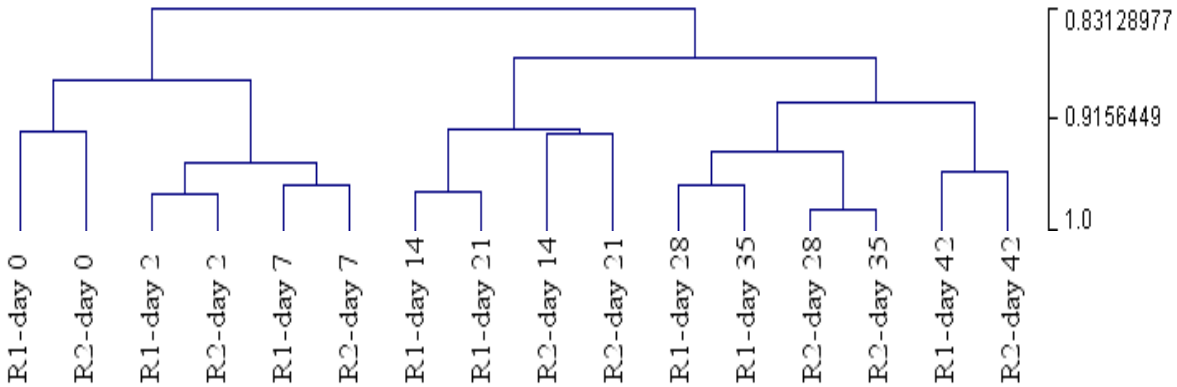

Figure 1: Hierarchical clustering linkage of retentostat 1 (R1) and 2 (R2) samples. Complete clustering linkage was performed for samples days on 0, 2, 7, 14, 21, 28, 35, and 42 of duplicate retentostat cultivations based on Pearson correlation with using MeV.

To identify gene expression patterns during the course of the experiment, absolute expression levels of all genes (2533 genes in *L. lactis* KF147) were subjected to expression cluster analysis using the Short Time-series Expression Minor (STEM) module, which employs an enforced process of statistical clustering of time-series datasets into pre-composed patterns of expression over time (16). STEM analysis with the maximum unit change set to one between each time-point over the first time-point (ratio change of 2 on $\log_2$-scale), divided the expression patterns into 50 time-resolved model expression profiles, which were sorted on basis of the number of genes assigned to the profile. In total 66.9 % and 64.4 % of the annotated *L. lactis* KF147 genes in retentostat 1 and retentostat 2 were clustered by STEM into eight and eleven statistically significant model profiles, respectively (Fig. S2A & S2B). Since the congruency between the transcriptomes obtained in the replicate experiments was

very high, and the highly similar STEM profiles obtained for the duplicate retentostat cultivations, the data presented in this study are exemplary data from retentostat 1, but the congruency of every transcriptome response reported here was confirmed in the dataset obtained from retentostat 2. Among all model profiles, because, profile 7, 8, 40, and 41 display a shape of expression that coincides with the metabolic shifts of carbon metabolism that was previously observed, we focused on genes in these model profile 7, 8, 40, and 41 that were specifically up- or down- regulated in both retentostat cultures (Table 1 and Fig. S3). Among these, model profile 8 encompassed the highest number of statistically significant (*p*-value ≤ 0.05) gene ontology (GO) terms, such as "cellular amino acid metabolic process", "fatty acid metabolic process", "organic acid metabolic process", "lipid metabolic process", etc., in retentostat cultivation 1 and 2 (Table 1).

Table 1: Gene Ontology (GO) enrichment analysis. This table contains the GO enrichment results for the set of genes shown in the figure S2A and S2B where the enrichment is computed based on actual size enrichment (*p*-value ≤ 0.05).

Model Profile #	**GO Category Name**
7	Membrane Integral to membrane Membrane part
8	Cellular amino acid metabolic process Fatty acid metabolic process Organic acid metabolic process Carboxylic acid biosynthetic process Lipid metabolic process Primary metabolic process Glutamine family amino acid metabolic process Branched chain family amino acid metabolic process Arginine metabolic process
40	Sequence-specific DNA binding transcription factor activity RNA biosynthetic process Regulation of RNA metabolic process Regulation of cellular biosynthetic process Regulation of gene expression Regulation of macromolecule metabolic process
41	Regulation of transcription, DNA-dependent Carbohydrate kinase activity Regulation of RNA biosynthetic process Regulation of biological process

Matching transcriptional patterns of central carbon metabolism during retentostat cultivation. STEM-Model profile 8, encompasses genes that were first low-expressed during the growth phase, then remained stable, and finally were highly-expressed during the near-zero growth phase (Fig. S3). Thereby the genes clustering in this model profile appeared to follow the timing of the metabolic fluctuations observed during the fermentation (14), which is in good agreement with the most striking overrepresented GO terms in this profile 8 being "organic acid metabolic process" and "carboxylic acid biosynthesis process". To investigate and visualize the expression levels of the genes associated with the "organic acid metabolic process", a heat map representation of the expression of central carbon metabolism associated genes in *L. lactis* KF147 was generated by using the MeV at chemostat culture (day 0) and during the retentostat cultivation (Fig. 2). Pyruvate dissipation associated genes displayed a remarkably consistent time-dependent transcription profile during retentostat cultivation. At days 0, 2, and 7, the lactate dehydrogenase encoding *ldhL* gene (involved in lactic acid production) had low level expression, whereas the genes encoding pyruvate dehydrogenase (*pdhABCD*), alcohol dehydrogenase (*adhAE*), pyruvate formate lyase (*pflA*), phosphotransacetylase (*eutD*), and acetate kinase (*ackA1A2*) involved in mixed-acid fermentation (production of ethanol, formic acid, and acetate) had high level expression (Fig. 2B). After 7 days, the transcript level of *ldhL* gene started to increase until day 28, while the transcript levels of *pdhABCD, adhAE*, *pflA*, *eutD*, and *ackA1A2* genes decreased until day 35. At the end of the retentostat cultivation, *ldhL* gene had again low level expression, whereas the mixed-acid production associated genes had again high level expression (Fig. 2B). These findings clearly support our earlier conclusion in the study of zero-growth physiology of *L. lactis* (14) and indicate that the previously observed metabolic fluctuations are reflected by the transcriptome profiles of genes involved in pyruvate dissipation during the retentostat cultivation, and are accurately corresponding with the timing of the switch between mixed-acid and homolactic fermentation.

Unlike the regulation of pyruvate-dissipation related genes, glycolysis pathway related genes (*glk*, *pgi*, *pfk*, *fbaA*, *tpiA*, *gapAB*, *pgk*, *pmg*, *enoAB*, and *pyk*) were inconsistently regulated in the first week of retentostat cultivation, where all glycolytic genes were repressed or did not change (*i.e.* < 2-fold change), except the *glk*, *pfk*, and *fbaA* genes (Fig. 2B). The regulation of these genes was expected as glucose depletion has been reported to induce repression of the

gene expression of central carbon metabolism associated genes (17). However, at days 14, 21, and 28 of retentostat culture, genes *glk*, *pgi*, *pfk*, *fbaA*, *tpiA*, *gapAB*, *pgk*, *pmg*, *enoAB*, and *pyk* were consistently highly expressed, whereas they were subsequently lower expressed during the last two weeks of the cultivation, except the *gapA*, *pmg*, and *enoA* genes (Fig. 2B).

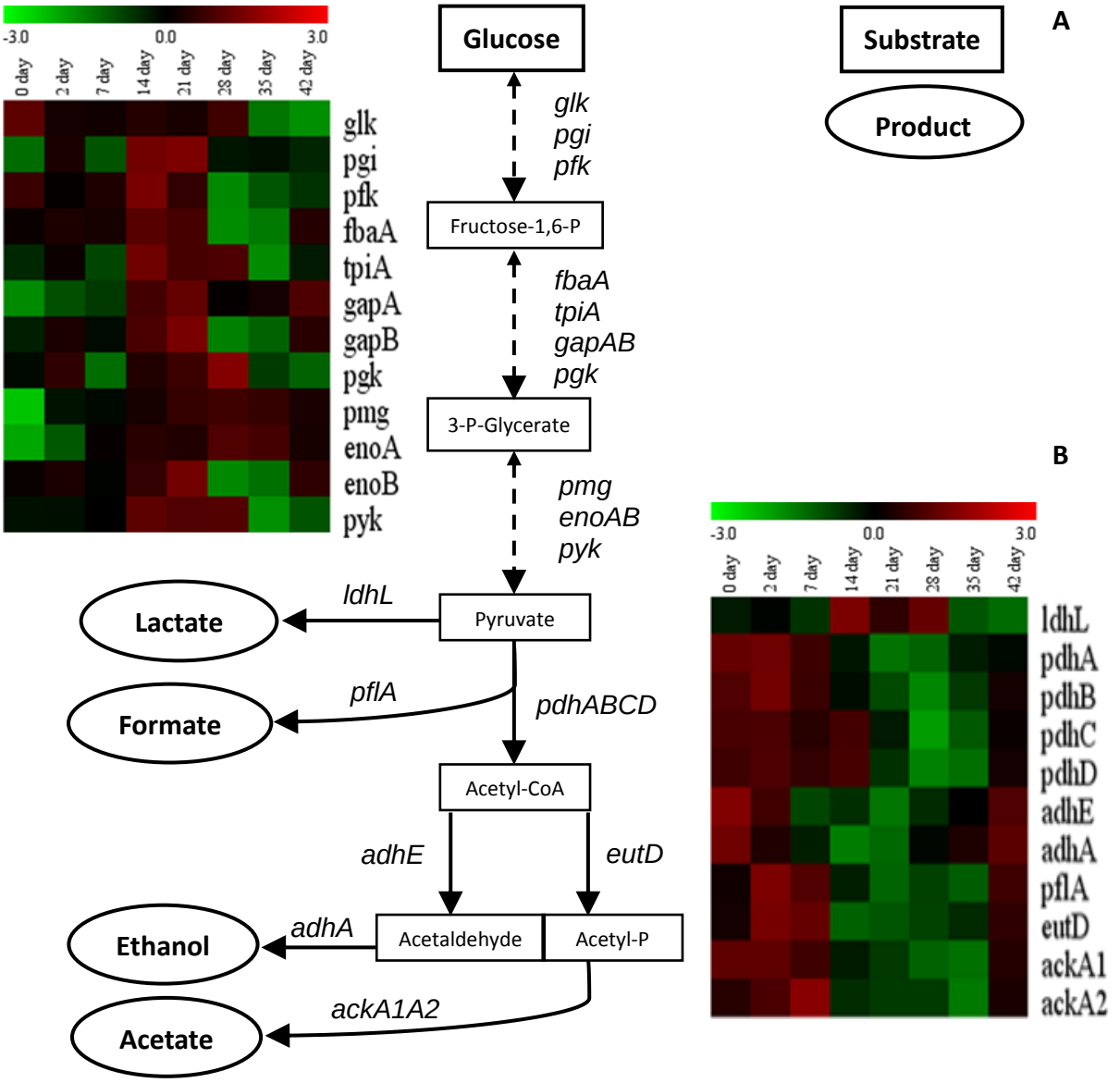

Figure 2: Overview of central carbon metabolism in *L. lactis* KF147. (A) Simple scheme of glycolysis and pyruvate dissipation pathways. One arrow represents one metabolic reaction and dashed line arrows correspond to more than one reaction. Genes are indicated beside the arrows. End-products are indicated in ellipses. (B) Heat map of *L. lactis* KF147 glycolysis and pyruvate dissipation genes differentially expressed (on $\log_2$-scale, *p*-value ≤ 0.05) during retentostat cultivation over the beginning of the chemostat (day 0) (retentostat 1). Similar transcriptome results obtained in retentostat 2 confirmed the consistency of these results in an independent experiment.

Consequently, the progressive reduction of the growth rate (Fig. S1; 14) appears to be paralleled by reduced glycolytic gene expression, although at the time-points that corresponded with increasing lactic acid production, the glycolytic gene expression was again transiently raised. Thereby, it seems that under these conditions, the expression pattern of the glycolytic genes appears to follow the previously observed metabolic fluctuations between mixed-acid and homolactic acid fermentation (14) rather than actual growth rates.

The increased expression of glycolytic genes might lead to increased glycolytic flux and pyruvate production rates in *L. lactis* KF147 at days 14, 21, and 28, which might have elicited the metabolic shift towards homolactic fermentation behavior. Intriguingly, the genes *gapA*, *pmg*, and *enoA* that are involved in production of glycolytic intermediates like 3-phosphoglycerate, phosphoenolpyruvate (PEP), and pyruvate, were constinuously expressed at elevated levels after 14 days of retentostat cultivation. The metabolites associated with these enzymes (substrates and products), also happen to be substrates for the inter-conversion to amino acids biosynthesis, and thereby appear to be consistent with the observed amino acid production at certain stages of the fermentation (see below).

Amino acid profiles and transcriptional regulations of amino acid metabolism pathways during retentostat cultivation. Amino acid concentrations were determined (Fig. 3 & S4), at each time-point after initiation of retentostat growth (day 0). The concentrations of branched chain amino acids (BCAAs) valine, leucine, and isoleucine remained almost constant during the first week of the retentostat cultivation. However, after the first week, the BCAA concentrations gradually decreased during days 21 and 28 of the retentostat cultivation, reaching a minimum of 150 ± 100 µM (Val), 510 ±160 µM (Leu), and 305 ± 105 µM (Ile) (Fig. 3A). Interestingly, prolonged retentostat cultivation beyond 28 days led to a slow increase again of the BCAA concentration to 385 ± 25 µM (Val), 710 ± 90 µM (Leu), and 360 ± 40 µM (Ile) at the end of the cultivation. Analogously, the concentration of the aromatic amino acids (AAAs) phenylalanine, tyrosine, and tryptophan in the fermenter effluent appeared to display similar patterns of concentrations as compared to the BCAAs (Fig. 3B).

The GO terms “cellular amino acid metabolic process” and “branched chain family amino acid metabolic process” were also overrepresented in transcriptome model profile 8 in both retentostat cultivations (Table 1), and thereby also adequately reflected the pattern of amino

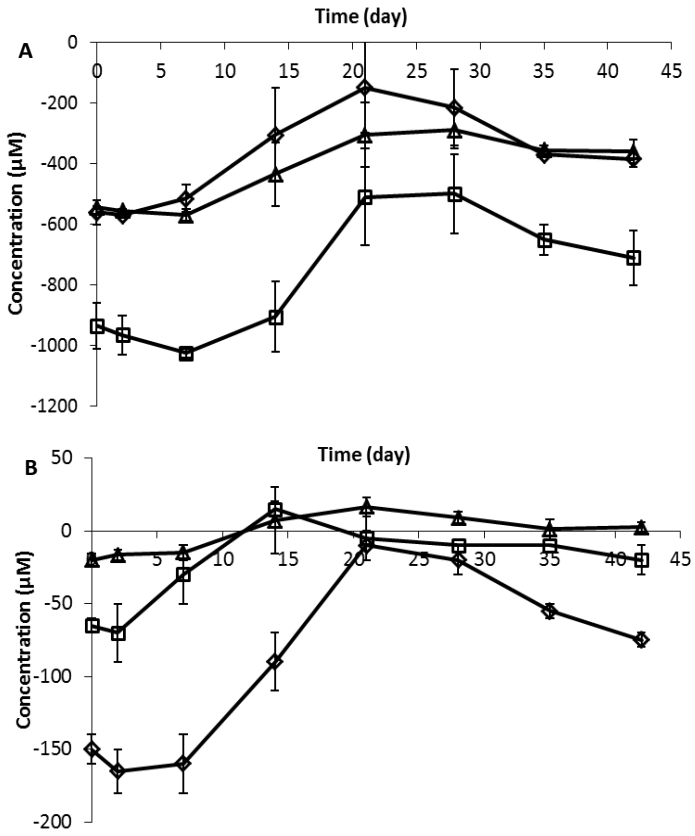

Figure 3: Concentration of branched chain amino acids (BCAAs) (A) and aromatic amino acids (AAAs) (B) in *L. lactis* KF147 in retentostat culture. Data points represent average ± mean deviation of measurements of two independent cultures. All concentrations in panels A and B are expressed as the difference between the measured concentration in the medium feed and the measured concentration in the filter line samples. Negative numbers indicate net-consumption; positive numbers indicate net-production.
(A) Concentration of Val (diamonds), Leu (squares), and Ile (triangles).
(B) Concentration of Phe (diamonds), Tyr (squares), and Try (triangles).

acid consumption/production during retentostat cultivation. To examine the transcript levels of BCAAs biosynthesis related genes in *L. lactis* KF147 during retentostat cultivation in more detail, a heat map representation of the significantly assigned genes in branched chain family amino acid metabolic process term was constructed by using the MeV tool (Fig. 4). The genes *ilvABCDH*, *leuABCD*, *als*, *bcaT*, and *aspC* encoding enzymes responsible for BCAAs biosynthesis depicted similar transcription patterns during retentosat cultivation. These genes fluctuated between up- and down-expression in the first week of the retentostat cultivation, whereas their expression levels were consistently decreased after 7 days, followed again by increased expression after 35 days (Fig. 4B). Moreover, genes related to the synthesis of tryptophan (*trpCDFGS*) and histidine (*hisABDFGHIKZ*) were also significantly clustered in model profile 8 (Fig. 5A & 5B). The expression pattern of the *trpCDFGS* genes displayed strong similarity with those involved in BCAA synthesis (Fig. 5A). Overall, these findings illustrate a relatively good match between the gene expression patterns and the consumption/production of specific amino acids.

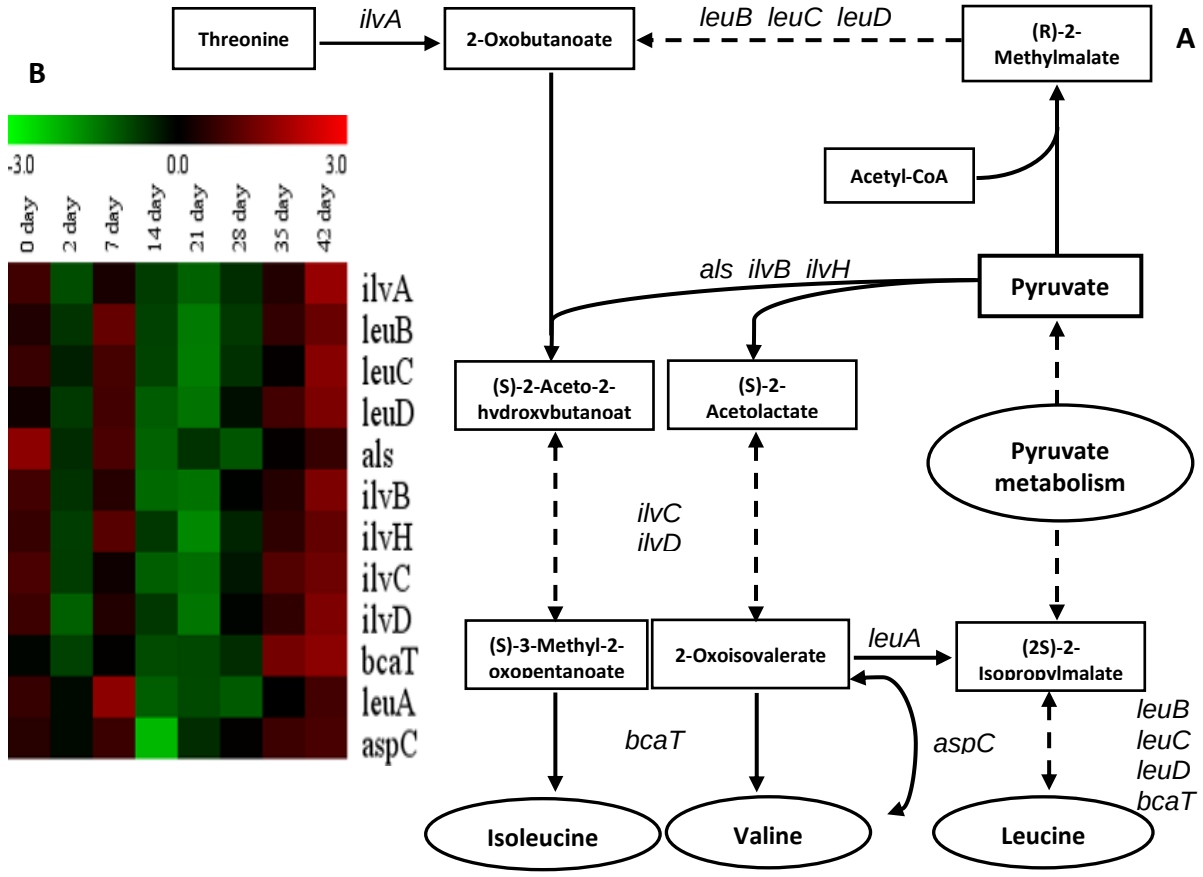

Figure 4: Overview of branched chain amino acids (BCAAs) biosynthesis in *L. lactis* KF147. (A) Simple scheme Ile, Val, and Leu production pathways. One arrow represents one metabolic reaction and dashed line arrows correspond to more than one reaction. Genes are indicated beside the arrows. End-products are indicated in ellipses. (B) Heat map of *L. lactis* KF147 BCAAs biosynthesis genes differentially expressed (on $\log_2$-scale, p-value ≤ 0.05) during retentostat cultivation over the beginning of the chemostat (retentostat 1). Similar transcriptome results obtained in retentostat 2 confirmed the consistency of results in an independent experiment.

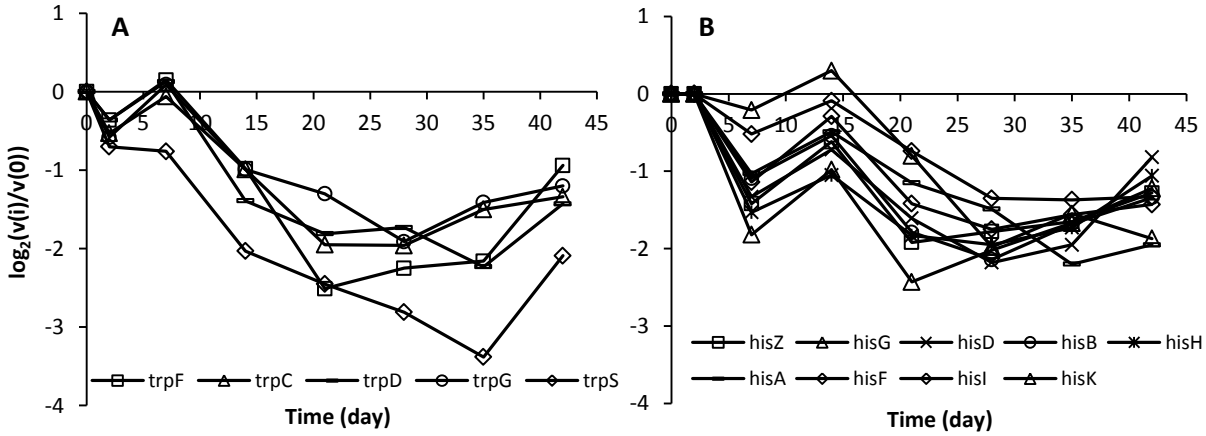

Figure 5: Expression graphs of genes involved in (A) Try (AAA) and (B) His metabolism in *L. lactis* KF147 during retentostat cultivation over the beginning of the chemostat in model profile 8 (retentostat 1). Similar transcriptome results obtained in retentostat 2 confirmed the consistency of results in an independent experiment.

Although the effluent concentration pattern over time of aspartic acid, glutamic acid, lysine, threonine, serine, proline, and cysteine (concentration fluctuated between -29 and +37 µM) looked similar to the concentration profiles of BCAAs and AAAs during retentostat cultivation, these amino acids were produced by *L. lactis* KF147 at days 14, 21, and 28 (Fig. S4A & S4B), reaching a maximum during day 21. At the time-points of net production (days 14 and 21) of glutamic acid, cysteine, and threonine, the genes encoding glutamate synthase, a glycerol-3-phosphate transporter, and a glutamate ABC transporter (*gltBDPS*), as well as cysteine synthase (*cysK*), and homoserine kinase and threonine synthase (*thrBC*) were lowly expressed while these genes were highly (days 0, 2, and 7) or at least somewhat higher (days 28, 35, and 42) expressed at the time-points for which net-uptake of these amino acids was observed (Fig. S5). Moreover, while the gene encoding the lysine specific permease (*lysQ*) displayed a similar pattern of expression as *gltBDPS*, *cysK*, and *thrBC* genes, the diaminopimelate decarboxylase encoding *lysA* gene displayed an opposite profile of expression (Fig. S5). Consequently, the production of glutamic acid, cysteine, threonine, lysine during the period that the *L. lactis* KF147 retentostat culture displayed a homolactic fermentation pattern could be controlled by the *gltBDPS*, *cysK*, *thrBC*, *lysAQ* genes, respectively.

In contrast, other amino acids, like histidine, ornithine, and methionine appeared to be consistently produced by the strain during retentostat cultivation (Fig. S4C). The concentration of histidine, ornithine, and methionine in the spent medium appeared to be more or less constant during the first 7 days of retentostat cultivation, but subsequently gradually increased to reach a maximum concentration of 201 ± 15 µM, 618 ±10 µM, and 130 ± 10 µM, respectively, after 21 days (Fig. S4C). After 28 days of retentostat cultivation, the concentrations of histidine, ornithine, and methionine decreased gradually towards 107 ± 59 µM, 498 ± 20 µM, and 45 ± 28 µM, respectively, at the end of the retentostat cultivation.

Comprehensive, time-resolved transcriptome mining of *L. lactis* KF147 during retentostat cultivation. Model profile 7 clustered genes that were expressed at continuously declining levels during retentostat cultivation (Fig. S3). In this profile, the most strongly overrepresented functional categories were membrane associated function-clusters such as “membrane”, “integral to membrane”, and “membrane part” (Table 1). These clusters

included many ATP-binding cassette (ABC) transporter encoding genes (*cbiQ*, *choS*, *feuB*, *malF*, *malG*, *optBCDF*, *phnC*, *potBC*, *rgpC*, *ychE*, *ydcBC*, *ylbB*, *yngFG*, *ypcH*, *ypdA*, *ysfF*), and phosphotransferase systems (PTS) encoding genes (*e.g*. including, *bglP*, *celB*, *fptBC*, *yidB*, *yedEF*). Low level expression of these genes appears to reflect the responses of the strain to the strongly limited carbon and energy sources during retentostat cultivation. In addition, genes encoding enzymes involved in exopolysaccharide (EPS) synthesis (*epsABCDEFGHIJ* and *ycbABCDFHIJK*) were also assigned to model profile 7 (Fig. S6A & S6B). This observation is in agreement with the previously proposed notion that LAB produce and secrete EPSs into their environment only during growth (30), and therefore could be expected to be lower expressed in *L. lactis* KF147 during prolonged retentostat cultivation that coincides with a near-zero growth state.

Model profile 8, encompassed also some genes that are assigned to the GO term "fatty acid metabolic process", in particular the fatty acid biosynthesis associated *fabDFGHZ* and *accABCD* genes, which were expressed at low level during the first three weeks of retentostat cultivation reaching a minimum value after 21 days. Subsequently, their expression level remained very low during prolonged retentostat cultivation (Fig. S6C). This observation indicates that *L. lactis* KF147 adapted to near-zero growth by repression of genes related to fatty acid production, thus down regulating the synthesis of one of the main building-blocks of the cell membrane.

Model profile 40, contains genes that were initially increasing in terms of level of expression during the stages of cultivation associated with continued growth and remained stably expressed during the transition towards the near-zero growth stage of the retentostat cultivation (Fig. S3). In this model profile, significantly enriched functional categories related to "sequence-specific DNA binding transcription factor activity", "RNA biosynthetic process", "regulation of cellular biosynthetic process", etc. (Table 1). The pattern of the expression profile clustered in model profile 41 resembles that of model cluster 40 and contains genes of which the expression levels consistently increased during retentostat cultivation (Fig. S3) and encompasses the overrepresentation of functional categories similar to those found in model profile 40 (*i.e*. regulation of transcription, carbohydrate kinase activity, regulation of RNA process, and regulation of biological process; see Table 1). These

findings indicate that the retentostat cultivation sustained relatively high expression levels of the genes related to RNA biosynthesis, DNA replication, transcription and translation.

Identification of a potential *cis*-acting DNA-motif involved in retentostat gene regulation. Transcriptional regulators strongly control expression levels of genes by binding to TFBSs. To identify candidate DNA-motifs that are potential TFBSs involved in adaptation to near-zero growth conditions, we searched for overrepresented DNA sequences in the upstream regions of genes that showed correlated expression. As a result, a highly conserved motif, encompassing the palindromic sequence element 5'-CTGWCAG-3' (Fig. 6) was identified in profile 7 and 8, in which the GO terms "cellular amino acid metabolic process", "branched chain family amino acid metabolic process", and "fatty acid metabolic process" were overrepresented. The motif is present upstream of genes related to synthesis of BCAAs (*ilvAB*, *ileS*, *leuC*), histidine (*hisB*), cysteine (*cysK*), arginine (*argBF*, *arcD*), serine (*serS*), tryptophan (*trpG*), and fatty acid (*accD*, *fabZ*); and peptide uptake (*dtpT*, *pepC*) (Fig. S9). This observation implies that the identified motif could play a key role in the regulation of nitrogen metabolism in *L. lactis* KF147, and is proposed to be an important *cis*-acting element in the adaptation to near-zero growth conditions.

Figure 6: Weblogo visualization of the postulated CodY upstream binding sequence found in front of nitrogen metabolism related genes. The CTGTCAG palindrome sequence that forms the core of the motif is positioned from nucleotide 2 to 8. The thymidine at position 5 appears to be conserved as well.

Genome level prediction of enhanced catabolic flexibility of *L. lactis* KF 147. Model profiles 40 and 41 also encompassed many genes involved in uptake and metabolism of alternative carbon sources such as ribose, mannitol, galacturonate, raffinose, sucrose, and fructose that were expressed at elevated levels upon prolonged cultivation linked to conditions of severely restricted carbon source availability (Fig. 7 & S7). The transcript levels of genes encoding proteins for uptake and metabolism of ribose (*rbsABCDKR*) and mannitol

(*mtlADFR*) fluctuated between slightly elevated and low expression (0- and 4-fold) during the first 14 days of retentostat cultivation (Fig. 7). However, upon prolonged retentostat cultivation, their transcript levels were strongly induced after 35 days, and remained highly expressed after 42 days (Fig. 7A & 7B). Similar gene expression levels were obtained from

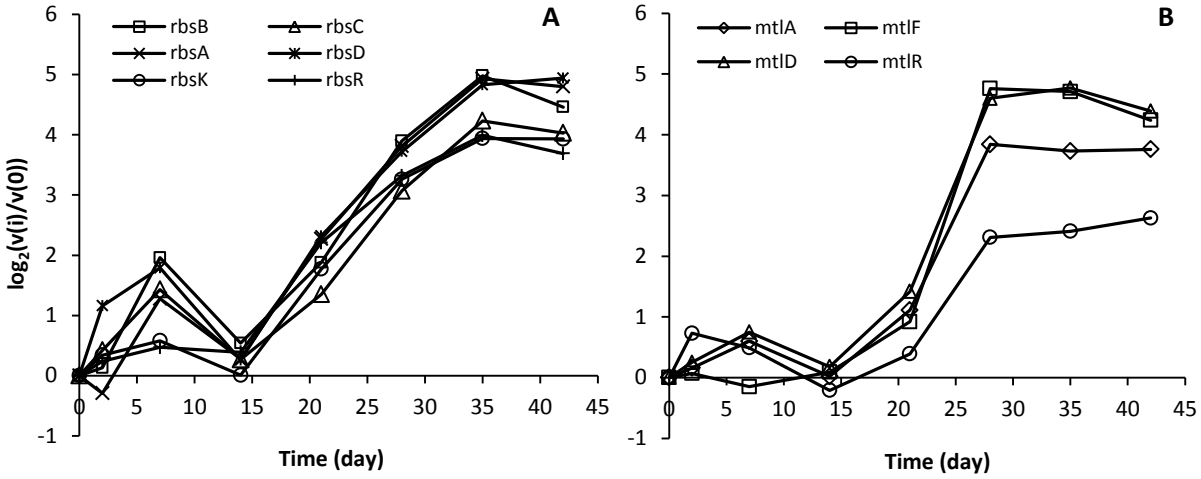

Figure 7: Expression graphs of genes involved in (A) ribose and (B) mannitol uptake and metabolism during retentostat cultivation over the beginning of the chemostat (day 0) from model profiles 40 and 41 (retentostat 1). Similar transcriptome results obtained in retentostat 2 confirmed the consistency of these results in an independent experiment.

genes encoding proteins involved in uptake and catabolism of galacturonate, raffinose, sucrose, and fructose (Fig. S7). These observations illustrate specific transcriptional responses to extremely low carbon source availability during prolonged retentostat cultivation, leading to the derepression, and /or activation of expression of a number of genes required for the catabolism of alternative carbon sources in *L. lactis* KF147. These responses raised two questions: i) whether *L. lactis* KF147 culture samples extracted from prolonged retentostat cultures display significantly enhanced catabolic flexibility and ii) whether end-time point retentostat culture samples would show faster-fermentation of alternative carbon sources as compared to samples taken from earlier time-points of the retentostat culture. To address these questions, the acidification rates on alternative sugars by non-growing bacterial suspensions of *L. lactis* KF147 withdrawn from the retentostat culture on days 14, 21, 35, and 42 were determined.

To this end, the maximum acidification rate (V_{max}) was determined in triplicate *L. lactis* KF147 cell-suspensions derived from the retentostat culture on days 14, 21, 35, and 42 by incubation under anaerobic conditions in phosphate-buffered saline (PBS) with glucose, ribose, mannitol, sucrose, fructose, or raffinose as the fermentable substrate. Acidification rates were monitored (see Materials and Methods), and V_{max} values of the different carbon sources were calculated using an arbitrary, pH-based unit (pH $Ux10^{-3}min^{-1}$) (Table 2). The highest V_{max} values were obtained from the sample day 42 for all carbon sources. Specifically, V_{max} of the cell suspension generated from the retentostat culture on day 42 was several folds higher than the V_{max} of the suspension based on day 14 cultures for the carbon sources ribose (3-fold), mannitol (5-fold), sucrose (2-fold), and raffinose (3-fold), respectively (Table 2). Only the acidification rates obtained for fructose and glucose were similar between all retentostat derived suspensions tested (days 14, 21, 35, and 42), where glucose functions as the positive control and was consistently associated with the highest acidification rate for all of the suspensions tested (Table 2).

These results are in good agreement with the transcriptome data and confirm that the induced transcription of genes involved in alternative sugar utilization pathways enables the cells to rapidly adjust to the utilization of alternative carbon sources when these would become available. Notably, the enhanced derepression of metabolic pathways dedicated to the use of alternative carbon and energy sources clearly exceeds the basal levels of expression that can be seen upon initial carbon limitation conditions, *e.g.* during carbon-limited chemostat cultivation.

Table 2: Maximum acidification rates on different carbon source of *L. lactis* KF147 samples taken on days 14, 21, 35, or 42 of the retentostat cultivation period.

Carbon sources	Vmax (pH $Ux10^{-3}min^{-1}$)*			
	14	**21**	**35**	**42**
Glucose	3.9 ± 0.34	2.7 ± 0.69	3.7 ± 0.41	5.6 ± 0.43
Ribose	0.2 ± 0.05	0.1 ± 0.06	0.5 ± 0.05	0.6 ± 0.06
Mannitol	0.2 ± 0.03	0.2 ± 0.04	0.7 ± 0.03	1.0 ± 0.02
Sucrose	0.5 ± 0.02	0.5 ± 0.01	1.1 ± 0.04	1.1 ± 0.03
Fructose	2.3 ± 0.05	1.8 ± 0.06	2.3 ± 0.04	2.5 ± 0.05
Raffinose	0.3 ± 0.01	0.3 ± 0.01	0.6 ± 0.02	0.9 ± 0.02

* R^2, regression coefficient > 0.99

Discussion

This paper presents the molecular adaptation of *L. lactis* to near-zero growth rates induced by carbon-limited retentostat cultivation to expand our previous analysis of quantitative physiology and metabolic behavior of this bacterium (14). Genome-wide transcriptional data were integrated in a global physiology study that included metabolite datasets of organic- and amino-acid production and consumption under retentostat conditions. Furthermore, the transcriptome data allowed identification of transcription signatures that reflect the near-zero growth conditions within the extended retentostat cultivation. These included the confirmation of the repression of several growth associated functions that predominantly appear to be related to macromolecule biosynthesis, including membrane components as well as extracellular polysaccharides. Notably the retentostat conditions did not induce responses analogous to the stringency response and as a consequence, the genes encoding the components of the machineries for DNA replication, transcription and translation remain relatively highly expressed.

Previously reported observations (14) highlighted that *L. lactis* KF147 retentostat cultures display intriguing metabolic switches within their central carbohydrate and energy metabolism, fluctuating between mixed-acid and homolactic fermentation. Notably, in the present study we show that the fermentation end-product analyses (14) are congruent with the transcriptional patterns of the genes involved in the cognate pathways, implying that the distribution of pyruvate among the different dissipation pathways available in *L. lactis* KF147, is controlled at the transcriptional level rather than through alternative allosteric or redox-balance driven control (9, 21). Intriguingly, Carbon-starved batch cultures of *L. lactis* showed that genes encoding enzymes involved in carbon metabolism were strongly suppressed in the stationary phase while glycolytic and pyruvate dissipation functions were continued to be expressed at high levels (36). In the current experiments, genes encoding glycolytic enzymes were expressed at elevated levels at days 14, 21, and 28 when cells were approaching the near-zero growth stage. These transcriptional adaptations are proposed to lead to enhanced glycolytic flux and also appeared to lead towards a change of pyruvate dissipation towards increased lactic fermentation rather than the mixed-acid fermentation seen in slowly growing cells. The transcriptional control of these pathways is in apparent contradiction with the proposed regulation of glycolysis and pyruvate metabolism, which has

been postulated to be predominantly controlled by enzyme level allosteric control by glycolytic intermediates, ATP demand and carbon source uptake (8, 21, 36, 45). Many glycolytic enzymes have been assigned key roles in the regulation of glycolytic flux in *L. lactis*, on the basis of the strain and nutritional condition, for instance pyruvate kinase in *L. lactis* subsp. *cremoris* MG1363 during batch cultivation on maltose (42) or glyceraldehyde-3-phosphate dehydrogenase in *L. lactis* NCDO 2118 during batch cultivation on lactose (18) or phosphofructokinase in *L. lactis* subsp. *cremoris* MG1363 (1). Although the regulation of glycolytic flux in *L. lactis* is not completely understood, it has been shown that its flux is not controlled by an individual and rate-limiting glycolytic enzyme nor dictated by sugar transport across the membrane (33), although glycolytic flux may be controlled by sugar transport rates under conditions of low carbon flux (8). Several studies have evaluated the control of glycolytic flux by the cellular energy state by modulating the expression level of the F_1F_0-H^+-ATPase in *L. lactis* leading decreasing ATP levels that led to decreasing growth rate of the culture (28). These cultures displayed a 3-fold enhanced glycolytic flux in non-growing cells, but yielded reduced biomass, illustrating that glycolytic flux can be uncoupled from biomass formation (29). Mathematical modeling demonstrated that glycolytic flux can be predicted on basis of the kinetic parameters of the enzymes that constitute the pathway, although the kinetic parameters are commonly obtained from *in vitro* experiments and therefore could neglect many potentially relevant interactions and modifications, including transcriptional regulation, protein phosphorylation, or allosteric modulation (33). The present study indicates that transcriptional regulation contributes to glycolytic flux and pyruvate dissipation control in *L. lactis* KF147. This could be specific for lactococci isolated from plant origins, like KF147, which would be supported by the many studies that address metabolic control in dairy *L. lactis* strains that reach alternative but not necessarily consistent control-conclusions. Alternatively, this could also be specific for the conditions employed in this study, and thus depend on the specific metabolic characteristics induced by near-zero growth conditions.

The response of *L. lactis* KF147 to extreme low-growth rates also included some remarkable fluctuations of amino acid metabolism. As anticipated, during the period of retentostat cultivation that is associated with the continuous reduction of the growth rate, *i.e.* during the first 14 days, the overall consumption of amino acids appeared to gradually decline. This is

most likely a result of the reduced requirement for these biomass building-blocks as a consequence of the declining growth rate, which is in agreement with the observation that in batch cultures the amino acid consumption rates reduce during the growth-deceleration phase and approach zero in the stationary phase (36). Intriguingly, the transcription level of the genes encoding the glycolytic enzymes glyceraldehyde phosphate dehydrogenase, phosphoglycerate mutase, and enolase which are involved in the production of the intermediates 3-phosphoglycerate, phosphoenolpyruvate (PEP), and pyruvate, were continuously overexpressed after 14 days of retentostat cultivation. These glycolytic intermediates are known to serve as substrates in reactions that link the central carbon metabolism to amino acid synthesis, and are involved in the Ser, Cys, and Gly; Phe, Trp, and Tyr; Ala, Ile, Leu, Val, and Thr pathways, respectively (20, 35). The suggested role of these intermediates is in agreement with the observation that retentostat cultivation appeared to induce the production of certain amino acids at the stages of cultivation that coincided with the elevated expression level of these glycolytic genes (days 14-28).

In several low-GC Gram-positive bacteria, including *L. lactis*, the transcriptional regulatory protein CodY controls the expression of degradation of oligopeptides, uptake and metabolism of di/tripeptides and amino acids, especially BCAAs, Asn, Glu, His, and Arg, in response to the availability of amino acids or peptides (6, 12). When lactococcal cultures reach the stationary phase, and nutrients become limited, CodY-dependent repression of peptide and amino acid transporter systems is relieved to maintain nitrogen metabolism in the cells (12). Moreover, den Hengst and co-workers reported that *L. lactis* MG1363 CodY might have a role in regulation of not only nitrogen metabolism, but also carbon metabolism since the expression of citrate synthase (*gltA*), isocitrate dehydrogenase (*icd*), and aconitase (*citB*) in the incomplete Krebs oxidative cycle was derepressed in a *codY* deficient derivative of *L. lactis* MG1363 (12). Notably, the incomplete Krebs cycle present in *L. lactis* can support the production of α-ketoglutarate, which can be used for the formation of glutamate and as a substrate for the first step of BCAA catabolism (12, 51). Consequently, α-ketoglutarate creates a connection between BCAA and glutamate metabolism and may explain how CodY regulation connects nitrogen- to carbon-metabolism (12). Similarly, under near-zero growth conditions that include severe carbon source limitation, nitrogen metabolism in *L. lactis* could be predominantly regulated by CodY, which could include control of the connection to central

carbon metabolism via the glycolytic intermediates 3-phosphoglycerate, PEP, and pyruvate (see above) in *L. lactis* KF147. Furthermore, the biosynthesis pathway of BCAAs is connected to pyruvate metabolism (Fig. 4A), implying that CodY might indirectly regulate pyruvate dissipation and could play a role in attenuating homolactic versus mixed-acid fermentation behavior in *L. lactis* KF147 under retentostat conditions. In *L. lactis* MG1363, a 15-bp *cis*-element with the consensus AATTTTCWGAAAATT has been identified as a high affinity binding site for CodY (12). The DNA binding motif that we identified in *L. lactis* KF147 resembles this CodY motif identified in *L. lactis* MG1363 (12). Although the consensus sequence of both motifs is not identical, most of the residues appear to be conserved. In addition, the motif identified in KF147 contains a palindromic element and is frequently present in duplicate in a head-to-tail orientation, which expands the palindromic nature of the composite *cis*-acting element. Based on these findings we propose that the motif identified in this study represents the high affinity binding site for CodY in *L. lactis* KF147, and that CodY regulation plays a prominent role in nitrogen metabolism adaptation in *L. lactis* KF147 at extremely low-growth rates, and may indirectly also control the typical carbon-metabolism fluctuations observed under these conditions. The relevance of nitrogen metabolism regulation in the context of industrial applications of *L. lactis* is obvious since it has been well established that flavor formation for example in cheese ripening is driven largely by amino acid conversions that involve nitrogen metabolism associated enzymes (40).

The transcription signatures obtained in this study also established that genes associated with pathways for import and utilization of alternative carbon sources (instead of glucose) were progressively increased in expression by prolonged retentostat-growth. Moreover, it could be shown that these transcriptional adaptations enabled the bacteria to more rapidly switch to alternative energy and carbon resources upon their availability in the environment. This adaptation may reflect the evolutionary advantage for bacteria to be able to scavenge trace amounts of utilizable energy sources in nutrient-limited environments. These observations go beyond previous findings that indicate that a relief of global carbon catabolite repression (CCR) is advantageous in environments with low concentrations of energy and carbon sources (13, 19, 31, 38, 49) since such CCR relieving conditions are already present in the carbon-limited chemostat. Bacteria employ CCR controlled expression of genes involved in the utilization of carbon sources to maximize their efficiency for growth, which involves

specific regulation of gene expression by the availability of 'preferred' carbon sources (19). In low-GC Gram-positive bacteria, including LAB, global CCR is achieved through transcriptional control of catabolic operons by global regulators, such as catabolite control protein A (CcpA), in response to the availability of preferred carbon sources, whereas the intracellular availability of specific carbohydrate-derived inducers can activate the expression of the corresponding catabolic pathways, thereby adding a more specific level of gene expression control (19). Intriguingly, in *L. lactis* MG1363, CcpA was found to activate the *las* operon that encodes three key-enzymes of the glycolysis pathway, phosphofructokinase (*pfk*), pyruvate kinase (*pyk*) and lactate dehydrogenase (*ldh*), thereby, creating an additional connection to central metabolism control (32, 42). Near-zero growth conditions were shown to elicit high level induction of alternative carbon source import and utilization systems. The role of CcpA in the near-zero-growth associated derepression of gene expression, or the possible involvement of possibly additional regulators (*e.g.* HPr, ATP/ADP levels etc.), remains to be determined.

In conclusion, transcriptomic and metabolic adaptations of *L. lactis* KF147 to near-zero growth rates implemented by using retentostat cultivation is different from that of starvation-induced or stationary phase cultures. Metabolic behavior (*i.e.* the glycolytic flux control and the interaction between central metabolism and amino acid pathways) appears to be fluctuating in retentostat cultures. The regulation of nitrogen metabolism, and possibly in an indirect manner also the fluctuations in lactic- and mixed-acid fermentation patterns, might be regulated by CodY, for which we identify its candidate *cis*-acting motif that resembles the previously reported CodY-box. Moreover, the retentostat conditions triggered the progressive relief of carbon catabolite repression and the activation of pathways associated with the utilization of alternative substrates, which appears to go beyond the canonical CcpA mediated carbon-catabolite regulation and illustrates a remarkable form of "anticipation" of these bacterial cultures. Intriguingly, recent work in *Bacillus subtilis* established that CcpA and CodY can form a complex that interacts with RpoA, which clearly underpins the interactions between the gene-regulation networks involved in carbon- and nitrogen-metabolism regulation (50). The gene regulation profiles identified in this study include several CcpA and CodY target genes that could be controlled by a similar regulator complex that encompasses both CodY and CcpA.

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Supplementary information for chapter 3

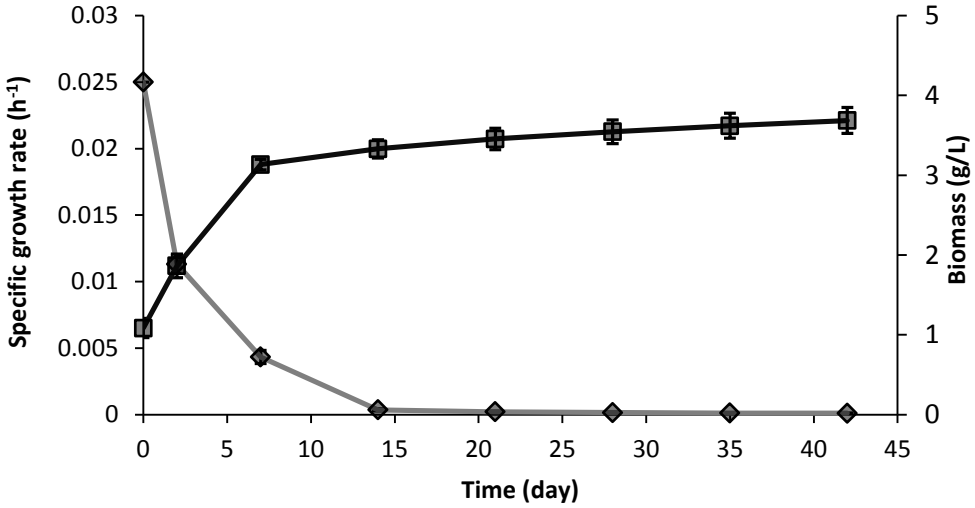

Figure S1: Growth of *L. lactis* KF147 in retentostat culture. A steady-state anaerobic chemostat culture was switched to retentostat mode at time zero. Data points represent average ± mean deviation of measurements of two independent cultures. Specific growth rate (h^{-1}) (grey line) and biomass accumulation (g/L) (black line) of *L. lactis* KF147 under retentostat conditions (adapted from Ercan, *et al*., 2013).

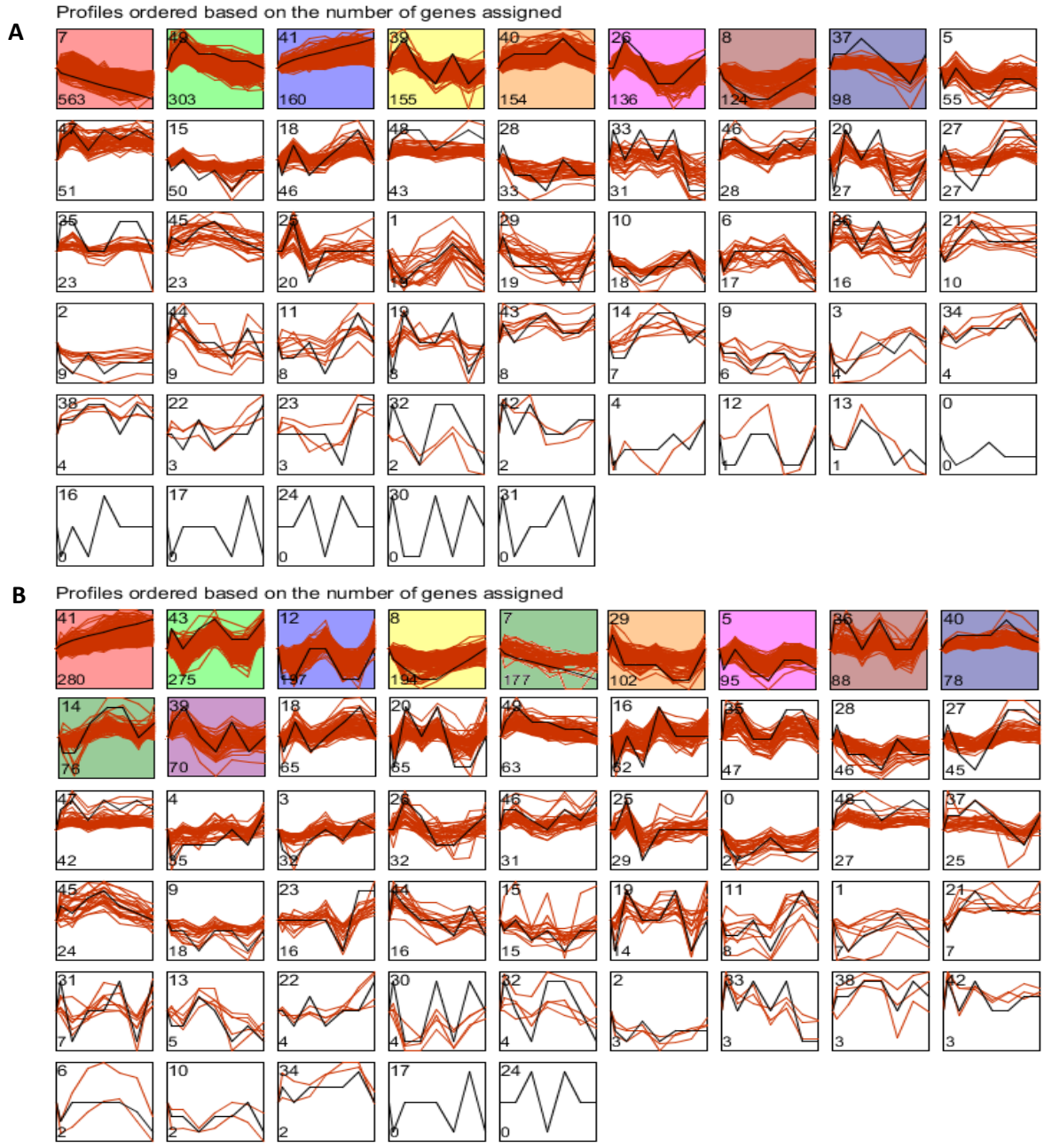

Figure S2: STEM clustering profiles from retentostat cultivation (A) 1 and (B) 2. 50 clusters contain the genes filtered on the basis of the expression ratio ≥ 1 (in $\log_2$-scale) between the time-points and p-value ≤ 0.05. The number in the top left-side corner of a profile box is the profile ID number and lower left-side corner is the number of genes. The colored profiles have a statistically significant number of genes assigned.

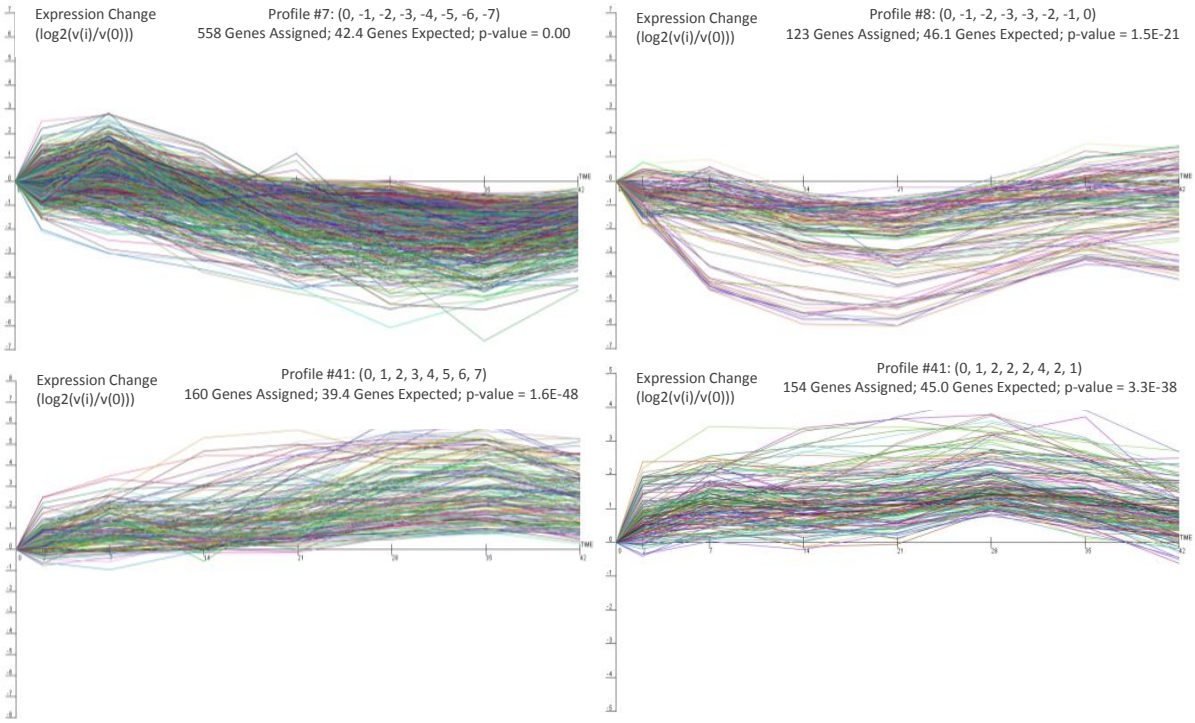

Figure S3: Four detailed model profiles. All four model profiles, which are profile 7, 8, 40, and 41, were significant. Along the top of each profile are statistics on the number of genes assigned to the profile, the number of genes expected, and the enrichment *p*-value (retentostat 1). Similar transcriptome analyses in retentostat 2 confirmed the consistency of the results.

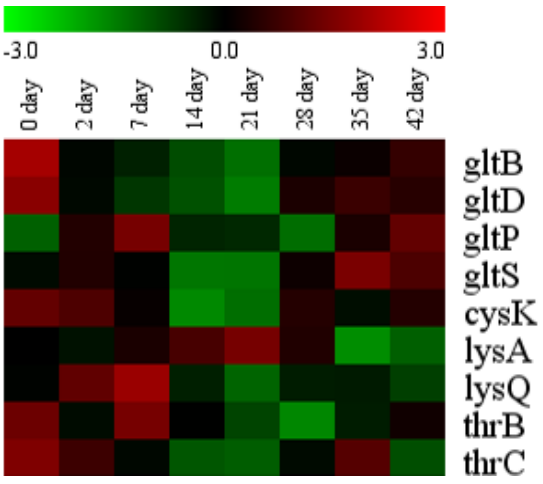

Figure S5: Heat map of *L. lactis* KF147 Glu, Lys, Cys, and Thr biosynthesis genes differentially expressed (in $\log_2$-scale, p-value ≤ 0.05) during retentostat cultivation over the beginning of the chemostat (day 0) (retentostat 1). Similar transcriptome analyses in retentostat 2 confirmed the consistency of the results.

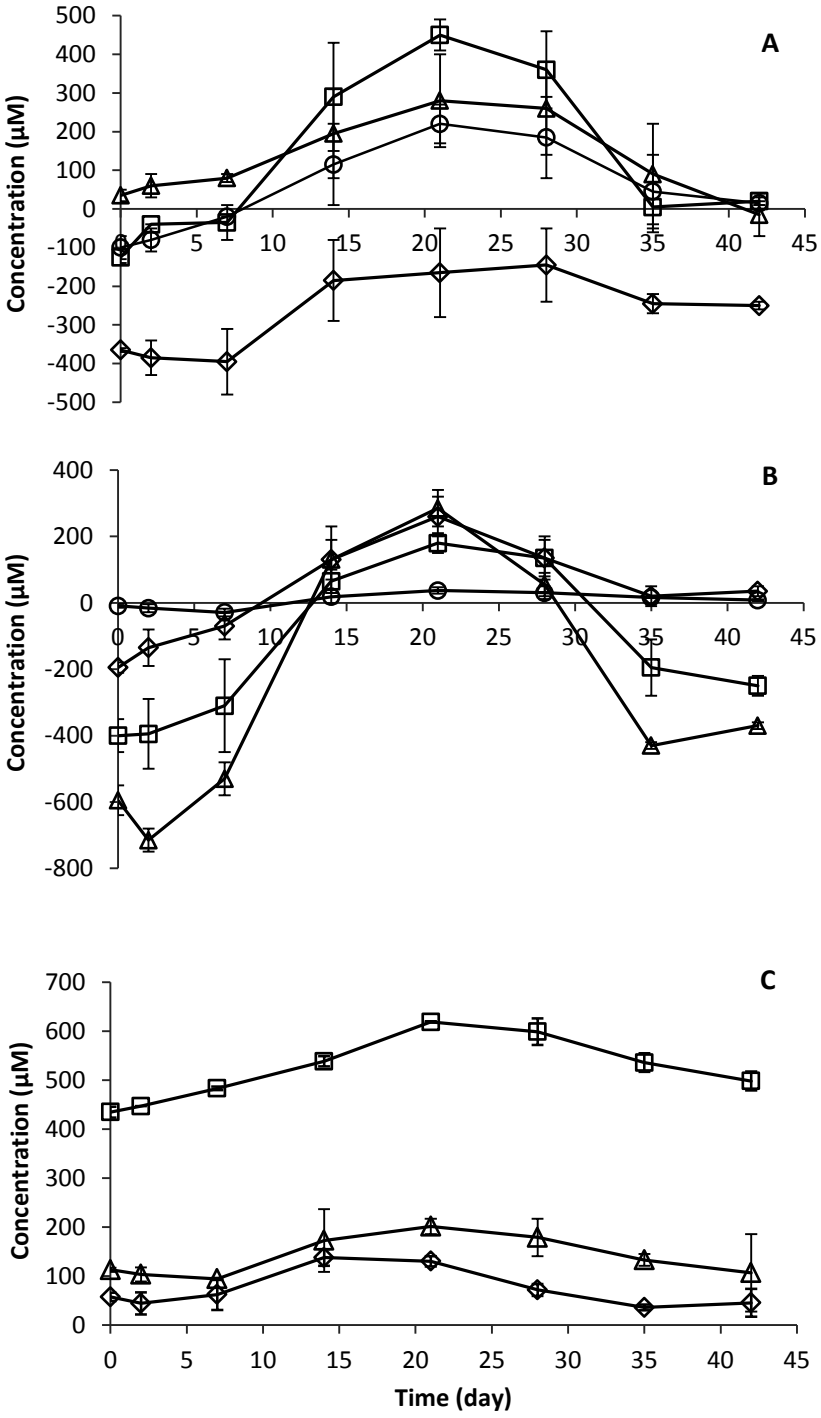

Figure S4: Concentration of amino acids in *L. lactis* KF147 in retentostat culture. Data points represent average ± mean deviation of measurements of two independent cultures. (A) Concentration of Gly (diamonds), Asp (squares), Glu (triangles), and Lys (circles); (B) Thr (diamonds), Ser (squares), Pro (triangles), and Cys (circles); (C) Met (diamonds), ornithine (squares), and His (triangles). All concentrations in panels A, B, and C are expressed as the difference between the measured concentration in the medium feed and the measured concentration in the filter line samples. Negative numbers indicate net-consumption; positive numbers indicate net-production.

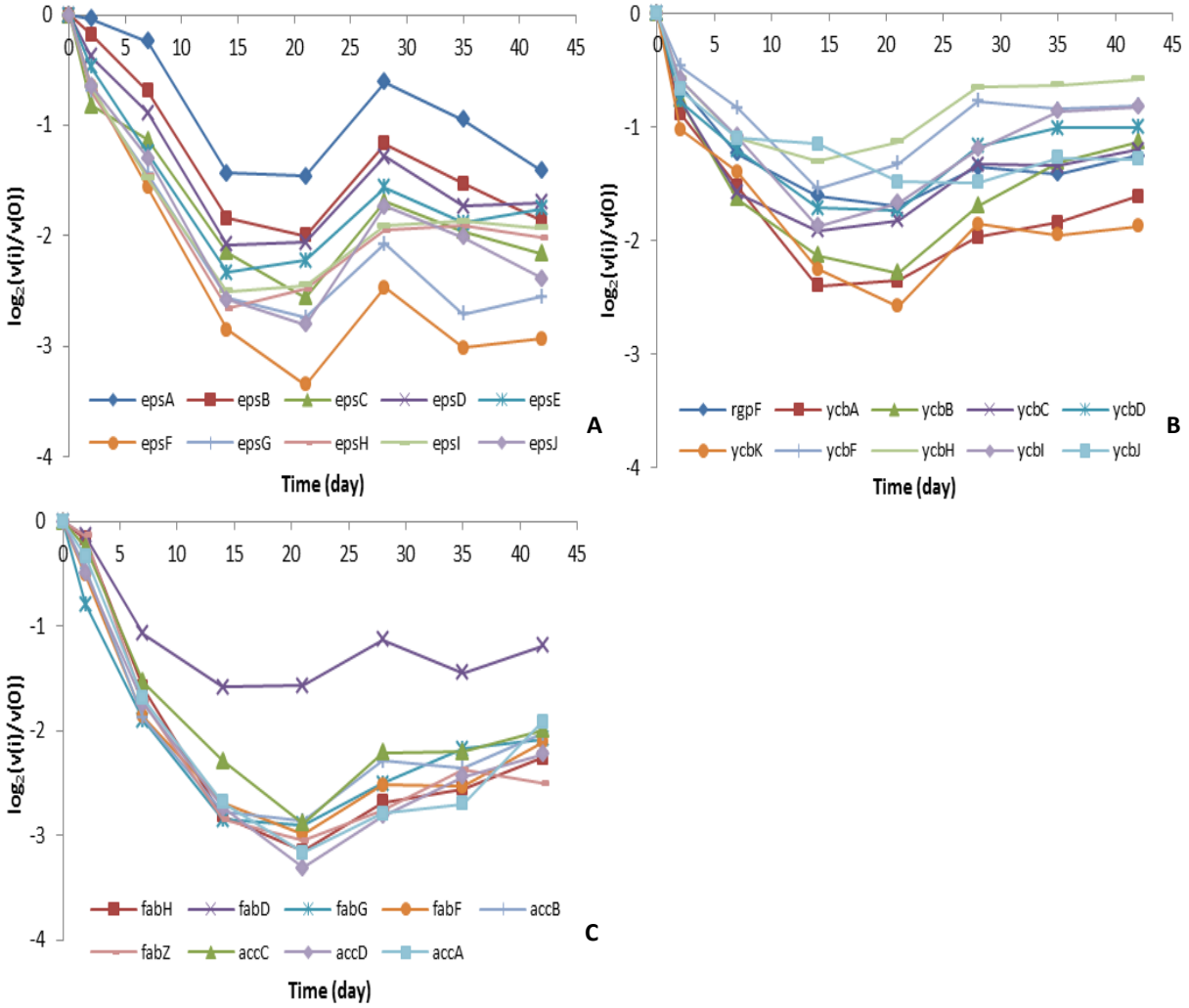

Figure S6: Expression graphs of genes involved in (A) exopolysaccharide synthesis and (B) glycosyltransferase (model profile 7); (C) fatty acid biosynthesis (model profile 8) during retentostat cultivation over the beginning of the chemostat (day 0) (retentostat 1). Similar transcriptome analyses in retentostat 2 confirmed the consistency of the results.

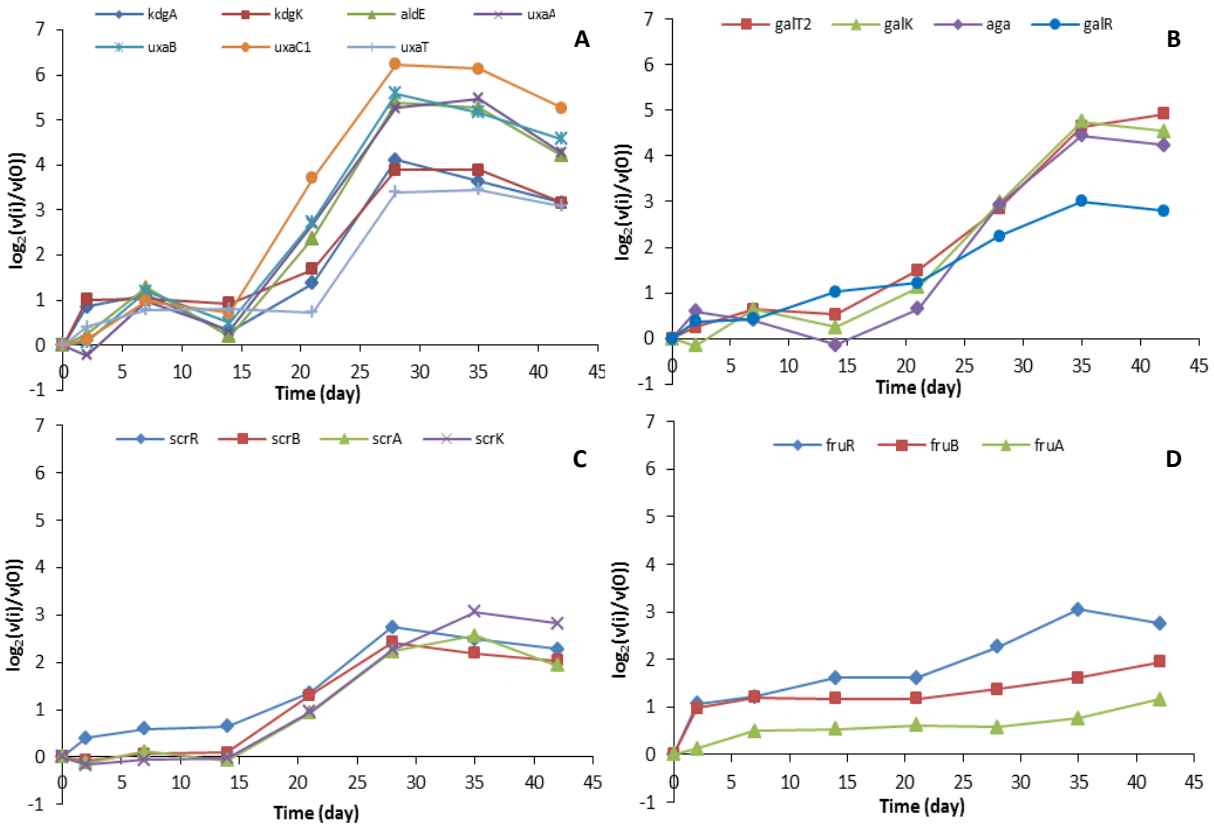

Figure S7: Expression graphs of genes involved in (A) galacturonate, (B) raffinose, (C) sucrose, and (D) fructose uptake and metabolism during retentostat cultivation over the beginning of the chemostat (day 0) from model profiles 40 and 41 (retentostat 1). Similar transcriptome analyses in retentostat 2 confirmed the consistency of the results.

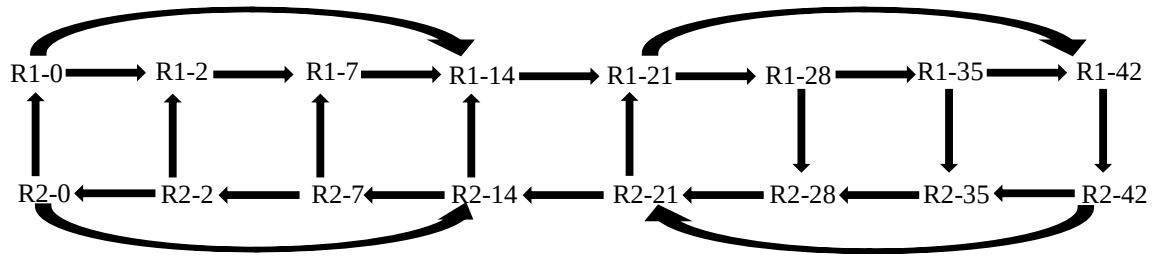

Figure S8: Experimental design and hybridization scheme. R1 and R2 represent retentostat 1 and retentostat 2 at days 0, 2, 7, 14, 21, 28, 35, and 42, respectively. Arrow represents Cyanine 3 (tail) and Cyanine 5 (head) label.

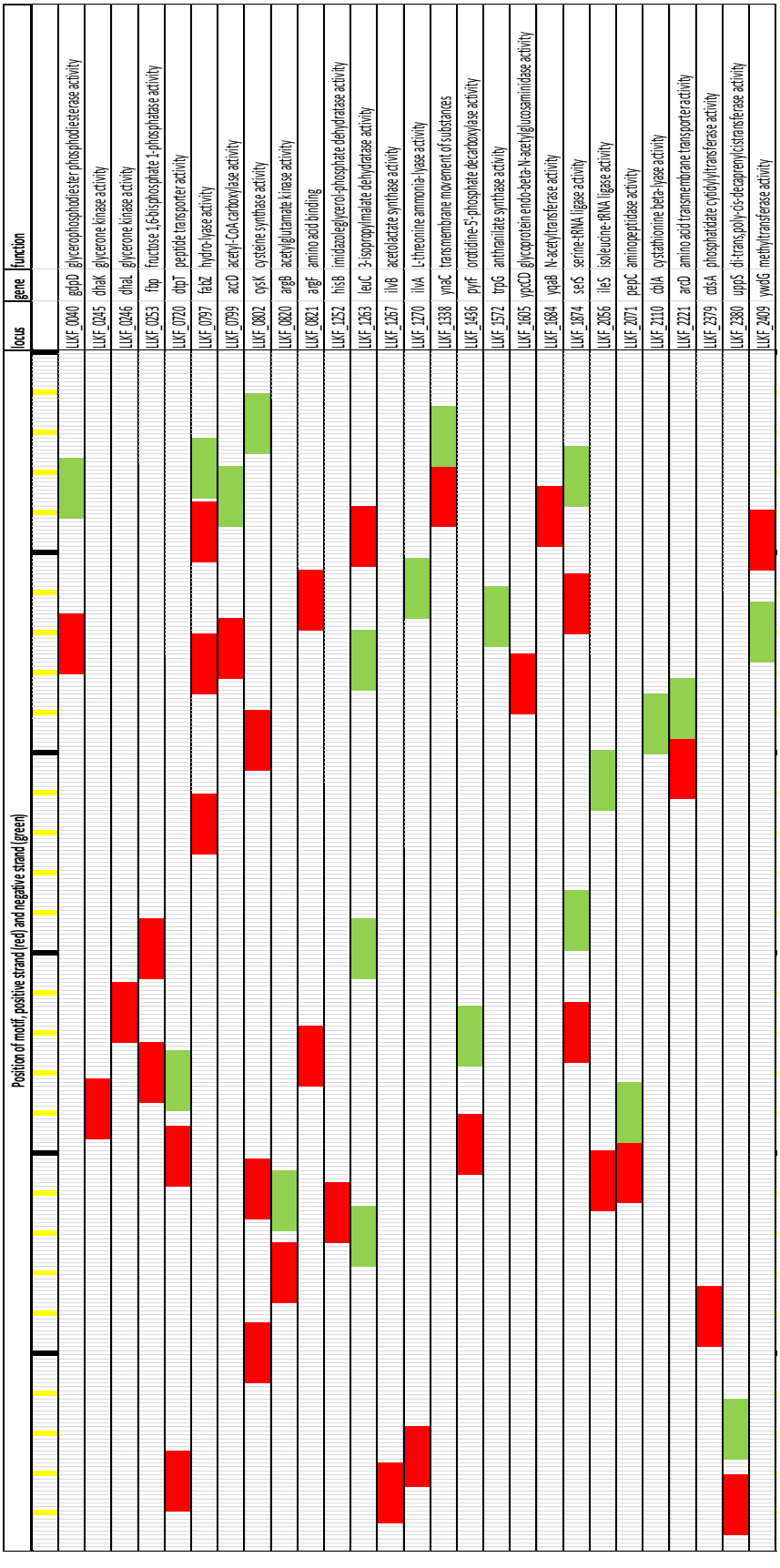

Figure S9: Motif positioning diagram. Genes in the genome of *L. lactis* KF147 that are regulated by a sequence element with significant sequence homology to the putative regulatory element identified in this study using MEME. Motif blocks show the order and spacing of non-overlapping matches to the motifs in each sequence. The ruler at the bottom gives an indication of the actual length of the sequence. Black blocks indicate positive strand and gray blocks indicate negative strand on the sequence for the motifs upstream of the genes (*p*-value < 1.0e-06).

Chapter 4

Improved stress robustness in *Lactococcus lactis* by adaptation to near-zero growth rates

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Manuscript in preparation

Abstract

This paper describes the increased robustness of *Lactococcus lactis* KF147 under various stress conditions such exposure to heat, acid, and oxidative conditions, by the adaptation to extremely low-specific growth rate (0.0002 h^{-1}) using carbon-limited retentostat cultivation. Culture samples withdrawn from chemostat and different periods of retentostat cultivation were exposed to heat, acid, and oxidative challenges and survival curves were determined during a period of 2 hours. This cultivation method resulted in remarkably reproducible stress robustness features. Transcriptome adaptation of *L. lactis* during retentostat cultivation encompassed the progressive enhancement of expression of stress related functions, which was shown to correlate with a progressive improvement of multiple-stress tolerance levels. Maximal stress tolerance levels occurred at the lowest specific growth rate that was obtained after 29 days of retentostat cultivation. Furthermore, the experimental survival data were fitted using the Weibull microbial inactivation model to estimate the first decimal reduction time and to compare the shape of the kill-curves. Correlation analyses of the kinetic parameter of inactivation and the transcriptome data confirmed that expression of genes involved in multiple stress response factors enabled the bacterium at the extremely reduced growth rates to have increased evolutionary fitness upon exposure different stress conditions. Additionally, the proposed correlation between the stress-gene expression levels and robustness of cells implies that genes that are related to the heat, acid, and oxidative stress responses allow quantifying the robustness level of lactococci under specific stress conditions. This quantitative stress-robustness study translates the molecular adaptations of *Lactococcus lactis* KF147 under extremely slow-growth rates, to environmental stress tolerance in a quantitative manner and correlates the growth rate, robustness and transcriptional expressions of the multiple stress response genes at near-zero growth rates.

Keywords: *Lactococcus lactis*, zero-growth, survival, stress response, cross-resistance, microbial survival model

Introduction

Lactococcus lactis is commonly found in milk fermentations and is considered a key species in many starter cultures applied in the manufacturing of sour cream, cheese, and various other fermented milk products. Starter cultures of *L. lactis* play a key role in preserving the product by acidification and also contribute to the formation of flavor and texture of cheese that is determining the product's sensory quality (26, 27). Next to these industrial (and artisanal) food fermentation applications, this bacterium is also commonly encountered in other habitats, for example in (decaying) plant materials, where they commonly co-inhabit this niche with other microbes like yeast and fungi that may liberate accessible nutrients for lactococci from polymeric plant structures (25).

Both in industrial fermentations and in environment of decaying-plant, *L. lactis* encounters a wide diversity of environmental stress conditions such as extremes in temperature, pH, nutrient starvation or osmotic pressure (23). Importantly, the endogenous fermentative activity of the lactic acid bacteria (LAB) imposes an intrinsic environmental stress condition, *i.e.* low pH and high levels of organic acids. Industrial processing may include exposure to several additional stress conditions like brining, freezing, thawing, drying, and/or nutrient starvation that may negatively affect the cultures' fermentation performance (35). Thus, selection of strains that perform well in fermentation and resist the harsh industrial processing conditions is an important innovation driver in the fermented-food industry (23). Consequently, in order to optimize starter culture performance under industrial fermentation conditions, it is important to understand the stress-sensing and -response mechanisms of *L. lactis*, to eventually exploit such understanding for the improvement of its survival capacity and *in situ* performance.

Stress response mechanisms depend on the strictly regulated expression of multiple genes, which modify different cellular processes, including core functions like cell division, DNA metabolism, general housekeeping, membrane composition, and transport that act in concert to increase bacterial stress tolerance (28). The coordination of stress response gene expression is achieved by (partially overlapping) regulatory networks that allow the cell to adequately respond to various environmental changes (32). The best studied stress conditions in *L. lactis*

are heat, cold, acid, and oxidative stresses, although most of the studies centered on a specific family of stress-genes or -proteins rather than the overall adaptation (3, 29).

In order to prevent or counteract heat stress induced loss of function due to protein denaturation, lactococci produce chaperons that are involved in maturation of newly synthesized proteins and in (re-)folding of denaturing proteins, and/or produce a protein degradation machinery to remove denatured and aggregating proteins (29). *L. lactis* encodes two canonical chaperone complexes composed of DnaK-GrpE-DnaJ and GroES-GroEL, which are members of the class I stress response (29). In *L. lactis*, stress response genes of class I are controlled by the repressor HrcA that binds to the palindromic operator sequence CIRCE (controlling inverted repeat of chaperone expression) located in the promoter regions of the *groEL-groES* and *hrcA-dnaK-grpE-dnaJ* operons (29). Additionally, in *L. lactis* class III heat shock genes are regulated by the repressor CtsR that binds to a heptanucleotide direct repeat called the CtsR-box that is present in the promoter regions of the *clpB*, *clpC*, *clpE*, and *clpP* genes (16). The ClpP protease plays a key role in degradation of damaged proteins that cannot be refolded by chaperons, which is facilitated by the four Clp-ATPase (ClpB, ClpC, ClpE, and ClpX) chaperones that are involved in recognition of abnormally folded proteins (16, 29).

L. lactis can cope with substantially lower pH values, and can still grow at a pH of approximately 4.5. Notably, at pH values below 4, the viability of *L. lactis* is generally quickly lost (22), although this critical pH depends on the environmental conditions, which is illustrated by the observation that rich laboratory media may enhance acid tolerance in *L. lactis* to pH values of 3.0 or even 2.5 (18). Lower pH leads to an increase in the proportion of protonated weak organic acids (*e.g.* lactic acid), which can passively diffuse into the cells and dissipate the proton motive force by dissipating of the transmembrane ΔpH (19), thereby compromising an important energy source in several transmembrane transport processes (21, 32). Concomitantly, the intracellular pH will decrease, which can negatively affect acid-sensitive enzymes and may contribute to protein and/or DNA damage (3). Acid-tolerance and internal pH homeostasis can be supported by enhanced F_0F_1-ATPase activity, or the arginine deiminase (ADI) and glutamate decarboxylase pathways (20). Additionally, pH induced DNA damage can in various lactic acid bacteria be counteracted by the acid inducible Uvr system

(*uvrB*) (14). These concerted acid stress responses can suppress intracellular acidification, and support maintenance of function under low pH conditions.

When exposed to molecular oxygen, lactococci can use oxygen through the closely coupled NADH oxidase/NADH peroxidase system that controls a shift from homolactic towards mixed-acid production, which generates an additional ATP (32). The use of molecular oxygen in lactococci comprises the formation of reactive oxygen species (ROS) that are toxic, including superoxide (O_2^-), hydroxyl radicals ($OH^\bullet$), and hydrogen peroxide (H_2O_2), which can damage virtually all cellular components (protein, RNA, DNA, membranes) and can lead to cell death (12, 32). To eliminate these toxic molecules, *L. lactis* possesses a NADH oxidase/NADH peroxidase system, through which O_2 oxidizes NADH into NAD^+ by NADH oxidase, and the H_2O_2 produced in this reaction is reduced to H_2O by NADH peroxidase, and a flavoprotein NADH oxidase (*noxCDE*) reduces O_2 directly to water without producing H_2O_2 as an intermediate (12). In addition, lactoccoci encode a superoxide dismutase (*sod*A), which converts O_2^- to the less-damaging H_2O_2 (12).

In previous studies, we have described the physiological as well as the metabolic and molecular adaptations of the plant isolate *L. lactis* KF 147 to near-zero growth rates induced by carbon-limited retentostat cultivation (9, 10). Retentostat cultivation is an adaptation of chemostat cultivation in which a growth-limiting carbon source is continuously supplied at a steady rate, while the microbial biomass is retained in the fermentation vessel by continuous removal of spent medium through an external cross-flow filter effluent channel (10, 33). Extended retentostat cultivation leads to growth rates that approximate zero and induces a cellular physiology in which the generated metabolic energy is completely invested in maintenance related processes, which support high cell viability over extended periods of time (10, 31).

In this study, we investigated the stress response and corresponding tolerance development in *L. lactis* KF147 at near-zero growth rates. Here we present that besides the molecular and metabolic adaptations elicited by extended retentostat cultivation in *L. lactis* (9), also induced the expression of a broad spectrum of stress related functions, including factors predicted to be involved in acid-, heat-, and cold- tolerance. To validate these transcriptional data the present study determines stress tolerance and survival rates of cells derived from different

stages of retentostat cultivation. Non-linear modeling of the stress survival curves established that extended retentostat cultivation and the corresponding extremely low-growth rates quantitatively correlate with the enhanced resistance towards various stress challenges in *L. lactis*. Furthermore, the kinetic parameters of stress-survival could be accurately correlated with the previously determined gene expression patterns of the subclasses of stress-related functions Taken together, extended retentostat cultivation led to very reproducible culture features, allowing to quantitatively correlate the growth rate, inactivation rate, and transcription level changes of stress related gene in *L. lactis* at slow-growth rates.

Materials and Methods

Bacterial isolates, media and cultivation conditions. *Lactococcus lactis* subsp. *lactis* strain KF147 originates from mung bean sprouts, and its genome sequence has been determined (25). Pre-cultures for retentostat cultivations (10) were inoculated in 50 ml M17 broth (30) supplemented with 0.5% glucose (w/v; GM17) and incubated overnight at 30°C. Subsequently, overnight cultures were harvested by centrifugation (6,000 x g, 10 min., 4°C), washed twice with physiological salt solution (0.9% NaCl in water), and the culture was inoculated into CDM containing 25 mM of glucose (13). To retain the medium composition constant during long-term cultivation, 120-liter batches of medium were prepared, filter sterilized, and stored at -20°C.

Two independent, carbon source-limited retentostat cultivations with 1.5 L working volume were performed under anaerobic conditions, initiating from chemostat cultivation at dilution rates of 0.025 h^{-1}, as previously described (10).

Biomass, lactic acid concentration, and viability determination. During fermentations, culture samples were withdrawn at specific time-points to measure cell dry weight (CDW), lactic acid concentration, and viable cell concentration. Since the removal of samples during the retentostat fermentation could interrupt biomass accumulation, sample volume and sampling frequency were kept to a minimum. CDW was determined as previously described (10). Samples for the determination of lactic acid concentration in the culture supernatant were immediately centrifuged (20,000 x g; 2 min.; at 4°C) and supernatants were stored at -20°C until analysis. The concentration of lactic acid was quantified with the enzymatic assay kits no. 10139084035 (R-Biopharm, Darmstadt, Germany), according to the manufacturer's

protocols. The viability of cells in the culture was assessed by quantification of colony forming units (CFUs) on M17 agar plates (24), using serial dilutions (10-fold dilution in M17 broth) in triplicate samples.

Determination of lethal level of heat, acid and oxidative stresses. Late-exponential phase *L. lactis* KF147 cells were harvested from a GM17 culture with an OD_{600} of 1.7, for the development of all stress assays. Samples were exposed to heat, acid, and oxidative stress conditions for 120 minutes and samples were withdrawn from the stress treatment condition at 0, 15, 30, 60, and 120 minutes for CFU enumeration. Serial dilutions of triplicate samples in M17 broth were prepared immediately after withdrawing the samples from the stress exposure condition, and these dilutions were directly plated on M17 agar in triplicate. Plates were incubated at 30°C for 2 days for CFU enumeration (24), of which detection limit of CFU was 10^4 cfu ml^{-1}. To determine the most appropriate lethal stress temperature, 1 ml of culture was directly incubated in a thermo-mixer (Eppendorf, Hamburg, Germany) at 45, 47, and 50°C. The appropriate levels of lethal acid and oxidative stresses were determined by suspending 100 µl of *L. lactis* KF147 culture in 900 µl M17 broth with an adjusted pH of 2.0, 2.5, and 3.0 (acidified by 5 M HCl) and M17 broth containing 10 and 20 mM hydrogen peroxide, respectively, followed by incubation at 30°C for 120 minutes.

Stress tolerance analysis of retentostat derived culture samples. For all stress tolerance assays, samples were withdrawn from chemostat (day 0) and retentostat cultivation at days 14, 21, and 29 of cultivation, and the above mentioned stress treatments and CFU enumeration by plate counting, for which samples were withdrawn from the stress treatment condition at 0, 15, 30, 45, 60, 90, and 120 minutes, were performed. For heat stress resistance, 1 ml of culture sample was directly incubated in a thermomixer (Eppendorf, Hamburg, Germany) at 50°C. Acid and oxidative stress tolerance were determined by suspending 100 µl of the retentostat grown *L. lactis* KF147 culture in 900 µl M17 broth with an adjusted pH of 2.5 (acidified by 5 M HCl) and M17 broth containing 20 mM hydrogen peroxide, respectively, followed by incubation at 30°C for 120 minutes.

Microbial survival model and its application. The Weibull model was used to fit the survival data. The Weibull model (4) describes linear survival or curves with convex and concave curvature. The Weibull model is formulated as follows:

$$\log_{10}\left(\frac{N(t)}{N(0)}\right) = -\left(\frac{t}{\delta}\right)^{\beta} \quad (1)$$

where δ is the first decimal reduction time (min) and β is a fitting parameter which defines the shape of the curve. β values of < 1 correspond to concave upward survival curves, β values of > 1 correspond to concave downward curves, and a β value of 1 corresponds to a straight line (6).

The Weibull model was fitted to the survival data for each of the experimental stress exposure conditions applied, using Microsoft Excel Solver add-in and checked in TableCurve2D. The statistical criteria used to choose the most suitable model to fit the experimental data were MSE_{model}, *p* value, and *F* test (6). The experimental reproductions were fitted individually and the mean and standard deviation of the parameters were calculated. The data fitting of the average δ value in $\log_2$-scale as a function of the relative specific growth rate data in $\log_2$-scale (Fig. 4 and Fig. 5) was done using Microsoft Excel.

Transcriptome analysis. Microarray data used in this study is part of a large dataset described in (9) and accessible at Gene Expression Omnibus (GEO) with accession number GPL17806 (http://www.ncbi.nlm.nih.gov). In our earlier study, time-resolved transcriptome analyses were performed on two independent retentostat cultures, from which samples were taken before starting the retentostat cultivation regime (t = 0 day; chemostat conditions), and 2, 7, 14, 21, 28, 35, and 42 days during the retentostat regime, and analyzed on a total of 26 genome-wide microarrays (9).

Results

Stress response induction during retentostat cultivation. Molecular and physiological adaptations of *L. lactis* KF147 at near-zero growth rates have been studied using long-term (42 days) anaerobic, carbon-limited retentostat cultivations (10). Previously, we have reported on the quantitative physiology (10) and metabolic and molecular adaptations (9) that are induced in *L. lactis* KF147 when it is grown under retentostat conditions for extended periods of time. Genome-wide gene expression profiling of this strain during retentostat growth supported the metabolic adaptations the strain underwent under these conditions, including

the validated relief of repression of several alternative carbohydrate import and utilization pathways (9). In addition, these transcriptome profiles revealed that retentostat cultivation and the corresponding reducing growth rate, led to the induction of expression of several genes linked to different stress responses, including heat, cold, acid, and cell membrane related stresses (Fig. 1A & S1). The profiles of genes encoding proteins involved in cell membrane associated stress (*hemG*, *lmrA*, *rmaB*, and *yhbF*) were expressed at elevated levels from 7 days of retentostat cultivation onwards, and their expression levels steadily increased during extended cultivation (Fig. 1A1 & S1A). Moreover, the expression of the gene encoding UvrB that is involved in SOS response (*uvrB*), the universal stress protein A (*uspA*), and cAMP receptor protein (*rcfB*) that are part of the acid stress response, were initially fluctuating up to 14 days of retentostat cultivation, but with further decreasing growth rates during prolonged retentostat cultivation were found to be transcribed at significantly higher levels (Fig. 1A2 & S1B). The expression of several genes encoding cold-shock proteins (CSPs; CspA1, CspB1, CspC1, CspD, CspA2, CspB2, and CspC2) were rapidly increasing during the first week of the retentostat cultivation, and remained at a high expression level during extended retentostat cultivation (Fig. 1A3 & S1C). Finally, the expression level of the heat shock proteins (HSPs) DnaK, DnaJ, GrpE, GroEL, and GroES as well as the ClpB protease was gradually increasing during the first 28 days of retentostat cultivation, and remained high at later stages (Fig. 1A4 & S1D). These observations demonstrate that prolonged retentostat cultivation enhances the expression of a panel of stress response associated genes in *L. lactis* KF147, which supports the adaptation of the cells to enhance maintenance related functions under these conditions (9, 10). Importantly, the expression values of these stress genes inversely correlated with the growth rate of the corresponding cell cultures that gradually decreased during retentostat culture (Fig. 1B; growth rate data were used from reference 10).

The elevated expression of a range of stress associated genes during retentostat cultivation (near-zero growth) poses the question whether these conditions enhance the stress-robustness of *L. lactis* KF147, as compared to the same strain grown under chemostat (growing) conditions. To address this question, appropriate stress tolerance assays were required to effectively investigate the percentage of viability loss of *L. lactis* KF147 upon exposure to various levels of stress.

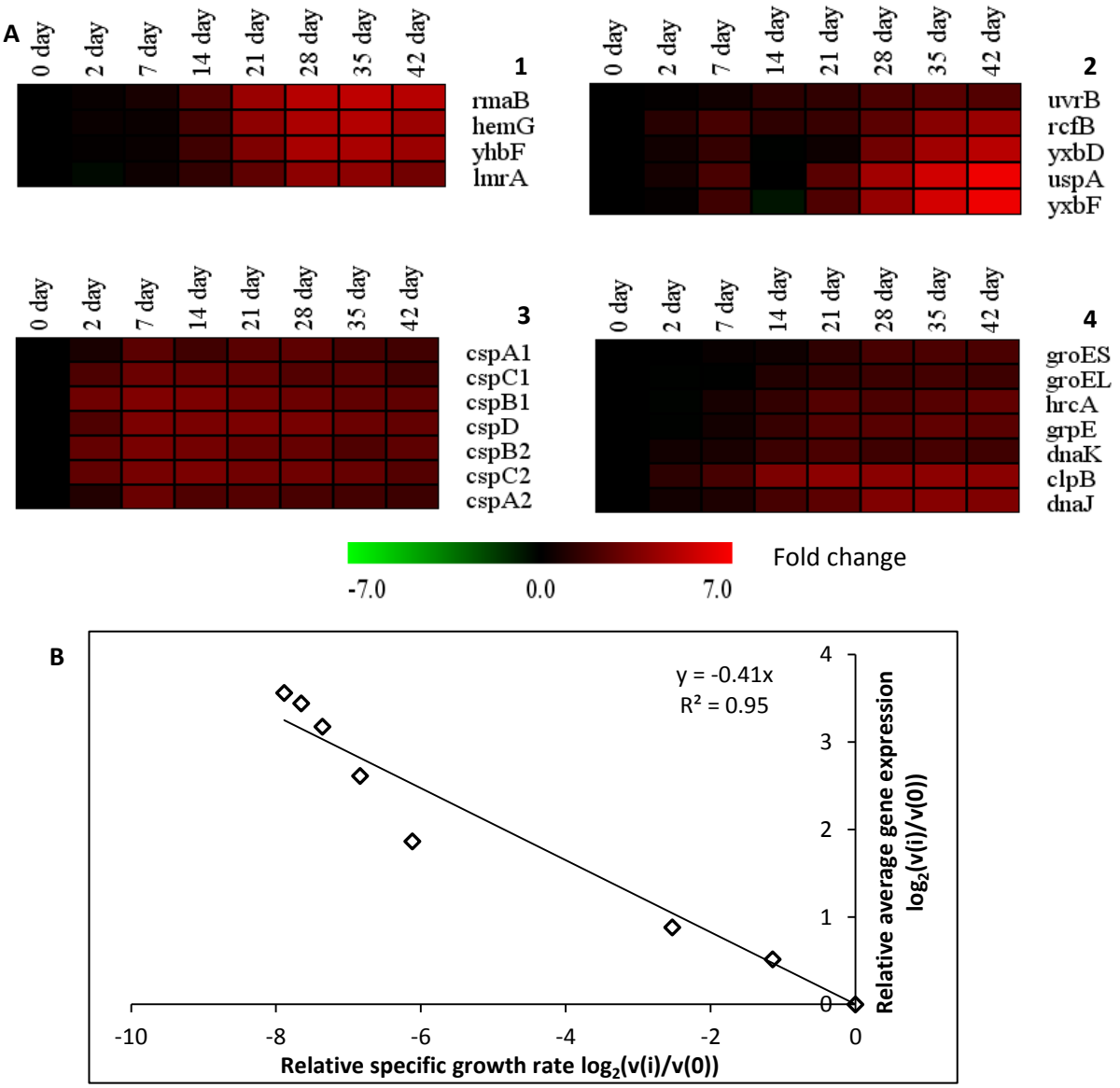

Figure 1: Expression of stress related genes and correlation with specific growth rate during retentostat cultivation. (A) Heat map of *L. Lactis* KF147 (A1) cell membrane, (A2) acid, (A3) cold-shock, and (A4) heat stress responses genes differentially expressed (in $\log_2$-scale, p-value ≤ 0.05) during retentostat cultivation on days 2, 7, 14, 21, 28, 35, 42 over the beginning of the chemostat (day 0) (made public in Ercan, *et al*., 2014). (B) Correlation of relative average of overall expressions of genes involved in stress responses (heat, acid, and oxidative stress responses) and relative specific growth rate during retentostat cultivation (obtained from Ercan, *et al*., 2013) over the chemostat cultivation in $\log_2$-scale (retentostat 1). Similar transcriptome results obtained in retentostat 2 confirmed the consistency of these results in an independent experiment (Ercan *et al*., 2013; 2014).

Stress tolerance of late-exponentially growing *L. lactis* cells. To estimate the specific stress conditions that are appropriate to quantify the stress tolerance levels of cells derived from chemostat- and retentostat- cultivation, bacteria were isolated from the late-exponential phase

of batch cultured *L. lactis* KF147 and immediately exposed to different stress temperatures (45, 47, and 50°C), low-pH conditions (pH of 2, 2.5, and 3), and oxidative stress (10 and 20 mM H_2O_2), and the percentage of viability loss over time under these conditions was determined (kill-curve analysis) (Fig. 2). After 120 minutes of the different heat stress exposures, the remaining viability was estimated to be 2.9±0.9 and 0.4±0.1% at 45 and 47°C, respectively, while the detection limit was reached at 50°C (Fig. 2A). Sixty minute acid stress treatments elicited an approximate 1.5-log, 3.5-log, and 5.5-log reduction in viability, in M17 medium adjusted to pH of 3, 2.5, and 2, respectively (Fig. 2B). Oxidative stress treatments displayed an estimated remaining viability of 0.02 ± 0.003% at 10 mM H_2O_2 after 120 minutes of exposure, while the detection limit was reached at 20 mM H_2O_2 after 120 minutes (Fig. 2C). Stress conditions were selected that allow for feasible kinetic determinations of killing rates under stress conditions within a 2 hour timeframe, leading to the selection of 50°C, pH 2.5, and 20 mM H_2O_2 as the heat, acid, and oxidative stress conditions, respectively.

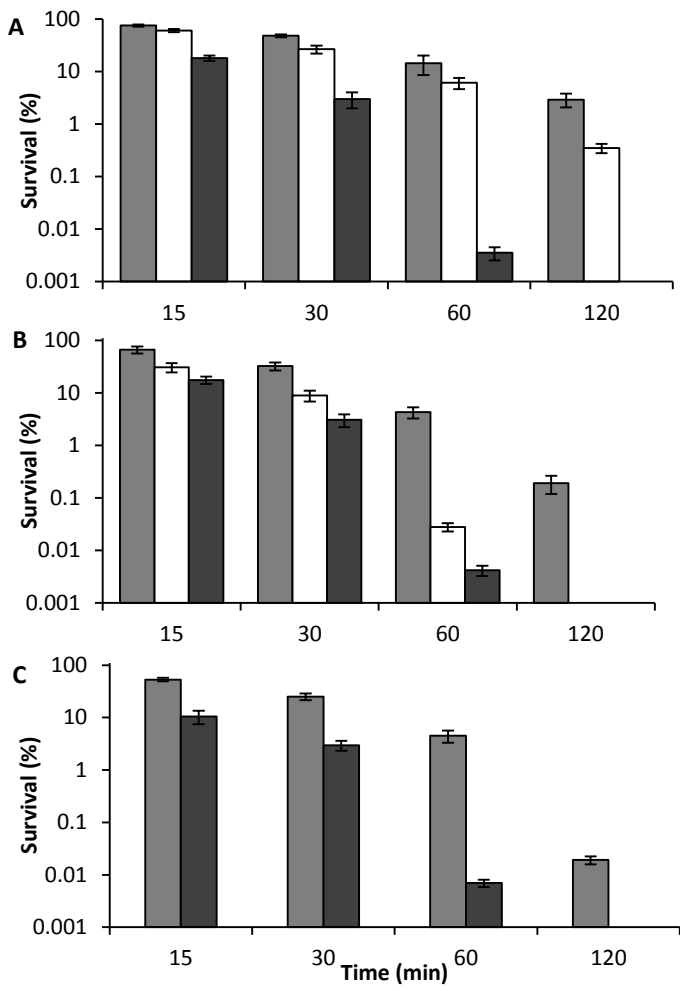

Figure 2: Relative survival kinetics of late-exponential phase *L. lactis* KF147 against direct exposure to different (A) heat, (B) acid, and (C) oxidative stress conditions for 120 minutes. Data points represent average ± standard deviation of measurements of independent triplicate cultures.
(A) Survival percentage of late-exponential phase cells in M17 medium broth at 45°C (light gray), 47°C (white), and 50°C (dark gray).
(B) Survival percentage of late-exponential phase cells at 30°C in M17 medium broth with a pH of 2 (dark gray), 2.5 (white), and 3 (light gray).
(C) Survival percentage of late-exponential phase cells 30°C in M17 medium broth containing 10 (light gray) and 20 mM H_2O_2 (dark gray). Notably, untreated and non-stressed cells remained fully viable (100 %) over a timespan of 2 hours.

Retentostat cultivation and enhanced stress tolerance. To test stress tolerance development under retentostat conditions, two independent, anaerobic, carbon-limited retentostat cultivations using *L. lactis* KF147 were performed with an overall running time of 29 days. During the retentostat cultivation, biomass accumulation, culture viable log counts, and the concentration of lactic acid in the spent-medium effluent were determined (Fig. S2 & S3). Analogous to previous observations (10), biomass accumulation reached a maximum and remained stable after approximately 14 days, and the growth rate of the culture decreased from 0.025 h^{-1} (initiating chemostat cultivation) to nearly 0.0002 h^{-1} after 29 days of retentostat cultivation (Fig. S2). The viability of the culture remained high throughout the retentostat cultivation level (Fig. S3) and the fermentation metabolite production pattern displayed the typical fluctuating between mixed acid and lactic acid fermentation that has been reported before (Fig. S4; ref. 9, 10).

Stress resistance assays were performed with samples taken from chemostat- (day 0) and retentostat culture (days 14, 21, and 29) using the pre-determined heat, acid, and oxidative stress exposure conditions (Fig. 3). The reproducibility of the inactivation curves of the two retentostats was remarkably high in all conditions, which likely reflects the highly consistent culture conditions (chemostat followed by retentostat) employed during these experiments. Exposure to 50°C heat stress of chemostat- and retentostat-grown cells, revealed a significantly higher survival percentage for retentostat-grown culture-samples. Moreover, prolongation of retentostat cultivation led to consistent improvement of stress tolerance reaching a maximum survival percentage in the sample obtained after 29 days of retentostat cultivation (Fig. 3A). During the first 30 minutes of stress treatment, the remaining viability of the retentostat-grown cells extracted after 29 days of cultivation was approximately 50%, which is substantially higher (3-fold) as compared to samples taken from chemostat cultivation conditions. Relative survival percentages in the sample taken after 14 and 21 days of retentostat cultivation were also enhanced, but to a lesser degree than the sample taken at 29 day of retentostat cultivation (Fig. 3A). Prolonged heat-stress exposure expanded the difference of stress tolerance between the different culture samples. This is apparent from the almost 3-log reduction of viability (approximately 0.1% remaining viability) in the culture sample taken during chemostat-growth, after 90 minutes of heat exposure, whereas the culture samples taken after 14, 21, and 29 days of retentostat-growth displayed approximately 2, 5

and 13% of remaining viability, respectively. Moreover, further extension of the heat-stress exposure period beyond 90 minutes led to a clear reduction in the percentage of viability loss in the retentostat grown cells extracted after 29 days of cultivation, leading to a 10% remaining viability after 120 minutes of heat stress exposure (Fig. 3A).

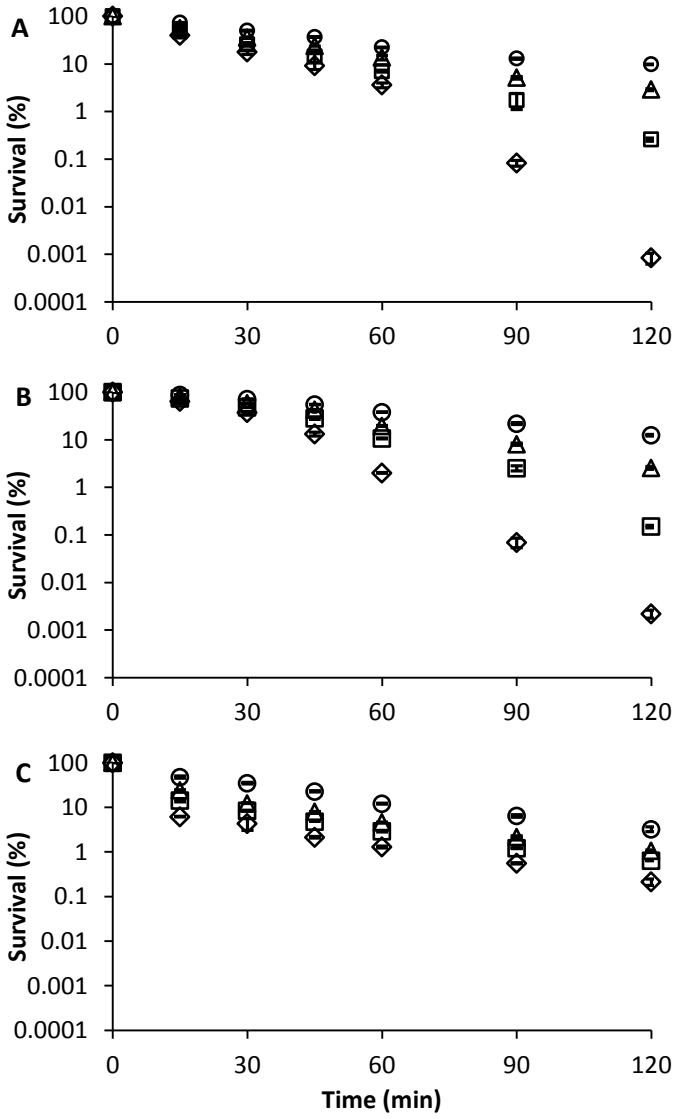

Figure 3: Relative survival kinetics of chemostat- and retentostat-grown *L. lactis* KF147 cells against direct exposure to (A) heat stress at 50°C, (B) acid stress, pH of 2.5, and (C) oxidative stress by incubation in 20 mM H_2O_2. Survival percentage of chemostat-grown cells (diamonds); retentostat-grown cells on day 14 (squares), 21 (triangles), and 29 (circles). Data points represent average ± standard deviation of measurements of two independent retentostat cultures. Notably, untreated and non-stressed cells remained fully viable (100 %) over a timespan of 2 hours.

Analogous to what was observed for heat-stress tolerance enhancement in retentostat derived culture samples, relative survival upon exposure to a lethal level of acid stress (pH 2.5) was significantly enhanced in all retentostat culture samples as compared to the chemostat derived culture sample. Again, the magnitude of the acid tolerance enhancement was highest in culture samples taken after 29 days of retentostat cultivation, while the shorter retentostat

cultivation period elicited intermediate tolerance improvement compared to chemostat derived cultures (Fig. 3B).

Exposure to oxidative stress (20 mM H_2O_2) led to very fast killing (within 15 minutes) of the vast majority (93%) of the cells present in culture samples derived from the chemostat cultivation. Although exposure to this stress condition was also quite detrimental for the culture samples obtained from the retentostat cultivation, still after 15 minute of exposure to this level of oxidative stress, 85, 75, and 50% of the cells were inactivated in culture samples obtained after 14, 21, and 29 days of retentostat cultivation, respectively (Fig. 3C). Prolongation of oxidative stress exposure roughly sustained the magnitude of this relative survival difference between the different samples (Fig. 3C).

Taken together these experiments clearly establish that the previously determined elevation of the expression levels of stress associated genes during (extended) retentostat cultivation confer a substantial improvement of stress robustness to *L. lactis* KF147. Especially cells derived from prolonged retentostat cultivation, where growth rates approximated zero, appeared to be highly resistant to stress exposure.

Stress tolerance modeling. To better quantify the stress resistance of *L. lactis* KF147 culture samples derived from chemostat and different time-points of the retentostat cultivation, inactivation curves were analyzed by model fitting using two microbial survival models, which were the first-order kinetic and the Weibull model. The model-fitting performances of the two inactivation models were statistically compared and evaluated with the mean square error of the model (MSE_{model}), *f*, and *p* value (6). Notably, the first-order kinetic model fitted well in only 2 experimental conditions (out of 12 experimental conditions), according to the *f*-test (data not shown). Moreover, the Weibull model described the experimental results better and was acceptable for most of the experimental conditions (9/12), which was confirmed by *f*-test (Table 1). The measuring error was low for those conditions where the Weibull model fitting was not acceptable according to the *f*-test (3/12). Visual inspection of the fitting performance of the Weibull model showed that the adequacy of the Weibull model was sufficient. Furthermore, an additional *f*-test (36) confirmed that the parameter β of those acceptable experimental conditions significantly improved the description of the inactivation

data. Therefore, the Weibull model was considered the most suitable model to compare the *L. lactis* KF147 culture samples upon their exposure to different stress conditions.

Table 1: Statistical indices of the Weibull model for fitting the inactivation data for *Lactococcus lactis* KF147. Bold values indicate that the *F* test was accepted.

Sample / Stress condition		Heat (50˚C)	Acid (pH 2.5)	Oxidative (20 mM H_2O_2)
Chemostat (day 0)	RSS	0.312	0.174	0.117
	MSEmodel	0.026	0.015	0.010
	F	6.82	**2.73**	**1.38**
	F table	3.57	3.57	3.57
	p value	0.004	0.078	0.371
Retentostat (day 14)	RSS	0.069	0.047	0.015
	MSEmodel	0.006	0.003	0.001
	F	**0.99**	11.72	**3.14**
	F table	3.57	3.57	3.57
	p value	0.583	0.0004	0.052
Retentostat (day 21)	RSS	0.023	0.024	0.005
	MSEmodel	0.002	0.002	0.0004
	F	**1.94**	4.30	**1.22**
	F table	3.57	3.57	3.57
	p value	0.188	0.019	0.449
Retentostat (day 29)	RSS	0.028	0.006	0.025
	MSEmodel	0.002	0.0005	0.002
	F	**1.81**	**2.15**	**1.56**
	F table	3.57	3.57	3.57
	p value	0.22	0.148	0.30

In the Weibull model, δ represents the first decimal reduction time and β defines the shape of the curve (see Materials and Methods). As it was already apparent from the experimental data, the highest δ value was obtained using the stress-exposure kill-curves determined for the culture sample derived from the 29-days retentostat culture, irrespective which stress condition was evaluated. Specifically, the δ value of the retentostat-grown cells on day 29 were 2.5-3 folds higher than the δ value of chemostat-grown cells, when exposed to heat and acid stress conditions (Table 2). This increased heat and acid stress tolerance was already apparent, albeit to a lesser extent, in the comparison of the δ value calculated for earlier retentostat cultivation time-points relative to the chemostat culture values (Table 2).

Table 2: Comparision of Weibull model parameter estimates. *L. lactis* KF147 samples obtained from chemostat (day 0) and retentostat cultivation on day 14, 21, and 29 were exposed to heat, acid, and oxidative stress conditions and inactivation data were fitted using the Weibull model. Data points represent average ± standard deviation of measurements of two independent retentostat cultures.

Condition	Parameter	Chemostat	Retentostat		
		Day 0	Day 14	Day 21	Day 29
Heat (50˚C)	δ (min.)	44.4 ± 0.9	51.6 ± 0.7	71.7 ± 1.9	111.9 ± 1.3
	β	1.62 ± 0.05	1.10 ± 0.03	0.89 ± 0.01	0.85 ± 0.02
Acid (pH 2.5)	δ (min.)	44.3 ± 0.5	64.1 ± 0.2	84.3 ± 1.1	128.5 ± 2.2
	β	1.56 ± 0.0	1.63 ± 0.01	1.33 ± 0.01	1.23 ± 0.0
Oxidative (20 mM H_2O_2)	δ (min.)	12.4 ± 1.3	24.3 ± 0.9	36.2 ± 0.2	72.1 ± 2.5
	β	0.42 ± 0.01	0.49 ± 0.01	0.57 ± 0.01	0.80 ± 0.0

Modeling of the experimental data obtained when exposing culture samples to oxidative stress conditions confirmed the anticipated higher δ value for the samples taken from the retentostat cultivation relative to those taken from the chemostat cultivation (Table 2). Remarkably, robustness of cells expressed in δ values inversely associated with the growth rate of the related cell cultures that approached zero during retentostat cultivation, and bacterial survival increased in exponential fashion under heat, acid, and oxidative conditions (Fig. 4). These results indicate that prolonged exposure to extreme carbon-limitation can lead to a degree of induction of stress response mechanisms that clearly exceeds the basal levels of expression that can be observed upon initial carbon-limitation conditions (Fig. 4).

Discussion

This paper presents the increased stress tolerance of *L. lactis* at a near-zero growth rate, which is induced by carbon-limited retentostat cultivation. The stress tolerance evaluations were inspired by the observation that the transcription (10) of stress related genes was significantly induced during previous retentostat cultivations, where the expression level of the stress related genes was inversely correlated with the growth rate. The stress tolerance analyses established that these transcriptional changes confer strongly enhanced broad spectrum stress robustness to *L. lactis* cells at near-zero growth rates. The Weibull microbial survival model was used to determine the kinetic data of the survival curves under stress conditions. The

obtained δ values were negatively correlated with the growth rate of the culture samples, and when combining all datasets, could be tentatively correlated with the stress-gene expression values, creating a quantitative relationship between the transcriptional activities of these genes with cellular stress robustness.

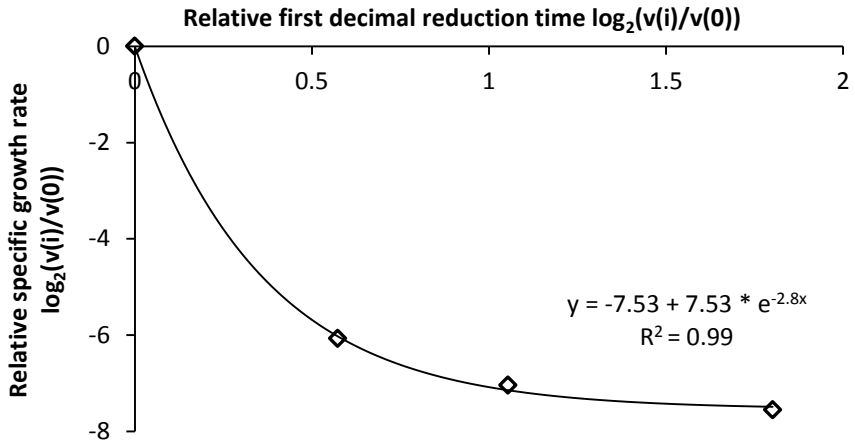

Figure 4: Correlation of robustness and the relative specific growth rate in *L. lactis* KF147 during retentostat cultivation. Robustness is determined with average of first decimal reduction time (δ) of retentostat-grown *L. lactis* KF147 cells over average δ of chemostat-grown cells under heat, acid, and oxidative stress conditions in $\log_2$-scale. The curve represents the best-fit model. The data fitting of the average δ value in $\log_2$-scale as a function of the relative specific growth rate data in $\log_2$-scale.

Bacterial cells can adapt to specific environmental stress condition by launching stress response reactions, these stress responses can provide cross-resistance to other stress conditions (7, 32). Cross-resistance is provided by the elevated expression of generic adaptive stress responses that provide survival advantage when cells are exposed to a single or a combination of stress factors. A well-described example of cross-resistance is the generic stress tolerance that cells can develop during the stationary phase of growth. In *L. lactis*, acid tolerance increases during logarithmic phase in batch cultivation without pH control, reflecting the adaptive response to the decreasing pH and the increasing levels of environmental lactic acid that is due to growth of the culture. Upon entry of the stationary phase, acid tolerance rises further as a consequence of the induction of a general stress response (15, 21). It has been reported that *L. lactis* cells taken during logarithmic, transition from logarithmic to stationary, and 3 hours into the stationary phase of growth, displayed a significantly improved resistance against heat- (30 minutes exposure to 52°C), acid- (2 hours at pH 4.0), and oxidative- (15 mM hydrogen peroxide exposure for 60 minutes) stress (15).

Cross-resistance studies have most commonly been performed using batch cultures that proceed from the exponential phase of growth to the stationary phase of growth (15, 34). However, these batch systems include the continuous increase of the concentration of end-metabolites as well as the accumulation of cell-material derived from deteriorating cells due to autolytic activity of *L. lactis* that stimulates cell-death (8, 9, 11, 15). As an alternative, adaptive responses of microorganisms to multiple stress conditions at low-growth rates can be achieved using chemostat cultivation, enabling cultivation at a specific, predetermined growth rate with constant environmental and/or nutritional conditions (8, 11). However, chemostat cultivations cannot readily be executed at extreme low dilution rates, because dilution rates of $< 0.05\ h^{-1}$ have been reported to result in heterogeneity of the population (2). Therefore, the examination of adaptive stress responses and cross-resistance of microorganisms using retentostat cultivation has great advantages compared to batch and chemostat approaches. The magnitude of acquired stress robustness achieved by prolonged retentostat cultivation appears to exceed that observed in stationary phase compared to logarithmically growing cells. As an example, the stationary phase *L. lactis* cells displayed a 1-log loss of viability after 30 minutes of heat (50°C) (17) or H_2O_2 (15 mM) exposure, or after 120 minutes of exposure to pH 4 (15), whereas the retentostat derived cells tested in the present study appeared to be more resistant (1-log reduction was only reached after more than 2 hours at 50°C, or pH of 2.5, and more than 70 minutes of exposure to 20 mM H_2O_2). Furthermore, the late-exponential phase *L. lactis* cells tested in this study displayed a 3.5-4.5-log loss of viability after 60 minutes of heat (50°C), H_2O_2 (20 mM), or acid (pH 2.5) exposure, whereas the chemostat (dilution rate of $0.025\ h^{-1}$) derived cells displayed only a 2-log reduction in viability after 60 minutes of exposure to the same stress conditions. Taken together, these results imply that prolonged retentostat culture samples that exhibit extremely slow-growth rates are considerably more tolerant to various stress conditions as compared to, not only late-exponential and stationary phase culture derived samples, but also chemostat derived samples. Moreover, carbon-limited retentostat cultivation encompasses a gradual transition from a growing to a near-zero growth state under stable environmental conditions and sustains cell-viability (10), illustrating that this approach can deliver high viable biomass yields of cells with high stress robustness.

Quantitatively linking microbial responses at molecular and phenotypic levels facilitates mechanistic understanding of stress adaptation and allows the identification of cellular

indicators for bacterial robustness (1, 5). For this reason, numerous mathematical models have been developed to quantitatively describe microbial survival curves, which depend on different factors including the type of bacteria, the composition of medium, stress intensity, and the stress treatment regime. In this study, the Weibull model was employed to compare the kill-curves, using concave upward or downward curve-descriptions for each of the stress exposure kill-curves, and thereby estimated accurately the first decimal reduction time of culture samples that were exposed to stresses.

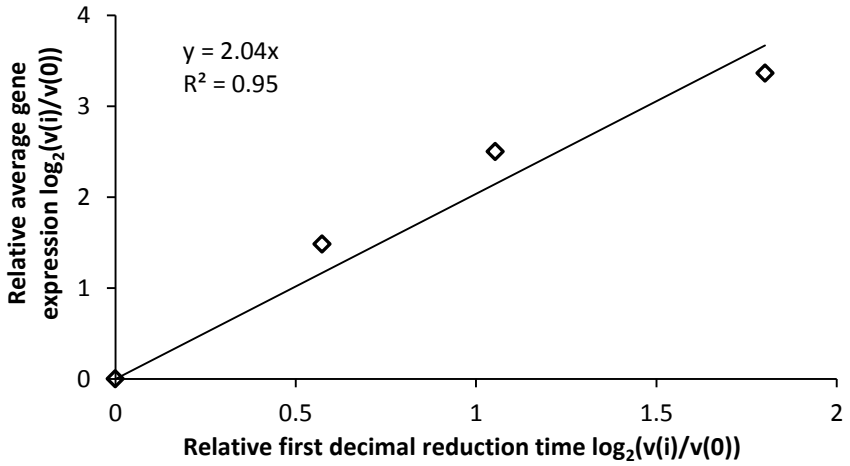

Figure 5: Relative expressions of stress response genes and robustness correlation in retentostat-grown *L. lactis* KF147. Robustness is determined with average of the first decimal reduction time (δ) of retentostat-grown *L. lactis* KF147 cells over average δ of chemostat-grown cells under heat, acid, and oxidative stress conditions in $\log_2$-scale. Expressions of genes involved in heat, acid, and oxidative stress responses were made public in Ercan, *et al*., 2014.

The tentative relationship between the expression levels of stress related genes and the observed first decimal reduction time showed that the genes typically associated with heat, acid, and oxidative stresses directly correlated with the increasing stress tolerance levels (Fig. 5 & 6). Furthermore, each correlation analysis, including growth rate-stress gene expressions, growth rate-robustness, and stress gene expression-robustness, was quantitatively modeled with high accuracy, identifying correlation coefficients of growth, robustness and gene expression levels (Fig. 6). These observations imply that genes that are typically associated with heat (*e.g. groEL-groES*, *hrcA-dnaK-grpE-dnaJ*)-, acid (*e.g. uvrB*, *rcfB*)- and oxidative-stress responses (*e.g. rmaB*, *lmrA*) could be defined as biomarkers for enhanced robustness in *L. lactis* under prolonged retentostat cultivation. In addition, these candidate biomarkers could

allow quantifying the robustness level of retentostat-grown lactococci under specific stress conditions.

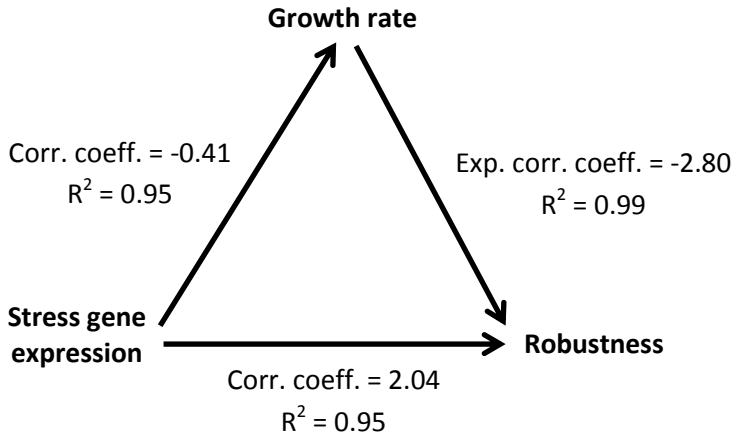

Figure 6: Basic scheme of correlation between specific growth rate and expression of stress related genes and robustness of *L. lactis* KF147. Correlation coefficients (Corr.coeff.) were calculated based on the direction of arrows.

Next to the physiological and metabolic adaptations reported earlier (9, 10), the present study illustrates that stress tolerance mechanisms are highly expressed in cultures that reach near-zero growth conditions during retentostat cultivation. These gene expression changes confer a substantial phenotypic robustness to *L. lactis* KF147 when it is exposed to heat, acid and oxidative stress conditions, and exemplify the importance of cross-resistance in stress responses. The enhancement of stress robustness at near-zero growth rates is likely to provide a great evolutionary advantage for bacteria in natural ecosystems, where near-zero growth rates are likely to occur frequently, and stress robustness can contribute to environmental persistence in a viable form, awaiting the improvement of the environmental conditions that allow the regain of growth and proliferation. Enhanced stress robustness of lactococci may benefit their industrial application through higher survival percentages of starter cultures during their production and processing, but may also impact on their *in product* functionality by supporting their prolonged survival, which may enhance their contribution to flavor formation during ripening or may extend the shelf-life of products that are marketed to deliver health beneficial probiotic bacteria.

Acknowledgements

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Supplementary information for chapter 4

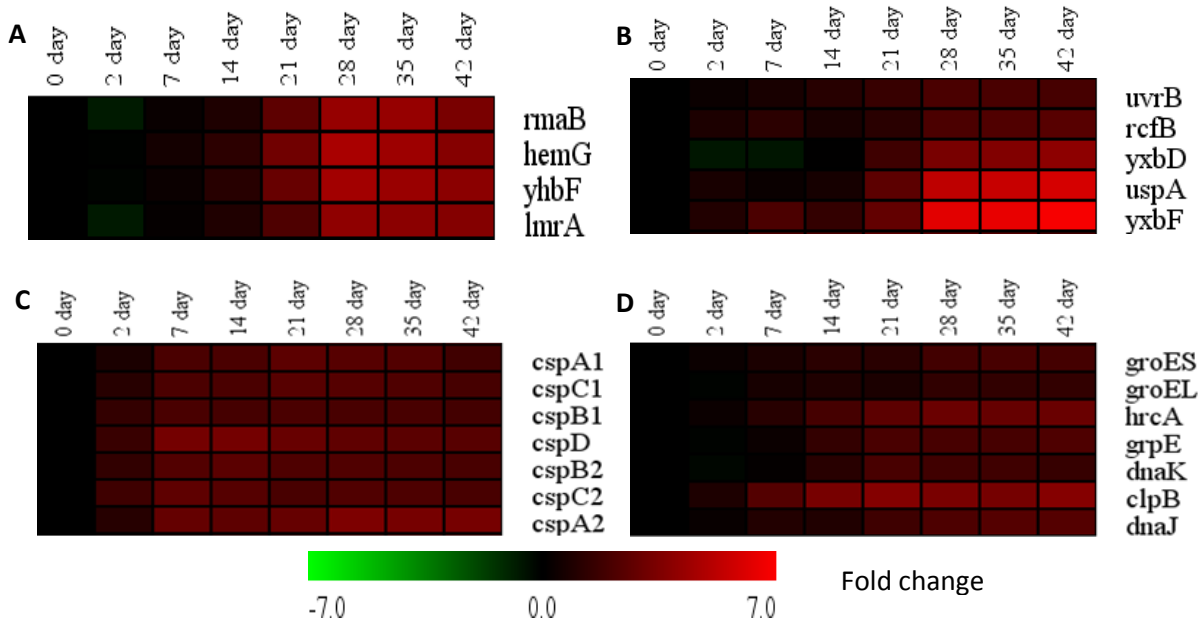

Figure S1: Heat map of *L. Lactis* KF147 (A) cell membrane, (B) acid, (C) cold-shock, and (D) heat stress responses genes differentially expressed (in $\log_2$-scale, p-value ≤ 0.05) during retentostat cultivation on days 2, 7, 14, 21, 28, 35, 42 over the beginning of the chemostat (day 0) made public in Ercan, *et al*., 2014).

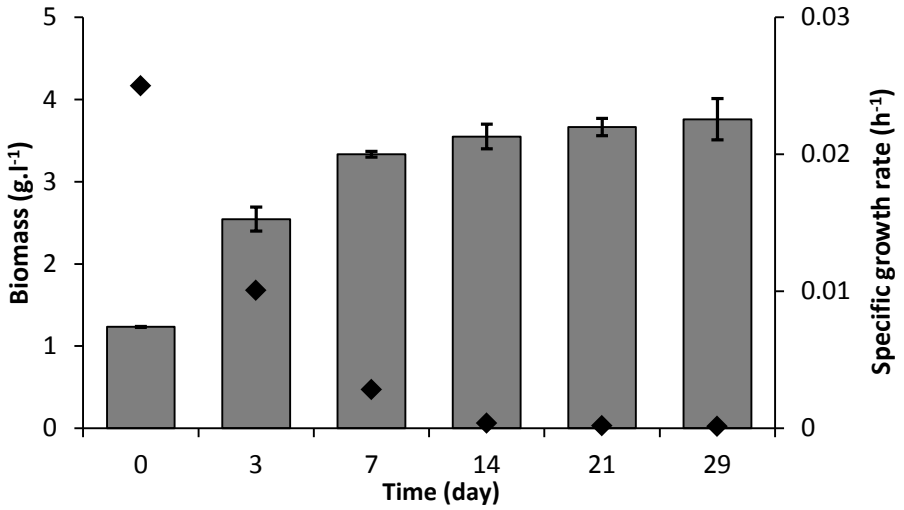

Figure S2: Growth of *L. lactis* KF147 in retentostat culture. A steady-state anaerobic chemostat culture was switched to retentostat mode at time zero. Biomass accumulation (bars) and specific growth rate (diamonds) of *L. lactis* KF147 under retentostat conditions. Data points represent average ± standard deviation of measurements of two independent retentostat cultures.

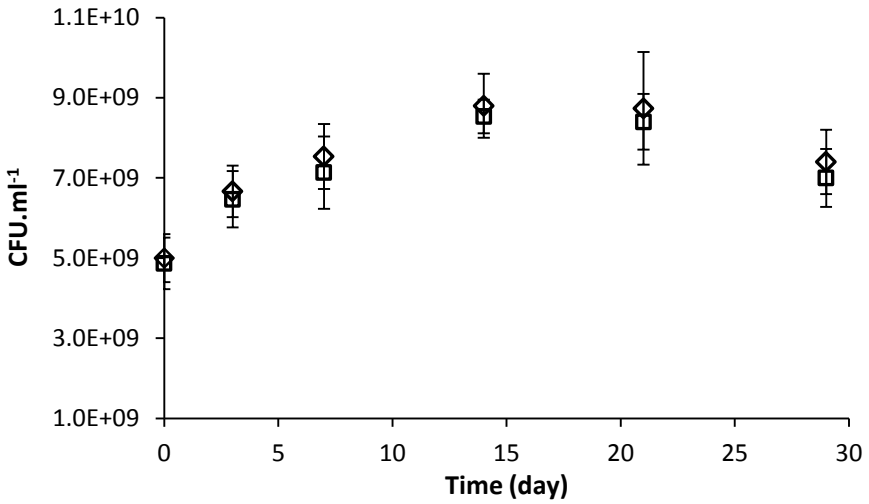

Figure S3: Viability of *L. lactis* KF147 under retentostat conditions. Culturable cell estimation in retentostat 1(diamonds) and 2 (squares) by plate count (cfu.ml^{-1}) on solid medium. A steady-state anaerobic chemostat culture was switched to retentostat mode at time zero. Data points represent average ± standard deviation of measurements of two independent retentostat cultures.

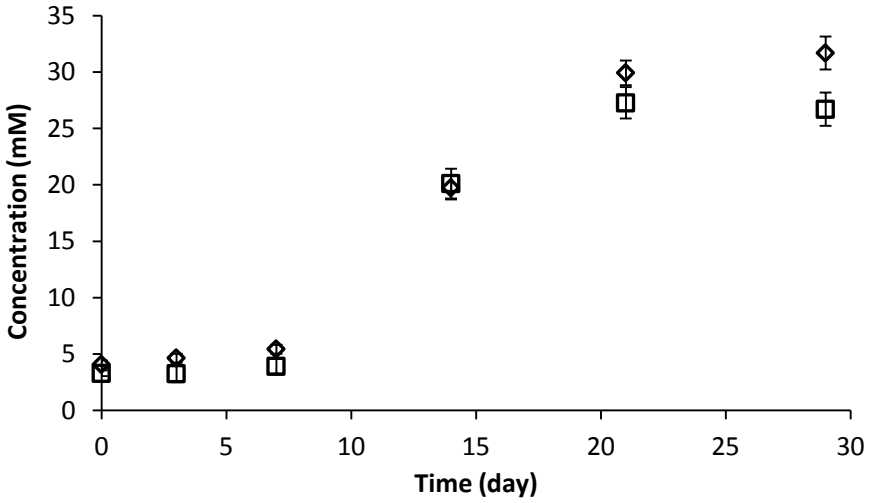

Figure S4: Lactic acid profile of *L. lactis* KF147 in retentostat culture. Concentrations are expressed as the difference between the measured concentration in the medium feed and the measured concentration in the filter line samples from retentostat 1(diamonds) and 2 (squares). A steady-state anaerobic chemostat culture was switched to retentostat mode at t = 0. Data points represent average ± standard deviation of measurements of two independent retentostat cultures

Chapter 5

Molecular responses of *Lactococcus lactis* to severe carbon starvation in non-growing cells

O. Ercan, M. Wels, E. J. Smid, and M. Kleerebezem

Manuscript in preparation

Abstract

This paper describes the adaptations of non-growing, retentostat cultures of *Lactococcus lactis* to severe starvation elicited by switching off the medium supply. Near-zero growth cultures (μ=0.0001 h^{-1}) obtained by extended retentostat cultivation were exposed to starvation by termination of the medium supply for 24 hours, followed by a recovery period of another 24 hours by re-initiating the medium supply to the retentostat culture. During starvation, the viability of the culture reduced only slightly, and the expression of genes involved in transcription and translational machineries, cell division, and cell membrane energy metabolism was strongly repressed. Expression of these genes was largely recovered following the re-initiation of the medium supply. Starvation triggered the elevated expression of genes associated with branched chain amino acids, histidine, purine, and riboflavin biosynthesis, which appeared to remain expressed at an elevated level after re-initiated of the medium supply. Notably, starvation also strongly induced the complete gene set predicted to be involved in natural competence in *L. lactis* and expression was sustained during the subsequent recovery period, but we failed to demonstrate natural transformation in these cells. A conserved *cis*-acting element that resembles the lactococcal CodY motif and includes a highly conserved palindromic sequence (CTGWCAG) was detected in the upstream regions of several regulated genes associated with the transcription and translational machinery, purine biosynthesis, and natural transformation in *L. lactis*, suggesting a bimodal role of the master-regulator CodY in the observed transcriptome adaptations to severe starvation in non-growing cells.

Keywords: *Lactococcus lactis*, transcriptome, starvation, stringent response, CodY-regulation

Introduction

Lactococcus lactis has a long history of use in fermented food manufacture and food preservation. Starter cultures of *L. lactis* have a great economic impact in the production of cheese due to their role in conserving the product by acidification. In addition, *L. lactis* contributes to the texture and formation of flavor in cheese, which are key-determinants of the product's sensory quality (32, 33). Next to these industrial food fermentation applications, this species is frequently found in other environments such as in (decaying) plant material, especially when nutrients become more available due to primary degradation of plant-polymers by other microorganisms, including yeasts and fungi (31).

Natural microbial populations mostly live in famine conditions due to limited carbon and energy source availability (22). Similarly, microbes may encounter extremely restricted access to carbon sources for longer periods in industrial fermentation applications. For instance, lactic acid bacteria (LAB) encounter long periods of severely low or no carbon availability during the ripening process of dry sausage productions (19) and cheese (33), but are able to survive in these conditions during months of ripening (5, 12, 19).

Physiological and molecular level responses of microorganisms to growth stagnation as a consequence of nutrient starvation have been studied in batch cultures that proceed from the logarithmic phase of growth to the stationary phase of growth. An alternative design to study starvation in bacteria is at a steady-state continuous cultivation in which the medium supply is turned off (11, 26). However, stationary phase induced by batch and continuous cultures of LAB encompasses a major bottleneck that is generally not encountered in natural habitats. Both cultivation systems of LAB undergo continuous changes in culture conditions due to the increasing concentrations of fermentation end-metabolites (*e.g.* lactic acid) and eventually the accumulation of dead cells that emerge from stimulated cell-death that involves an autolysis process (14). Consequently, it is difficult to disentangle the effects elicited by nutrient depletion from those elicited by end-metabolite accumulation and cell debris exposure.

Retentostat cultivation provides an alternative way to study starvation in microorganisms. Retentostat cultivation is an adaptation of chemostat cultivation in which complete biomass retention in the fermenter is achieved by continuous removal of spent medium through a cross-flow filter effluent channel (37). As a result of cell retention, the microbial biomass

concentration increases over time while the carbon source supply remains constant, which results in a decreasing amount of carbon source availability for each individual cell (3, 11, 16). Prolonged retentostat cultivation leads to growth rates that approximate zero and induces a cellular physiology in which the metabolic energy is completely invested in maintenance associated processes (*e.g*. osmoregulation, turnover and repair of damaged cellular components, activation of defense mechanisms and proofreading) (11, 36), which support high cell viability, and is different from stationary phase induced starvation (11). Recent retentostat studies with *L. lactis* at a near-zero growth rate illustrated that lactococcal adaptation to these conditions includes the de-repression of genes involved in alternative carbon source import and metabolism and stress response functions, as well as the repression of cell membrane metabolism (9, 10). Some of these adaptations were also observed in batch cultures that entered the stationary phase of growth (27). However, contrary to batch culture stationary phase conditions, retentostat cultivation did not induce repression of functions related to central metabolism, cell division, and macromolecule synthesis, or the induction autolytic processes and cell death. These findings illustrate the difference of these two approaches, and illustrate that retentostat cultivation enables the study of nutrient starvation in non-growing cells without the confounding effects of metabolite accumulation and the induction of autolysis and cell-death.

In the present study, we determined the transcriptional responses of *L. lactis* KF147, which was originally isolated from mung bean sprouts, to severe carbon starvation in zero-growth cultures obtained by retentostat cultivation. Initially, *L. lactis* was grown under anaerobic, carbon-limited retentostat conditions for 42 days (11). The obtained near-zero growth cultures were exposed to severe starvation by terminating the medium supply for 24 hours, followed by a recovery phase induced by restarting of the medium supply for another 24 hours. During severe starvation the expression of genes related to cell division, the transcription and translational machinery and cell membrane energy metabolism were significantly decreased, whereas viability of the culture remained high. Subsequent re-initiation of the medium supply led to the recovery of initial expression levels of the translational machinery and energy metabolism associated genes. Strikingly, the gene repertoires predicted to be involved in natural transformation were highly induced during the starvation phase, which was sustained during the recovery period. The transcriptome adaptations are evaluated in the context of their

regulation, where we identify a conserved *cis*-acting regulatory motif and propose a regulatory role of CodY in controlling expression of the translational machinery and natural competence.

Materials and Methods

Bacterial isolates, media and cultivation conditions. *Lactococcus lactis* subsp. *lactis* strain KF147 originates from mung bean sprouts, and its genome sequence was determined (31). Frozen stock cultures from -80°C were used to inoculate 50 ml M17 broth (35) supplemented with 0.5% glucose (w/v) and grown overnight at 30°C, which were used as pre-cultures for retentostat cultivations (11). After overnight cultures had been harvested by centrifugation (6,000 g, 10 min., 4°C) and washed twice with physiological salt solution (0.9% NaCl in water), the culture was inoculated into CDM containing 0.5% glucose (w/v) (16). To retain the medium composition constant during long-term cultivation, 120-liter batches of medium were prepared, filter sterilized, and used during retentostat cultivations.

Duplicate carbon-limited retentostat cultivations with 1.5-l working volume were performed under anaerobic conditions, commencing from chemostat cultivation at a dilution rate of 0.025 h^{-1} as earlier described (11). When the estimated specific growth rate decreased to 0.0001 h^{-1} after 42 days of retentostat cultivation (11), the supply of fresh medium was switched off for 24 hours. This caused immediate and severe carbon-source starvation since the retentostat cultivation was already performed under carbon limiting conditions (11). After 24 hours of starvation, the supply of fresh medium was switched on again, and retentostat cultivation was continued for another 24 hours. During these starvation and retentostat conditions, all other fermentation parameters were kept constant; including temperature of 30°C, nitrogen flow at 15 ml/min, stirring speed of 100 rpm, and pH of 5.5 (11). Since removal of samples could potentially influence the fermenter-conditions and the bacterial culture, sample volume and sampling frequency were minimized.

Biomass, substrate and metabolites determination. During the 24 hour starvation period and subsequent re-initiation of 24 hour retentostat cultivation, culture samples were taken at regular intervals to measure cell dry weight (CDW), glucose, and organic acid concentrations. CDW was measured using pre-dried and -weighted membrane filters with a pore size 0.45µm (Merck Millipore, Darmstadt, Germany) as previously described (11). Concentrations of

organic acids and residual glucose in the culture supernatant were determined by high performance liquid chromatography (HPLC) (11). Supernatant organic acid, ethanol, and glucose patterns were determined by HPLC using a Rezex ROA-organic acid column (300 x 7.8 mm) (Phenomenex Inc., CA, USA), with sulphuric acid (5 mM; 0.6 ml min^{-1}) as mobile phase at 60°C, lactate, acetate, formate, ethanol, and glucose were detected and quantified by refractive index detector (Shimadzu Scientific Instruments, MD, USA) (20).

Cell viability and culturable cell estimation. The viability of cells in the culture was assayed using the LIVE/DEAD *BacLight*™ Bacterial Viability and Counting Kit (L34856) (Molecular Probes Europe, Leiden, The Netherlands), according to the manufacturer's instructions, using the BD FACSAria II flow cytometer (BD Biosciences, San Jose, CA, USA). The kit contains two fluorescent dyes, which intercalate with DNA: Red fluorescent propidium iodide (PI) and green fluorescent SYTO9. While SYTO9 can diffuse into cells through intact cell membranes, PI can only penetrate cells when cell membranes are permeabilized. In addition, viability was also assessed by enumeration of colony forming units (CFUs) on GM17 agar plates, using serial dilutions of the cultures (10-fold dilution in M17 broth) in triplicate (30).

RNA isolation and transcriptome analysis. Microarray data used in this study has been merged into the dataset described in (10) and represented as day 42 (the end of retentostat cultivation; t=0 hour of carbon starvation), 43 (after 24 hour of starvation condition; t=24 hours), and 44 (after 24 hour of re-initiated retentostat cultivation; t=48 hours). The microarray hybridization scheme for the transcriptome analyses of this experimental set-up consisted of a compound loop design with 7 arrays (Fig. S6). Array data and the experimental procedure are accessible at Gene Expression Omnibus (GEO) under accession number GPL17806 and GSE51494 (http://www.ncbi.nlm.nih.gov), respectively.

The gene expression intensities were compared and clustered using Short Time-series Expression Minor (STEM) (version 1.3.6, http://www.cs.cmu.edu/~jernst/stem/) (13). The STEM Clustering Algorithm enriched with Gene Ontology (GO) terms was applied with Bonferroni correction method, a maximum number of model profiles of 8, a minimum unit change in model profile between time points of 2 (ratio change of 1 in log_2-scale). Profiles with statistically significant numbers of gene assignments were selected and significantly

changed genes expression was visualized using heat maps using the MultiExperiment Viewer (MeV) (http://www.tm4.org/mev/) (28).

The correlation of the transcriptome data at each time-points in duplicate retentostat cultivations were calculated by Pearson correlation coefficient in Microsoft Excel by using Data Analysis ToolPak add-in and hierarchical clustering linkage was performed using the MeV tool.

DNA motif mining. Gene expression ratio data from the significant model profiles of the STEM clustering was used as a data source to mine for transcription factor binding sites (TFBSs) in the genome of *L. lactis* KF147. The PePPeR database was used as a source of literature based regulon clusters (6). Binding sites searches were performed for sequences within 300 bp upstream of the translational start site of each gene. To identify upstream binding sites the algorithm for fitting a mixture model by expectation maximization (MEME) was performed (1), using the parameters mod anr (unlimited number of motifs per upstream sequence), revcomp (allowing motifs to be present on both "+" and "-" strand), and allowing for maximally 3 motifs to be found and without any restriction on the total number of motifs to be found.

Results and Discussion

Biomass accumulation and viability in retentostat-induced carbon starvation. In our previous study, near-zero growth cultivation of *Lactococcus lactis* KF147 was achieved using an anaerobic, carbon-limited retentostat set-up (11). During retentostat cultivation, the carbon source was fed at a constant rate, while biomass accumulation increased, reaching a steady-state after 14 days and growth rates declined to approximately 0.0001 h^{-1} after 42 days of retentostat cultivation (11). Consequently, no carbon source was available for production of biomass, as illustrated by the extremely extended culture doubling time of more than 260 days, at which stage the entire carbon feed was employed for maintenance related process in the cells (11). To contrast and compare the physiological and transcriptional responses of *L. lactis* KF147 at near-zero growth (retentostat) and strict carbon starvation conditions, the medium supply was switched off after 42 days of retentostat cultivation, thereby exerting a virtually immediate and severe carbon starvation. During the 24 hour starvation period (t=24), biomass concentration, culture viability, and fermentation metabolite levels were determined

at the starting point (stable retentostat condition; t=0) and after 6, 14, and 24 hours (t=6, 14, and 24). Subsequently, the medium supply was switched on again (recovery phase) and the same parameters were determined after an additional 24 hours of retentostat cultivation (t=48). In two independent experiments the biomass concentration remained constant during 24 hour of starvation and subsequent 24 hour of recovery (Fig. 1). In parallel, viability of the culture was assessed using the enumeration of colony forming units (CFUs) (Fig. 1) and LIVE/DEAD staining linked with fluorescent activated cell sorting (FACS) analysis (Fig. 2). CFU enumeration by plate counting established only a very slight decrease of CFU during the 24 hours of starvation, which remained stable during the subsequent period of re-initiated retentostat cultivation (Fig. 1). Previously, the viability assessment by discriminative staining

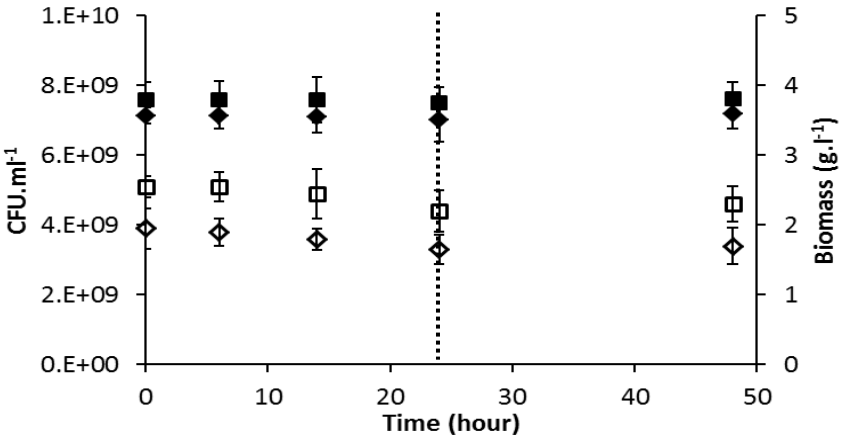

Figure 1: Biomass and CFU enumeration of *L. lactis* KF147 grown under extensive retentostat conditions (t=0), and medium starvation (up to 24 hours), and subsequent recovery period in retentostat cultivation (t=48). The dashed line indicates the end of the starvation. Biomass concentration ($g.l^{-1}$) of independent cultures 1 (closed diamonds) and 2 (closed squares), and colony forming unit enumerations ($cfu.ml^{-1}$) of independent cultures 1 (open diamonds) and 2 (open squares) are indicated. Error bars indicate the standard deviation of triplicate measurements.

and FACS analysis of *L. lactis* culture samples from the extended retentostat fermentation revealed a division of subpopulations on the basis of their SYTO9 and propidium iodide (PI) staining characteristics, which were classified into live, dead and damaged cell populations. The latter state was tentatively considered as a transition state that could no longer form a colony on solid media, but was still intact (11). Using the same approach in the present study revealed that the live population decreased slightly (form approximately 76% to 69 %) during the starvation period and remained stable during the subsequent recovery period, which is in

good agreement with the CFU enumeration analysis (Fig. 2). In parallel, the damaged cell population also decreased during the starvation period from approximately 21% to 15%, whereas the population that stained with both dyes (*i.e.* the dead population) increased from 4% to almost 17% of the total population and these damaged and dead population estimates remained stable during the recovery period (Fig. 2).

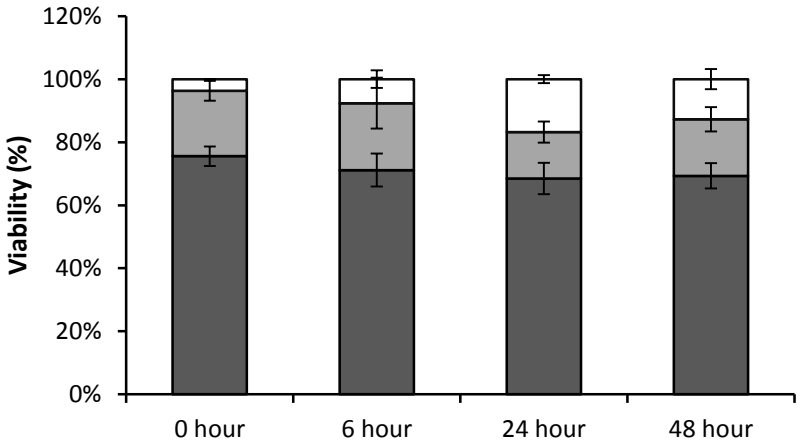

Figure 2: Viability assessment of *L. lactis* KF147 after extended (42 days) retentostat cultivation (t=0 hour) and subsequent severe carbon starvation for 6 and 24 hours, followed by 24 hours of recovery period in retentostat cultivation (t=48 hour). Percentages of biomass viability were assayed by LIVE/DEAD *BacLight* kit using FACS during the cultivations. This method enables discrimination of percentages of live (dark gray), damaged (light gray), and dead (white) cell populations. Error bars indicate the standard deviation values of two independent cultivations.

Metabolic profiling in retentostat and starvation conditions. The levels of the main fermentation metabolites lactate, formate, acetate and ethanol, were determined (Fig. S1). Due to extremely low-growth rate of *L. lactis* at the end of retentostat cultivation, a mixed-acid fermentation was observed with a relative metabolic distribution of 82% oriented towards formation of formate, acetate and ethanol and 18% towards formation of lactate (Fig. S1). The organic acid levels remained constant during the 24 hour starvation and recovery periods in the current experiment, suggesting that during the starvation period *L. lactis* is not utilizing its fermentation products in secondary fermentation processes that may generate additional energy (*e.g.*, lactate conversion to acetate). Moreover, these data also illustrated that upon re-initiation of the medium supply the culture executes the same carbon flux distribution as observed for the initial near-zero growth retentostat culture (Fig. S1). Notably, at any stage during the experiment, all carbon sources available in the medium (glucose and citrate) were

completely consumed and carbon balances calculated on basis of the major fermentation end-products were consistently above 95% (data not shown).

Transcriptomic adaptations to starvation and recovery. To investigate genome-level adaptation of near-zero growth *L. lactis* KF147 to severe starvation conditions, transcriptome analyses were performed in samples derived from the two independent retentostat cultures. Samples were withdrawn before initiating starvation (t=0; at the end of 42 days of retentostat cultivation), after 24 hours of starvation (t=24; starvation), and after 24 hours of subsequent re-initiated medium supply in retentostat cultivation mode (t=48; recovery). The initial analysis of the normalized transcriptome datasets included the comparative analysis of the two retentostat fermentations, revealing that samples were strongly discriminated by their time-point of sampling, while the samples taken at the same time-point in the two independent cultivations were highly similar (Fig. 3).

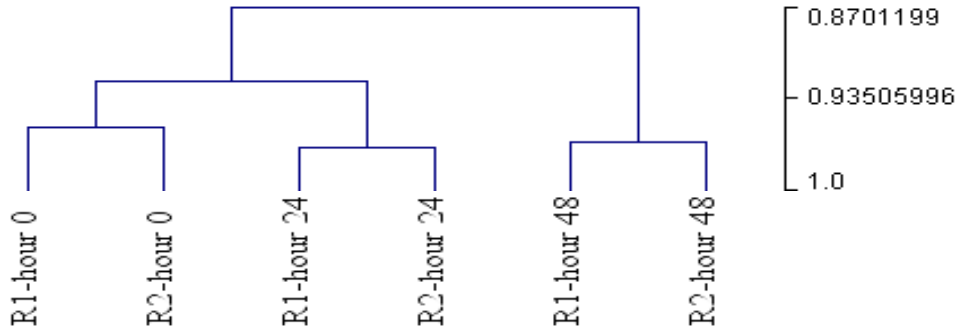

Figure 3: Hierarchical clustering linkage of transcriptome patterns obtained from retentostat 1 (R1) and 2 (R2) samples. Complete clustering linkage was performed for samples hours at 0, 24, and 48 of duplicate cultivations initiated with near-zero growth retentostat cultures after 24 hour of starvation and re-initiation of nutrient supply (48 hours) conditions based on Pearson correlation analyses using MeV.

To identify gene expression changes during the course of the experiment, absolute expression levels of all genes (2533 annotated genes in the *L. lactis* KF147 genome) were subjected to expression cluster analysis using the Short Time-series Expression Minor (STEM) module, which uses a process of statistical clustering of short time-series datasets into pre-composed patterns of time dependent expression (13). STEM clustered 53% and 46% of the annotated *L. lactis* KF147 genes into three statistically significant model profiles (Fig. S2A & S2B; and Table 1), using the datasets derived from the two independent cultivations, respectively. To explore specific transcriptional responses to starvation and recovery conditions, we focused

on gene profiles with similar characteristics; *i.e.* model profile 3, characterized by repressed expression during the starvation period (t=24) and recovered expression during the recovery period (t=48), and model profile 6 and 7 that are characterized by induction of gene expression during the starvation period (t=24), followed by respectively sustained high expression or dampening of expression, during the recovery period (Fig. S2A & S2B).

Table 1: Gene Ontology (GO) enrichment analysis. This table contains the GO enrichment results for the set of genes shown in the figure S2 where the enrichment is computed based on actual size enrichment (*p*-value ≤ 0.01).

Model Profile #	**GO Category Name**
3	Cellular macromolecule metabolic process Protein metabolic process Primary metabolic process Ribosome Nucleic acid binding Gene expression Signal transducer activity Proton-transporting ATP synthase complex Transferase activity Cell division
6 7	Purine nucleoside monophosphate metabolic process Purine ribonucleoside monophosphate metabolic process Integral to membrane Membrane Organic acid transmembrane transporter activity Amino acid transmembrane transporter activity Heterocycle biosynthetic process

Model profile 3 encompasses 328 and 360 genes in the two independent experiments, respectively, and 252 of those genes are shared in both experiments (Fig. S3). Within these model profiles the gene ontology (GO) terms that were strongly overrepresented included "cellular macromolecule metabolic process", "protein metabolic process", "ribosome", "gene expression", "proton-transporting ATP synthase complex", "cell division" (Table 1). Many transcriptional regulator encoding genes (*bglR*, *glnR*, *hslR*, *nisRK*, *tcsKR*, *truA*, *uxaR*, etc.) (Fig. 4A & S4A) and the complete membrane associated ATP synthase encoding operon (*atpABCDEFH*) were included in these model profiles (Fig. 4E & S4E). In addition, many

genes involved in DNA replication (*dcm*, *dnaAC*, *infC*, *lemA*, *papL*, *parC*, *rexB*, *topA*, *xerC*), components of the RNA polymerase (*rpoAEZ*) (Fig. 4B & S4B), several ribosomal proteins (*rplIKOQU*, *rpmBEFGIHJ*, *rpsJKLMO*) (Fig. 4C & S4C), and cell division and autolysis associated genes (*ddl*, *divIVA*, *engB*, *ftsAEWXZ*, *murDFGI*, *acmAD*) (Fig. 4D & S4D) were also assigned to the model profile 3. These genes appear to be repressed in response to the severe carbon and energy starvation when the medium supply is shut-down, but they recover their expression upon re-initiation of the medium supply. These observations indicate that *L. lactis* KF147 adapted to starvation conditions through stringent responses, which suppress the translational machinery, DNA replication, and cell division. Notably, members of the *acm* gene family play prominent roles in stationary phase induced autolysis (4), but the *N*-acetyl muramidases (*acmAD*) assigned to the profile 3 has also been reported to play a central role in cell wall repair and remodeling (34). The observation that only a marginal reduction of CFU enumeration is observed during these experiments implies that under these conditions the autolytic phenotype may not be induced, which contrasts the typical induction of autolysis in stationary phase cells obtained from batch cultures.

Remarkably, stringent responses have also been associated with the induction of stress response genes, including heat-, acid-, and cold-shock genes (27), and also starvation conditions during batch-culture stationary phase induced several stress responses in *L. lactis* (18, 27). These responses were not observed in the experiments performed here, and this apparent difference is likely explained by the fact that retentostat-preculturing and near-zero growth conditions already led to very strong induction of a panel of stress related functions (9) in the cultures that were used as the starting point in the current experiment (t=0). This retentostat induced high level of expression of stress-related genes appeared to be sustained during the starvation and recovery periods of the current experiments. This implies that these stress responses in *L. lactis* are important for maintenance of cellular function and viability in near-zero growth conditions, as well as extreme starvation conditions, which may reflect the evolutionary advantage of stress-robustness for bacteria that encounter conditions that do not permit growth and include (temporal) severe nutrient starvation (24, 29).

Model profiles 6 and 7 were clustered together because these profiles are both characterized by elevated expression levels during starvation (t=24), which was either remained at high

level of expression (profile 7) or declined level of expression (profile 6) during the subsequent recovery period. The profiles 6 and 7 of the two replicate experiments collectively shared many genes, but their precise distribution between profiles 6 and 7 appeared to be more inconsistent as illustrated by the observation that profile 7 of experiment 1 (748 genes) shared a substantial amount of genes with both model profiles 6 (291 of 504 genes are shared) and 7 (267 of 296 genes are shared) of its replicate, experiment 2 (Fig. S2A, S2B, and S3). These

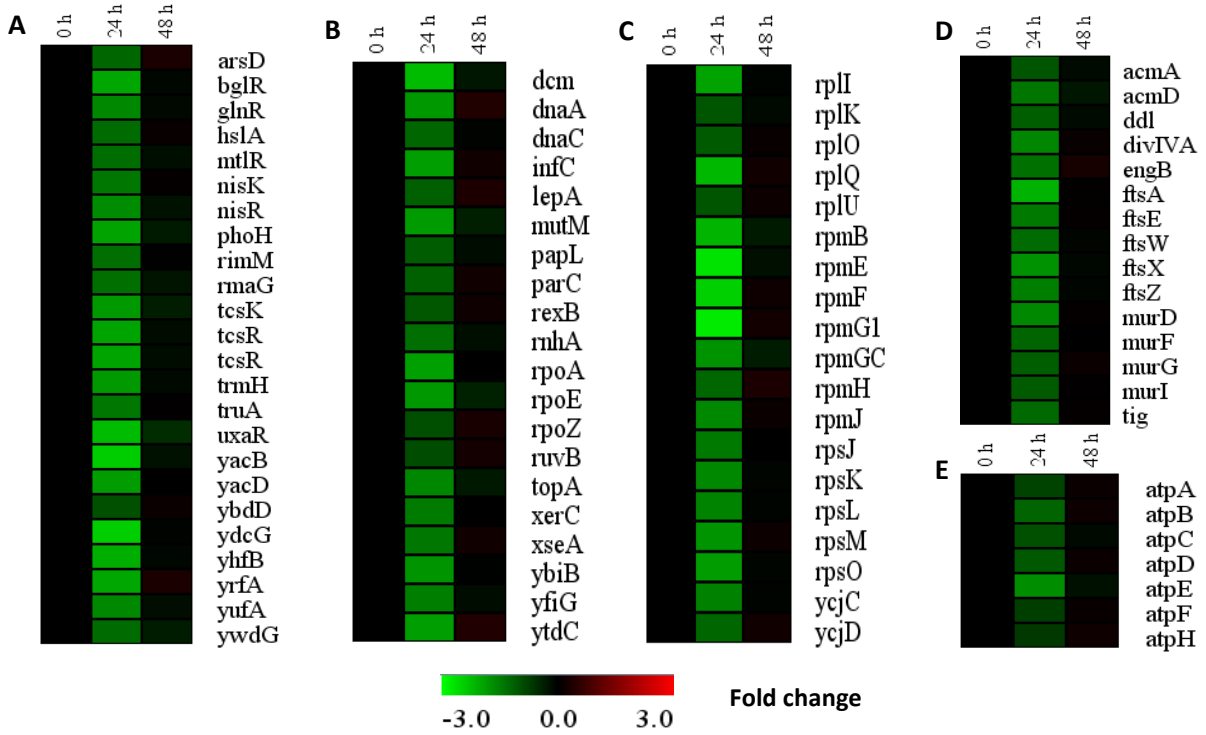

Figure 4: Heat map of *L. lactis* KF147 genes differentially expressed (in $\log_2$-scale, p-value ≤ 0.05) under medium starvation (24 h) and recovery period in retentostat cultivation (48 h) compared to the initial retentostat culture (0 h) that are clustered in model profile 3 using the STEM module. Data are derived from one of two replicate cultures (culture 1), and the corresponding dataset obtained in the replicate culture is provided in Supplementary figure S4. Genes are grouped in statistically significant GO categoreis (A) transcriptional regulators, (B) replication, (C) ribosome functions, (D) cell division and lysis, and (E) ATP synthases.

combined model profiles were significantly enriched for the functional categories related to "purine nucleoside monophosphate metabolic process", "integral to membrane", "organic acid transmembrane transporter activity", "amino acid transmembrane transporter activity", and "heterocycle biosynthetic process" (Table 1). Particularly, histidine, branched chain amino acids valine, leucine, and isoleucine, purine, and riboflavin biosynthesis associated *hisACDGHBFIK*, *ilvBD*, *leuA*, *purCDEFHKLMNQ*, and *ribABH* genes, respectively, were

highly expressed during starvation, which was sustained during recovery (Fig. 5A & S5A). In addition, the genes encoding spermidine/putrescine (*potA2B1B2C1C2D1*), potassium (*kdpBCE*), cobalt (*corA* and *cbiQ*), gamma-aminobutyric acid (GABA) (*yibEFG*, *ysjA*, *yshA*) metabolism, and multidrug resistance transporters (*blt*, *cydCD*, *ydiCD*, *ypgD*, *sugE*) were strongly induced during the starvation period (Fig. 5B & S5B).

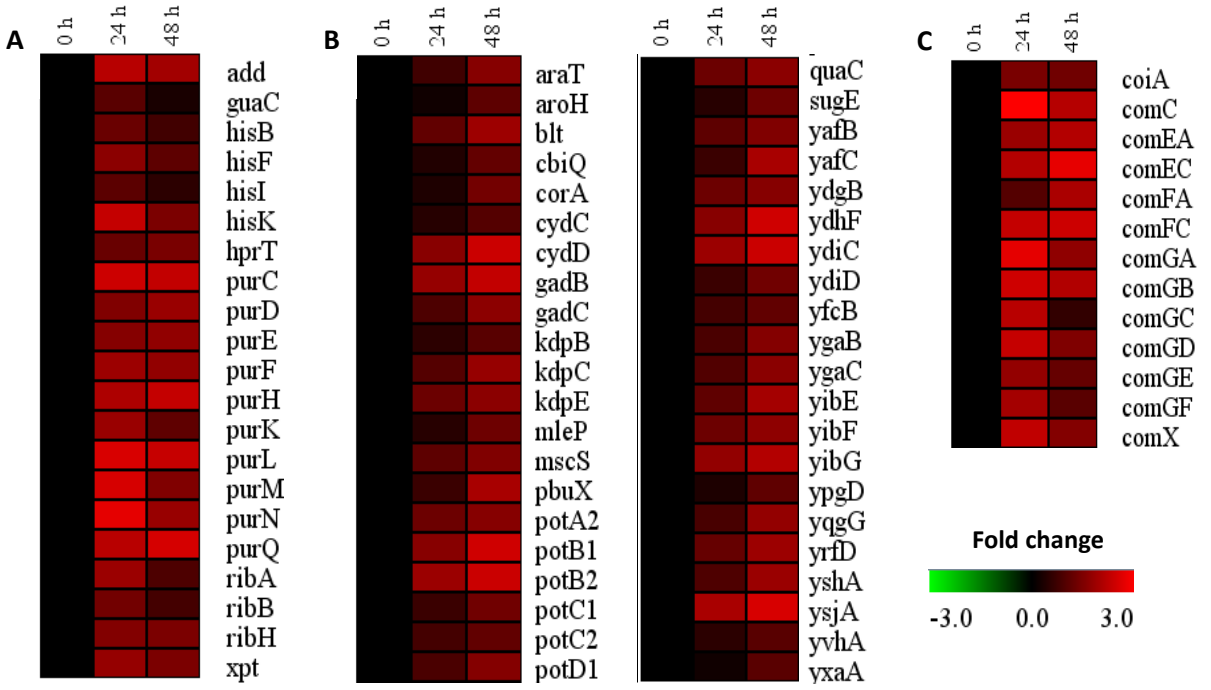

Figure 5: Heat map of *L. lactis* KF147 genes differentially expressed (in $\log_2$-scale, p-value ≤ 0.05) under medium starvation (24 h) and recovery period in retentostat cultivation (48 h) compared to the initial retentostat culture (t=0 h) that are clustered in model profiles 6 and 7 by the STEM module. Data are derived from one of two replicate cultures (culture 1), and the corresponding dataset obtained in the replicate culture is provided in Supplementary figure S5. Genes are grouped in statistically significant GO categoreis (A) purine metabolic process, (B) membrane and transmembrane transporter activity, and (C) competence protein related genes.

A remarkable molecular adaptation of *L. lactis* KF 147 to the carbon starvation and recovery regime executed here was the enhanced transcription level of genes predicted to play a role in natural competence (*coiA*, *comC*, *comEA*, *comEC*, *comFA*, *comGA*, *comGB*, *comGC*, *comGD*, *comGE*, *comGF*, *comX*) (Fig. 5C & S5C), a phenotype that has not been observed in *L. lactis* strains, to date (39). Natural competence enables cells to import exogenous genetic material to either repair damaged endogenous genes, provide additional genetic diversity or simply serve as a nutrient source (21). Notably, Redon and co-workers reported that five out of thirteen genes related to natural transformation pathway were induced during the stationary phase of

growth of batch-cultured *L. lactis* IL1403 (27), whereas our experiments revealed the induction of all genes of *L. lactis* KF147 that are associated with competence. The starvation induced in the high density retentostat culture by the closing of the medium supply (t=0) is instant and quite severe, which may explain the more complete expression of pathways that could facilitate the import of exogenous DNA as a potential nutrient source. In this context it is interesting that the cells adapted to near-zero growth conditions by extended retentostat cultivation (the state of the culture at t=0) highly expressed the capacity to import alternative carbon sources, and could be shown to more rapidly utilize these carbon sources when they were provided to the cells (10). Apparently, the severe starvation that was elicited during the current experiments led to an expansion of this quest for alternative nutrients, including the machinery to import exogenous DNA.

Identification of a potential *cis*-acting DNA-motif involved in starvation and recovery gene regulation. To identify candidate *cis*-acting DNA-motifs that are potentially involved in controlling transcriptional adaptation to strict starvation and recovery, we searched for overrepresented DNA sequences in the upstream regions of genes that showed correlated expression. Remarkably, the previously identified, highly conserved sequence element containing the palindromic 5'-CTGWCAG-3' motif, and resembling the CodY binding site (6, 10) was also identified in profile 3, 6, 7 in the present study (Fig. 6). The motif is present upstream of genes related to transcriptional and cell division machinery (*ddl*, *murCDFG*, *mutM*, *papL*, *parC*, *rpmF*, *rexB*, *truA*, *yacB*), purine metabolic process (*guaC*, *purHL*), and natural transformation protein (*coiA*, *comEA*, *comEC*, *comGC*) (Fig. S7). This finding implies

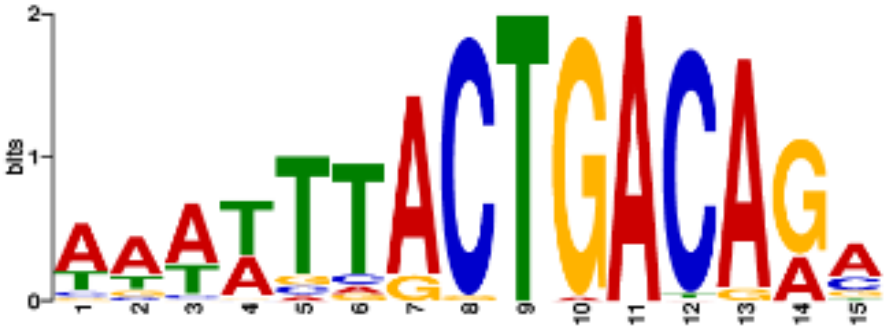

Figure 6: Weblogo visualization of the postulated CodY upstream binding sequence found in front of the transcription and translational machinery, purine biosynthesis, and natural transformation related genes. The CTGACAG palindrome sequence that forms the core of the motif is positioned from nucleotide 8 to 14. The adenine at position 11 appears to be conserved as well.

that the previously identified motif could play a prominent role in the regulation of stringent response, purine metabolism, and natural competence in *L. lactis* KF147 in the adaptation to strict starvation.

The lactococcal CodY has been reported to be a global regulator that contributes to the expression control of a variety of cellular functions, including macromolecular degradation, nutrient transport, and, amino acid and peptide metabolism (7, 17). CodY repression is alleviated in nutrient-limited environments, allowing the expressions of many genes involved in adaptation to these restrictive conditions. In addition, several CodY-regulated genes are co-regulated by additional mechanisms, allowing their fine-tuned modulation in response to other environmental conditions (17). In our earlier study, we proposed that the identified motif represents high affinity binding site for CodY in *L. Lactis* KF147, and that CodY orchestrates transcriptional adaptation of nitrogen- and carbon-metabolism in *L. lactis* KF147 at near-zero growth rates (10). In the present study, we expand the regulatory role of CodY towards the control of stringent response, purine metabolism, and natural transformation in non-growing *L. lactis* KF147 upon its exposure to severe starvation conditions. Notably, some of the proposed CodY mediated responses lead to down-regulation of expression while other genes are upregulated, implying that the CodY regulon may be subject to multiple mechanisms of dualistic and bimodal regulation.

Concluding remarks and Perspective

This paper presents the physiological and molecular responses of near-zero growth cultures of *L. lactis* obtained by carbon source restricted retentostat cultivation to severe starvation elicited by closure of the medium supply. In addition, this study evaluates the recovery capacity of this starved culture upon re-initiation of the medium supply after 24 hours of starvation.

Previous studies have shown that anaerobic retentostat cultivation of *L. lactis* mimics zero-growth conditions in which high culture viability is sustained, and where metabolic energy is exclusively used for maintenance related processes rather than cell-growth related processes (11). Notably, the maintenance related adaptations included significant metabolic adaptations of both carbohydrate and nitrogen metabolism, as well as the gene expression patterns that support an expanded carbohydrate source flexibility (10) and high stress tolerance (9).

Contrary to the retentostat cultivation, the induction of severe starvation conditions strongly repressed typical growth-associated functions, including translational, DNA replication and transcription as well as cell division machineries, but also including the ATP synthase function. This response can be classified as a typical stringent response (27), which was not elicited under near-zero growth conditions, but was apparent only upon subsequent severe starvation. These observations are in clear contrast to the stringency response that is already significantly induced during retentostat cultivation of another Gram-positive bacterium, *Bacillus subtilis* (25), but also in yeast cultures of *Saccharomyces cerevisiae* (2, 8). Despite the starvation induced stringency response, the *L. lactis* culture viability remained relatively high and it was capable of rapid recovery of metabolic activity upon subsequent re-initiation of the medium supply, which also led to a relief of the stringency response.

Intriguingly, among the response observed in the starved cultures was the strong induction of the genes predicted to play a role in natural competence, which under these conditions may serve as an adaptation to sequester additional nutrients (*i.e.* exogenous DNA) rather than a system to expand the strains genetic repertoire, although that option cannot be excluded. Our attempts to validate the proposed natural competence of the cells obtained from these cultures failed to proof that these cells could import, and/or chromosomally integrate (linearized) plasmid DNA. To date, the functionality of the competence system and natural transformation in *L. lactis* has not been experimentally validated, which is in agreement with our failing transformation experiments. Nevertheless, the observed activation of the complete competence gene set within the KF147 genome is intriguing and may offer possibilities to activate and study this relevant phenotype in lactococci. Finally, TFBSs mining of the transcriptome datasets identified a highly conserved motif 5'-CTGWCAG-3', which we previously also detected as a potential-regulatory motif involved in the adaptation to near-zero-growth conditions (10) and is likely to represent the target site for CodY. This finding appears to indicate a dualistic and bimodal role of CodY mediated regulation in response to changing environments, which may imply the involvement of additional co-regulatory mechanisms to fine-tune these responses within the CodY regulon. Notably, structural analysis of the CodY of *B. subtilis* indicated that GTP acts as a ligand for this conserved regulator and that CodY may react to (p)ppGpp levels during the stringent response (15, 23 38). The proposed role of CodY regulation in the stringent response gene regulation patterns

observed in this study strongly support an interaction between CodY and the alarmone (p)ppGpp which may explain the dualistic and coordinated role of CodY in gene expression control during the adaptation to zero-growth conditions (retentostat; ref. 10) and during the adaptation to severe starvation in which its interaction with (p)ppGpp may drive the stringent responses observed in the present study.

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Supplementary information for chapter 5

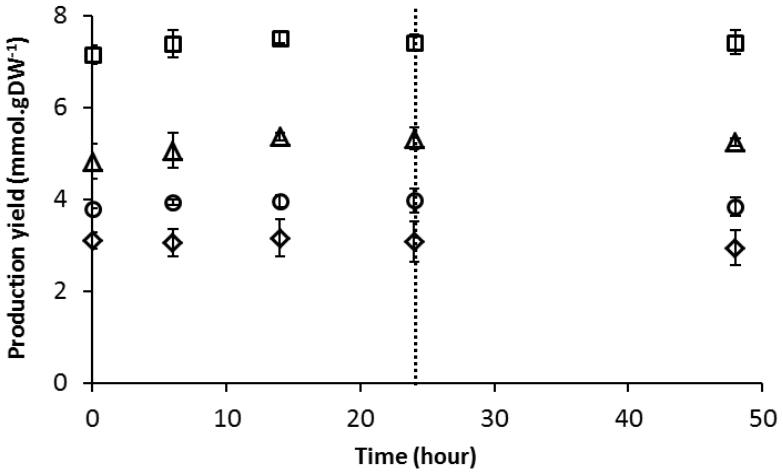

Figure S1: Metabolic profile of *L. lactis* KF147 under extensive retentostat conditions (t=0), and subsequent starvation (up to 24 hours), and re-initiation of medium supply (t=48) to the retentostat culture. Two independent retentostat cultures at near-zero growth was switched to starvation condition at time zero. The dashed line indicates the end of the starvation. Levels of the major fermentation metabolites lactate (diamonds), formate (squares), acetate (triangles) and ethanol (circles) are indicated (mmol.g_{DW}^{-1}). Error bars indicate the standard deviation values from two independent cultivations.

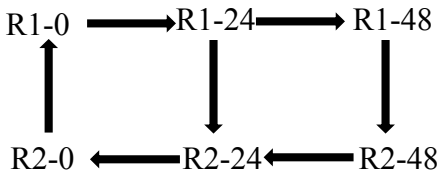

Figure S6: Experimental design and hybridization scheme. R1 and R2 represent retentostat 1 and retentostat 2 at hours 0, 24, and 48, respectively. Arrows indicate Cy3 (tail) and Cy5 (head) labeling.

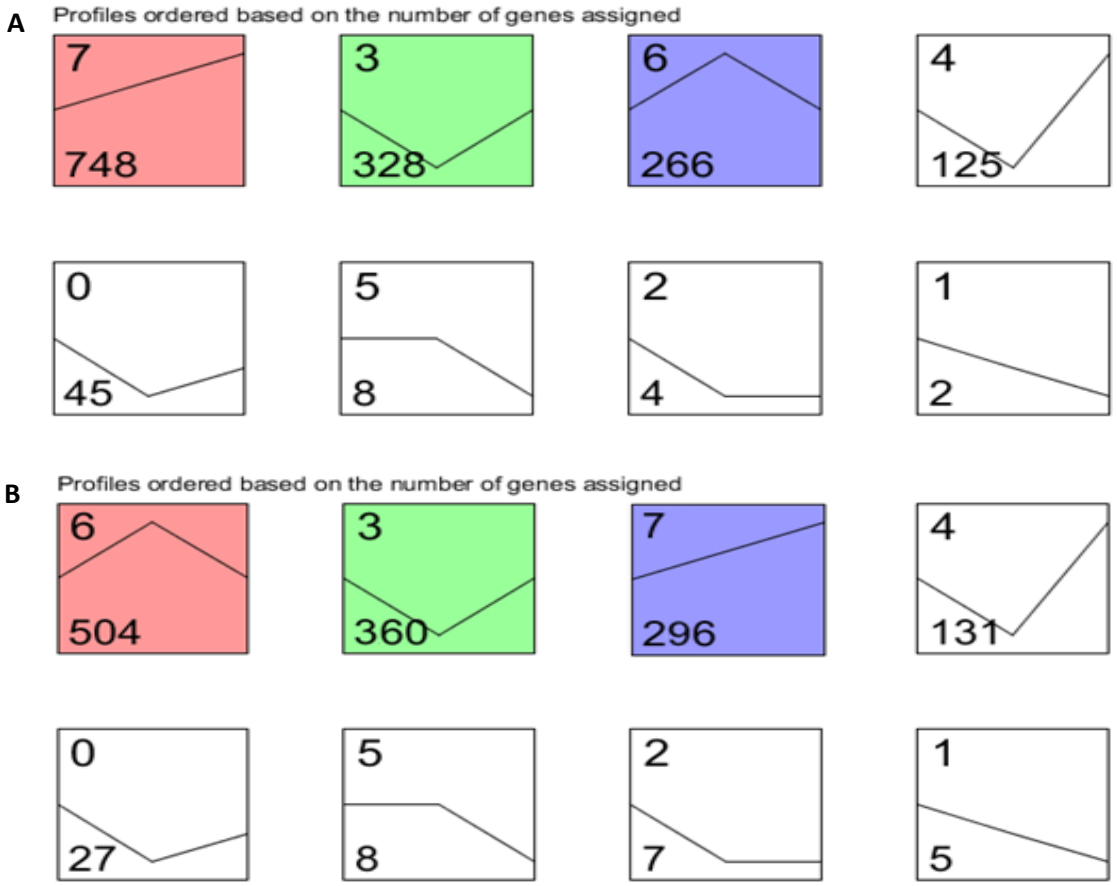

Figure S2: STEM clustering profiles from retentostat 1 (A) and 2 (B). 8 clusters contain the genes filtered on the basis of the expression ratio ≥ 1 (in $\log_2$-scale) between the time-points. The number in the top left-side corner of a profile box is the profile ID number and lower left-side corner is the number of genes. The colored profiles have a statistically significant number of genes assigned.

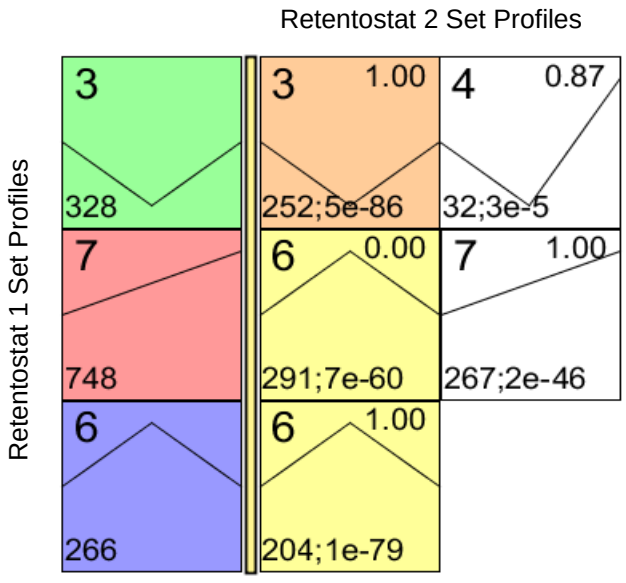

Figure S3: Model profiles comparison. The profiles to the left of the yellow line are from the retentostat 1 and to the right of the yellow line are from the retentostat 2. The number in the top left-side corner of the profile is the profile ID number and in the top right-side corner indicates the correlation of expression patterns between the compared profiles. The number in the lower left-side corner of the profiles to the left yellow line is the number of assigned genes. The numbers in the lower left-side corner of the profiles to the right yellow line are the number of the shared genes in the both experiments and *p*-value for the number of the genes in the intersection, respectively.

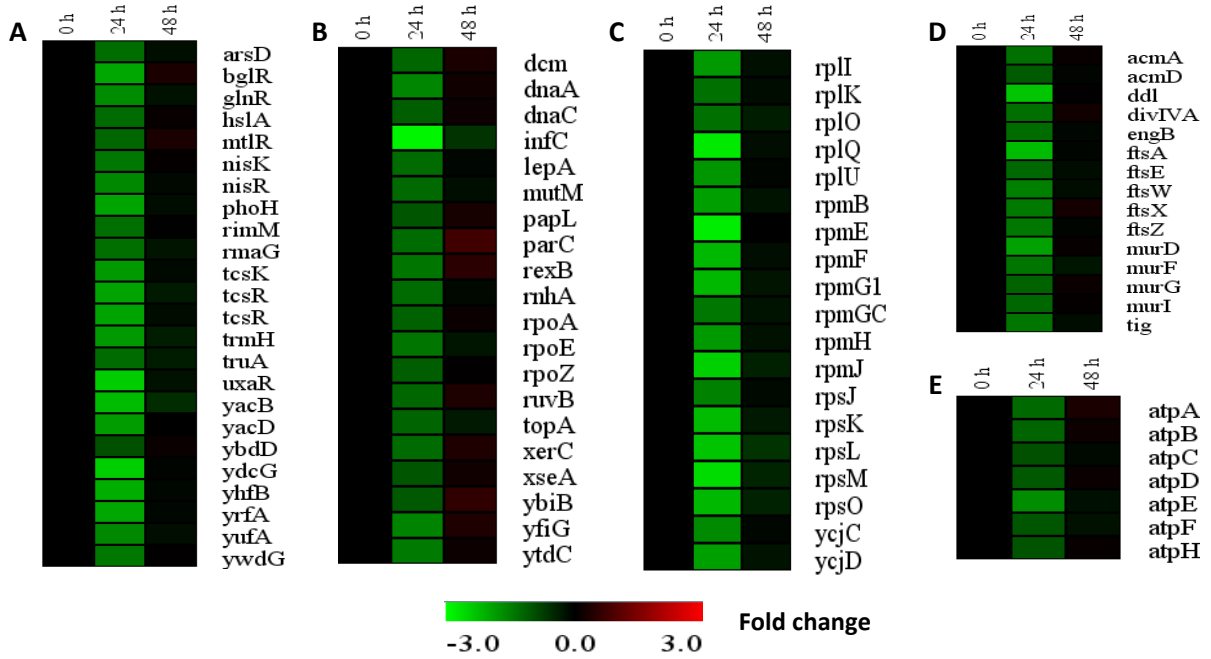

Figure S4: Heat map of *L. lactis* KF147 genes differentially expressed (in $\log_2$-scale, p-value ≤ 0.05) under medium starvation (24 h) and recovery period in retentostat cultivation (48 h) compared to the initial retentostat culture (t=0 h) that are clustered in model profile 3 by the STEM module. Genes are grouped in statistically significant GO categoreis (A) transcriptional regulators, (B) replication, (C) ribosome, (D) cell division and lysis, and (E) ATP synthases (culture 2).

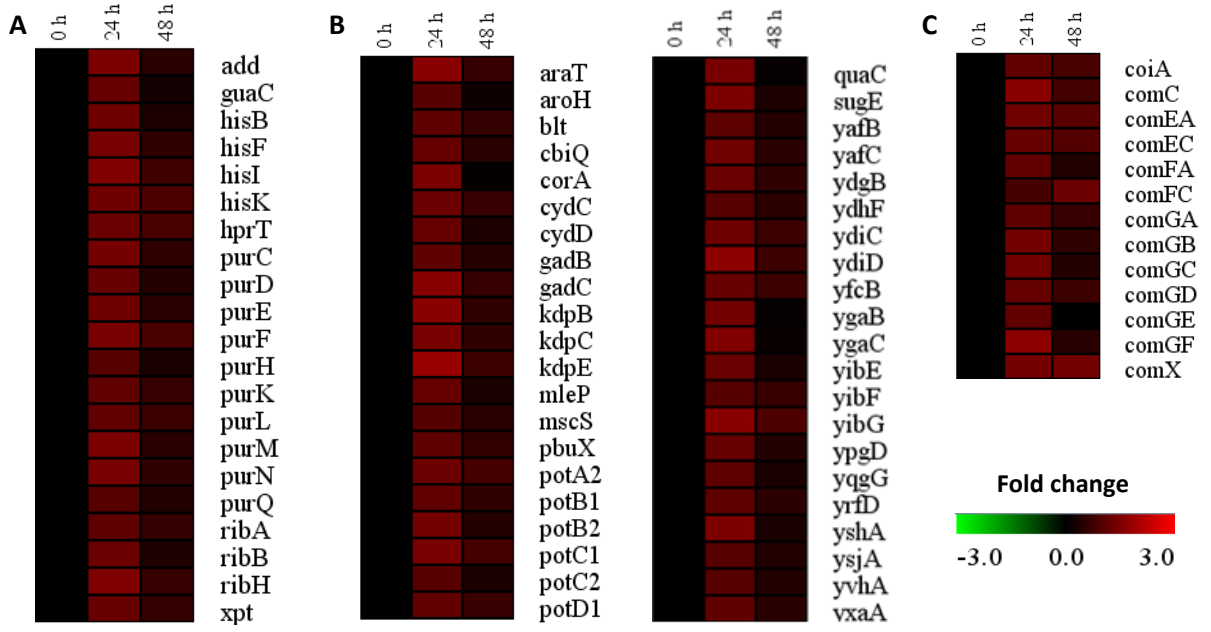

Figure S5: Heat map of *L. lactis* KF147 genes differentially expressed (in $\log_2$-scale, p-value ≤ 0.05) under medium starvation (24 h) and recovery period in retentostat cultivation (48 h) compared to the initial retentostat culture (t=0 h) that are clustered in model profiles 6 and 7 by the STEM module. Genes are grouped in statistically significant GO categoreis (A) purine metabolic process, (B) membrane and transmembrane transporter activity, and (C) competence protein related genes (culture 2).

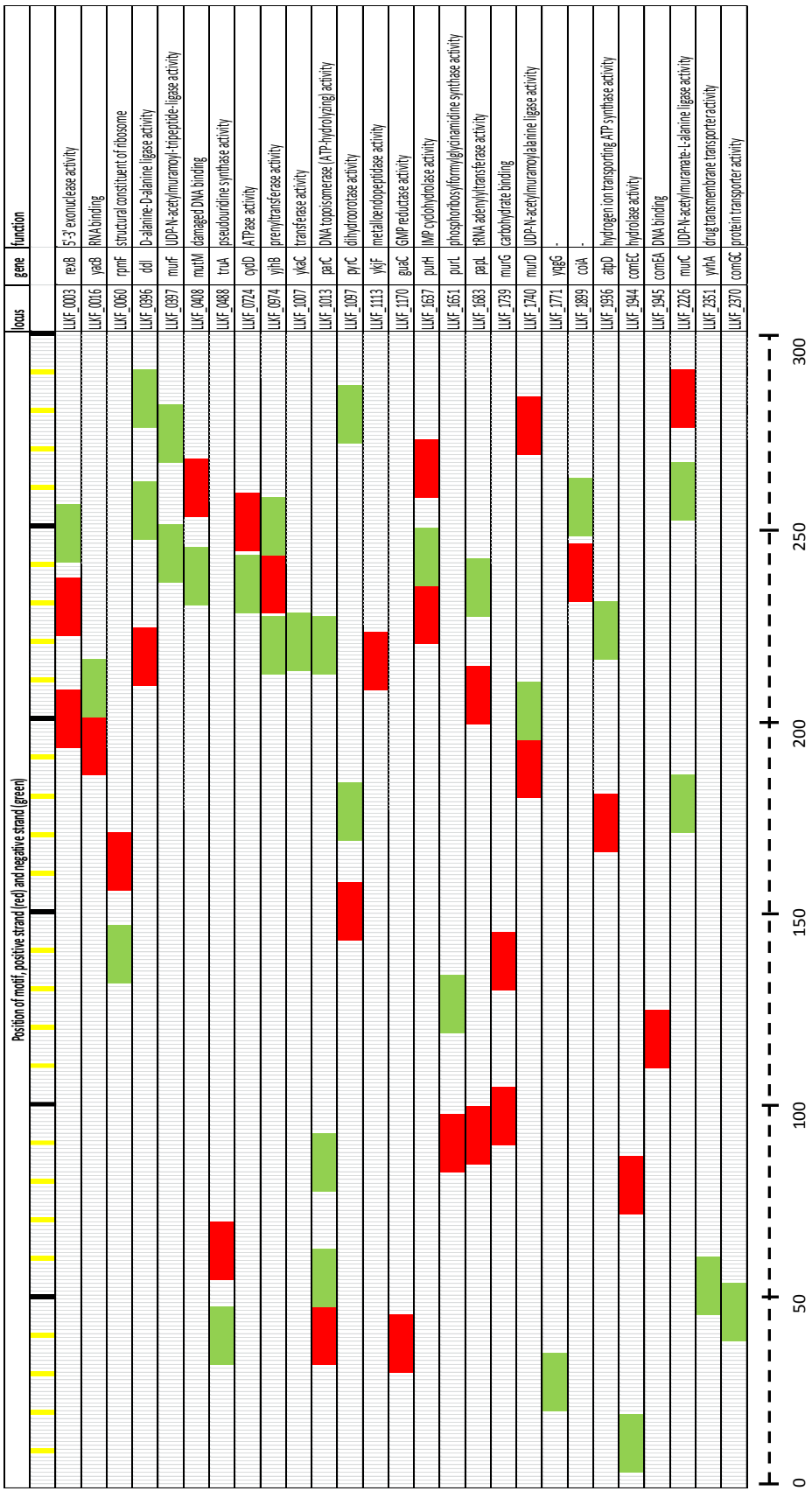

Figure S7: Motif positioning diagram. Genes in the genome of *L. lactis* KF147 that are regulated by a sequence element with significant sequence homology to the putative regulatory element identified in this study using MEME. Motif blocks show the order and spacing of non-overlapping matches to the motifs in each sequence. The ruler at the bottom gives an indication of the actual length of the sequence. Black blocks indicate positive strand and gray blocks indicate negative strand on the sequence for the motifs upstream of the genes (*p*-value < 1.0e-06).

Chapter 6

Physiology and transcriptomes of different industrial microbes at extremely low specific growth rates

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Manuscript in preparation

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Abstract

Our understanding of the physiology of industrial microorganisms is largely based on laboratory studies under conditions that result in rapid growth and high metabolic activity. However, natural microbial ecosystems and industrial processes are often severely calorie-restricted. As a consequence, microbial growth rates in such settings can be extremely slow and may even approach zero. From a process economy point of view, the ability to uncouple microbial growth from product formation, while maintaining cellular integrity and activity, offers interesting perspectives. Retentostat cultivation has been developed to investigate microbial physiology at near-zero growth rates. This minireview compares information on from recent physiological and gene-expression studies on retentostat cultures of the industrial microorganisms *Lactobacillus plantarum*, *Lactococcus lactis*, *Bacillus subtilis*, *Saccharomyces cerevisiae* and *Aspergillus niger*. Shared responses of these organisms to near-zero growth rates include increased stress tolerance and a down-regulation of protein synthesis genes. Other adaptations, such as changes in morphology and (secondary) metabolite production, were species specific. This comparison underlines the industrial and scientific significance of further research on near-zero growth physiology.

Keywords: Industrial microbes, zero-growth, quantitative physiology, transcriptome

Introduction

Most research in microbial physiology focuses on growing cells, although under natural and industrial conditions microbes frequently encounter in a state in which neither growth nor deterioration of cells occurs. However, the experimental design to study microbes in this clearly relevant physiological state is far from trivial.

In the context of this mini-review, we define 'zero-growth' as a non-growing state in which viability and metabolic activity of a microbial culture are maintained over prolonged periods. As such, zero-growth differs from starvation, which is inevitably coupled to cellular deterioration, loss of activity and, ultimately, cell death (39, 51) It also differs from differentiated survival states such as bacterial or fungal spores, in which metabolism comes to a standstill (16). Conversely, under zero-growth conditions, microbes exclusively use available substrates for processes that contribute to maintenance of cellular integrity and homeostasis. Such processes, for example, include homeostasis of transmembrane gradients of protons and other solutes, osmoregulation, protein turn-over, as well as defense and repair systems (7, 24, 29, 34, 62).

In many classical microbial food fermentation processes, near-zero growth occurs as a consequence of prolonged periods of extremely restricted availability of energy substrates. Examples include cheese ripening (2, 56) by lactic acid bacteria (LAB), wine fermentation by *Saccharomyces cerevisiae* (43) and natto fermentation by *Bacillus subtilis* (58). Despite the severely energy-limiting conditions, microbes manage to survive in these processes for many weeks, while continuing to produce aroma and flavor compounds in the product matrix (2, 25, 43, 58). A further incentive for studying zero-growth physiology of industrial microorganisms is related to their application as cell factories for production of food ingredients, enzymes, chemicals and biofuels. In such applications, biomass is essentially a non-desirable by-product, especially when costs have to be incurred for inactivation of genetically modified biomass (11, 31, 38, 48). Ideally, metabolic product formation in such applications should be uncoupled from growth, using 'zero-growth' cultures that retain high productivity and product yields over prolonged periods.

The physiology of non-growing microbes is currently mostly studied in batch cultures that proceed from exponential growth to stationary phase (54, 66). However, the fast transition

from exponential growth to stagnation of growth in batch cultures does not allow researchers to capture the physiology of sustained 'zero-growth'. Slow growth of microbes under tightly defined, constant experimental conditions can be studied in chemostats grown at low dilution rates (27, 30, 42). However, chemostats cannot be used to assess extremely low specific growth rates (< 0.01 h^{-1}), due to technical limitations related to discontinuous substrate addition, the time required for reaching 'steady state', and the emergence and selection of adapted mutant variants (13, 17).

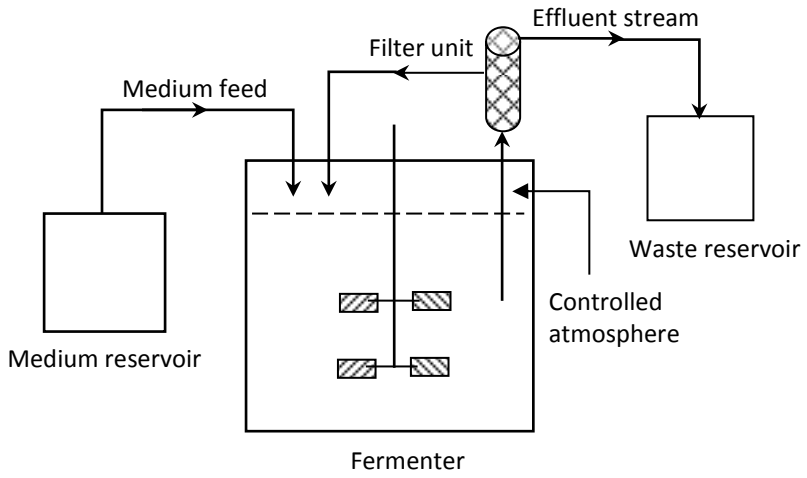

Figure 1: Basic scheme of retentostat cultivation set-up. Temperature, pH and working volume of cultures were sustained constant during cultivations in all studies.

The retentostat is a continuous cultivation set-up that was specifically developed to study microbial physiology at extremely low specific growth rates (30, 64). Contrary to the situation in chemostats, biomass cannot leave a retentostat via the effluent line, but is retained in the bioreactor by a membrane filter (Fig. 1). Prolonged energy-source-limited retentostat cultivation at a constant dilution rate leads to a progressive build-up of biomass (Fig. 2) that coincides with a progressive lowering of the specific rate of substrate consumption. Eventually, this leads to a situation where growth is no longer possible because all energy substrate provided to the cultures is used to meet cellular maintenance requirements (8, 24, 29, 64; Fig. 3). Retentostat cultivation therefore allows a gradual transition from exponential growth to a defined near-zero growth state in which sufficient energy substrate is provided for maintaining cellular activity and integrity, thereby preventing cell death.

Already before the advent of genomics techniques, retentostat cultivation was used to study microorganisms such as *Escherichia coli* (15), *Bacillus polymyxa* (1), *Paracoccus denitrificans*, *Bacillus licheniformis* (64), *Nitrosomonas europaea* and *Nitrobacter winogradskyi* (59). As part of a multi-laboratory collaboration, our groups have combined retentostat cultivation with quantitative physiological analysis and genome-wide expression studies to analyze 'zero-growth' of several industrial microorganisms (*Lactococcus lactis*, *Lactobacillus plantarum*, *Bacillus subtilis*, *Saccharomyces cerevisiae*, and *Aspergillus niger*). The aim of this mini-review is to compare physiological and gene-expression responses of different industrially important microbes at near-zero growth rates in energy-limited growth retentostat cultures.

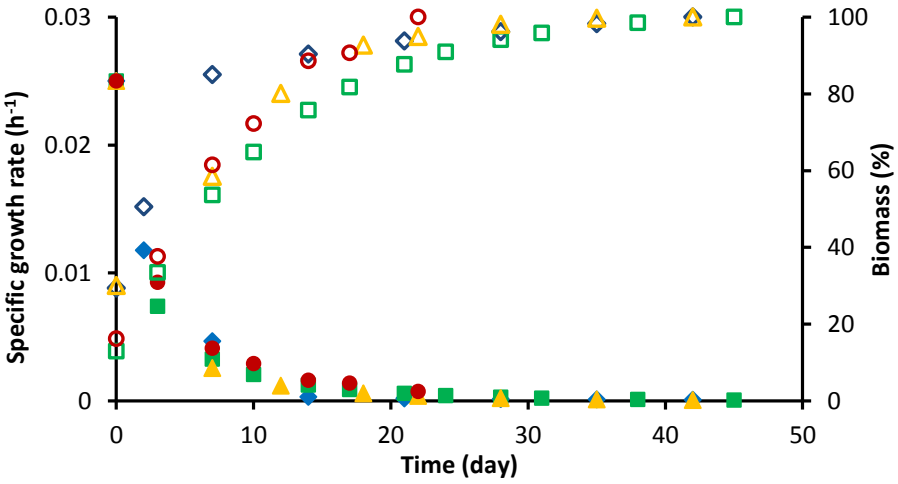

Figure 2: Specific growth rate (h^{-1}; closed symbols) and biomass accumulation (%; open symbols) of *L. lactis* (diamonds), *Lb. plantarum* (squares), *B. subtilis* (triangles), and *S. cerevisiae* (circles) during prolonged retentostat cultivation. Biomass concentrations at the end of retentostat cultivations are accepted as 100%. Biomass accumulation of *L. lactis*, *Lb. plantarum*, *B. subtilis*, and *S. cerevisiae* reached their maximal of 3.7, 6.6, 3.2, and 22.7 $g.l^{-1}$ after 42, 45, 42, and 22 days of retentostat cultivation, respectively.

Physiology of microbes in retentostat cultures

Retentostat cultures are generally started from energy-limited chemostat cultures. After establishing steady-state at a low dilution rate, the effluent line is connected to a biomass retention device and the specific growth rate starts to decrease (64). Depending on the dilution rate of the system and the physiological characteristics of the microorganism, it can take several days or weeks to reach extremely low specific growth rates. Typical growth-rate profiles and quantitative data on specific growth rates for carbon-limited retentostats of

different industrial microorganisms (Fig. 2, Table 1) show that growth rates below 0.001 h^{-1} (corresponding to doubling times of several hundred hours) can be reproducibly achieved in retentostats.

Table 1: Specific growth rate and doubling time of different microorganisms grown in energy-limited retentostat cultures.

Microorganism		Time in retentostat (day)	Specific growth rate (h^{-1})	Doubling time (day)
L. lactis KF147	(24)	42	0.00011	286
Lb. plantarum WCFS1	(29)	45	0.00006	472
B. subtilis 168 *sigF::spec amyE::*P_{rrnB}*-gfp+*	(46)	42	0.00006	470
S. cerevisiae CEN.PK113-7D	(8)	22	0.00063	46
A. niger N402	(34)	10	0.003	10

Due to the complete cell retention in chemostat cultures, even a low death rate will lead to a significant accumulation of dead cells upon prolonged cultivation. Analysis of viability, for example by counting colony forming units and/or fluorescence-based live/dead staining (8, 24, 29, 46) is therefore essential. After prolonged retentostat cultivation, viability of Firmicutes like *Lb. plantarum*, *L. lactis* and *B. subtilis* remained above 90% (24, 29, 46). Retentostat cultures of the yeast *S. cerevisiae* showed a slow accumulation of non-viable cells, leading to a viability of ca. 80% after three weeks of retentostat cultivation (8). In many microorganisms, severe energy limitation triggers the onset of sporulation. Overkamp *et al*. (manuscript in preparation) and Boender *et al.* (2009) circumvented this problem by using a sporulation-negative strain of *B. subtilis* and a haploid strain of *S. cerevisiae*, respectively. However, in a study on maltose-limited retentostat cultivation of *A. niger*, extensive conidiation already occurred 4 days after the onset of biomass retention (34). Since conidiospores passed the specially designed biomass retention device for this filamentous fungus, retentostat culture of *A. niger* did not reach the extremely low specific growth rates found for other microorganisms.

Microscopy of non-sporulating retentostat-grown cultures revealed significant morphological changes at near-zero specific growth rates (Fig. 4). The morphology of *L. lactis* changed from the typical coccoid form to a rod shape as the specific growth rate declined during retentostat cultivation (Fig. 4A & 4B). This morphological change caused an approximate 2.4-fold

increase of cell surface/volume ratio after 42 d of retentostat cultivation estimated from flow-cytometry analysis (24). Similarly, the morphology of *B. subtilis* changed from its characteristic short rod shape to substantially elongated cells after 42 days of retentostat cultivation (Fig. 4E & 4F). Conversely, microscopic examination of chemostat- and retentostat-grown *Lb. plantarum* did not reveal significant changes in morphology (29; Fig. 4C & 4D), showing that such a response is not a general phenomenon in Firmicutes. Electron microscopy of retentostat-grown *S. cerevisiae* showed massive intracellular accumulation of glycogen and, in addition, an increased content of lipid droplets in the yeast cytosol (7; Fig. 4H1). Accumulation of these storage polymers likely contributed to a concomitant increase in cell size and mass.

Energy metabolism at near-zero growth rates: impact of maintenance energy requirements

In heterotrophs, a single growth-limiting carbon- and energy substrate (*e.g.*, a sugar) is often used for growth as well as for maintenance processes. The distribution of substrate between these two processes can be derived from relationship between the biomass-specific substrate consumption rate (q_s) and specific growth rate (μ). When the biomass-specific rate of substrate consumption for maintenance processes (the so-called maintenance coefficient m_s) is growth-rate independent, this relation is linear and known as the Herbert-Pirt relation ($q_s = m_s + \mu/Y_{sx}^{max}$; 50, 63; Fig. 3). Conventionally, m_s is predicted by extrapolation of measurements

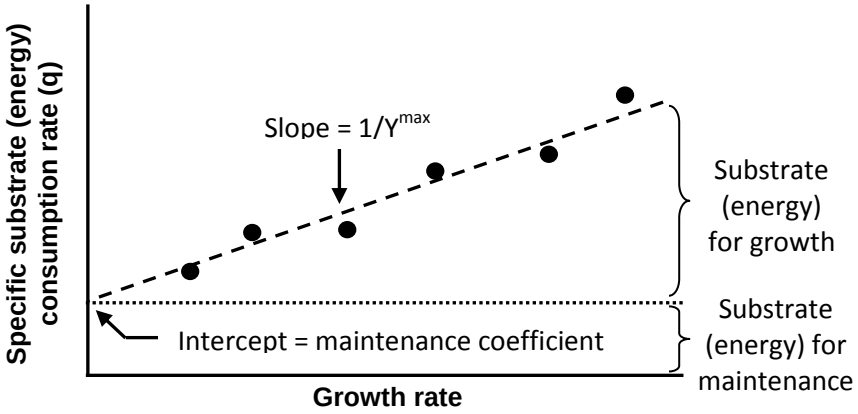

Figure 3: Determination of energy- and substrate-related coefficients with the plot of the specific substrate (energy) consumption rate (q) against growth rate in chemostat and/or retentostat cultivation. The intercept (extrapolation to a zero growth condition) gives the maintenance coefficient (m) and the slope of the plot (dashed line) gives the inverse of the maximum growth yield (Y^{max}). Below the dotted line indicates metabolic energy or substrate for maintenance-related processes.

obtained from chemostat cultures grown at relatively high specific growth rates (*e.g.* $\mu \geq 0.05$ h^{-1}) and the slope of the linear relation represents $1/Y_{sx}^{max}$ (Y_{sx}^{max} is the maximum growth yield, the biomass yield on the growth-limiting nutrient at very high μ, where $m_s << q_s$) (8, 24, 29, 49).

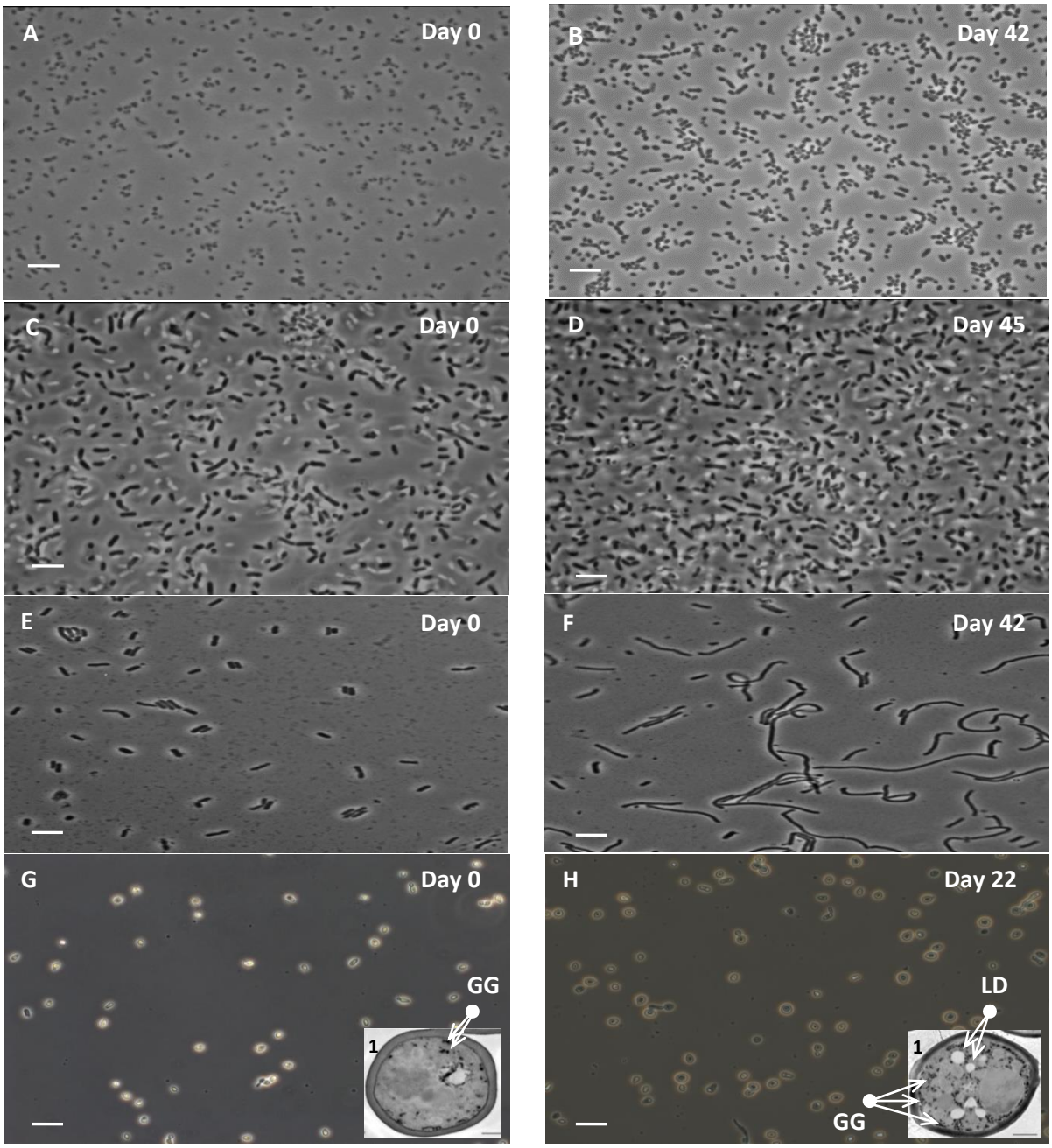

Figure 4: Phase contrast microscopic images at chemostat (day 0) and at the end of retentostat cultivations of *L. lactis* (A & B), *Lb. plantarum* (C & D), *B. subtilis* (E & F), and *S. cerevisiae* (G & H). Scale bars show 0.5 cm. Electron micrographs of *S. cerevisiae* at chemostat (G1) and after 22 days in retentostat cultivation (H1). GG and LD indicate glycogen granules and lipid droplets, respectively. Scale bar of picture G1 & H1 represents 1 µm.

While, in most microorganisms, growth-rate independent maintenance energy requirements adequately describe the stoichiometry of biomass and product formation at low to intermediate specific growth rates (61), it is unclear to what extent this concept is generally applicable at near-zero growth rates. It is well known that several prokaryotes activate a so-called 'stringent response' at low specific growth rates. This alarmone-mediated response results in a down-regulation of energy-intensive cellular processes (*e.g.* protein turn-over) at low specific growth rates, which reduces the ATP demand for maintenance processes (12, 14, 26, 54). Retentostat cultivation enables a much closer approximation of a zero growth rate than chemostat cultivation and, thereby, a better assessment of the growth-rate (in)dependency of m_s in different microorganisms.

In recent studies, m_s values were estimated from retentostat cultures of the industrial microorganisms *S. cerevisiae* (8), *Lb. plantarum* (29), *L. lactis* (24), and *B. subtilis* (46) (Table 2). In contrast to earlier retentostat studies employing other bacterial species (1, 15, 59, 64), these experiments did not reveal a stringent response, *i.e.* m_s values calculated from near-zero growth retentostats were in good agreement with those estimated by extrapolation from chemostat cultivations at high dilution rates (8, 24, 29). *B. subtilis* represented a notable exception, which may be related to strain differences and/or different culture conditions used to obtain the chemostat data (46). For all four organisms, the fraction of the carbon and energy source used for maintenance-associated processes increased from 13-30% at the initial specific growth rate of 0.025 h^{-1} to 97-99% after prolonged chemostat cultures (Table 2). This virtually exclusive allocation of energy substrate to maintenance is entirely consistent with the near-zero growth rates that were reached at the end of the retentostat experiments.

In *S. cerevisiae*, *B. subtilis*, and *Lb. plantarum* the relative concentrations of catabolic products remained essentially constant during the transition from chemostat to retentostat cultivation. Major catabolic products were lactate and low concentrations of acetate, formate, and ethanol for *Lb. plantarum* (29), CO_2 and H_2O for *B. subtilis* (46), and ethanol and CO_2 for *S. cerevisiae* (8). In contrast, substantial changes in fermentation products were observed in *L. lactis* that were also reflected by modulations in the expression of the corresponding genes. However, these metabolic changes were not predicted to influence the overall energy flux in these cells (23, 24).

Although growth was limited by the energy substrate, *L. lactis* displayed major adaptations of its nitrogen metabolism during extended retentostat cultivation (23). By integration of transcriptome analysis and metabolic modeling, *Lb. plantarum* was predicted to produce plant-growth stimulating metabolites, including indole compounds and phenylacetate, at near-zero growth rates (29). Although the *A. niger* retentostats did not achieve a true near-zero growth because of the efflux of conidiospores from the bioreactor, short-term retentostat fermentation of this species elicited significant metabolic changes, including the production of the secondary metabolites flavasperone, aurasperone B, tensidol B, fumonisins B_2, B_4, and B_6 (33).

Taken together, retentostat studies on these four microbial species demonstrated the concept of a growth-rate-independent maintenance energy-requirement. In two of the four species investigated, near-zero growth elicited the production of secondary metabolites.

Table 2: Substrate-related maintenance coefficient (m_s; $mmol_{carbon}\ g_{DW}^{-1}\ h^{-1}$) and maximum specific growth yield (Y_{sx}^{max}; $mg_{DW}\ mmol_{carbon}^{-1}$) for the species reported in this review, and the relative distribution of energy and substrate costs to maintenance-associated processes during chemostat with a dilution rate of 0.025 h^{-1} and extended retentostat cultivation under anaerobic conditions, while aerobic conditions for *S. cerevisiae*.

Microorganism		Carbon source	m_s	Y_{sx}^{max}	Distribution of energy and substrate for maintenance in chemostat (%)	Distribution of energy and substrate for maintenance in retentostat (%)
L. lactis KF147	(24)	Glucose and citrate	1.11	9.80	30.3	99.0
Lb. plantarum WCFS1	(29)	Glucose and citrate	0.65	5.54	12.8	98.4
B. subtilis 168 *sigF::spec amyE::*P_{rrnB}-*gfp+*	(46)	Glucose	1.43	2.89	29.2	97.2
S. cerevisiae CEN.PK113-7D	(8)	Glucose	2.88	2.90	20.4	99.6

Similar transcriptional responses in pathways involved in nutrient sensing, anabolism and response to stress

Besides adaptations in metabolic fluxes, also transcriptional adaptations have been observed in response to the decreasing growth rate and nutrient availability (Fig. 5). Both in the

eukaryotic and prokaryotic kingdoms, dedicated glucose sensing and signalling cascades (such as the PKA- and TOR-pathways in eukaryotes and catabolite control protein A (CcpA) in gram-positive bacteria) result in transcriptional reprogramming, including the repression of genes involved in alternative carbon source utilization in glucose-rich environments (18, 36, 60, 69; Fig. 5). During extended retentostat cultivation, severe glucose limitation led to the progressive relief of repression of CcpA targets in prokaryotes and of glucose-repressible genes in *S. cerevisiae*, thereby demonstrating a gradual alleviation of carbon catabolite repression (CCR) when approaching near-zero growth (7, 23, 29, 46). In *L. lactis* KF147, this CCR transcriptional relief was shown to enable retentostat derived cultures to more rapidly ferment carbon sources other than glucose, illustrating the functional consequence of this transcriptional adaptation (23). Notably, also *A. niger* retentostat cultures displayed increased expression of genes involved in nutrient mobilization, including carbohydrate transporters. These evolutionary conserved adaptive responses enable the microbial species to rapidly scavenge alternative carbon and energy sources when they appear in their environment.

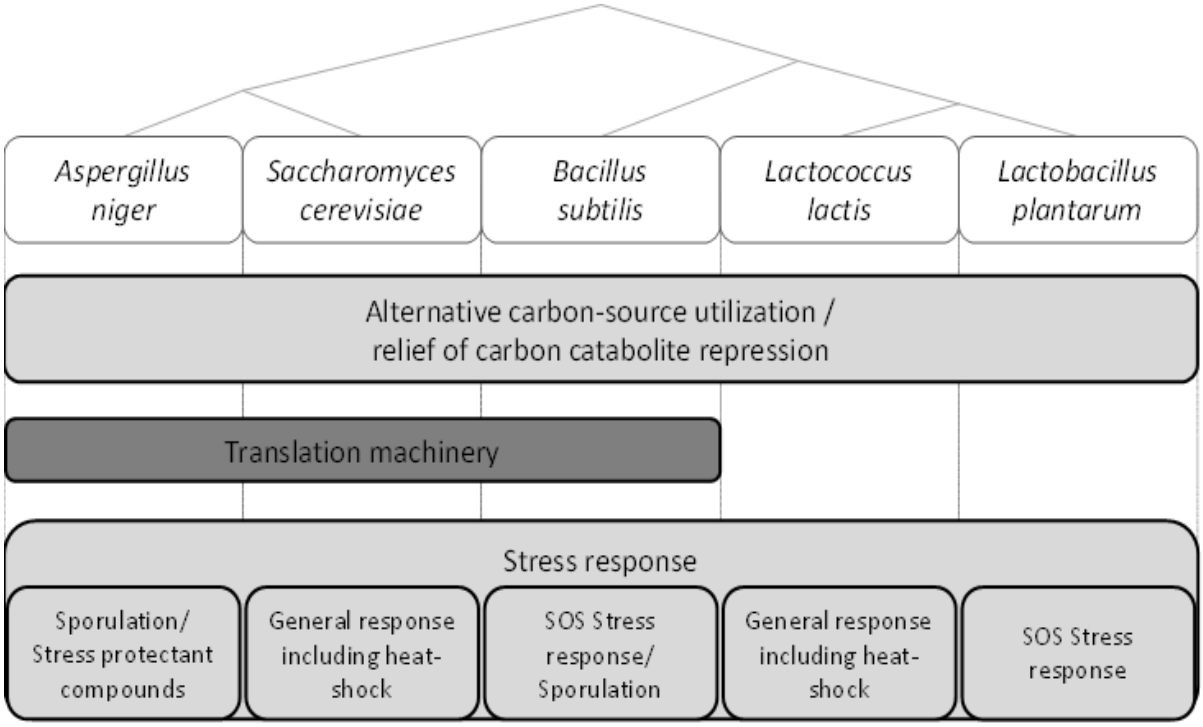

Figure 5: Cross-kingdom shared transcriptional adaptation to zero growth. Light gray boxes indicate increased expression of the mentioned functional categories; dark gray box indicates decreased expression during retentostat cultivation.

When microbial cultures approach near-zero growth rates, the cellular requirement for biosynthetic building blocks strongly decreases. This reduced anabolic demand is reflected in the transcriptome of most organisms by a decreased expression of genes involved in biosynthetic routes. Protein synthesis is the most energy-demanding biosynthetic process (13). Under the severe caloric restriction in retentostat cultures, *B. subtilis*, *S. cerevisiae*, and *A. niger* cells decreased expression of components of the translation machinery, including ribosomal proteins an amino acyl tRNA synthases (7, 34, 46, 65; Fig. 5). Although chemostat studies of *L. lactis*, at growth rates above 0.09 h^{-1} revealed the existence of a positive correlation between growth rate and expression of genes involved in translation (20), such a correlation was not observed in retentostat cultures of *L. lactis*, nor of *Lb. plantarum* (23, 29).

The severe energy limitation in retentostats can be expected to represent a stress factor. Indeed, a third conserved transcriptional response observed in the five microorganisms mentioned above encompassed an increased expression of genes involved in stress responses. Although observed during retentostat cultivation of all five microbes, responses were diverse (Fig. 5). *L. lactis* and *S. cerevisiae* showed an increased expression of genes involved in stress resistance, including heat-shock proteins. These responses were shown to confer a drastically increased tolerance of the yeast cells to heat stress and, in the case of *L. lactis*, to heat-, acid- and oxidative stress conditions (6, 7, 22). Although general stress response regulons were not significantly induced in retentostat cultures of *B. subtilis* and *Lb. plantarum*, these bacteria did induce expression of genes involved in the SOS response, including error-prone DNA-polymerases (29, 46). By increasing mutation rates, this response might drive evolutionary adaptation, and thereby increase chances of survival. However, the number of generations in the retentostat was too small to directly observe adaptive evolution, and genome resequencing of retentostat-grown *B. subtilis* cultures did not reveal marked changes (46). *A. niger* increased expression of genes involved in stress-protectant compound synthesis during retentostat cultures (34), while the induction of sporulation in this fungus can also be interpreted as an adaptation to the stress imposed by near-zero growth (45, 68). Interestingly, a sporulation-deficient *B. subtilis* strain grown in retentostat cultures still activated expression of genes involved in initiation of the sporulation cascade, suggesting that wild-type *B. subtilis* strains will probably sporulate during retentostat cultivation (46). A similar approach, using a

non-sporulating mutant of *A. niger* (see for example ref. 37) could be exploited to reach a near-zero growth condition.

Microbe-specific transcriptional responses at near-zero growth rates

In addition to responses that were identified in two or more microorganisms, retentostat cultivation was also found to induce species-specific transcriptional responses. In situations of nutrient scarcity, *B. subtilis* cells are known to increase expression of genes involved in motility and chemotaxis, in order to enable migration to nutrient-rich environments (53). However, *B. subtilis* retentostat cultures down-regulated expression of genes involved in motility, including flagella synthesis, a response also observed in stationary phase cultures (5, 44, 46). Reduced autolysin expression in growing cultures of *B. subtilis* has been associated with cell elongation (35, 46). Reduced expression of this function during retentostat cultivation may explain the pronounced cell elongation in *B. subtilis*.

Exopolysaccharide production in LAB has been reported to be strongly growth-related, which is in agreement with the repression of these genes during retentostat cultivation of *L. lactis* KF147. Increased exopolysaccharide expression at near-zero growth rates may facility adhesion and/or biofilm formation and affect susceptibility to polysaccharide recognizing bacteriophages (40, 41). In addition to the above mentioned production of plant-growth stimulating compounds, retentostat cultures of *L. plantarum* revealed further transcriptome response that could relate to plant-environment associated conditions, including the activation of genes that are shared with other plant-associated bacteria and putatively involved in the degradation of plant-derived material (29).

In anaerobic *S. cerevisiae* retentostat cultures, many genes involved in mitochondrial functions, including respiration, were up-regulated at the transcript as well as at the protein level, despite the absence of oxygen. This transcriptional reprogramming could not solely be attributed to the alleviation of glucose repression (3, 7) and therefore probably reflects a preparation for environmental changes. In general, many yeast genes that were previously shown to be characteristic for quiescent cells (66), *i.e.* post-mitotic cells, were up-regulated in a gradual manner during retentostat cultivation, suggesting a growth-rate dependent expression of these genes rather than an ‘on-off’ switch of quiescence upon cessation of growth.

Consistent with the observed sporulation of *A. niger* retentostat cultures, genes involved in conidiation and related processes showed the strongest concerted up-regulation in retentostat cultures of this filamentous fungus. This response also included an increased expression of genes encoding small cysteine rich proteins, such as hydrophobins, and of gene clusters involved in secondary metabolism. Conversely, expression of genes encoding secreted hydrolases was decreased (34), possibly to reduce the energy-intensive production of proteins that do not exclusively benefit the producing cells.

Conclusions and Future perspectives

In this minireview, we compared physiological, metabolic and genome-wide transcriptional adaptations of the industrially important microbes *L. lactis*, *Lb. plantarum*, *B. subtilis*, *S. cerevisiae* and *A. niger* to the near-zero growth rates, imposed by energy-limited retentostat cultivation. Retentostat cultivation enables the uncoupling of growth and non-growth associated processes while maintaining a relatively high culture viability, which allows the estimation of the relative distribution of substrate and energy to maintenance-processes under non-growing conditions.

A down-regulation of genes involved in protein synthesis was the most prominent conserved response to near-zero growth conditions. In *L. lactis* and *S. cerevisiae*, an even stronger down-regulation of protein synthesis genes was observed when retentostat cultures were switched to complete carbon- and energy-source starvation by terminating the medium supply (6, 21). Substrate expenditure for growth is considered ‘as a loss’ in the industrial enzyme, protein or peptides manufacture by microbial production (19, 48, 64) and a similar situation occurs and some of the high-value flavor, texture, and health metabolite production processes that employ LAB and *S. cerevisiae* (31, 38, 47, 57, 67). The observed down-regulation of native protein synthesis genes in near-zero growth retentostats, as well as the scarcity of ATP in these severely calorie-restricted conditions, provides a key challenge for synthesis such ‘anabolic’ products, which involves a large net input of ATP. Rather than constraining growth by a limited supply of the energy source, it seems more promising to base such processes on growth limitation by a non-energy substrate, provided that overflow metabolism and reduced efficiency of energy source utilization under such ‘energy excess’ conditions (9, 55) can be prevented.

A second conserved response related to stress adaptation, which, in several organisms, involved activation of the generic chaperonin-type heat-shock protein response. These stress responses are in agreement with an increased focus of overall cellular activity to maintenance (supporting survival rather than growth) associated processes. In *L. lactis* and *S. cerevisiae*, transcriptional responses were shown to correlate with strongly enhanced stress tolerance (robustness) of these cells. These observations are relevant for industrial application, for instance in optimization of fermentation protocols to improve survival under industrial processing and storage conditions. Eliciting increased heat-stress tolerance by introducing a near-zero growth phase could, for example, be exploited in the production of robust starter cultures with enhanced spray-drying survival.

As mentioned above, energy-limited retentostat cultivation represents a challenging starting point for production of compounds whose synthesis yields ATP. A different picture emerges for catabolic products, whose synthesis is accompanied by ATP production. In anaerobic retentostat cultures of *S. cerevisiae* and lactic acid bacteria, virtually all energy substrate was converted to the main fermentation products, leading to near-theoretical yields. For example, yields of yeast biomass and glycerol, which are the major byproducts of bioethanol production by *S. cerevisiae* (11), were negligible in retentostat cultures. This observation confirms the power of retentostat cultivation or other means of cell retention in energy-limited cultures to improve yields of catabolic products. An alternative scenario to uncouple growth from product formation is to genetically engineer strains for efficient conversion of the available carbon source into the desirable end-product, in a growth-uncoupled fashion. Such applications have been demonstrated for *L. lactis* for the production of its endogenous metabolites (10, 28, 32, 52) as well as the alternative metabolite L-alanine (31).

This study highlights the power of combining controlled cultivation in retentostats with genome-scale analytical techniques. Although setting up and running prolonged retentostat cultures is labor-intensive, this cultivation method yields quantitative information on an important domain of microbial physiology that is not experimentally accessible via other methods. We hope that this minireview will alert colleagues to the possibilities of this approach, not only for uncoupling growth and product formation in industrial microbes, but

also for understanding microbial lifestyles and ecology in their natural habitats and, more tentatively, for the study of cellular ageing (4).

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Chapter 7

General Discussion

Introduction

In traditional industrial biotechnological applications of microbes, they are employed for the manufacture of food products, *e.g*. cheese, yoghurt, wine, and bread. In addition, industrial biotechnological application of microbes also encompasses their use as cell factories for the production of food ingredients, enzymes, pharmaceuticals, and (fine) chemicals from renewable resources (biomass from agriculture and/or forestry). Especially the declining petroleum resources, increasing oil prices, and concerns about sustainable production and ecological effects of the oil industry, has led to the development of strategies that aim at the replacement of fossil fuels for renewable feedstocks (*i.e.* carbon sources) (48). Industrial biotechnology (also referred to as white biotechnology) uses microbial 'cell factories', such as bacteria, yeast, and filamentous fungi, to manufacture a large variety of products, which include biofuels, but also encompass commodity products and (fine) chemicals (*e.g.* bioplastics), food ingredients, and pharmaceuticals. An important advantage of microbial production processes as compared to classical chemical manufacturing is the fact that the catalysts (*i.e*. the microbial cells) used in such biotechnological applications are self-replicating, which implies that the process can be started with a low amount of catalyst and cost. On the other hand, in some industrial applications, biomass formation due to intrinsic coupling of microbial metabolism and growth is considered as a loss, since at least part of the substrate flux is involved in supporting growth rather than product formation (5). For example, substrate expenditure for growth is considered a loss in some of the high-value flavor, texture, and health metabolite production processes that employ lactic acid bacteria (LAB) (27, 32, 52). Therefore, in non-biomass driven industrial applications, uncoupling of product formation and microbial growth would be an efficient solution to retain high productivity and product yields over prolonged periods. Moreover, product formation that is uncoupled from growth is also an intrinsic characteristic of microbes that are used in extended ripening processes of fermented foods, such as cheese and dry sausages ripening by LAB. The microbes involved in these ripening processes encounter strongly limited availability of energy sources that do not permit growth (3, 28, 51). Analogously, due to variable- but mostly restricted nutrient accessibility in natural ecosystems, microbes commonly live in a state of famine that is not the same as strict starvation, and permits only very low-specific growth- and metabolic- rates as a result of restricted nutrient availability.

One of the aims of this thesis was to examine the metabolic adaptations and quantitative physiology of *L. lactis* at near-zero growth rates. This was achieved by extended carbon source limited retentostat cultivation, enabling the uncoupling of growth- and maintenance-related processes in *L. lactis* while maintaining high cell-viability (Chapter 2). During extended retentostat cultivation, the bacteria are supplied with just enough energy substrate to sustain maintenance-related processes, while growth and complete starvation are prevented by a biomass retention filter and continuous medium supply, respectively. Integrated transcriptome, metabolome, and gene regulatory analyses allowed the deciphering of the molecular adaptations of *L. lactis* that underlies the physiological observations under retentostat cultivation conditions and provided an expanded view of the molecular metabolism of the bacterium at near-zero growth rates (Chapter 3). The molecular adaptations of the strain during extended retentostat cultivation encompassed the progressive induction of stress related functions, which led us to quantify the stress-tolerance of *L. lactis* cells obtained from near-zero growth rate cultures. These experiments underpinned that the observed induction of stress-gene expression correlated strongly with a generic increase of robustness of *L. lactis*, which was apparent in heat-, acid- as well as oxidative stress tolerance levels. In addition, by modeling of the data, highly significant quantitative correlations between growth rate, stress-gene expression and robustness could be determined (Chapter 4). Following extended retentostat cultivation, the obtained near-zero growth cultures were exposed to severe starvation and the transcriptome responses to this intervention were determined, revealing a strong downregulation of the cell's capacity for macromolecule synthesis, and the intriguing induction of the complete set of genes associated with natural competence development in *L. lactis* (Chapter 5). Finally, the collective activities within the joint-project 'Zero-growth Product formation' of the Kluyver Centre for Genomics of Industrial Fermentation were integrated in a minireview that compares and contrasts the retentostat cultivation, its quantitative physiology and gene-expression responses of the industrial microbes that form the pillars under the Kluyver Centre's approach, *Lactococcus lactis*, *Lactobacillus plantarum*, *Bacillus subtilis*, *Saccharomyces cerevisiae*, and *Aspergillus niger* (Chapter 6). The different findings described in this thesis are discussed in more depth in the following sections of the general discussion.

Physiological adaptations of *L. lactis* at near-zero growth rate

In this thesis, the *L. lactis* culture progressed from a growing to a near-zero growth state under carbon source (glucose and citrate) limited retentostat cultivation, which allowed the decoupling of growth and maintenance-associated cellular processes in *L. lactis*. Due to extremely low carbon availability for prolonged periods of time in retentostat cultivation (42 days), the determination of culture viability was a key parameter. Moreover, if dead cells would accumulate in the culture, they would confound the estimations of specific growth rate or maintenance energy coefficients. Notably, it has been well established that non-growing, stationary phase cultures of *L. lactis* undergo substantial autolysis during the stationary phase of growth in batch cultures. This process is strongly driven by the peptidoglycan hydrolase AcmA (9), and cell envelope associated proteolytic functions (24), and could also occur during prolonged retentostat cultivation. Therefore, viability of the retentostat culture was determined using the classical enumeration of colony forming units (CFU) as well as fluorescence-based LIVE/DEAD staining combined with flow-cytometer. The latter technology indicated that a culture-viability of approximately 93% was maintained in the retentostat culture, although 21% of these viable cells were classified as 'damaged' after prolonged retentostat cultivation (Chapter 2). The staining property of these damaged cells implies that they are partially permeable and it is likely that they have lost the capacity of colony formation on solid media. Still, in terms of staining properties they are substantially different from dead cells, and could reflect a cell population that has entered a dormant state. Dormancy allows microbes to resist temporal changes of environmental conditions and dormant microbial cells can contribute significantly to the overall microbial richness in natural microbial communities (30). Remarkably, although the proportion of dormant cells was relatively low in nutrient-rich habitats, the fraction of the dormant population could reach up to 40% of a microbial community in nutrient-poor ecosystems, and thereby serve as a seed-bank upon condition improvement (30). Although the dormant state, is observed in a broad taxonomic spectrum of microbes, still a variety of microbes is not able to use dormancy as an adaptive survival strategy. In erratically fluctuating environments, the formation of a dormant subpopulation may be energetically inefficient and an expensive investment for a microbial species (11), whereas in environments characterized by limited fluctuating conditions, microbial communities with the capacity to form a dormant state have a great

advantage to increase their chances of survival over prolonged periods of time (12). Cells in this dormant-like state have also been referred to as viable but nonculturable cells (VBNC) that were identified in *L. lactis* cultures exposed to severe and extensive periods of nutrient starvation (19). Therefore, carbon-poor condition induced by extended retentostat cultivation may have driven a fraction of lactococci to enter the state of VBNC, which could have contributed to the community's increased fitness in the retentostat culture.

These observations raised the question whether the increasing damaged and dead cell populations may serve as, or release substrates that provide growth-supporting fermentable substrates to the viable cell population. To address this question, growth rates on different concentrations of heat-killed *L. lactis* KF147 suspensions as the sole supplemented substrate for energy and growth were determined for non-growing, retentostat-derived *L. lactis* KF147 culture samples (Fig. 1). These experiments showed that the supplementation with cellular components was not supporting growth of *L. lactis* KF147, suggesting that the damaged or dead cell subpopulations do not provide readily fermentable substrates that support energy generation or growth of their viable community members. These observations imply that

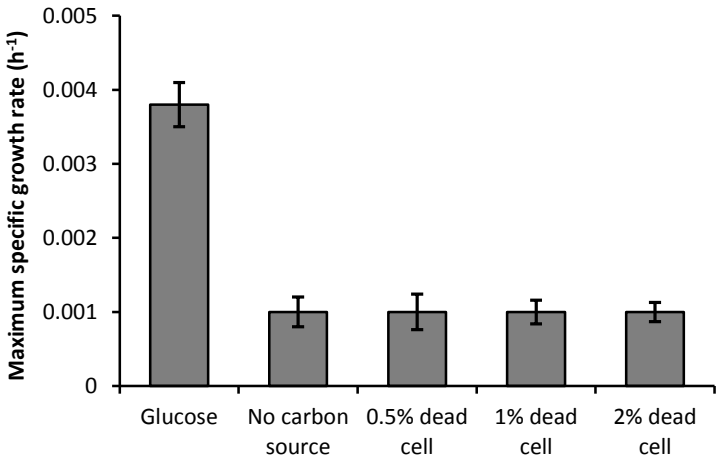

Figure 1: Maximum specific growth rates on glucose; 0.5, 1, and 2% of heat-killed *L. lactis* cells as the carbon source of *L. lactis* KF147 samples obtained from retentostat cultivation on day 14, 21, 35 and 42. Data bars represent average ± standard deviation of triplicate samples.

lactococci lack the metabolic capability for biomass recycling, and appear to exclude the possibility that the damaged and dead subpopulation serve as a substrate that drives a substantial amount of cellular turn-over during extended retentostat cultivation.

Earlier studies have suggested that in order to survive in nutrient-poor ecosystems, microbes minimize their maintenance energy and substrate requirements as an adaptive strategy, thus, slow-growing bacteria can import and use trace amount of a variety of carbon sources that may be available in the environment (15). In this study, however, the maintenance coefficients estimated from the anaerobic, carbon-limited *L. lactis* KF147 retentostat culture at extremely low growth rates (0.0001 h^{-1}) was virtually identical to the maintenance coefficient extrapolated from anaerobic, carbon-limited chemostat cultures at high dilution rates (0.025 to 0.1 h^{-1}) (see Chapter 2 for the estimation of the maintenance coefficients). This identity indicated that *L. lactis* cannot adjust its maintenance energy requirements and specifically minimize them to sustain extremely low-growth rates in the low-carbon ecosystem of the retentostat. Nevertheless, *L. lactis* KF147 did activate high level expression of a multitude of carbon import and utilization systems, implying that it does adjust its nutrient capacities to harness any environmentally available carbon source (see also below). Notably, the maintenance energy coefficient calculated for *L. lactis* KF147 was significantly lower as compared to the maintenance energy demand calculated, not only for several other microbes like *B. polymyxa* (2), *B. licheniformis* (61), *N. winogradskyi* (54), *S. cerevisiae* (5), and *B. subtilis* (42), but also for other strains of *L. lactis* that were isolated from the dairy environment (36, 41, 55). Plant-related ecosystems are considered as habitats with highly dynamic chemical and physical conditions, and are commonly regarded as poor conditions for microbes (*e.g.* low nutrient concentrations, pH, and high osmolarity). It can therefore, be proposed that plant-isolated LAB have adapted to more severe conditions in their ecosystems by minimizing their maintenance energy requirement. Unlike plant isolates, dairy-lactococci can be considered to live in a nutrient-rich habitat (*e.g*. milk), which may have allowed their evolutionary adaptation (*i.e.* which you could actually characterize as energy-sloppiness) towards increased substrate energy demands for maintenance.

Discriminatory phenotypes of plant- and dairy-isolated *L. lactis*

Nutrient-variability in the relatively hostile and benign conditions provided by the plant and dairy ecosystem, respectively, is reflected in niche-specific physiological and metabolic adaptations observed in plant and dairy isolates of the species *L. lactis* (47, 60). For example, compared to dairy isolates, plant isolates of *L. lactis* can ferment a more diverse spectrum of carbohydrate sources, commonly produce higher amounts of antimicrobial compounds, have

fewer amino acid auxotrophies, and can tolerate higher levels of various stresses, *e.g*. high pH and salt concentration (39, 49).

To further explore the phenotypic differences that may reflect niche-adaptations of plant- and dairy-isolates of *L. lactis*, we compared the growth characteristics of plant and dairy isolates in media with different salt or osmolite concentrations (osmotic stress), different temperatures (heat stress), which are among the discriminatory phenotypic traits that allow the separation of *L. lactis* spp. *lactis* from ssp *cremoris*. To this end, maximum growth rates (μ_{max}) of *L. lactis* KF147 (isolated from mung bean sprouts) and *L. lactis* SK11 (dairy isolate) were determined under anaerobic conditions in M17 medium supplemented with 1 % glucose at different temperatures (37, 42, and 45°C), and with different NaCl (2, 3, and 5%) or non-fermentable D-arabinose (3, 5, 7, 9, and 10%) concentrations (Fig. 2). The highest μ_{max} values were obtained from the non-stressed cultures for both strains. With increasing levels of stress, the μ_{max} values of both strains gradually decreased, but this decrease at a certain stress level was consistently larger for strain SK11 as compared to KF147 (Fig. 2). In addition, KF147 was still able to grow at levels of stress (*e.g*. 45°C, 10% NaCl, and 5% D-arabinose) that did already completely eliminate the growth of SK11 (Fig. 2). These findings confirmed that plant-isolated KF147 can withstand, and grow in, conditions that include higher levels of several stress conditions as compared to the dairy-isolated strain SK11. These observations raised the question whether these discriminatory growth characteristics were strain specific or a reflection of conserved discriminatory traits for plant- and dairy-isolated *L. lactis* strains. To address this question, growth kinetics of 20 plant- and 15 dairy-isolates of *L. lactis* (obtained from NIZO culture collection) were determined using the aforementioned stress-media (data not shown). These experiments could not consistently discriminate between the plant and dairy isolates on basis of their stress tolerance, and revealed that differences in these phenotypic traits appeared to be predominantly strain-specific and independent of the origin of isolation of these strains. These experiments fail to expand the discriminatory stress-tolerance repertoire of plant and dairy lactococci, but they do provide an intriguing dataset that could be employed for genotype-phenotype matching (4), using the genome sequences of these lactococcal strains, which are currently determined in a joint effort of Kluyver Centre, TI Food and Nutrition, NIZO food research (Herwig Bachmann, personal communication).

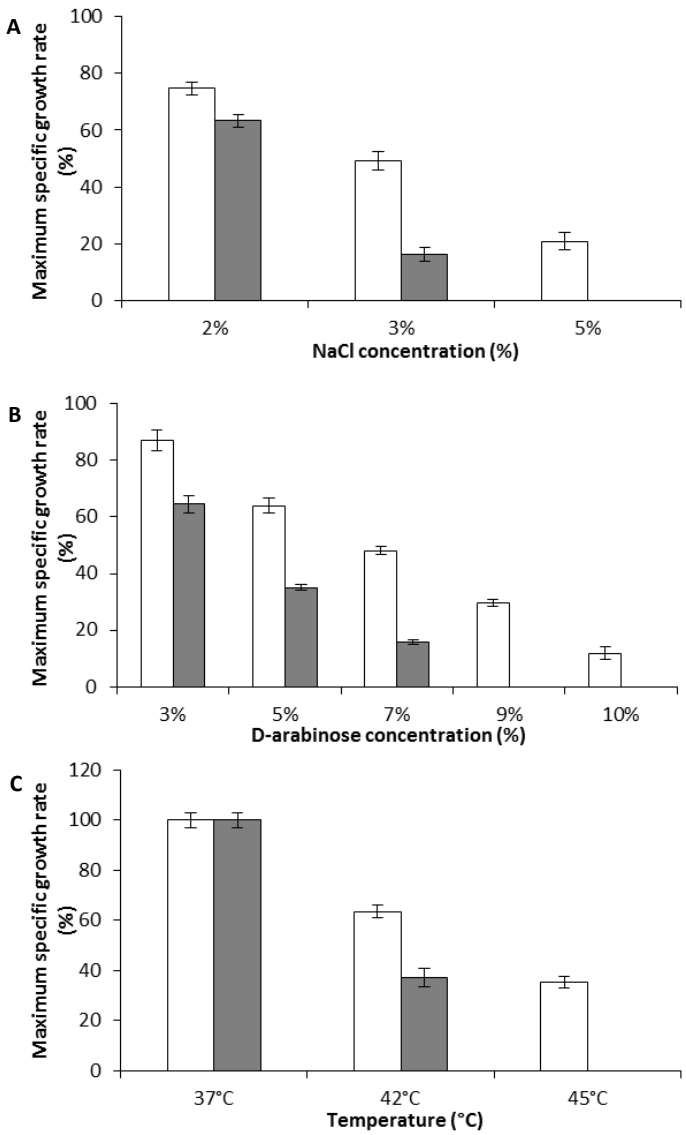

Figure 2: Relative maximum specific growth rates (μ_{max}) of plant-isolated *L. lactis* KF147 (white) and dairy-isolated *L. lactis* SK11 (gray) under different concentrations of NaCl (A), D-arabinose (B), and different temperatures (C). Cells were incubated in M17 medium broth contaning glucose (GM17) for overnight. Data points represent average ± standard deviation of measurements of triplicate cultures.
(A) Cells were grown in M17 medium broth containing 2, 3, and 5% NaCl.
(B) Cells were grown in GM17 containing 3, 5, 7, 9, and 10% D-arabinose. The highest μ_{max} values were obtained from untreated and non-stressed cultures, of which μ_{max} values were normalized as 100%.
(C) Cells were grown in GM17 at 37, 42, and 45°C.

Correlation analysis of the orthologous gene matrix of these strains and their phenotypic differences may reveal genetic markers that play a role in these discriminatory phenotypes, and could be employed in genetic screening of culture collections aiming to select stress-robust *L. lactis* strains. Previous efforts in genotype-phenotype matching have succeeded to discover genes encoding cell wall anchored adhesion factors (38, 44) and immune-modulatory effector molecules (37, 59) in another lactic acid bacterium, *Lactobacillus plantarum*, which illustrates the discovery potential of this approach. Alternatively, the phenotypes of interest may rely on differential gene regulatory patterns that generate distinct levels of expression of conserved genes in different strains, leading to the observed stress tolerance discrimination. These type of relations may be uncovered by performing phenotype-transcriptome matching

as have been applied in the identification of genes and their transcripts that play a role in the persistence of *L. plantarum* under conditions that mimic those encountered in the gastro-intestinal tract (8, 57). Overall, although these studies did not discover strongly discriminatory phenotypic traits between plant and dairy isolated *L. lactis* strains, the data generated may still serve as a valuable dataset for gene-function discovery approaches using recently developed post-genomic approaches.

Post-genomic discovery of molecular adaptations of *L. lactis* at near-zero growth rates

In 2001, *L. lactis* was the first LAB to have its complete genome sequenced. Subsequently, LAB genomics has rapidly developed further and includes the implementation of various functional genomics technologies, including trancriptomics, proteomics and metabolomics. These technologies massively expanded the average scope of current experimental approaches in microbiology, and are also successfully implemented in industrial microbiology research. Especially, they have provided insight in the molecular characteristics of the fermentation process executed by LAB at an unprecedented resolution, which can be exploited to improve their fermentation performance (1, 58). Multivariate analyses and pattern recognition software suits enabled the reduction of dataset-complexity by focusing on the underlying determinants in a reduced variation-space that can be visualized and interpreted. These analyses also discovered unexpected relations between variables, which can provide useful and novel biological relationships that drive the generation of new and experimentally testable hypotheses. In this thesis we have employed transcriptomics to unravel the molecular and metabolic adaptations of *L. lactis* at near-zero growth rates (Chapter 3 and 4).Time-resolved whole genome transcriptome analysis during retentostat cultivation was performed and analyzed using one of the pattern recognition tools (STEM) to cluster genes into predetermined expression pattern and evaluate the enrichment of functional gene categories into specific profiles of expression (17). Moreover, the transcriptome data were integrated with the metabolome analysis of organic and amino acids produced and/or consumed by the culture.

The transcriptome analyses revealed that the expression patterns of pyruvate dissipation related genes were in good agreement with the metabolic fluctuations observed in the *L. lactis* retentostat culture, which appeared to reproducibly fluctuate between mixed acid and lactic

fermentation during retentostat cultivation (Chapter 2 and 3). Notably, retentostat cultivation studies using other LAB, *S. cerevisiae*, or *B. subtilis* revealed virtually constant metabolic behavior of these organisms during retentostat cultivation. Retentostat fermentations using other LAB consistently reported that the metabolic end-product pattern formed from pyruvate was strongly dominated by lactate (23, 26, 35, 64), whereas complete aerobic conversion of glucose to CO_2 was observed for *B. subtilis* (42), and consistent patterns of ethanol and CO_2 formation were seen in *S. cerevisiae* retentostat cultures (5). However, these observations are in clear contrast with the product formation by *L. lactis*, which produced lactate, acetate, formate, and ethanol under retentostat cultivation in variable ratios at different time points during the extended cultivation. *L. lactis* retentostat cultures showed highly reproducible metabolic shifts within its central carbon metabolism, between mixed-acid and lactic fermentation. Remarkably, these shifts appeared to be regulated at the transcriptional level of the genes encoding the corresponding pyruvate-dissipation enzyme pathways as well as the central energy generating pathway (glycolysis) (Chapter 3). Although the control of glycolytic flux and pyruvate dissipation in *L. lactis* is not well understood, it has been proposed that their regulation is predominantly controlled by allosteric modulation of enzyme activity and/or sugar import rates in conditions of low carbon flux (10, 20). Transcriptional regulation of glycolytic flux and pyruvate metabolism in *L. lactis* has not been extensively reported before, although it has been described that the three glycolytic genes encoded within the *las* operon (*pfk, pyk, ldh*) are controlled by carbon catabolite control in *L. lactis* MG1363, which contributes to the control of mixed acid and homolactic pyruvate dissipation in this strain (34). The high level of conservation of the glycolytic pathway and its downstream pyruvate dissipation repertoire in different strains of *L. lactis* makes it relatively unlikely that the metabolic fluctuations observed for strain KF147 during retentostat cultivation are unique for this strain, and we propose that these observations are more likely a conserved response of *L. lactis* to the growth conditions induced by carbon-limited retentostat cultivation.

At extreme low-growth rates, some of the amino acid metabolism pathways (*e.g.* branched chain and aromatic amino acids) displayed fluctuations similar to those seen in the fermentation end-product fluctuations. Again, these amino acid metabolism fluctuations appeared to be at least in part controlled at the transcriptional level (Chapter 3). Interestingly, the glycolytic intermediates 3-phosphoglycerate, phosphoenolpyruvate, and pyruvate provide

direct connections of the central carbon metabolism pathway with amino acid synthesis pathways (40), and during retentostat cultivation, it is remarkable, that transcription of the genes related to the synthesis of these carbon metabolism intermediates and certain amino acids displayed similar profiles (Chapter 3).

The transcriptional regulator CodY is the key player in controlling transport and catabolism of oligopeptides, di/tripeptides and amino acids in several Gram-positive bacteria, including lactococci (13). Under nutrient limited conditions, CodY-mediated repression of genes encoding peptide transporter systems is relieved to enhance peptide and amino acid import capacity, aiming to sustain nitrogen metabolism in the cells. In addition, CodY has been reported to play a role in regulation of carbon metabolism through regulation of the genes of the incomplete Krebs cycle in *L. lactis*, which connects carbon metabolism with branched chain amino acid and glutamate metabolism via the intermediate α-ketoglutarate. Thereby, CodY controls an important linkage between nitrogen- and carbon-metabolism in *L. lactis* (13). Analogously, at extremely low-growth rates resulting from severely limited carbon availability, CodY regulation may mainly affect nitrogen metabolism but may also indirectly influence carbon metabolism and pyruvate dissipation in *L. lactis* KF147. Such a role of CodY is supported by the identification of a conserved *cis*-acting sequence motif upstream of several amino acid synthesis related genes that contains a palindromic sequence and bears similarity to the previously reported target site for the *L. lactis* MG1363 CodY (13). We propose this motif as the target sequence for CodY regulation in *L. lactis* KF 147 (Chapter 3), and postulate a central role for this CodY mediated regulation in the tuning of carbon and amino acid metabolism in *L. lactis* at extremely low growth rates enforced by retentostat cultivation. Intriguing in that perspective is a study in *B. subtilis* that reports that CodY can interact with the central regulator of carbon catabolite control CcpA (63). CcpA controls a large repertoire of genes through its interaction with the conserved catabolite response element *cre*, which is present upstream of a variety of genes involved in import and utilization of different carbon sources and also controls the expression of the lactococcal *las* operon (see above). Such an interaction of CodY and CcpA provides further evidence for the strong intertwinement of carbon and nitrogen metabolism control, and could play a prominent role in the transcriptional adaptation of *L. lactis* KF147 under retentostat conditions (Fig. 3). Moreover, the modulation of CcpA activity under near-zero growth conditions is strongly

supported by the observed progressive induction of expression of gene-clusters involved in the import and utilization of alternative carbohydrate sources (other than glucose). After extended retentostat cultivation, *L. lactis* KF147 expresses these systems for the use of alternative carbohydrates at a very high level, and the functional consequence of this expression could be confirmed by the higher acidification rates of the predicted alternative carbon sources in cells derived from long-term retentostat cultivation (Chapter 3; Fig. 3). CcpA control has been shown to be co-regulated by additional factors including HPr and intracellular ATP/ADP levels, and the role of these co-regulators of CcpA regulon under retentostat conditions deserves further attention.

The relieved repression of alternative carbohydrate utilization systems illustrates some kind of anticipation of these bacteria that appear to expand their carbohydrate utilization repertoire to more effectively harness eventual carbohydrates that they encounter in their environment. Such 'multivorous' responses have been proposed to be an important strategy of microbes to cope with conditions characterized by very low nutrient availability, and it has been proposed that these adapted, 'mutivorous' cells can simultaneously import and metabolize a variety of different carbohydrate substrates providing these cells with a kinetic advantage and expanded metabolic flexibility (15). These gene regulatory characteristics and their functional consequences could provide an evolutionary advantage for *L. lactis* (and other bacteria) in severely nutrient-restricted environments (15, 16, 18).

Enhanced stress response in *L. lactis* to low-growth rates

Comprehensive transcriptome mining revealed that the expression level of genes involved in several stress responses was gradually induced in *L. lactis* at near-zero growth rates. In chapter 4, stress associated transcriptome data were combined to phenotypic stress robustness analyses of the cells taken from the retentostat cultivation at different timepoints (*i.e*. heat, acid, and oxidative stress). Notably, the high degree of reproducibility achieved by subsequent chemostat and retentostat cultivation procedures is very strongly illustrated by the highly comparable stress survival curves obtained in these analyses. To further verify and quantify the experimental inactivation data, kinetic parameters of the kill-curves obtained were estimated using the Weibull inactivation model. Additionally, correlations were quantitatively modelled and accurately described the relationships between growth-rate, stress-robustness,

and stress-gene expression levels, generating correlation coefficients for each of the correlations involved (Chapter 4).

Microbes can adapt to environmental stress conditions by activating stress response systems, which can trigger cross-resistance to other stresses. When a bacterium is exposed to a single or multiple stress treatments, cross-resistance is provoked by the increased expression of generic adaptive stress responses that deliver survival advantage to the bacterium (14). Cross-resistance studies have commonly been performed using batch cultures, which progress from the logarithmic phase to the stationary phase of growth, or chemostat cultivation under several stress conditions at low-growth rates. Cells obtained from extended retentostat cultivation and displaying extremely low specific growth rates were considerably more resistant to diverse stress conditions as compared to chemostat-, and stationary phase-associated samples. Moreover, the findings in this thesis propose that genes that are typically associated with the heat-, acid- and other stress responses can serve as molecular biomarkers for increased robustness in *L. lactis*. Such biomarkers could enable the predictive estimate of the robustness level of lactococci grown under specific conditions, which may be employed to design starter culture production regimes for the production of robust cultures that can better withstand the variety of stresses associated with industrial processing. Furthermore, enhanced robustness of LAB may influence product characteristics that depend on long term maintenance of cellular integrity, like some flavor and aroma formation pathways that play a key role during product ripening (50), but may also enhance prolong the shelf-life of LAB containing products in which viability of the bacteria is a prerequisite like for example probiotic products.

In lactococci, the repressor protein HrcA, which binds to the palindromic operator sequence CIRCE (controlling inverted repeat of chaperone expression), and CtsR, which binds to a heptanucleotide direct repeat called the CtsR-box, play key roles in the control of canonical chaperone complexes composed of DnaK-GrpE-DnaJ / GroES-GroEL and the protease complexes ClpB-ClpC-ClpE-ClpP, respectively (31, 53). Under heat stress conditions, HrcA- and CtsR-associated repression of genes encoding heat-shock proteins is relieved to produce chaperons that assist the (re-)folding of denaturing proteins and/or produce a protein degradation machinery to remove denaturing and aggregating proteins (53). Analogously, under extreme restricted carbon source availability, the HrcA- and CtsR-regulons were completely activated to enable *L. lactis* to sustain cellular viability at these low growth rates.

The induction of chaperone functions that support protein function maintenance, illustrate that under these energetically challenging conditions the intrinsic gene-regulatory network wiring in these bacterial is geared to invest substantial amounts of energy in the build-up of additional maintenance associated functions like the chaperones. Besides the HrcA and CtsR regulons, some other undefined regulators may also be involved in controlling some other stress-gene repertoires, such as cold shock proteins that have been proposed to act as nucleotide chaperones that can increase the efficiency of translation or transcription and/or replication, respectively. Alternatively, we may not yet fully understand the extent by which HrcA and CtsR repressors may govern gene expression levels of genes that are not directly controlled by either of these two regulators through the CIRCE- or *ctsR*-box *cis*-elements, but through so-far unknown regulatory mechanisms. Such expanded gene expression control by these canonical stress regulators has recently been illustrated in *L. plantarum* by the comparative whole genome transcriptome analyses of the regulatory consequences of single *hrcA* or *ctsR* deletion-mutations, or mutation of both stress regulators, which elicited a plethora of gene regulatory consequences that exceed the classical *hrcA* and *ctsR* regulons (56).

Prolonged retentostat cultivation massively activates the cellular chaperone machineries and thereby supports the maintenance of the structure and function of cellular macromolecular components (*e.g*. nucleic acids and proteins). This finding further strengthens the notion of Chapter 2 that postulates that adaptation to extremely low specific-growth rate is dominated by a complete dedication of energy sources to maintenance-related functions, which appears to include maintenance supporting chaperone functions (Chapter 4; Fig. 3).

Adaptations of *L. lactis* to severe starvation in non-growing cells

In chapter 3 and 4, we investigated the several transcriptional responses of *L. lactis* at near-zero growth rates, such as the relief of genes involved in different sugar uptake and catabolism and stress response functions, but also the repression of genes related to cell membrane metabolism. Similarly, stationary phase lactococci in batch cultures display also some of these responses, but also repress the transcription of genes involved in central metabolism, cell division, and macromolecule synthesis, while they also induce the genes associated with the autolysis process (25, 46). These latter responses were entirely absent in

retentostat-grown lactococci, corroborating that the retentostat conditions are substantially different from those encountered in the stationary phase of batch growth. Moreover, these findings supported the suitability of the retentostat cultivation system for the study of the lactococcal responses to severe nutrient starvation in non-growing cells that are not confounded by the accumulation of metabolic end-products, or the emergence of large amounts of dead and lysed cells. Therefore, following 42 days of retentostat cultivation (Chapter 2), the medium supply was switched off, leading a virtually instant and severe carbon starvation of the culture in the fermenter vessel. After 24 hour of starvation, the medium supply was switched on again to allow the culture to recover from the induced starvation. Notably, cell viability was largely sustained throughout the starvation and recovery phases, although the typical damaged population present after long term retentostat cultivation appeared to at least partially lose its viability during the starvation period (Chapter 5). Under strict starvation large sets of genes related to translational, DNA replication and transcription as well as cell division machineries were strongly repressed (*i.e*. stringent response), which is in clear contrast to the sustained high level expression of these functions during prolonged retentostat cultivation. During the subsequent recovery phase, the expression of these stringency response associated functions was rapidly recovered to, after 24 h of recovery, reach levels that were very similar as those observed prior to the starvation phase (Chapter 5). Intriguingly, repression of genes associated with typical stringent responses (translation, transcription, etc.) did already occur during prolonged retentostat-cultivation of *B. subtilis* (42) and *S. cerevisiae* (5). A remarkable transcriptional response of *L. lactis* KF147 to starvation was that these non-growing cells induced the complete set of genes associated with natural competence, which may provide starved-cells with additional nutrient (*i.e*. exogenous DNA) and explain this response from a purely nutritional perspective. Alternatively, expression of these functions may enable cells to expand their genetic repertoire by the uptake of exogenous intact DNA strands and their chromosomal incorporation. This may provide these cells with additional genetic capacities that contribute to their survival under these harsh conditions. However, although we tried to experimentally verify this natural competence for transformation using both intact and linearized plasmid DNA, we failed to verify this phenotypic trait in these bacteria.

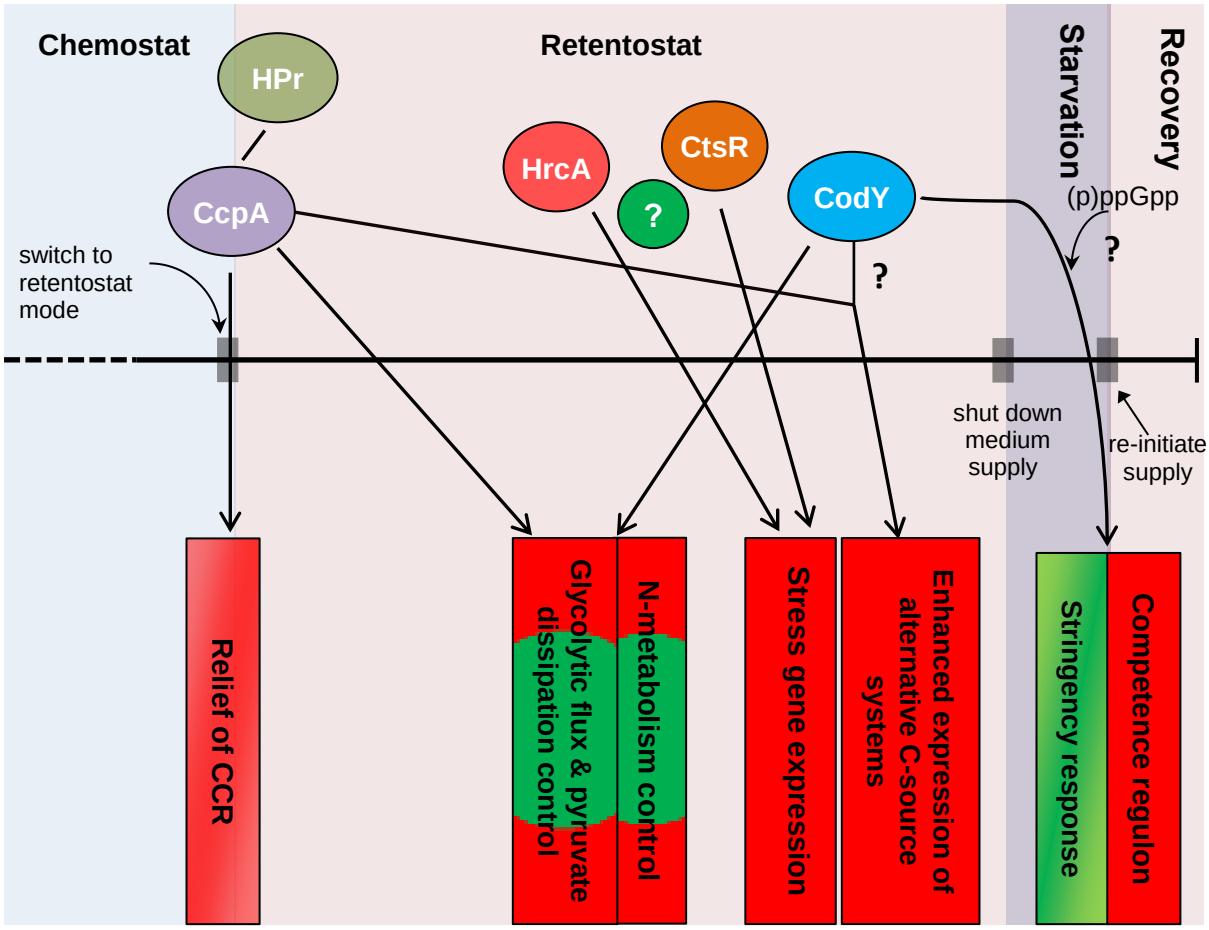

Figure 3: Integrated view of adaptive regulations of *L. lactis* KF147 to near-zero growth rates and subsequent severe starvation. In rectangular boxes, red and green indicate increased and decreased expression of the mentioned functional categories, respectively.

In Chapter 5, a highly conserved motif, which resembles the lactococcal CodY motif, was identified to be overrepresented in the upstream regions of genes of which the expression was affected during the starvation and/or recovery phases. At severe starvation condition, CodY repression is apparently relieved, leading to induction of various genes associated with natural competence, purine biosynthesis, and the transcription- and translational-machineries (Fig. 3). This observation implies a dualistic (different regulation role under different conditions) and bimodal (both up and down regulation of target genes) role of CodY in the adaptation of lactococcal gene expression patterns during its adaptation to near-zero growth, which is distinct from its role in the adaptation to severe starvation conditions, and encompasses both up and down regulation of a variety of genes within the *L. lactis* KF147 genome. These findings may suggest that CodY regulation is co-regulated in concert with other regulatory

cascades in the cell, which play a role in fine-tuning of the regulation of gene expression by CodY (Fig. 3). These co-regulators may include CcpA, which in acting in concert with CodY connects C- and N-metabolism control in *L. lactis* (see above). Moreover, in *B. subtilis*, GTP has been shown to act as a ligand for CodY and during the stringency response CodY could react to alarmone (p)ppGpp levels in this species (22, 33, 62). A similar role of CodY in controlling the alarmone mediated responses in *L. lactis* provides an attractive co-regulatory model to explain the identification of the CodY regulatory motif upstream of many genes induced and repressed during the starvation and recovery experiments (Fig. 3). It could be very interesting to evaluate the survival and molecular behavior of a *L. lactis* CodY mutant derivative in extended retentostat cultivation and or severe starvation conditions. One would anticipate that many of the adaptation described in this thesis would be dysregulated in such a strain, which may render it much less adaptive to slow or extremely-low growth rates.

Different industrial microbes at near-zero growth rates

The findings of the different projects within the joint-program 'Zero-growth Product formation' of the Kluyver Centre for Genomics of Industrial Fermentation were combined in a minireview that compares physiological, metabolic and genome-wide transcriptional adaptations of the industrially important microbes *L. lactis*, *Lb. plantarum*, *B. subtilis*, *S. cerevisiae*, and *A. niger* to the near-zero growth rates, induced by energy-limited retentostat cultivation. The shared responses of these microbes to near-zero growth rates include increased stress tolerance genes. Other adaptations, such as changes in morphology and (secondary) metabolite production, appeared to be species specific (Chapter 6).

Adaptation of central energy metabolism pathways did not occur in any of the industrial organisms, except *L. lactis*, which displayed substantial metabolic adaptations of its central energy metabolism pathway expression as well as product formation. Metabolic adaptations in the other organisms were apparently restricted to their secondary metabolite production, where *Lb. plantarum* was predicted to enhance its production of plant-growth stimulating metabolites, while a variety and highly significant activation of secondary metabolite pathways was observed in *A. niger*. Although the induction of stress related functions appeared to be a conserved response to retentostat growth among these industrial microbes, the exact nature of that stress response was still quite species specific. Not in all organisms

did stress responses include the generic chaperone-type heat-shock protein activation, which appeared to occur only in *L. lactis* and *S. cerevisiae*, where also these responses could be verified at the level of enhanced stress tolerance levels in near-zero growth cultures. Other microbes did still elicit stress responses but these were of a different nature (*e.g.* SOS response; see Chapter 6). Elevated robustness of microbial cells is of clear industrial relevance, since these cells are likely to survive better under the harsh industrial processing and storage conditions (Chapter 6). Possibly starter culture producing industries could harness the zero-growth induced robustness enhancement by incorporating a stage to their production process that induces the adjustment of the starter culture to non-growing conditions, and thereby raise its robustness level. Obviously, retentostat cultivation is incompatible with large scale industrial starter culture production, but it may be possible to still induce analogous molecular responses as those observed under retentostat cultivation in an industrial setting. In such approaches the biomarker quality of the identified stress response gene sets may be used as guidance for the design of industrial conditions that enhance starter-robustness in a post-production procedure.

For industrial processes that exploit microbial catalysis in cell factories for the production of desirable metabolites it is preferable to employ non-biomass driven fermentation applications. For example, in bioethanol production by *S. cerevisiae* (7), in the industrial enzyme, protein or peptide ingredient production by *B. subtilis* and *A. niger* (43, 61), and some of the high-value flavor, texture, and health metabolite production applications that use LAB (27, 32, 52), cell biomass the major by-product. Therefore, uncoupling of growth and metabolite production is of great relevance for industrial cell factories. This may in some cases be achieved by engineering strains to efficiently convert the available carbon source into the desirable end-product, in a growth-uncoupled fashion. Such applications have been demonstrated for *L. lactis* for the production of its endogenous metabolites, such as diacetyl, mannitol, acetaldehyde, and citrate, (6, 21 29, 45) as well as the alternative metabolite L-alanine (27). Retentostat cultivation in combination with engineered microbes may provide highly efficient industrial cell factories that can sustain production over extended periods, due to the robustness of the cultures that sustains cell-viability.

Concluding remarks

In this thesis, the quantitative physiology of *L. lactis* at near-zero conditions was studies, employing both metabolic and transcriptome analyses in an experimental set-up of carbon-limited retentostat cultivation. To comprehensive understand the metabolic adaptation of *L. lactis* to retentostat cultivation; the transcriptional responses of the culture were determined in combination with its metabolic adaptations. Furthermore, the increased robustness to several stresses of *L. lactis* after its adaptation to carbon-limited retentostat cultivation was investigated at molecular and physiological level, as well as stress-survival data were analyzed by using the Weibull model. The relationships between growth-rate, stress-robustness, and expression level of stress-genes were quantitatively correlated. Moreover, the distinction between the transcriptional responses to extreme low carbon source availability induced by retentostat cultivation, severe starvation, and recovery phase conditions was investigated. The summary of adaptive regulations of *L. lactis* KF147 to near-zero growth rates, subsequent strict starvation as well as recovery phase in retentostat cultivation has been compiled in Figure 3. In this context, the nitrogen metabolism associated functions of *L. lactis* are of great industrial importance based on their prominent role in flavor formation in product matrices (50). The proposed transcriptional control by master-regulator CodY in cells growing at extremely low specific growth rates is highly relevant for industrial applications, since the CodY activity may be influenced by the addition of specific amino acids or peptides, which may allow targeted modulation of the flavor forming capacity of these lactococci as a function of the peptide composition. Thereby modulation of cheese flavor formation may be controlled by the addition of specific peptides and/or amino acids, which drive the altered regulatory role of CodY and may thereby, change the overall N-metabolic output, including the significant flavor compounds.

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Appendices

Summary

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Summary

Lactococcus lactis is an important lactic acid bacteria (LAB) species that is used for the manufacture of dairy products, such as cheese, buttermilk, and other fermented products. The predominant function of this bacterium in dairy fermentation is the production of lactic acid, as its major fermentation end-product that contributes to preservation and microbial safety of the product. In addition, *L. lactis* hydrolyses casein and other milk proteins, and some strains of the subspecies spp. *diacetylactis* can contribute to citric acid fermentation, whereby these starter cultures of *L. lactis* influence important product properties, *i.e.* flavor. Next to industrial (and artisanal) food fermentation applications, *L. lactis* is also frequently encountered in natural ecosystems such as in (rotting) plant material, especially when plant materials are degraded by co-inhabitants of the same niche, like yeasts and fungi, that liberate nutrients from plant polymer structures and thereby make them accessible for the lactococci.

Due to restricted energy source availability, natural microbial communities commonly live in a situation that can be characterized as 'hunger', which is different from strict nutrient-starvation because concentrations of fermentable energy sources are usually not absolutely zero in ecosystems. As a consequence environmental microbes commonly grow at very low-growth rates as compared to laboratory cultures. Analogously, microorganisms can experience such nutrient-poor conditions in diverse industrial fermentation applications. For example, LAB encounters extreme low or no energy source availability during the extended ripening process of cheeses or dry sausages, which can take months. Despite these harsh environmental conditions, many LAB are able to remain viable in these processes for months and sustain a low-level metabolic activity, which plays an important role in their contribution to flavor and aroma formation in the product matrix. In this thesis, the quantitative physiology of *L. lactis* at near-zero specific growth rates was studies, employing both metabolic and genome-wide transcriptome studies in an experimental set-up of carbon-limited retentostat cultivation. The work described specifically addressed some of the research questions summarized below.

The initial target in this thesis was to uncouple growth and maintenance-related cellular processes in *L. lactis*, using carbon-limited retentostat cultivation. This culture set-up allowed the adjustment of a growing *L. lactis* culture to a non-growing state, while retaining high

culture viability. This enabled us to study the physiological and metabolic adaptations of this bacterium to near-zero growth rates in a comprehensive manner. The maintenance energy coefficients of *L. lactis* KF147 were deduced from the parameters determined during retentostat cultivation and compared to those extrapolated from chemostat cultivations at 'high' dilution and growth rates. The maintenance coefficients determined by these two approaches were virtually identical, illustrating that maintenance energy requirements are constant and apparently independent of the specific growth-rate under the conditions used. In addition, biomass accumulation during retentostat cultivation could be accurately predicted on basis of the calculated maintenance coefficient, illustrating the appropriateness of the growth-and-energy models employed to mathematically describe biomass accumulation under non-growing conditions. Moreover, the estimated energy and substrate cost distributions between maintenance- and growth-associated processes further confirmed that the retentostat culture had reached a near-zero growth state after extended cultivation. Intriguingly, the metabolic patterns of *L. lactis* during retentostat cultivation appeared to reproducibly fluctuate over time between mixed-acid and lactic fermentation.

To further understand the metabolic adaptation of *L. lactis* to retentostat cultivation, the transcriptional responses of the culture were determined in combination with its metabolic output. The transcriptome data revealed that the metabolic fluctuations between mixed-acid and lactic fermentation were consistent with adaptation of the transcriptional profiles of the genes encoding the corresponding carbohydrate metabolism genes. This implied that pyruvate dissipation in *L. lactis* KF147 is at least partially controlled at the transcriptional level. Additional transcriptome mining led to the identification of a candidate conserved *cis*-acting element, resembling the previously identified CodY binding-motif in *L. lactis* MG1363, which led us to postulate the involvement of CodY in the control of several genes associated with amino acid synthesis pathways in cells that are maintained at extremely-low specific growth rates. Furthermore, the restricted carbon availability during retentostat cultivation led to the progressive relief of carbon catabolite repression and the high-level induction of alternative carbon-source catabolic functions, which enabled the bacteria to ferment these alternative energy sources at accelerated rates. The integrated transcriptome and metabolome analyses supported reflect the considerable intertwinement of carbon- and nitrogen-

metabolism in *L. lactis*, and proposed a role of the global regulator CodY in the tuning of these transcriptional and metabolic adaptations to extremely low growth rates.

Transcriptome profiles also revealed that at near-zero growth rates the expression of several genes associated with different stress responses, including, heat, cold, acid, and cell membrane stresses, were induced in *L. lactis*. Notably, these transcriptome results could be confirmed at physiological level by stress resistance analyses, revealing substantially increased survival of cells obtained from extended retentostat cultivation to heat, acid, and oxidative challenges as compared to cells obtained from growing, chemostat cultures. The experimental stress-survival data were analyzed using the Weibull inactivation model to obtain kinetic parameters of stress-induced inactivation for the different culture samples. This enabled the determination of the quantitative correlations that describe the relationships between growth-rate, stress-robustness, and expression level of stress-genes. Taken together, these analyses established the quantitative correlation between stress-gene expression levels, growth-rate and robustness, implying that these parameters, including stress-gene expression levels, may serve as robustness biomarkers in *L. lactis* KF147.

Several transcriptional responses that are associated with adaptation of lactococci to the stationary phase of growth were also elicited in near-zero growth retentostat cultures, including the activation of alternative sugar import systems as well as stress response functions. However, other typical stationary phase associated responses, like the repression of genes related to central metabolism, cell division, and macromolecule synthesis were absent in retentostat-cultures, which is a clear illustration of the clear distinction between retentostat conditions and those encountered during the stationary phase of growth in batch cultures. To further study the distinction between the transcriptional adaptation to extremely low specific growth rates induced by carbon limited retentostat cultivation and strict starvation conditions, the extended retentostat cultivation, was followed by a closing of the medium supply for 24 hours for induce strict starvation, and subsequently, the cultures were allowed to recover from this challenge by re-initiation of the medium supply. During starvation, the expression of genes associated to transcription and translational machineries, cell division, and cell membrane energy metabolism were massively repressed, and the expression of these genes was rapidly recovered upon re-initiation of the medium supply. Remarkably, strict starvation

conditions elicited the induction of the complete set of genes related to natural competence in near-zero growth cells, which may provide these starved-cells with an additional nutrient supply (*i.e.* exogenous DNA). Analogous to what was found in the transcriptome datasets related to the adaptation of *L. lactis* to retentostat cultivation, the transcriptional responses to strict starvation were predicted to involve a role of the CodY master regulator. This was concluded from the fact that the CodY motif was enriched within the upstream regions of strict starvation-regulated genes, including those encoding components of the transcription and translational machinery, the purine biosynthesis pathway, and the operons encoding natural competence in *L. lactis*. These findings suggest a bimodal role of the global regulator CodY in orchestration of the *L. lactis* transcriptome responses to near-zero-growth conditions as well as to the subsequent strict starvation.

The observations of the different projects within the joint-programme 'Zero-growth product formation' of the Kluyver Centre for Genomics of Industrial Fermentation were brought together in a minireview. The aim of this review was to compare the physiological and molecular adaptations to carbon-limited retentostat conditions in industrially important microbes, including *Lactobacillus plantarum*, *Lactococcus lactis*, *Bacillus subtilis*, *Saccharomyces cerevisiae*, and *Aspergillus niger*. The comparison addresses adaptations of physiological characteristics, such as specific minimal growth-rates, cell morphology, maintenance energy requirement, but also evaluates conserved and species-specific adaptations of the near-zero growth associated transcriptional profiles in these industrial workhorses.

In conclusion, this thesis describes the quantitative physiological, metabolic, and genome-wide transcriptional adaptations of *L. lactis* at near-zero specific growth rates induced by carbon source limited retentostat cultivation, and compares these molecular adaptations to those elicited by strict carbon-starvation conditions. Moreover, the physiological and molecular responses studied in *L. lactis* were compared to those observed in other industrially important microorganisms when they are cultured under near-zero growth conditions.

Samenvatting

Lactococcus lactis is een belangrijke melkzuurbacterie soort die wordt gebruikt voor de vervaardiging van zuivelproducten, zoals kaas, karnemelk en andere gefermenteerde producten. De belangrijkste functie van deze bacterie in de fermentatie van melk is de productie van melkzuur, dat als fermentatie eindproduct bijdraagt aan de houdbaarheid en microbiële veiligheid van het product. Daarnaast hydrolyseert *L. lactis* caseïne en andere melkeiwitten en sommige stammen van de subspecies spp. diacetylactis kunnen bijdragen aan citraat fermentatie waardoor deze *L. lactis* startercultures belangrijke producteigenschappen en met name smaak kunnen beïnvloeden. Naast toepassingen in industriële (en ambachtelijke) voedselfermentatie wordt *L. lactis* ook vaak in natuurlijke fermentatie ecosystemen aangetroffen, zoals in (rottende) plantmaterialen, waar *L. lactis* bijdraagt aan de afbraak van plantaardige stoffen die veelal door andere microbiële bewoners van diezelfde niche, zoals gisten en schimmels, zijn vrijgemaakt uit de plantaardige polymeren en daarmee de groei en fermentatie van *L. lactis* faciliteren.

Vanwege de beperkte beschikbaarheid van energie, leven de meeste microbiële gemeenschappen in de natuur in een situatie die kan worden gekarakteriseerd als 'nutriënt gelimiteerd' en die is anders dan strikte nutriënt depletie, omdat de concentraties van fermenteerbare energiebronnen meestal niet helemaal nul zijn in natuurlijke ecosystemen. Als gevolg, groeien micro-organismen in natuurlijke omgevingen vaak met een extreem lage groeisnelheid. Dit is zeker zo in vergelijking met cultures die in het laboratorium worden gekweekt. Soortgelijk, kunnen micro-organismen nutriënt gelimiteerde condities tegenkomen gedurende industriële fermentatie toepassingen, zoals gedurende de langdurige rijpingsprocessen die een onderdeel zijn van de productie van sommige soorten kaas en worst, waar de betrokken melkzuurbacteriën meerdere maanden kunnen worden blootgesteld aan een productmatrix met extreem lage of helemaal geen energiebron. Ondanks deze zware omstandigheden, slagen sommige melkzuurbacteriën erin om maandenlang levensvatbaar te blijven gedurende deze processen en ze behouden een lage metabolische activiteit, die een belangrijke rol speelt in de bijdrage die deze bacteriën leveren in de smaak en aroma vorming in het product.

In dit proefschrift wordt de kwantitatieve fysiologie van *L. lactis* bestudeerd bij specifieke groeisnelheden die bijna nul zijn, die aan de culture worden opgelegd door cultivering onder zogenaamde koolstof-gelimiteerde retentostat condities. Door daarnaast gebruik te maken van zowel metabole en transcriptome studies konden de moleculaire reacties van *L. lactis* op deze extreem lage groeisnelheden worden bepaald. De beschreven werkzaamheden en de specifieke onderzoeksvragen die ten grondslag liggen aan dit proefschrift worden hieronder kort toegelicht.

De initiële doelstelling van dit proefschrift was om de groei en onderhoud gerelateerde cellulaire processen in *L. lactis* te ontkoppelen, met behulp van de retentostat kweekmethode. Deze kweekmethode geeft de mogelijkheid om de aanpassingen van *L. lactis* te bestuderen gedurende extreem lage groeisnelheid, terwijl de bacteriën wel levensvatbaar blijven. Hierdoor konden de fysiologische en metabole aanpassingen van deze bacterie op een globale en kwantitatieve manier in kaart worden gebracht, onder condities waarbij de cellen vrijwel niet meer groeiden. De energie die *L. lactis* KF147 nodig heeft voor onderhoud-gerelateerde processen werd afgeleid uit de parameters die bepaald werden gedurende extreem lage groeisnelheid en deze waarden bleken zeer vergelijkbaar met soortgelijke berekeningen op basis van extrapolatie vanuit cultures die met een vaste en constante snelheid groeiden. Dit betekent dat de onderhoudsenergie eisen van de cel constant zijn en blijkbaar onafhankelijk van de specifieke groei-snelheid. Daarnaast bleek dat biomassa accumulatie gedurende retentostat cultivering nauwkeurig kan worden voorspeld op basis van de berekende onderhoud energie behoeften. Dit onderschrijft dat de gebruikte groei-en energie-modellen voor de kwantitatieve beschrijving van groei, biomassa en energie conversie een accurate beschrijving van niet groeiende cultures kunnen geven. Een opvallende observatie was het feit dat het metabole gedrag van *L. lactis* tijdens retentostat cultivering onverwacht bleek te fluctueren, waarbij de bacteriën wisselend melkzuurvergisting en gemengd-zuur vergisting (azijnzuur en melkzuur) vertoonden.

Om de aanpassingen van *L. lactis* gedurende retentostat cultivering op moleculair niveau te kunnen beschrijven werden metabolieten profielen en transcriptomen van de cultures bepaald. Uit de transcriptome gegevens bleek dat de metabole fluctuaties tussen melkzuurvergisting en gemengd-zuur vergisting in overeenstemming waren met de transcriptie-profielen van de

genen die coderen voor de enzymen die betrokken zijn bij de bijbehorende vergistingreacties. Dit impliceerde dat de omzetting van pyruvaat in *L. lactis* KF147 tenminste gedeeltelijk gereguleerd wordt op het niveau van gentranscriptie. Aanvullende analyse van de transcriptoom data leidde tot de identificatie van de mogelijke regulatie van dit proces, waarin de eerder geïdentificeerde regulator CodY een rol lijkt te spelen. In een andere *L. lactis* stam (MG1363) is aangetoond dat deze regulator betrokken is bij de controle van een aantal genen die geassocieerd zijn met aminozuur metabolisme, terwijl de gegevens in dit proefschrift aangeven dat CodY waarschijnlijk ook een rol speelt in de aanpassing aan extreem lage groeisnelheden. Daarnaast leidde de beperkte beschikbaarheid koolstof tijdens retentostat cultivering ook tot de geleidelijke opheffing van koolstof gestuurde katabole repressie waardoor systemen die andere koolstof-bronnen kunnen gebruiken verhoogd tot expressie kwamen. De geïntegreerde metabole en transcriptoom analyse bevestigt de grote verwevenheid van de regulatie patronen van het koolstof-en stikstof-metabolisme in *L. lactis*, waarbij de centrale regulator CodY een rol lijkt te spelen in de afstemming van de transcriptionele en metabole aanpassingen die in de bacterie plaatsvinden gedurende extreem lage groeisnelheden.

Transcriptoom analyse toonde ook aan dat de expressie van genen betrokken bij verschillende stress-responses ook verhoogd tot expressie kwamen gedurende de aanpassingen aan extreem lage groeisnelheden in *L. lactis*. Bovendien konden deze transcriptoom resultaten op fysiologisch niveau worden bevestigd door stressbestendigheids analyses, waarbij sterk toegenomen stress-overleving werd gemeten in cellen die waren verkregen uit retentostat cultures die waren aangepast aan extreem lage groeicondities. Deze bacterie cellen bleken veel beter bestand te zijn tegen hitte-, zuur-, en oxidatieve-stress condities vergeleken met cellen die waren verkregen uit groeiende cultures. Met behulp van modelleringen konden de kinetische parameters van stress-overleving worden geanalyseerd voor iedere geteste bacteriële culture, waardoor de wiskundige relaties tussen de specifieke groeisnelheid, het expressie niveaus van stress-gerelateerde genen, en de overleving onder stress condities konden worden bepaald. Deze benaderingen geven aan dat genexpressie niveaus van stress-gerelateerde genen kunnen dienen als robuustheids biomarkers in *L. lactis* KF147.

Verscheidene transcriptionele reacties die geassocieerd zijn met de aanpassing van lactococci aan de stationaire groeifase werden ook waar genomen in cultures die aangepast waren aan extreem lage groeisnelheden, zoals de activering van alternatieve suikerimport systemen en stressrespons functies. Echter, andere typische stationaire fase geassocieerd aanpassingen werden niet gezien, zoals de onderdrukking van genen betrokken bij het centraal energie metabolisme, de celdeling, en de synthese van verschillende macromoleculen. Dit geeft een duidelijk onderscheid aan tussen de condities zoals die tijdens stationaire fase van 'batch-culture' groei optreden en de condities gedurende retentostat cultivering. Om verder te bestuderen wat het onderscheid is tussen de transcriptionele aanpassing aan extreem lage specifieke groeisnelheden onder constante nutient limitatie en strikte nutrient depletie condities, werd aan het eind van de retentostat cultivering de medium toevoer van het systeem dichtgedraaid voor 24 uur waardoor er momentane en extreme nutriënt depletie optrad in de culture. Vervolgens werd de medium toevoer weer opengedraaid voor wederom 24 uur om te bestuderen hoe de culture zich zou kunnen herstellen. Tijdens nutriënt depletie werden de genen betrokken bij transcriptie, translatie, celdeling, en celmembraan processen alsmede het energie metabolisme sterk onderdrukt. Na herstel van de medium toevoer nam de expressie van deze genen echter weer snel toe zodat deze processen weer opnieuw actief werden gedurende het herstel van nutriënt depletie. Opvallend was ook dat de nutriënt depletie ervoor zorgde dat de complete set genen betrokken bij natuurlijke competentie werd geactiveerd, hetgeen zou kunnen betekenen dat deze uitgehongerde-cellen een nieuwe voedingsbron proberen aan te spreken, d.w.z. extracellulair DNA. Evenals de aanpassing aan extreem lage groeisnelheden, bleek ook de transcriptie aanpassing aan nutriënt depletie een rol van de centrale regulator CodY te voorspellen. CodY werd voorspeld een rol te spelen bij de regulatie van genen die coderen voor componenten van de transcriptie en translatie machinerie, de purine biosynthese route en de operons coderend voor natuurlijke competentie. Deze bevindingen suggereren een dualistische en tweeledige rol van de centrale regulator CodY in de afstemming van de *L. lactis* transcriptie patronen aan extreem lage groeisnelheden en strikte nutriënt depletie.

De bevindingen van de verschillende projecten binnen het gezamenlijke programma 'Zero-growth product formation', dat werd uitgevoerd binnen het Kluyver Centre for Genomics of Industrial Fermentation, werden samen gebracht in een minireview. Het doel van deze review

was om de fysiologische en moleculaire aanpassingen aan koolstof gelimiteerde extreem lage groeisnelheid te vergelijken in industrieel belangrijke micro-organismen, waaronder *Lactobacillus plantarum*, *Lactococcus lactis*, *Bacillus subtilis*, *Saccharomyces cerevisiae*, en *Aspergillus niger*. De vergelijking richtte zich op aanpassingen van fysiologische kenmerken, zoals specifieke minimale groei-snelheden, cel morfologie, onderhouds-energie behoeften, maar keek daarnaast ook naar geconserveerd en soort-specifieke transcriptie aanpassingen gedurende de aanpassing aan extreem lage groeisnelheden in deze industriële 'werkpaarden'.

In het kort, dit proefschrift beschrijft de kwantitatieve fysiologische, metabole en genoom-brede transcriptie aanpassingen van *L. lactis* aan extreem lage groeisnelheden opgelegd door koolstof gelimiteerde retentostat cultivering, en vergelijkt deze aanpassingen met de aanpassingen betrokken bij extreme nutriënt depletie. Bovendien zijn de aanpassingen gemeten in *L. lactis* vergeleken met de aanpassingen in andere micro-organismen aan soortgelijke condities van extreem lage groeisnelheid.

Acknowledgements

I hope that this section of my doctoral thesis is not the only page you are going to read, and that you have or will read the other sections, such as “About the author”, “List of publications” or “Summary”. Although a notable part of my time and energy in past years was employed to finish these sections and only my name is on the cover, I could never have completed all these chapters without invaluable support of my colleagues, friends, and family. Here, I would like to acknowledge all of those who have contributed to the work presented in this thesis.

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List of publications

Ercan O, Smid EJ, Kleerebezem M. 2013. Quantitative physiology of *Lactococcus lactis* at extreme low-growth rates. *Environ Microbiol* **15**:2319-2332.

Ucisik-Akkaya E*, **Ercan O***, Yesiladali SK, Ozturk T, Ubay-Cokgor E, Orhon D, Tamerler C, Cakar ZP. 2009. Enhanced polyhydroxyalkanoate production by *Paracoccus pantotrophus* from glucose and mixed substrate. *Fresen Environ Bull* **18**:2013-2022.

Ercan O, Wels M, Smid EJ, Kleerebezem, M. 2014. Molecular and metabolic adaptations of *Lactococcus lactis* at near-zero growth rates. *Appl Envrion Microbiol* (Submitted for publication)

Ercan O, Wels M, Smid EJ, Kleerebezem M. Molecular responses of *Lactococcus lactis* to severe carbon starvation in non-growing cells. (Manuscript in prepapration)

Ercan O, den Besten HMW, Smid EJ, Kleerebezem M. Improved stress robustness in *Lactococcus lactis* by adaptation to near-zero growth rates. (Manuscript in prepapration)

Ercan O*, Bisschops MMM*, Overkamp W, Jørgensen TR, Kuipers OP, Ram AF, Smid EJ, Pronk JT, Daran-Lapujade P, Kleerebezem M. Physiology and transcriptomes of different industrial microbes at extremely low specific growth rates. (Manuscript in preparation)

Overkamp W, **Ercan O**, Herber M, van Maris AJA, Kleerebezem M, Kuipers OP. Physiological and cell morphology adaptation of *Bacillus subtilis* at near-zero specific growth rates: a genome-wide analysis. (Manuscript in preparation)

*contributed equally

Overview of completed training activities

Discipline specific activities

Courses

Systems Biology: statistical analysis of ~omics data, VLAG, 2012, Wageningen, NL
Advanced Visualisation, Integration and Biological Interpretation of -omics Data, VLAG, 2011, NL
Advanced Course on Microbial Physiology & Fermentation Technology, BSDL, 2011, Delft, NL
Genetics and Physiology of Food-Associated Microorganisms, VLAG, 2010, Wageningen, NL
Bioreactor Design and Operation, VLAG, 2010, Wageningen, NL

Meetings and Conferences

EMBO Symposium New Approaches and Concepts in Microbiology, 2013, Heidelberg, Germany
Annual International Microbiology Conference of VAAM, 2013, Bremen, Germany
NCSB 2012 Symposium: Systems Biology, 2012, Amersfoort, NL
Microbial Stress Responses in Gordon Research Conferences, 2012, Holyoke, MA, USA
KNVM & NVMM 2012 Spring Meeting, 2012, Arnhem, NL
10th Symposium on Lactic Acid Bacteria, 2011, Egmond aan Zee, NL
8th, 9th, 10th Kluyver Centre Symposium, (2010, 2011, 2012), Noordwijkerhout, NL
4th, 5th Kluyver Centre Programme Meeting Day, (2011, 2012), Wageningen, NL

General courses

Career Perspectives, WGS, 2013, Wageningen, NL
Philosophy and Ethics of Food Science & Technology, WGS-VLAG, 2012, Wageningen, NL
Life Sciences Momentum 2012: Communicating Science, NGI, 2012, The Hague, NL
Scientific Writing, WGS, 2012, Wageningen, NL
PhD Week, VLAG, 2010

Optional courses and activities

Preparation PhD research proposal
PhD-PostDoc meetings, Wageningen, NL
TI Food and Nutrition Project Meetings (Kluyver Centre-matching), 2010-2012, Wageningen, NL
Microbiology Lunch Meetings, NIZO Food Research, 2010-2013, Ede, NL
VLAG PhD trip, Laboratory of Microbiology, China and Japan, 2011
Zero-Growth Project Meetings, Kluyver Centre, 2010-2013

About the author

Onur Ercan was born on March 11th, 1983, in Antalya, Turkey. His excitement to life sciences was developed with the birth of Dolly the sheep, which was the first mammal to be cloned from an adult cell, in the mid '90s. Thus, he attended the Department of Molecular Biology and Genetics in Istanbul Technical University in 2001, and graduated with his BSc diploma in 2005. Subsequently, he continued his MSc education at the Biotechnology Programme of the same university. In his MSc thesis, he worked on the physiology and metabolite production of wild-type and mutant *Saccharomyces cerevisiae* strains, which have previously been modified in their metabolic pathway for glycerol production by using metabolic engineering approaches, under supervision of Prof. Dr. Zeynep Petek Cakar and collaboration with Prof. Dr. Elke Nevoigt from Berlin University of Technology. Furthermore, he was also involved in the project of the investigation the growth physiology and polyhydroxybutyrate storage potential of *Paracoccus pantotrophus* from glucose and mixed substrate. In January 2008, he obtained his MSc degree.

Onur decided to pursue his carrer at industrial microbiology field in The Netherlands. He was appointed as a PhD candidate within the joint-project "Zero-growth product formation" of the Kluyver Centre for Genomics of Industrial Fermentation and conducted at Top Institute Food and Nutrition. He carried out his PhD research on the "Zero-growth of *Lactococcus lactis*" sub-project at NIZO Food Research and Laboratory of Microbiology in Wageningen University, under supervision of Prof. Dr. Eddy J. Smid and Prof. Dr. Michiel Kleerebezem (2009-2013). The output is presented in this thesis with the title: "Physiological and molecular adaptations of *Lactococcus lactis* to near-zero growth conditions".

To broaden his research scope and vision, Onur changed his research field and moved to Sweden. Since July 2014, he has been employed as a Postdoc researcher at the laboratory of Prof. Dr. Måns Ehrenberg in Uppsala University, working on proteome dynamics of *Escherichia coli* in response to changes in nutritional conditions.

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