# Comparative Functional Genomics of Amino Acid Metabolism of Lactic Acid Bacteria

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# Comparative Functional Genomics of Amino Acid Metabolism of Lactic Acid Bacteria

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**Abstract**

The amino acid metabolism of lactic acid bacteria used as starters in industrial fermentations has profound effects on the quality of the fermented foods. The work described in this PhD thesis was initiated to use genomics technologies and a comparative approach to link the gene content of some well-known lactic acid bacteria to flavor formation and to increase our general knowledge in the area of amino acid metabolism. The three well-known lactic acid bacteria that were used in these studies were *Streptococcus thermophilus, Lactococcus lactis* and *Lactobacillus plantarum*. The complete genomes of all these model bacteria have been sequenced and annotated in detail.

Comparative experimental and *in silico* studies of *Streptococcus thermophilus* with the other two lactic acid bacteria, revealed the low degree of amino acid auxotrophies of this species; it only needs two amino acids for (minimal) growth and this strain is able to produce a varied amount of flavors. *Lactococcus lactis* and *Lactobacillus plantarum* require more amino acids and produce fewer flavors than *S. thermophilus*. Furthermore, *S. thermophilus* has a simple primary metabolism; homolactic growth is the only possible route under anaerobic conditions and, remarkably, it does not have a complete pentose phosphate pathway in contrast to the other two studied bacteria. This latter property has important consequences for the redox metabolism of *S. thermophilus* and particularly its ability to produce NADPH. A genome-scale metabolic model was developed and predicted that amino acid metabolism, and especially glutamate degradation, and citrate metabolism are the most obvious alternatives for NADPH generation. Several of these predictions were confirmed by constructing a glutamate dehydrogenase mutant of *S. thermophilus*. This mutant revealed the importance of the citrate pathway (and other amino acid degradation pathways) in NADPH generation.

A comparative and functional genomics study of the three lactic acid bacteria showed that amino acid depletion not only affects amino acid metabolism, but also flavor formation and overall growth. The comparative genomics approach presented in this thesis can be used to understand the amino acid metabolism of different lactic acid bacteria and their potential to produce flavors under different conditions. Finally, it can be applied for optimization of industrial fermentations.

# Chapter 1

## Introduction and outline of this thesis

## Introduction

This chapter will provide an overview of lactic acid bacteria, general concepts of amino acid metabolism and the potential of a comparative genomics approach.
First, characteristics and applications of lactic acid bacteria will be described. Then amino acid metabolism in general is explained and the last part will focus on functional genomics techniques that can be used to study and compare different organisms. Finally, an outline of this thesis will be provided.

## Lactic acid bacteria

Lactic acid bacteria (LAB) belong to the order of *Lactobacillales*, a related group of Gram-positive bacteria that are descended form a common ancestor. LAB are catalase-negative, acid-tolerant, non-spore forming and are rod- or cocci shaped (55). The group of LAB include genera such as *Lactococcus, Lactobacillus, Oenococcus, Enterococcus, Streptococcus, Leuconostoc* and *Weissella* (Figure 1) (36, 55).

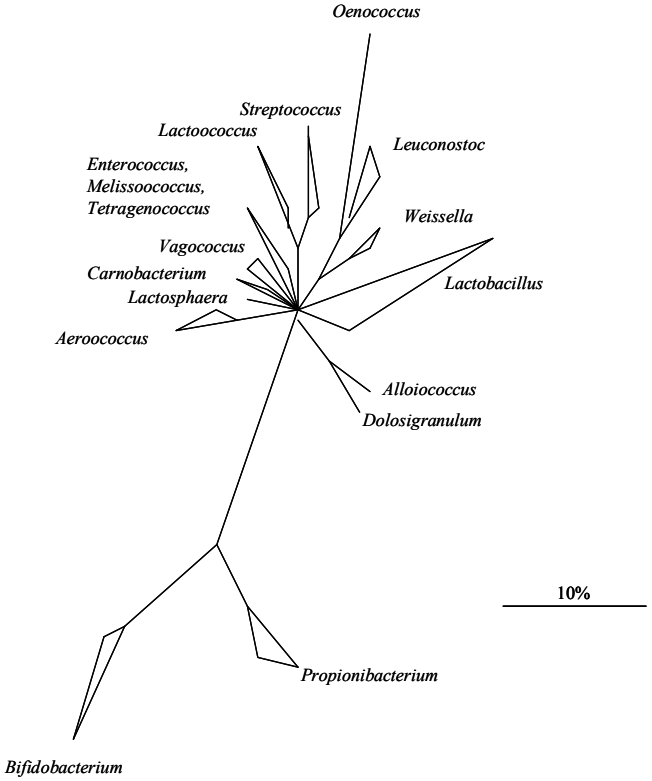



**Figure 1.** Major phylogenetic groups of LAB and related gram-positive bacteria with low (upper part) and high (lower part) GC% in the DNA (modified according to Stiles and Holzapfel (55))

LAB generally produce lactic acid as their major end product and are strictly fermentative. Ecological niches of LAB are milk (5, 64), the gastro-intestinal tract of humans and other animals and decaying plant material (66).

The LAB members can be divided in two groups based on their carbohydrate metabolism. The homofermentative group, including *Lactococcus, Enterococcus, Streptococcus* and some lactobacilli use the Embden-Meyerhof pathway in which glucose is completely converted into lactate (48). The heterofermentative LAB, composed of *Leuconostoc, Weissella* and some lactobacilli, produce lactate, acetate, $CO_2$ and ethanol using the hexose monophosphate pathway (48). Some homolactic LAB can also use the mixed acid fermentation for growth, resulting in the formation of mainly acetate, formate and ethanol (20, 59).

The economic and industrial value of LAB is demonstrated by the wide variety of applications (Table 1). Some LAB are also used as adjunct culture for flavor formation (21, 22, 56).

**Table 1.** Examples of some foods that are fermented with LAB, taken from Leroy and De Vuyst (34)

| **Type of fermented product** | **Lactic acid bacteria**[a] |
|---|---|
| Dairy products | |
| - Hard cheese without eyes | *L. lactis* subsp. *lactis, L. lactis* subsp. *cremoris* |
| - Cheeses with small eyes | *L. lactis* subsp. *lactis, L. lactis var.* subsp. *diacetylactis,* *L. lactis* subsp. *cremoris, Lc. mesenteroides* subsp. *cremoris* |
| - Swiss- and Italian- type cheeses | *Lb. delbrueckii* subsp. *lactis, Lb. helveticus, Lb. casei,* *Lb. delbrueckii* subsp. *bulgaricus, S. thermophilus* |
| - Butter and buttermilk | *L. lactis* subsp. *lactis, L. lactis var.* subsp. *diacetylactis, L. lactis* subsp. *cremoris, Lc. mesenteroides* subsp. *cremoris* |
| - Yoghurt | *Lb. delbrueckii* subsp. *bulgaricus, S. thermophilus* |
| - Fermented, probiotic milk | *Lb. casei, Lb. acidophilus, Lb. rhamnosus, Lb. johnsonii* |
| - Kefir | *Lb. kefir, Lb. kefiranofacies, Lb. brevis* |
| Fermented meats | |
| - Fermented sausage (Europe) | *Lb. sakei, Lb. curvatus* |
| Fermented fish products | *Lb. alimentarius, C. piscicola* |
| Fermented vegetables | |
| - Sauerkraut | *Lc. mesenteroides, Lb. plantarum,* |

| | |
|---|---|
| - Pickles | *Lc. mesenteroides, Lb. brevis, Lb. plantarum* |
| - Olives | *Lc. mesenteroides, Lb. pentosus, Lb. plantarum* |
| Soy sauce | *T. halophilus* |
| Fermented cereals | |
| - Sourdough | *Lb. sanfransiscensis, Lb. farciminis, Lb. fermentum, Lb. brevis, Lb. plantarum, Lb. amylovorus, Lb. reuteri, Lb. pontis, Lb. panis, Lb. alimentarius, W. cibaria* |
| Alcoholic beverages | |
| - Wine (malolactic fermentation) | *O. oeni* |
| - Rice wine | *Lb. sakei* |

[a] *C.= Carnobacterium, L.=Lactococcus, Lb.=Lactobacillus, Lc.=Leuconostoc, O.= Oenococcus, S.=Streptococcus, T.=Tetragenococcus, W.=Weissella.*

Fermentation enhances the shelf-life of a product and LAB influence the sensory properties of a product including the flavor development (3, 65). Fermented foods also have increased levels of nutrients and are sometimes easier to digest than the raw product (34). Some LAB produce bacteriocins, of which nisin is probably the best known member. Combined with the process of acidification, this inhibits the growth of other (spoilage) bacteria (39).

A few representatives of the LAB group are marketed as probiotics, defined as '*Live microorganisms which when administered in adequate amounts confer a health benefit on the host'* (17). Most probiotic products contain one or more strains of LAB. Some health-promoting effects of probiotics are: establishing immune tolerance (61), shortening the course of rotavirus infection (13), and preventing the development of atopic diseases and allergies (15, 16, 46). The mechanisms by which these probiotic strains exploit their beneficial effects and the specific interaction between probiotic bacteria and the human intestinal tract are currently the topic of many research projects (49, 61).

Because of their economic and industrial value, many genomes of different species of LAB have been sequenced and annotated and have become publicly available. A summary of sequenced and annotated LAB genomes that are important for the food industry and some of their features is shown below (Table 2).

**Table 2.** Features of some sequenced LAB genomes important for the food industry, adapted from (45)

| Species[a] | Genome size | Proteins | Refs |
|---|---|---|---|
| *Lb. acidophilus* NCFM | 1.9 Mb | 1864 | (1) |
| *Lb. brevis* ATCC 367 | 2.3 Mb | 2221 | (36) |
| *Lb. casei* ATCC 334 | 2.9 Mb | 2776 | (36) |
| *Lb. delbrueckii* subsp. *bulgaricus* ATCC 11842 | 1.9 Mb | 1562 | (62) |
| *Lb. delbrueckii* subsp. *bulgaricus* ATCC BAA-365 | 1.9 Mb | 1725 | (36) |
| *Lb. gasseri* ATCC 33323 | 1.9 Mb | 1763 | (37) |
| *Lb. johnsonii* NCC 533 | 1.9 Mb | 1821 | (47) |
| *Lb. plantarum* WCFS1 | 3.3 Mb | 3009 | (30) |
| *Lb.reuteri* F275 | 2.0 Mb | 1900 | (54) |
| *Lb. sakei* subsp. *sakei* 23k | 1.9 Mb | 1879 | (8) |
| *Lb. salivarius* subsp. *salivarius* UCC118 | 1.8 Mb | 1717 | (11) |
| *L. lactis* subsp. *cremoris* MG1363 | 2.5 Mb | 2434 | (67) |
| *L. lactis* subsp. *cremoris* SK11 | 2.4 Mb | 2509 | (36) |
| *L. lactis* subsp. *lactis* IL1403 | 2.3 Mb | 2321 | (6) |
| *Lc. mesenteroides* subsp. *mesenteroides* ATCC8293 | 2.0 Mb | 2009 | (36) |
| *O. oeni* PSU-1 | 1.8 Mb | 1701 | (36) |
| *S. thermophilus* CNRZ1066 | 1.8 Mb | 1915 | (6) |
| *S. thermophilus* LMD-9 | 1.8 Mb | 1710 | (36) |
| *S. thermophilus* LMG18311 | 1.8 Mb | 1889 | (6) |

[a], *L.=Lactococcus, Lb.=Lactobacillus, Lc.=Leuconostoc, O.= Oenococcus, S.=Streptococcus*

All LAB have relatively small genomes and the number of predicted protein-coding genes in the sequenced genomes differs from ~1700 to ~3000. Even though the LAB are closely related, this difference in gene number suggests a substantial gene loss and/or gain in evolution. The last common ancestor of *Lactobacillales* is predicted to have lost circa 600-1200 genes and gained <100 compared to the *Bacilli* ancestor (50). Evidence of the recent and

ongoing genome reduction of LAB is the presence of pseudogenes (genes that are not functional due to frameshift, nonsense, mutation, deletion or truncation (5)), often in relatively high numbers compared to other groups of bacteria (50). All LAB contain pseudogenes, although their number differs from < 20 in *Leuconostoc mesenteroides* to ~200 in *Streptococcus thermophilus* and *Lactobacillus delbrueckii.* Another example of gene-loss in LAB is the coding capacity for the electron transport chain which can be used for aerobic respiration and is present in only few LAB species (7). Most striking is the loss of most biosynthetic capacities. This is exemplified by the requirement of a variety of amino acids during cultivation of LAB on chemically defined medium requires supplementation (5, 26, 36).

**Amino acid metabolism**

Milk, one of the natural habitats of LAB, is a protein-rich environment. The protein fraction consists mainly of casein, whey proteins and free amino acids; the most abundant amino acid in milkprotein is glutamic acid (1.7 g/l) (38). In LAB, amino acid metabolism can play an essential role in maintaining the redox balance or serve as a NADPH source.

Glutamate dehydrogenase plays an important role in some LAB, as it catalyzes the reversible deamination of glutamate in α-ketoglutarate and free $NH4^+$, using $NAD^+$ or $NADP^+$ as co-factor (2). It is also the major pathway for the formation of α-amino groups directly from ammonia (56). The amino acid glutamate can serve as carbon backbone for many other amino acids (4). Therefore, glutamate dehydrogenase forms a bridge between the carbon and nitrogen metabolism and thus plays a key role in the metabolism of many organisms.

Amino acid catabolism has important effects on the quality of fermented foods. For fermenting microbes, the amino acid catabolism can be important for obtaining energy in a nutrient-limited environment (10). LAB need essential amino acids for growth, the number and type of essential amino acids is strain and even species dependent (19, 40, 65). *Lactococcus lactis* is probably the best studied LAB concerning amino acid metabolism (65). Non-dairy (plant-associated) strains of *Lactococcus lactis* are not associated with a nutrient-rich environment such as milk and they need fewer amino acids than milk-adapted strains. Some non-dairy *Lactococcus lactis* subsp. *cremoris* and subsp. *lactis* strains only require 1-3 amino acids (65). To obtain all essential amino acids, *Lactococcus lactis* is able to degrade

proteins into small peptides and amino acids which can be taken up from the environment by transporters (Figure 2) (10). The proteolytic system has been studied in much detail and is initiated by a single cell envelope-bound serine protease (Prt). Especially the proteases and peptidases of *Lactococcus lactis* and *Lactobacillus helveticus* are intensively studied. Although there are homologous enzymes between the two species, significant differences exist (10, 33). Peptidase mutants showed reduced growth rates in milk compared to the wild-type (10). Peptides are intracellular degraded by peptidases in amino acids (10). Amino acids are then converted by aminotransferases into the corresponding α-keto acids (53). Decarboxylases can convert α-keto acids into aldehydes and aldehydes can be dehydrogenated or hydrogenated to their corresponding alcohols and carboxylic acids, which are the substrates for (thio) esters. The hydrogenation of α-keto acids may act as a sink for excessive redox potential (NADH). A second conversion route for amino acids is initiated by lyases and aldolases (53), such as threonine aldolase which converts threonine into acetaldehyde (9). A third conversion route for amino acids is the deimination/decarboxylation to amines. These amines are studied extensively because of the health risk of biogenic amines (53).

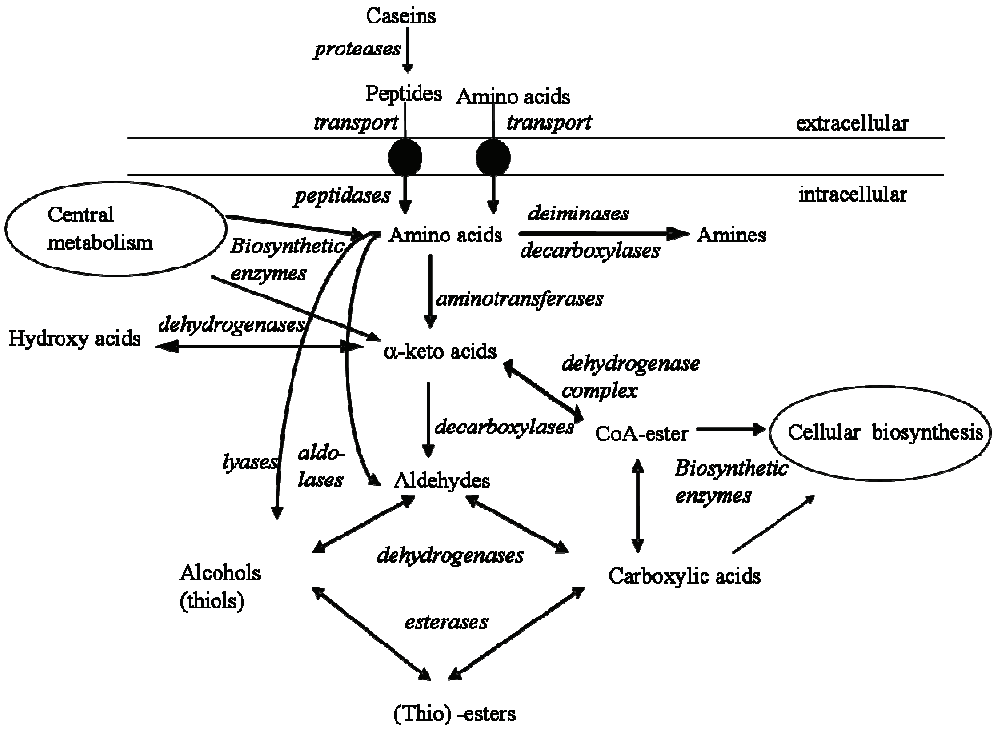



**Figure 2.** Summary of general protein conversion pathways by LAB, adapted from Smit (53)

The major flavor compounds produced during amino acid metabolism are aldehydes, alcohols, carboxylic acids and esters. Especially those derived from methionine, aromatic amino acids and branched chain amino acids are important for the final product flavor (Table 3).

**Table 3.** Examples and descriptions of important amino acid derived flavours, adapted from Smit (53).

| Flavour | Description | Metabolism |
|---|---|---|
| 2-methylpropanal | banana, malty, chocolate-like | valine |
| 2-methylbutanal | malty, chocolate | isoleucine |
| 3-methylbutanal | malty, powerful, cheese | leucine |
| 3-methylbutanol | fresh cheese, breathtaking, alcoholic | veucine |
| Acetaldehyde | yoghurt, green, nutty, pungent | threonine |
| Phenylacetaldehyde | rose, floral | phenylalanine |
| Phenol | medicinal | tyrosine |
| Methional | cooked potato, meat like, sulphur | methionine |
| Methanethiol | 'rotting' cabbage, cheese, vegetative, sulphur | methionine |
| Benzaldehyde | bitter almond oil, character, sweet cherry | phenylalanine |

**Pentose Phophate Pathway**

The pentose phosphate pathway meets the need of all organisms for a source of NADPH to use in reductive biosynthesis. This pathway contains two parts: an oxidative part and a non-oxidative part that interconverts phosphorylated sugars, as is shown in Figure 3 (4). In the oxidative part, NADPH is generated when glucose 6-phosphate is oxidized to ribulose 5-phosphate. NADPH is used for reductive biosynthesis and ribose 5-phosphate for the synthesis of nucleotides. The dehydrogenation of glucose 6-phosphate is controlled by the level of $NADP^+$ as the electron acceptor.

The second stage is the non-oxidative, reversible reaction of five-carbon phosphosugars into phosphorylated three-carbon and six-carbon intermediates of the glycolytic pathway. The non-oxidative branch can introduce riboses into glycolysis for catabolism of generate riboses from glycolytic intermediates (4). Most LAB, including *Lactococcus lactis, Lactobacillus plantarum* and *Lactobacillus delbrueckii* subsp. *bulgaricus*

possess a complete pentose phosphate pathway. However, *Streptococcus thermophilus* LMG18311 (6, 24) is predicted, based on the genome, to lack a complete pentose phosphate pathway. Likely, it needs alternative pathways for NADPH generation, and the available genome-scale model was applied to find these alternatives.

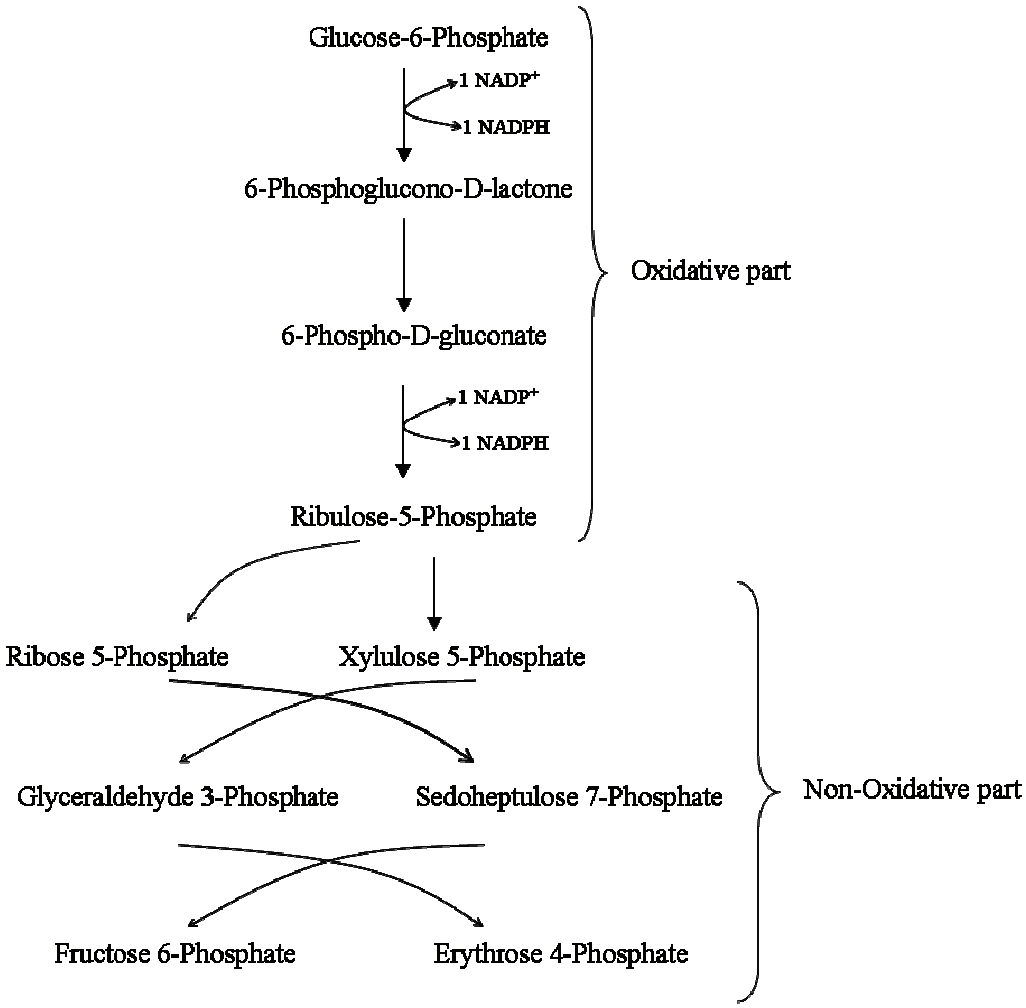



**Figure 3.** A schematic design of the Pentose Phosphate Pathway, adapted from (4). Only NADP(H) as reaction by-product is indicated

**Functional genomics**

The Netherlands Genomics Initiative (NGI) defines genomics as '*the mapping of the genes of humans, animals, plants and micro-organisms, by means of DNA sequencing, and wide-scale research into the function of genes and the manner in which hereditary characteristics contained in cells are translated into the function of a cell and ultimately into the function of the entire organism. This also includes high-throughput technologies, such as proteomics and metabolomics and the bioinformatics that enable the data processing and analysis of colossal amounts of data. Genomics is as set of technologies that has become an indispensable tool in the current field of life science research'* (41). Comparative genomics is the relationship of genome structure and function across different species or strains (23). Comparative genomics is useful for two reasons: (i) the availability of complete genomes enables us to identify sets of orthologs (orthologs are genes in different species that are similar to each other because they originated from a common ancestor (18)), and (ii) the comparison of complete genomes not only shows which genes are present, but also which ones are absent (32). The three major functional genomics approaches address global mRNA (transcriptomics), proteins (proteomics) and metabolites (metabolomics) (63). Comparative genomics can be performed with all these approaches and include strain and species level comparison (Figure 4).

The available sequenced and annotated LAB genomes (Table 2) facilitate the use of these functional genomics techniques to study the response of LAB cells under certain conditions (37). The total set of messenger RNA (mRNA), under a certain condition is studied during transcriptional analysis, for these kind of studies, microarrays can be applied. Gene-based microarrays contain spots representing DNA fragments of the (sequenced) organism(s) of interest (51).

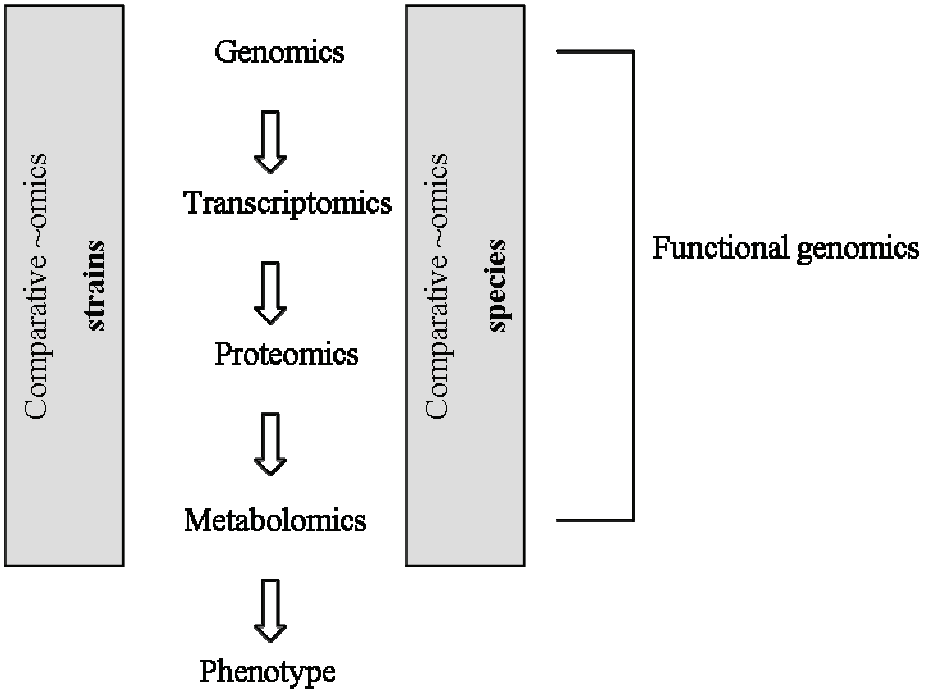
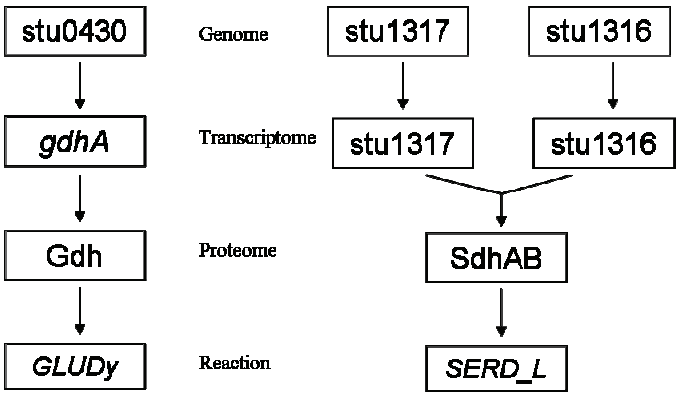



**Figure 4.** Overview of the different ~omics techniques

Comparative genomics of the available sequenced LAB genomes (Table 2) (28) showed a wide conservation of the essential housekeeping genes (genes that are transcribed at relatively constant levels under many or all conditions). Moreover, these studies established a high frequency of horizontal gene transfer (any process in which an organism incorporates genetic material from another organism, without being the offspring of that organism (25)) Comparative studies can give increased insight in their evolution, adaptation and potential for industrial applications (28). Computational models can be applied for a comparative genomics approach. Different kinds of computational models for data analysis exist (52, 57). Kinetic models contain kinetics of enzymes and mathematical descriptions. Since these models require detailed (and labor-intensive) information, they often study specific metabolic pathways, such as glycolysis. Black box models contain known inputs, outputs and functional performance, but the internal implementation is unknown or irrelevant. In a white box model, all necessary mechanistic information is implemented to compute functional performance on the basis of system parameters that represent properties of real objects or processes. Stoichiometric models are summarized in a stoichiometry matrix that depicts the participation of the metabolites in each reaction. One successful type of a stoichiometric model is the genome-scale model. These knowledge-based models not only contain stoichiometry of an

organism's metabolic network, but also contain information on the relationship between genes, proteins and reactions (Figure 5) (52, 57).




(A) (B)

**Figure 5. The structure of genome-scale models.** Relations between genes, proteins and reactions are reconstructed based on genomic information. The examples are taken from the *S. thermophilus* LMG18311 model, where STU means *S. thermophilus*. Part A shows how one gene is connected to one enzyme; glutamate dehydrogenase (GDH). Part B shows how two genes code for one functional enzyme; serine deaminase. Adapted from (52) and based on the Simpheny software environment (Genomatica Inc. San Diego, CA).

Genome-scale metabolic models are often applied to study cells on genome level. Such models are based on sequenced genomes and experimental data. These models can be used for biological interpretation and -the ultimate goal- prediction (58). Complete genome-scale models are available for an increasing number of micro-organisms (43), including different LAB; *Lactococcus lactis* (42) , *Lactobacillus plantarum* (59) and *Streptococcus thermophilus* (44).

**Comparative approach**

In this thesis, a comparative genomics approach is described for LAB. Not only different organisms, but also different genomics level approaches are compared. A comparative approach of complete genomes not only shows which genes are present, but also which ones are absent (32). For comparison of two or three similar genomes, sequence alignment is a standard procedure and this alignment can be graphical represented. Another method is to compare the number of tRNA's, rRNA's and protein-coding genes between

different strains. Also, much can be learned from a comparison of the same proteins across different organisms (60).

An example of comparative genomics is the development of an integrated database and bioinformatics platform called OGeR (Open Genome Resource). This platform was developed with 21 pathogenic streptococcal genomes (from the Strepto-DB platform). It was used for the prediction of the core-genome (fraction of genes that is shared among strains) and the pan-genome (core genome plus all distributed genes) (31). Claesson *et al* (12), performed a comparative phylogenomics study with 12 *Lactobacillus* strains. They suggested that GroEL (a molecular chaperone) is more suitable as a phylogenetic single-gene marker for large-scale analysis than the 16S rRNA gene (12). Klaenhammer *et al* (29) described a functional comparative genomics study of probiotic *Lactobacilli*. Differential Blast Analysis highlighted strain-specific genes and group-specific genes. *Lactobacillus*-specific genes include mucus-binding proteins involved in cell-adhesion and several transport systems for carbohydrates and amino acids (29).

Liu *et al* described the use of comparative genomics approaches to improve the functional annotation of the key enzymes in the formation of flavor compounds from amino acids. Comparative analysis of the various sequenced LAB resulted in an overview of differences in their capacities to form flavors (35). De Vos *et al* (14) used genome sequences and post-genomics techniques to compare different food-grade LAB and to investigate their function in the gut. Comparative genomics revealed some parallels between the different phylogenetic strains, that probably reflects the harsh and competitive environment in the human gut (14).

All these different examples show the strong potential of comparative genomics. It may reveal similarities or differences between different LAB. It can also provide insight in the (ongoing) evolution of the LAB. The available genome-scale models can play a role as powerful tool as part of comparative genomics. The ultimate goal of the comparative genomics will be a better understanding of the metabolism of these important industrial food starters and why they are so well-equipped for their respective food fermentations.

**Goal and outline of this thesis**

The work described in this PhD thesis was initiated to use genomics technologies and a comparative approach to link the gene content of some well-known LAB to flavor formation and to increase our general knowledge in the area of amino acid metabolism. The three well-known LAB that were used in these studies were *Streptococcus thermophilus* LMG18311 (5, 24), a yoghurt strain, *Lactococcus lactis* MG1363 (67), a cheese strain, and *Lactobacillus plantarum* WCFS1 (30), originally isolated from human saliva and used for vegetable fermentations. The complete genomes of all these model bacteria have been sequenced and annotated in detail (Table 2). Genome-atlas views of these strains are shown below. *S. thermophilus* has the smallest genome (1.8 Mb), but of these three LAB, it has the highest percentage of amino acid related genes (10%) compared to the total number of genes and the lowest percentage of genes involved in primary and sugar metabolism (7%). For *L. lactis* these percentages are 5.5% and 7.4% and for *Lb. plantarum* 8.5% and 13.4% respectively.

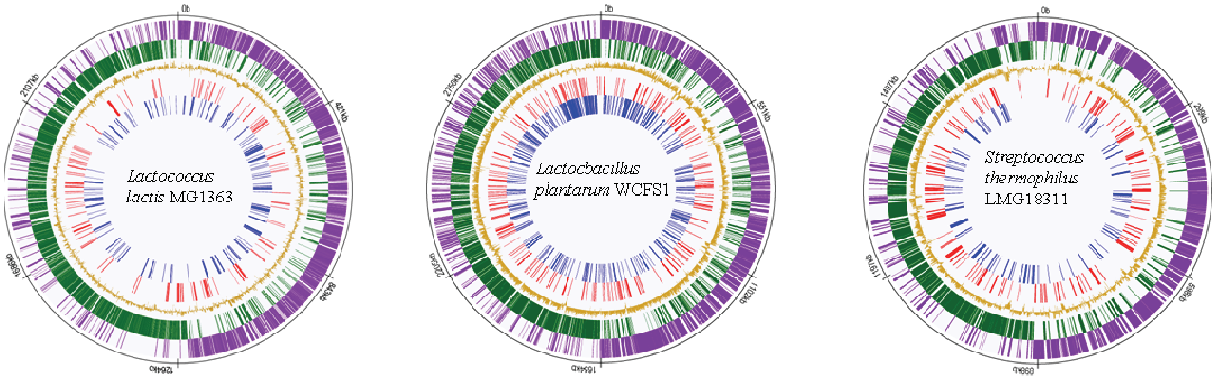



**Figure 6.** Genome-atlas view of the *L. lactis* MG1363, *Lb. plantarum* WCFS 1 and *S. thermophilus* LMG18311 chromosome. The outer to inner circles show (i) positive strand ORFs (purple); (ii) negative strand ORFs (green); (iii) GC% (yellow); (iv) genes in the COG group E (amino acid metabolism) (red); (v) genes in the COG group C and G (sugar and primary metabolism) (blue). Figures were made by Michiel Wels, according to (27)

The comparative genomics approach presented in this thesis can be used to understand the amino acid metabolism of different lactic acid bacteria and their potential to produce flavors under different conditions.

**Chapter 2** describes different high-throughput screening tools that can be used for the selection of flavor forming cultures. The headspace of fermentation samples was analyzed using GC-MS, different mixed and single cultures as well as industrial and defined media

were compared. Genome-scale metabolic models were used to predict the production of relevant (flavor) components and to expand our knowledge about flavor forming pathways.

In **Chapter 3** the amino acid-metabolism and amino acid-dependency of the dairy bacterium *Streptococcus thermophilus* LMG18311 is described and compared with that of *Lactococcus lactis* MG1363 and *Lactobacillus plantarum* WCFS1. Through the construction of a genome-scale metabolic model of *Streptococcus thermophilus*, the metabolic differences between the three bacteria were visualized by direct projection on a metabolic map.

In **Chapter 4** the growth of three different lactic acid bacteria (*Streptococcus thermophilus*, *Lactococcus lactis* and *Lactobacillus plantarum*) on a defined medium containing all amino acids is compared with that on the same with a minimal amount of amino acids. The cellular response towards a minimal amount of amino acids was studied on transcriptional level and data were visualized with the use of genome-scale models.

In **Chapter 5** the absence of a complete pentose phosphate pathway in *Streptococcus thermophilus* is discussed. This pathway is important for the generation of NADPH and therefore this strain needs alternative pathway(s). One of the alternatives can be glutamate dehydrogenase (encoded by the *gdhA* gene), and a knock-out of its gene was made. Growth on transcriptional level and fermentation behavior of this mutant were compared with the wild-type.

**Chapter 6** summarizes the main results obtained in this study with special attention towards the differences in amino acid biosynthesis pathways in different LAB and the effect of the completeness of the amino acid metabolism on the overall metabolism. Finally, concluding remarks and future perspectives are given in this chapter.

**Acknowledgements:** We thank Michiel Wels for his excellent design of Figure 6.

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# Chapter 2

## Genomics and high-throughput screening approaches for optimal flavor production in dairy fermentation

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**Abstract**

Most fermented dairy products are manufactured using mixed starter cultures. These cultures are usually a well-balanced mixture of several different lactic acid bacteria. For the development of novel or improved dairy products, flavor characteristics are generally a primary target. In this paper we describe the (GC-MS) analysis of the headspace of cultures of different lactic acid bacteria and of different growth media. These analyses showed that on industrial medium, different flavors are formed than on a defined medium. Furthermore, the flavor formation by mixed cultures is not just the sum of the single cultures due to metabolic interactions. Finally, genome-scale metabolic models were used to predict the production of relevant (flavor) components and to expand our knowledge about flavor forming pathways. The integration of these approaches is anticipated to strengthen culture development programs enabling the production of well-tasting, fermented dairy products.

## Introduction

Lactic acid bacteria (LAB) are important for the dairy industry, since they are used as (mixed) starter cultures for the production of fermented foods, such as yoghurt and cheese. During the fermentation process, LAB influence the sensory properties of a product including the flavor development (3, 47). Most flavor-related research has focused on cheese (46, 51). Flavor compounds are formed by the conversion of lactose and citrate (glycolysis and pyruvate metabolism), by lipolysis and by proteolysis and conversion of free amino acids (38, 42, 46, 47). Flavor production is strain dependent and therefore the composition of a starter culture can greatly influence flavor characteristics of the final product (25). Moreover, flavor formation in fermented food products largely depends on the substrate. Differences in the presence of flavor compound precursors as well as regulatory responses may result in different fluxes through flavor pathways (17, 18).

Most dairy fermentations are performed with mixed cultures and the final product properties are influenced by their composition. The yoghurt fermentation is probably the most extensively studied mixed dairy fermentation (52). Typically, mixed starters consisting of *Streptococcus (S) thermophilus* and *Lactobacillus (Lb.) delbrueckii* subsp. *bulgaricus* are used to ferment milk yielding the final yoghurt product. The bacteria stimulate each other's growth in a process called protocooperation (11, 14). Important functionalities that result from this are (I) increased acidification of milk by the conversion of lactose into lactic acid, (II) change in texture by the production of exopolysaccharides and by coagulation due to acidification, and (III) production of the typical yoghurt flavor (41). This yoghurt flavor is a combination of lactic acid and various carbonyl compounds like acetaldehyde and diacetyl (10, 52).

The process of finding suitable (combinations of) strains for optimal flavor production in dairy products can be accelerated by understanding of the metabolic processes leading to typical flavor compounds, the effects of different strains on flavor production and the ideal composition of different flavor compounds leading to the desired sensory effect. Combining these aspects is a major challenge in fermentation optimization.

Recent advances in technologies and approaches can support the development of improved flavor production in dairy fermentations. Here we show the results of recent experimental approach (GC-MS, genome-scale models, comparison of strains and different

media) and compare their outcome with examples from literature. It is envisioned that this knowledge-based selection of LAB will be a useful tool for the improvement of established (fermented) foods or for the development of novel (fermented) foods, based on their ability to produce flavor.

## Materials and methods

**Bacteria and growth conditions.** The strains used in this study were *S. thermophilus* LMG18311 (7) and *Lb.. bulgaricus* ATCC BAA-365 (29). Cells were grown anaerobically on Nilac skim milk (NIZO, the Netherlands) at 42°C. *S. thermophilus* was also grown anaerobically in Chemically Defined Medium (CDM) (28) at 42°C.

**GC-MS analyses**. The headspace of 5 mL batch cultures in milk (Nilac) and chemically defined medium (CDM) fermented with *S. thermophilus* LMG18311, *Lb. bulgaricus* ATCC BAA-365 (only milk) and the mixed culture (only milk) at 42 °C for 24 h were analyzed on GC-MS. Headspace volatiles of samples equilibrated at 60°C for 10 min were concentrated by Solid Phase Dynamic Extraction followed and focussed by cryofixation at -120°C. Subsequently the compounds were separated on a CP-SIL 5 CB column (60m x 0.32m) and detected on a mass spectrometer by scanning a mass range of 25-250 in 0.25s in the full scan EI ionization mode (70 eV). Data acquisition and processing were performed with the Xcalibur software and volatiles were identified using NIST MS Library.

**Model development**. Genome-scale models are based on annotated genomes and experimental data and have become available for an increasing number of organisms, including various LAB (34, 45). The construction of the genome-scale models is described elsewhere in much detail (chapter 3 of this thesis) (44, 45).

## Results and discussion: Approaches for selecting suitable starters

**Flavor profiling of fermentations by GC-MS.** It is important to realize that there is a large variation in flavor forming abilities among food fermenting LAB (1, 2, 37, 38, 48, 51).

This diversity does not only occur at the species level but also within species a large strain-to-strain diversity exists. Figure 1 (1, 49) shows an example of a GC-MS chromatogram of the flavors produced by a lactococcal wild strain and an industrial strain. The two chromatograms clearly show that different strains can produce different flavors. Differences between flavor profiles in culture fluid of growing cultures and the culture medium before the start of fermentation are mainly caused by metabolic activities of the fermenting microbes. Especially the wild strain produces high levels of methyl aldehydes and methyl alcohols. One of the identified compounds is 3-methylbutanal. This aldehyde is derived from leucine degradation and has been recognized as a key flavor compound in (semi-)hard cheeses like Proosdij and Parmesan, because of its malty, chocolate flavor (38). However, this aldehyde may also have a negative effect on the sensory properties of a cheese. Apparently, the contribution of 3-methylbutanal to the perception of cheese depends on the other volatiles present and the matrix composition (49). It is known that there is a large biodiversity among LAB in the activity of enzymes involved in the pathway leading to 3-methylbutanal (38) and this knowledge can be used for the selection of starter cultures for the food industry.

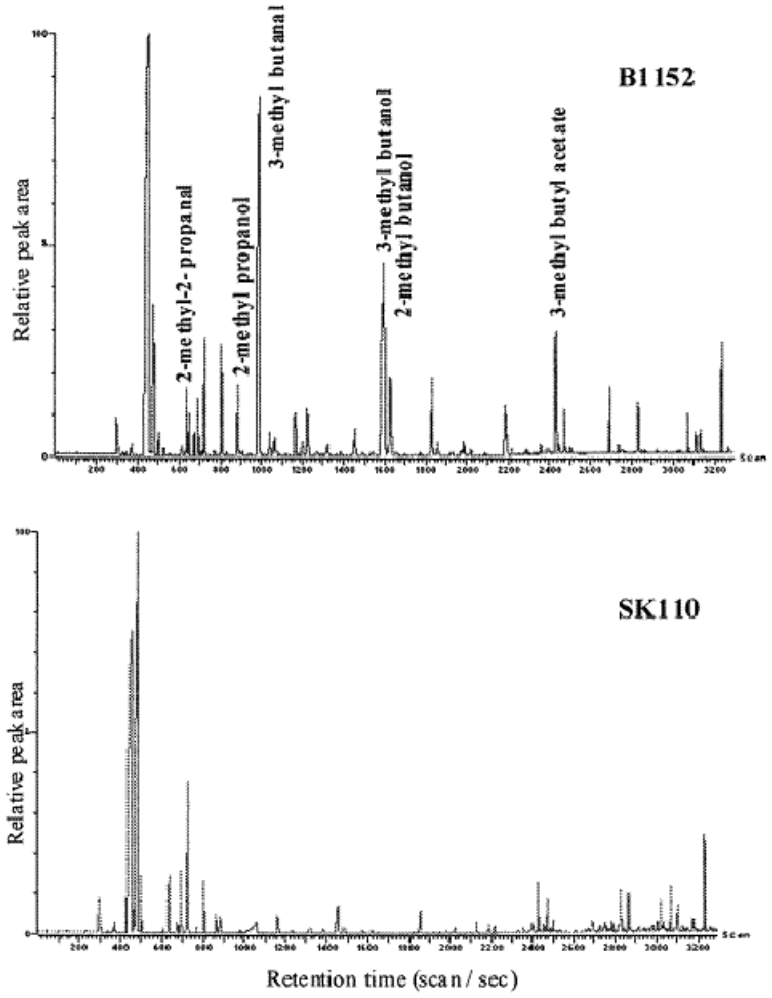


**Figure 1**. GC-MS aroma profiles of volatile compounds from a cheese model (Ch-easy) inoculated with the *Lactococcus lactis* subsp. *lactis* strains B1152 (wild strain) and SK110 (industrial strain) respectively. Relative peak areas are expressed in arbitrary units. Adapted from (1, 49).

The development of mixed cultures brings additional challenges. The flavor of a mixed culture is not necessarily the sum of the flavors produced by the individual pure cultures. Metabolic interactions may play a crucial role. For example, strains that have incomplete flavor producing pathways can complement each other. An elegant example for flavor formation by mixed cultures was provided by the work from Ayad and co-workers. They described mutual complementation of two *Lactococcus lactis* strains that have incomplete 3-methylbutanal-producing pathways. A combination of both strains was reported to produce this compound, whilst neither strain by itself did. It was shown that the one strain was highly proteolytic but lacked a decarboxylating enzyme necessary for producing 3-

methylbutanal and the other strain contained the decarboxylating enzyme but lacked proteolytic activity to provide sufficient amounts of leucine (Figure 2) (2, 4).

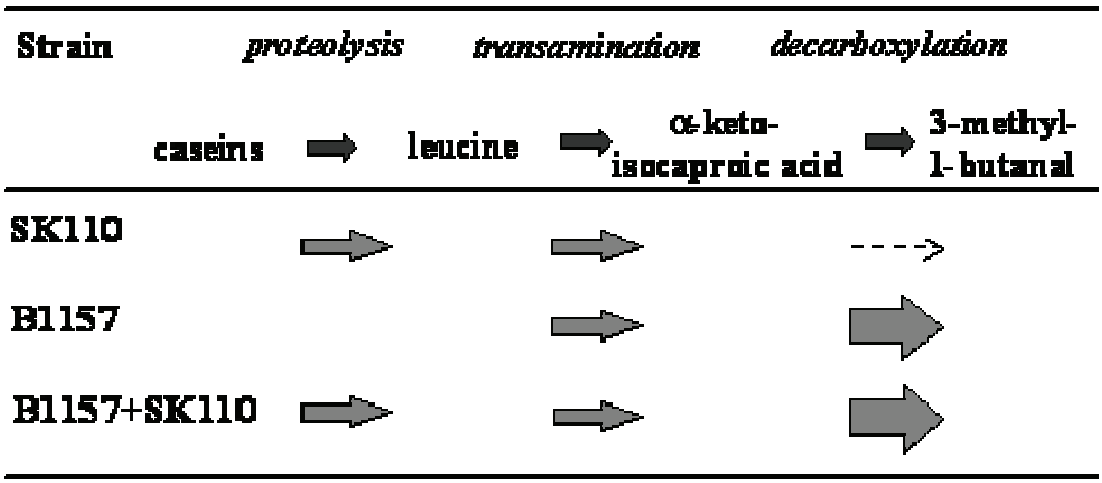


**Figure 2**. The completion of the 3-methylbutanal forming pathway by using a mixture of 2 *L. lactis* strains. Strain SK110 is proteolytic but lacks a decarboxylating enzyme necessary for producing 3-methylbutanal and B1157 contains the decarboxylating enzyme but lacks proteolytic activity. Arrows indicate the relative enzymatic activities. Adapted from (2).

Another example deals with the addition of mesophilic homofermentative lactobacilli to the cheese fermentation performed by *L. lactis* subsp. *lactis* and *L. lactis* subsp. *lactis* var. *diacetylactis* (31). Here, some strains of *Lactobacillus* spp. were reported to enhance formation of diacetyl and acetoin while other mixtures produced lower amounts. Volatile free fatty acid production increased in all combinations. For the yoghurt mixed culture, consisting of *S. thermophilus* and *Lb. bulgaricus*, a similar approach can be applied to study the performance of the mixture. In Table 1, an example is shown how GC-MS analysis can be used to recognize interactions between the two different yoghurt bacteria.

**Table 1.** Relative levels of important volatiles identified in the headspace of 5 mL batch cultures in milk (Nilac) and chemically defined medium (CDM) fermented with *S. thermophilus* LMG18311, *Lb. bulgaricus* ATCC BAA-365 (only milk) and the mixed culture (only milk) at 42 °C for 24 h. Identified+, detectable; ++, present at about 10 times higher concentration; +++, present at about 100 times higher concentration; -, not detectable.

| | Milk | *S. thermophilus* in milk | *Lb. bulgaricus* in milk | Mixed culture in milk | CDM | *S. thermophilus* in CDM |
|---|---|---|---|---|---|---|
| Acetaldehyde | + | ++ | ++ | ++ | ++ | +++ |
| Diacetyl | - | ++ | - | + | - | ++ |
| Methanethiol | + | + | ++ | ++ | ++ | ++ |
| Acetone | + | + | + | + | - | ++ |
| Ethanol | - | - | + | - | - | - |
| Acetic acid | + | + | + | + | + | ++ |
| Dimethylsulfide | +++ | +++ | +++ | ++ | + | + |
| Dimethyldisulfide | - | - | + | + | +++ | ++ |
| Dimethyltrisulfide | - | - | + | + | ++ | - |
| 2,3-Pentanedione | - | - | - | - | - | + |
| 2-Heptanone | + | - | ++ | ++ | - | - |

It is clear that for instance diacetyl is present at lower levels in the mixed culture than in the pure culture of *S. thermophilus* even though the cell counts of this strain are higher (data not shown). Ethanol is only detected in the mono culture of *Lb. bulgaricus*. Most compounds listed in Table 1 have also been identified in similar studies on yoghurt (16, 21), but never before have these mono cultures and mixed culture been compared in this way.

Flavor profile screening is an effective tool in acquiring (mixed) fermentations with the desired flavor. The effectivity of this process is enhanced by knowledge of the molecular mechanisms of flavor formation allowing rational improvement of existing cultures or development of new cultures.

**Mixed culture screening.** An important aspect in optimizing flavors produced in mixed cultures is the rapid analysis and quantification of the strains involved. As with flavor formation, the performance of mixed cultures, e.g. cell counts and acidification, is not the summation of the performances of the individual pure cultures due to molecular interactions between strains (11). High-throughput (HT) screening can play a major role in acquiring well-controlled mixed fermentations with desired end products. Various analytic tools are available for analysis and development of mixed cultures. These include a method for measuring real-

time pH in a 96 wells format (24), an efficient tool for fast and small-scale combinatorial screening based on acidification curves. Another key challenge relates to the analysis of population dynamics in mixed cultures. Traditionally, this analysis relies on the quantification of strains by plating dilutions of cultures onto selective media. Because this is very laborious and time-consuming, several attempts have been made to develop a HT plating method (20) and an automated colony counting method (12, 30). Recently, a miniaturized platform for plating microorganisms was developed (22, 23), which currently allows plating of thousands of micro colonies on a few squared centimeters. Alternatives for plating rely on the application of species or strain specific probes or primers as for instance quantitative PCR (19, 33) and fluorescent *in situ* hybridization (6) combined with flow cytometry.

These HT screening methods are useful for the analysis of the performances of (mixed) fermentations, but do not provide information on produced flavors or interacting pathways. For that, other tools can be used such as ~omics techniques. These include transcriptome analysis like DNA microarrays (27, 32), proteome analysis (9, 35) and fast volatile analysis, like GC-MS. Recently, a proteomics approach in mixed cultures was applied to identify the proteins involved in cheese ripening, amongst which several peptidases (15). Such an approach may also be useful for identification of proteins involved in flavor-forming pathways. However, proteomics techniques are still quite laborious. Phelps and co-workers argue that using bioinformatics to combine data derived from transcriptomics and metabolomics provides a powerful tool for the identification of gene function and pathways (36).

Having information on performances of cultures and metabolic pathways, it is possible to construct complete flavor producing pathways rationally by metabolic engineering, combining strains with incomplete pathways or addition of flavor precursors. The HT screening tools allow rapid testing of thousands of combinations for acquiring the desired fermentation. Additionally, combining different types of experimental data in a mathematical model may lead to a better understanding of a mixed culture. Kinetic models describing performances of mixed cultures have been made (5, 39) but these do not include the production of metabolites, including flavor components. Genome-scale metabolic models, as discussed later on, could be a very useful tool for describing and predicting the performances of mixed cultures.

**Defined media versus industrial media.** Flavor formation is not only strain and population dependent, but also depends on environmental conditions. Although many flavor components are produced independently of the growth medium, there are clear differences in flavor profiles between complex and defined media. An example of how the growth medium can affect flavor development is presented in Table 1. The headspace volatiles of batch cultures of the yoghurt bacterium *S. thermophilus* LMG18311 in skim milk (Nilac) and chemically defined medium (CDM) (28) are presented and compared. The components that are interesting for overall flavor are mentioned. Acetaldehyde and diacetyl are formed by *S. thermophilus* in both milk and CDM. However, 2,3-pentanedione and acetic acid are only produced in CDM and not in milk. Another example was recently published dealing with the optimization of acetoin formation by *Bacillus subtilis* CICC 10025 (50). Here, it was shown that acidified molasses and soy bean hydrolysate as culture media lead to higher amounts of acetoin than culturing in laboratory medium comprising sucrose, yeast extract and peptone. The increase in acetoin formation is at least partially due to increased growth in molasses and soy bean hydrolysate compared to laboratory medium. The authors argue that soy bean hydrolysate is a more optimal nitrogen source for (acetoin production in) *Bacillus subtilis*. Several more examples have been published on the effects of specific medium components, such as citrate, on the formation of C4-compounds such as acetoin and the butter flavor component, diacetyl. In most LAB, diacetyl is produced from pyruvate, via the intermediate acetolactate, deriving from several sources (40). In some cases, the production of diacetyl is clearly dependent on the presence of citrate (13). In summary, it is evident that the production of flavor components is greatly influenced by the composition of the cultivation medium.

**Understanding and improving flavor prediction with genome-scale metabolic models.** Genome-scale models are based on annotated genomes and experimental data and have become available for an increasing number of organisms, including various LAB (34, 45). An especially useful tool for the construction of these *in silico* models is the Simpheny™ software package (Genomatica Inc., San Diego CA, USA). The *in silico* models are based on a thorough metabolic reconstruction of well-annotated genome sequences (44). These models and other bioinformatics tools can be used to search in genomes for components in amino acid metabolism that contribute to flavor (43). If these models are combined with experimental

data, such as transcriptome and metabolome data, whole genome analysis can be used to expand our knowledge of flavor forming pathways and mechanisms in different bacteria, different mixed cultures and different environments. The volatile analysis of cultures, as described above, showing several similarities and differences in flavor formation between different strains, can be visualized on the different metabolic maps in the Simpheny models. The volatile analyses earlier in the results section for instance showed similarities and differences in flavor formation between different strains. These can be visualized on the different metabolic maps in the Simpheny models. An important key flavor in dairy products is acetaldehyde. It has been reported (10) that different strains use different pathways for acetaldehyde production. *L. lactis* produces acetaldehyde during lactose metabolism by pyruvate decarboxylation (8) (Figure 3). *S. thermophilus* can convert threonine into acetaldehyde and glycine by threonine aldolase activity (10) as is shown in Figure 4. Interestingly, the genome of *Lb. plantarum* showed no homologue of threonine aldolase, yet consumed threonine at significantly higher rates than needed for biomass production, indicating that degradation of threonine, possibly into acetaldehyde, occurs (45).

As mentioned earlier in the results section, kinetic models for mixed cultures are available to study the interactions during fermentations. An interesting development will be to combine such models with genome-scale metabolic models to get more insight into the metabolic changes that occur in mixed cultures.

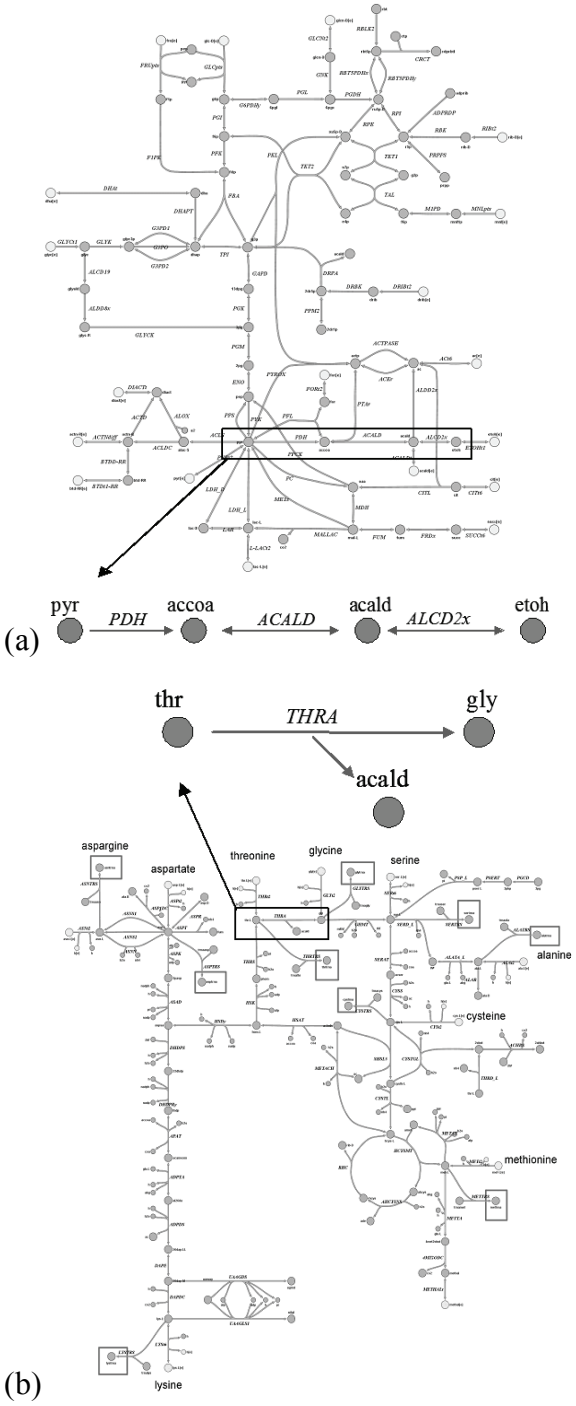


**Figure 3.** (a) Metabolic map (Simpheny) of primary metabolism in *Lactobacillus plantarum* WCFS1, the acetaldehyde pathway is highlighted. pyr = pyruvate, PDH = pyruvate dehydrogenase, accoa = acetyl coenzyme A, ACALD = acetaldehyde dehydrogenase, acald = acetaldehyde, ACALD2x = alcohol dehydrogenase, etoh = ethanol. (b) Metabolic map (Simpheny) of amino acid metabolism in *Lb. plantarum* WCFS1, the acetaldehyde pathway is highlighted. thr = threonine, THRA = threonine aldolase, acald = acetaldehyde, gly = glycine.

## Conclusion and future outlook

This overview discusses the application of different suitable tools such as GC-MS analysis and genome-scale metabolic modeling to discover differences in the flavor producing potential of separate strains and mixed cultures, as well as different media. Genome-scale models are nowadays available for various LAB and kinetic models are available for mixed cultures. These models can be combined with experimental data (e.g. the volatile analyses) and can help to increase our knowledge about flavor forming capabilities in different strains, mixtures and conditions. However, these models contain only the current state of affairs with respect to flavor pathways. To identify new routes and components, and to fill the knowledge gaps that become evident when constructing these models, ~omics techniques and HT methods for analyzing the performances of cultures are essential. In recent years, the genomes of many different LAB have been sequenced (26, 29). In order to get a better insight in the regulation of flavor forming pathways under different conditions, DNA microarrays of some of these sequenced strains have been developed. This provides a powerful tool for the understanding of interactions occurring in mixed LAB cultures, as well as for the understanding and control of flavor production by various LAB species. This knowledge will lead to better selection procedures of LAB and to rational improvement of starter cultures and (fermented) foods (Fig. 4).

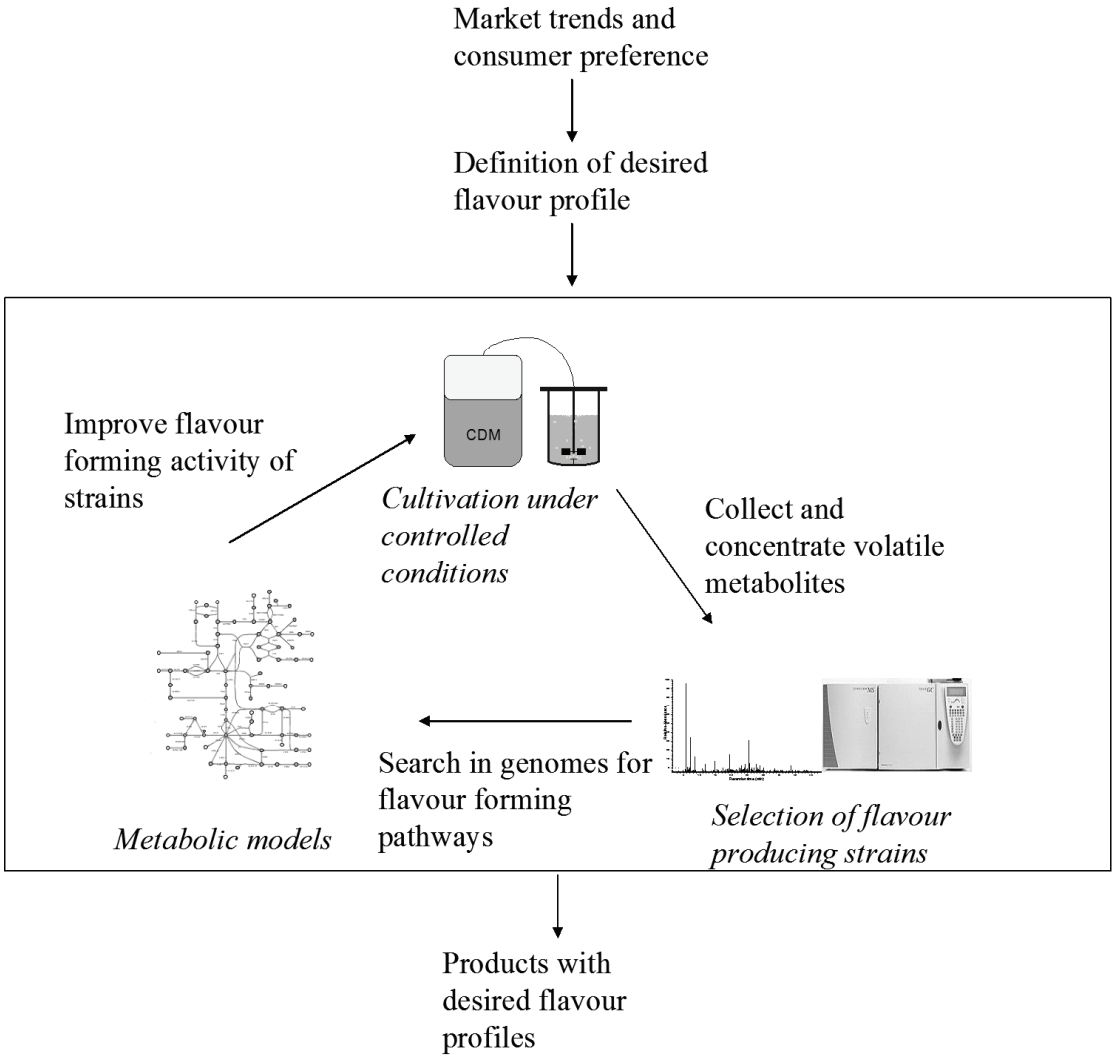


**Figure 4.** Schedule of knowledge-driven innovations in fermentative flavor formation. CDM = chemically defined medium.

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# Chapter 3

## Genome-scale model of *Streptococcus thermophilus* LMG18311 for metabolic comparison of lactic acid bacteria

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**Abstract**

In this report we describe amino acid-metabolism and amino acid-dependency of the dairy bacterium *Streptococcus thermophilus* LMG18311 and compare that with two other characterized lactic acid bacteria, *Lactococcus lactis* and *Lactobacillus plantarum*. Through the construction of a genome-scale metabolic model of *S. thermophilus*, the metabolic differences between the three bacteria were visualized by direct projection on a metabolic map. The comparative analysis revealed the minimal amino acid auxotrophy (only histidine and methionine or cysteine) of *S. thermophilus* LMG18311 and the broad variety of volatiles produced from amino acids compared to the other two bacteria. It also revealed the limited number of pyruvate branches, forcing this strain to use the homofermentative metabolism for growth optimization. In addition, some industrially-relevant features could be identified in *S. thermophilus* such as the unique pathway for acetaldehyde (yoghurt flavour) production and the absence of a complete pentose phosphate pathway.

## Introduction

Lactic acid bacteria (LAB) are of great importance in the food industry, because their lactic acid production and their characteristic impact (e.g. texture, flavor) on the final product (19). LAB, as fastidious organisms, require a complex medium (such as milk) and are dependent on their proteolytic system for their supply of essential amino acids (34). Amino acids are not only the building blocks for proteins and peptides, but they also serve as precursors for many other biomolecules (1). Amino acids are also important for the final flavor of a product. Most amino acids do not directly influence the product flavor, but they will contribute indirectly to it because they are precursors of aromatic compounds (36). The conversion of amino acids to flavor compounds is mainly initiated by amino acid transamination, which uses an α-ketoacid as an amino group acceptor for the aminotransferases (27). The presence (or absence) of the α-ketoacid either by endogenous production or by addition to the medium is an important factor in flavor formation (13). The α-ketoacids are decarboxylated into aldehydes, which are the precursors of other flavor compounds such as alcohols, esters and carboxylic acids (27). A large variation in flavor formation between strains and species is observed. Different studies have reported this biodiversity (25, 27, 32, 33); van Hylckama Vlieg *et al* studied for instance the difference between dairy and non-dairy lactococcal strains since the latter group has some unique flavor forming activities (33).

Amino acid catabolism and anabolism are complex processes and, thus, metabolic models will be helpful for their understanding. Genome-scale metabolic models provide an overview of all metabolic conversions in an organism, based on its genome sequence, and make it possible to visualize different metabolic pathways, such as amino acid metabolism. These models can be used to understand the metabolism and can then be applied for a directed study of functionality. For *Lactobacillus* (*Lb.*) *plantarum* and *Lactococcus* (*L.*) *lactis*, such genome-scale models have been already developed (18, 29); the construction of such a model for *Streptococcus (S.) thermophilus* LMG18311 is described in this paper. The characterization of the genome sequence of this *S. thermophilus* strain has revealed the presence of a large amount of incomplete or truncated genes. These so called pseudogenes amount to 10% of the total genes and most of them relate to carbohydrate metabolism,

transport and regulation (2, 11). *S. thermophilus* is an important starter for the dairy industry. It is used in combination with *Lactobacillus delbrueckii* subsp. *bulgaricus* for the production of yoghurt. It is also used for the manufacture of cheeses in which high cooking temperatures are applied (11). The objective of this paper is to study the metabolism of *S. thermophilus* with the use of genome-scale models and experimental data in a comparative way. This comparison with other LAB may reveal important differences. This study showed the simple primary metabolism and the extensive amino acid metabolism in *S. thermophilus.*

## Materials and methods

**Construction of the genome-scale model**. Genome-scale models are based on annotated genome sequences and experimental data and have become available for an increasing number of organisms, including various LAB (20, 30). A useful tool for the construction of these *in silico* models is the Simpheny$^{TM}$ software package (Genomatica Inc., San Diego CA, USA). The *in silico* models are based on a thorough metabolic reconstruction of well-annotated genome sequences (29). The reconstruction of the network of *S. thermophilus* LMG18311 (2, 11) was initiated by an automatic first reconstruction using the Autograph-method (AUtomatic Transfer by Orthology of Gene reaction Associations for Pathway Heuristics) as described in much detail elsewhere (18). The automatic output of Autograph was subsequently curated extensively to accommodate the available annotation and literature on metabolic pathways and enzymes, a process described in detail elsewhere (8). Also part of the curation was the comparison of the gene-reaction associations with the available annotations in KEGG (http://www.genome.jp/kegg/) and the ERGO bioinformatics suite (http://ergo.integratedgenomics.com/ERGO/) (26).

**Bacterial strains, media and growth conditions.** The strains used in this study were *S. thermophilus* LMG18311 (2), *L. lactis* MG1363 (35) and *Lb. plantarum* WCFS1 (14). Cells were grown anaerobically in Chemically Defined Medium (CDM, 15, 21, 23), containing the amino acids as listed in Table S1, at 42°C, 30°C and 37°C respectively.

**Amino acid omissions.** Cells of *S. thermophilus* were grown overnight in chemically defined medium (CDM, 15), containing all 20 amino acids in the concentrations shown in Table S5 of the Supplementary Materials. The overnight cultures were washed twice at 4°C in a Megafuge 1.0R (Heraeus Instruments, Germany) in Phosphate Buffered Saline (PBS).

CDM without amino acids was prepared freshly for each experiment. To this medium different combinations of amino acids were added. The amino acids were added in the same concentrations as used in complete CDM. We started with single omissions of amino acids followed by multiple omissions until we found the most minimal combination. In Table 2, the concentrations of the different amino acids supplied are listed for the different experiments. The different minimal defined media were inoculated 0.5% in triplicate with the washed overnight culture and growth was followed by measuring the $OD_{600}$.

**Growth on defined medium (chemostat).** Fermentations were performed in duplicate as described by Teusink *et al* (30). *S. thermophilus* LMG18311 was grown at 42°C in CDM in a 50-ml tube and used as inoculum of 500 ml pH-controlled (pH 6.5) CDM, the medium was 1% inoculated. Fermentations were performed in a 2-L fermentor (Applikon Biotechnology BV, The Netherlands). The fermentations were controlled by a Bio Controller ADI 1010 and by a Bio Console ADI 1025 (Applikon Biotechnology BV, The Netherlands). The headspace was flushed with nitrogen (10 ml $min^{-1}$) at a stirring speed of 100 rpm. At $OD_{600}$ of ~0.5, the medium pump was switched on to reach a dilution rate of 0.4 $h^{-1}$. Steady state conditions were achieved within five volume changes (30). The dilution rate was changed three times, so a total of 4 dilution rates was achieved (0.1 $h^{-1}$, 0.2 $h^{-1}$, 0.3 $h^{-1}$, 0.4 $h^{-1}$). At each steady state 4 x 50 ml samples were taken and spun down at 4°C in a Unicen MR (Herolab, The Netherlands). Supernatant was used for HPLC analysis of organic compounds (28).

**GC analyses.** For the identification of volatile components in the samples, purge and trap thermal desorption cold trap gas-chromatography (GC) was used as described before (7, 27). The headspace samples were concentrated on a Fisons MFA815 cold trap (CE Instruments, Milan, Italy), followed by separation on a GC-8000 top gas chromatograph (CE Instruments) equipped with a CIP-SIL 5 CB low-bleed column (Chrompack, Middelburg, The Netherlands) and detection by a flame ionization detector. The GC data were processed in

MetAlign, a tool (developed by Plant Research International, The Netherlands) to align spectra and to identify significant differences between the spectra (6, 16).

**HPLC analyses**. Extracellular metabolites present in the supernatant of fermentation samples were measured using reversed phase HPLC with a C18-column as described elsewhere (28).

## Results

**Genome scale model development.** A genome-scale metabolic model for *S. thermophilus* has been developed, based on the annotated genome of strain LMG18311 (2, 11). The available models of *Lb. plantarum* (30) and of *L. lactis,* which was constructed using the Autograph method (18), were used for the construction and development of the *S. thermophilus* model. Based on these models, many gene-protein relationships and non-gene associated reactions could be incorporated to our model, resulting in a metabolic map of *S. thermophilus* (Figure 1).

Different features of every gene such as correct annotation, function and EC number were checked manually, before they were included (or excluded) in the model. Examples of excluded genes are: truncated, hypothetical and non-metabolic genes. Excluded genes are not deleted and can be included again later when the function of such a gene has been identified. Genes coding for metabolic enzymes have been included and associated to the corresponding reactions (30). Also non-gene associated reactions, based on biochemical and experimental evidences (fermentations, amino acid omissions), were added to close gaps in the biochemical network, these included: (i) vitamin transport systems such as nicotinic acid uptake; (ii) specific *S. thermophilus* protein synthesis based on experimental data; (iii) different uptake systems such as oxygen diffusion a proton symporter for lactate.

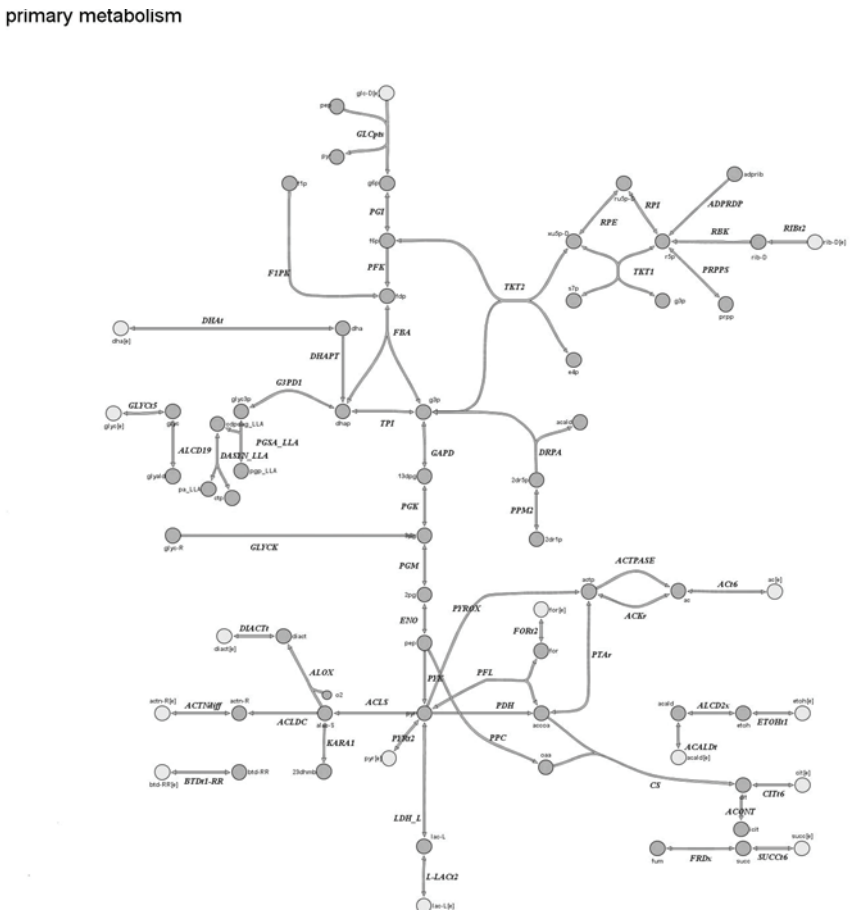


**Figure 1.** Primary metabolism of *Streptococcus thermophilus*. Part of the total genome-scale metabolic model developed for *S. thermophilus*. Large bold capital italics indicate the enzymes and normal small italics the metabolites. The complete model can be found in the supplementary material (Figure S1).

The current model consists of 429 genes (23% of the total number of genes) and 522 model reactions, 79 (15%) of which are non-gene associated. Moreover, the biomass composition of this strain was determined in this study and compared with two other LAB (Table 1). The closely related strains *L. lactis* and *S. thermophilus* have comparable amounts of protein. Organic compounds in fermentation samples were measured by HPLC, on the basis of which fluxes were calculated (30). Both biomass data and fluxes were used for *in silico* simulations. The model of *S. thermophilus* is now at a stage where *in silico* growth can be simulated under different conditions.

**Table 1.** Biomass composition of three different LAB: *L. lactis* (20), *Lb. plantarum* (30) and *S. thermophilus* LMG18311 (this study; average of 3 fermentations, samples taken at steady-state).

| Compound (% w/w) | Overall biomass composition | | |
|---|---|---|---|
| | *L. lactis* | *Lb. plantarum* | *S. thermophilus* |
| Proteins | 46 | 29.9 | 43.4 |
| Lipids | 3.4 | 6.3 | 6.1 |
| Polysaccharides | 12 | 9.9 | 24.1 |
| DNA | 2.3 | 1.9 | 1 |
| RNA | 10.7 | 9 | 8.2 |
| Other | 25.6 | 43 | 17.2 |

**Amino acid omissions.** Experiments with single amino acid omissions in *S. thermophilus* have shown that the number and type of essential amino acids is strain dependent (9, 15, 17). In general, *S. thermophilus* has a much lower degree of auxotrophy for amino acids than other LAB (4), showing no growth only in the absence of histidine and clearly reduced growth in the absence of cysteine (Table S2).

Multiple omissions of amino acids, performed in our laboratory, showed that *S. thermophilus* LMG18311 needs only histidine and one of the sulfur containing amino acids (cysteine or methionine) in the presence of citrate for (minimal) growth, (Table 2).

**Table 2.** Growth of *S. thermophilus* after 24 hours under multiple amino acid omissions. Data shown are the average of three parallel cultures. Additional data of these amino acid omission experiments are shown in the Supplementary material (Table S2)

| aa composition in CDM (g/l) Medium | OD600 |
|---|---|
| All AA | 1.55 |
| No AA | 0[a] |
| Only his (0.15) and cys (0.39) | 0.6[b] |
| Only his, cys, glu (0.4) | 0.69 |
| Only his, cys, glu, phe (0.28) | 0.75 |
| Only his, cys, glu, ser (0.34) | 0.83 |
| Only his, cys, glu, ala (0.24) | 0.44 |
| Only his, cys, glu, val (0.33) | 0.72 |
| Only his, cys, glu, phe, ser | 0.73 |
| Only his, cys, glu, phe, ala | 0.53 |
| Only his, cys, glu, phe, val | 0.61 |
| Only his, cys, glu, ser, ala | 0.69 |
| Only his, cys, glu, ala, val | 0.82 |

[a] negative control, should be 0
[b] growth after 48 hours

We have performed the growth experiments on a minimal defined medium with histidine, cysteine and glutamic acid, since the addition of glutamic acid improved the growth rate significantly and growth experiments showed that cysteine is preferred over methionine.

*In silico* predictions of the amino acid biosynthesis pathways of *S. thermophilus* LMG18311 were performed (11) and this strain indeed seems to contain all the genes coding for the enzymes required for the biosynthesis of all amino acids except histidine. This analysis also showed that *yhcE* is truncated by a conserved stop codon. The product of *yhcE* shows similarity to the vitamin B12-independent 5-methyltetrahydropteroyltriglutamate-homocysteine *S*-methyltransferase. Its orthologue in *L. lactis* is involved in the synthesis of cysteine from methionine. This gene inactivation may explain the auxotrophy for one of the two sulfur amino acids. Even though the genome of LMG18311 lacks a glutamate synthase gene, the strain shows (minimal) growth in the presence of citrate, when both glutamate and glutamine were depleted from the medium. However, *S. thermophilus* possesses a pathway for the synthesis of glutamate from citrate via 2-oxoglutarate involving glutamate dehydrogenase and glutamine synthetase for interconversion between glutamic acid and glutamine

Different LAB have different absolute requirements for amino acids; *S. thermophilus* only needs 2 amino acids as described above whereas *L. lactis* and *Lb. plantarum* need 6 and 11 amino acids for minimal growth respectively (Table 3) (12, 30).

**Table 3.** Essential amino acids for three different lactic acid bacteria: *L. lactis* MG1363 (12), *Lb. plantarum* WCFS1 (30) and *S. thermophilus* (this study, table 2)

| *L. lactis* MG1363 | *Lb. plantarum* WCFS1 | *S. thermophilus* LMG18311 |
|---|---|---|
| Glutamate | Arginine | Cysteine |
| Histidine | Cysteine | Histidine |
| Isoleucine | Glutamate | |
| Leucine | Isoleucine | |
| Methionine | Leucine | |
| Valine | Methionine | |
| | Phenylalanine | |
| | Threonine | |
| | Tryptophan | |
| | Tyrosine | |
| | Valine | |

**GC analyses.** In order to get an overview of flavor formation by the three different LAB, we compared fermentation samples using gas chromatography (GC). The headspace of steady state samples of *S. thermophilus* LMG18311, *L. lactis* MG1363 and *Lb. plantarum* WCFS1 grown on CDM (containing all amino acids) was compared. The metabolic activities of the fermenting microbes (22) was investigated through flavor profiles in the fermentation fluids, corrected for the medium components at the start of the experiments. An overview of the volatile metabolic products is shown in Figures 2, 3 and 4 and they show multiple differences in the volatile profiles of different strains.

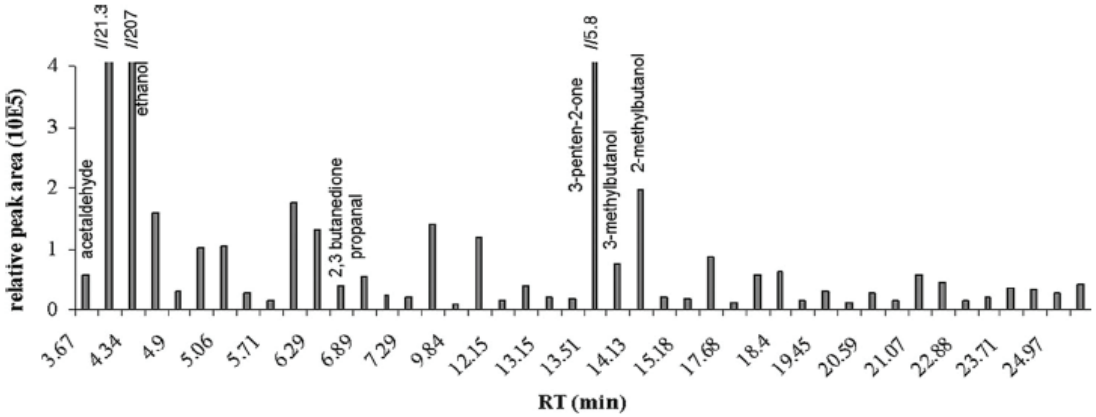


**Figure 2.** Major volatiles formed during growth by *L. lactis* on chemically defined medium. Relative peak areas are expressed as arbitrary units, the area of three peaks is indicated since they are beyond the scale. Some important peaks are indicated. Table S3 (Supplemental material) shows all the identified metabolites for *L. lactis*.

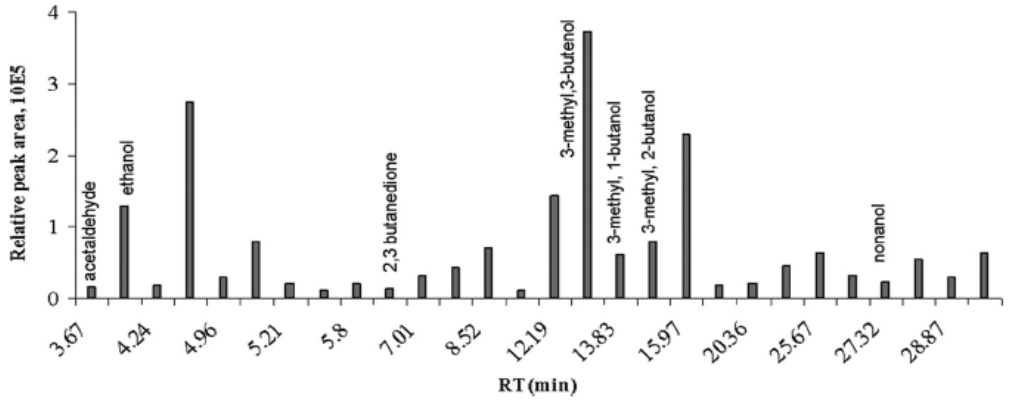


**Figure 3.** Major volatiles formed during growth by *Lb. plantarum* on chemically defined medium. Relative peak areas are expressed as arbitrary units. Some important peaks are indicated. Table S4 (Supplementary material) shows all the identified metabolites for *Lb. plantarum*.

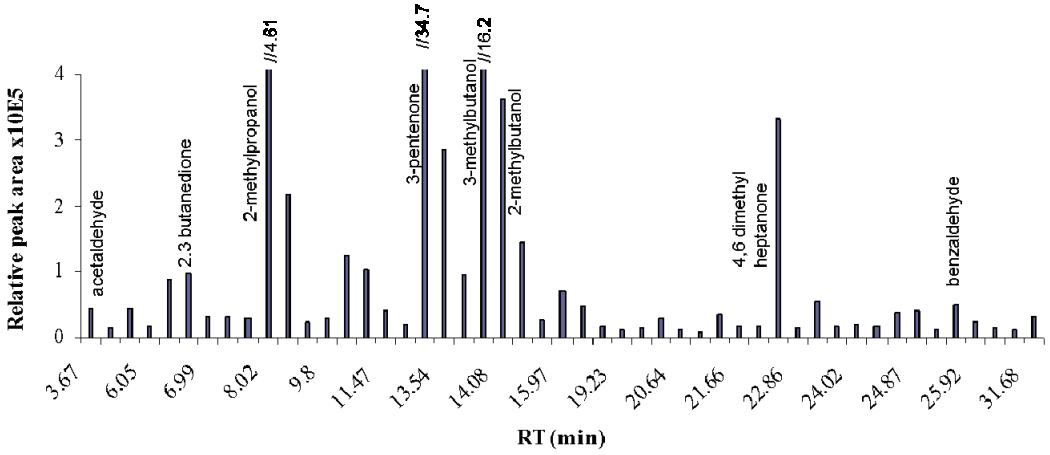


**Figure 4.** Major volatiles formed during growth by *S. thermophilus* on chemically defined medium. Relative peak areas are expressed as arbitrary units, the area of three peaks is indicated since they are beyond the scale. Some important peaks are indicated. Table S5 (Supplementary material) shows all the identified metabolites for *S. thermophilus*.

Many volatiles or flavors are produced during amino acid metabolism. When the results of the GC analyses of the three LAB are compared, they show that *S. thermophilus* is able to produce a broad variety of flavors. In combination with the low requirements of amino acids (only 2), this reflects a relatively complete set of amino acid biosynthetic and amino acid converting pathways. When *S. thermophilus* grows on CDM, all amino acids are consumed in small amounts (data not shown). *L. lactis* and *Lb. plantarum* need more amino acids (respectively 6 and 11) for minimal growth and especially *Lb. plantarum* produces less flavors.

One of the identified compounds produced by all three LAB is acetaldehyde. As described previously (5), *S. thermophilus* can convert threonine into acetaldehyde and glycine by threonine aldolase activity. *L. lactis* and *Lb. plantarum,* among others can produce acetaldehyde during lactose metabolism by pyruvate decarboxylation (3). This difference in pathways leading to the same compound, can also be visualized in the Simpheny models, as was shown in our previous paper (22).

**Homofermentative metabolism**. *S. thermophilus* was grown under chemostat conditions on a chemically defined medium containing all amino acids. Steady state fermentation samples

(dilution rate = 0.1 to 0.4 $h^{-1}$) of *S. thermophilus* were used for different analyses. The supernatant of these samples was analyzed on HPLC and was compared with the composition of the growth medium to determine which compounds are produced and consumed during growth (Table 4).

**Table 4.** HPLC analyses of fermentation cell supernatants; *S. thermophilus* was grown under chemostat conditions at a dilution rate (D) of 0.1 $h^{-1}$ to 0.4 $h^{-1}$ on CDM (5g $l^{-1}$ glucose) containing all amino acids. The table shows steady state concentrations of the various metabolites formed or utilized in mM.

| | | Compound (mM)[a] | | | | | |
|---|---|---|---|---|---|---|---|
| | | Citric acid | Pyruvate | Lactic acid | Formic acid | Acetic acid | Glucose |
| supernatant | CDM | 2.49 | ND | ND | ND | 12.11 | 25.46 |
| | D=0.1 | 1.41 | ND | 20.41 | ND | 9.90 | 0.09 |
| | D=0.2 | 1.39 | ND | 30.55 | 0.84 | 9.56 | 0.21 |
| | D=0.3 | 1.70 | 0.12 | 33.08 | 1.28 | 10.83 | ND |
| | D=0.4 | 1.99 | 0.21 | 34.70 | 1.83 | 12.01 | 0.36 |

[a] Average of two duplicates. ND, not detected

The HPLC-analysis shows that *S. thermophilus* consumes all the glucose and some of the citric acid. *S. thermophilus* produces mainly lactate and only small amounts of pyruvate, succinate and formate are formed. The model strongly suggests that homofermentative lactic acid production is the only primary metabolism operating in *S. thermophilus* and this is confirmed by our fermentation data and also by others (11). The mixed acid fermentation (acetate, formate and ethanol) is metabolically the most efficient route for lactic acid bacteria whereas the homolactic route is catalytically more efficient (10). Both *L. lactis* and *Lb. plantarum* can grow homolactic (high dilution rates) or via mixed acid fermentation (low dilution rates) (10, 30). Because *S. thermophilus* has pseudogenes in the primary metabolism that prevent the formation of ethanol, acetate formation will cause a redox problem, and hence, the only possible route is the homolactic fermentation at both high and low dilution rates.

Flux Balance Analysis (FBA) was carried out within the Simpheny software (30). FBA is an optimization technique that can be used as a tool to predict the metabolic possibilities given mass balance and capacity constraints (24). FBA correctly predicted homolactic fermentation in *S. thermophilus*, in contrast to what was found for *Lb. plantarum* (30) and *L. lactis* (20). Based on the sequenced genome of strain LMG18311, and visualized

on the model, it is known that this strain does not have the oxidative part of the pentose phosphate pathway (PPP). The absence of a complete PPP may have important consequences for the redox balance and thereby potentially influences primary metabolism.

**Discussion**

In this paper a comparative analysis of three lactic acid bacteria; *S. thermophilus, L. lactis* and *Lb. plantarum,* is described. Comparative analysis can provide extra insights in metabolism; such as flavor formation and growth rate and it can also reveal the absence of an important pathway in one of the strains, because it is present in the other strains and vice versa. An illustrative example of this is the extensive flavor forming potential of *S. thermophilus*. This was only noticed because we analyzed different strains simultaneously. Useful tools to compare different organisms are genome-scale metabolic models. Complete models are available for *L. lactis* and *Lb. plantarum* and in this paper, we describe the construction of such a genome-scale model for *S. thermophilus* LMG18311. These genome-scale models are of course never complete and can always be expanded with new insights. Growth can be simulated under different conditions with these models. With some given constraints such as lactose excess or different pH values, growth can be predicted and can give insights in optimal growth conditions.

The most obvious difference between the three bacteria and therefore also the models, is the size of the genome and thus the number of genes. The model of *Lb. plantarum* contains 3064 genes compared to 2563 genes in the *L. lactis* model and 1889 genes (or gene fragments) in the *S. thermophilus* model. This would suggest a more extensive metabolism for *Lb. plantarum* and *L. lactis.* But the total absolute number of reactions in the three models is nearly similar: 522 for *S. thermophilus*, 598 for *Lb. plantarum* and 598 for *L. lactis.* Based on the amino acid requirements and flavor analyses as described in the results section, it seems that *S. thermophilus* has a more extensive amino acid metabolism than the other two LAB. *S. thermophilus* only needs 2 amino acids, histidine and cysteine, for minimal growth, it can degrade all amino acids and is able to produce a varied amount of amino acid derived flavors. The genome-scale model, supported by the overall experimental data, suggests a rather complete set of amino acid biosynthesis pathways in *S. thermophilus*. This is unexpected

because *S. thermophilus* is used for centuries for the production of yoghurt. The LMG18311 strain is also a yoghurt strain (11). The assumption would be that *S. thermophilus* has evolved in this protein rich environment (milk) and therefore one may have expected loss of some or more amino acid biosynthesis pathways, but this is clearly not the case. It would be interesting to see if all these pathways are operated under all conditions during the different dairy fermentation processes. These studies in which expression data under different interesting conditions are involved, are currently under investigation. Intriguingly, *Lactobacillus delbrueckii* subsp. *bulgaricus*, an organism that is most often co-cultivated with *S. thermophilus* for yoghurt manufacturing, did follow this expected path and lost most of its amino acid biosynthetic capacity (31). An explanation for this unexpected behavior of *S. thermophilus* can be that amino acid metabolism is not only important for the synthesis of amino acids but also plays a role in maintaining the redox balance. Another explanation can be that *S. thermophilus* strains are selected for quick growth and acidification in milk, available amino acid are rate-limiting in milk. To support such a quick growth, maintenance of nearly all amino acid pathways is required.

In the result section, an *in silico* prediction of the amino acid biosynthesis pathways is described. This analysis showed that *ychE* is truncated by a conserved stop codon. It would be interesting to reconstitute this codon and study the effect of an activated codon. This mutated strain probably only needs one amino acid (histidine) and complete pathways for the sulfur amino acid metabolism may have important effects on the flavor formation.

A result from our experimental data, those described in the literature (11) and a prediction of the genome scale model is that *S. thermophilus* has a simple primary metabolism because the number of pyruvate branches is limited. Especially, those which are important for NAD+ regeneration for glycolysis, there is no real alternative to lactate dehydrogenase for NAD+ regeneration. Due to this, there is really only one possible route, leading to an equilibrated redox balance for glucose catabolism when *S. thermophilus* grows anaerobically, and that is the homolactic route. Therefore, Flux Balance Analysis does predict the right growth rate and products formation rates in *S thermophilus*. In *Lb. plantarum* and *L. lactis*, FBA invariably predicts the use of an alternative pathway with higher ATP yield (mixed acid fermentation), and homolactic fermentation cannot be predicted by FBA.

Another striking difference between *S. thermophilus* and *L. lactis* and *Lb. plantarum* is the absence of a complete pentose phosphate pathway. Three genes encoding for the enzymes glucose-6-Phosphate dehydrogenase, 6-phosphogluconolactonase and phosphogluconate dehydrogenase are missing, these 3 enzymes form the oxidative part of the pentose phosphate pathway. This might have important consequences for the NADPH generation, the ribonucleotides and aromatic amino acids synthesis. There might be a link between the simple primary metabolism (limited number of pyruvate branches and the absence of a complete PPP) and the complex amino acid metabolism via redox constraints, a hypothesis that is currently under investigation.

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## Supplementary material

**Table S1**. Amino acid composition (in grams/liter) of the full chemically defined medium

| | Concentration (g/l) | | |
|---|---|---|---|
| Amino Acid | *S. thermophilus* | *L. lactis* | *Lb. plantarum* |
| Alanine | 0.24 | 0.24 | 0.24 |
| Arginine | 0.13 | 0.125 | 0.125 |
| Aspartic acid | 0.46 | 0.42 | 0.42 |
| Asparagine | 0.35 | | |
| Cysteine | 0.25 | 0.13 | 0.13 |
| Glutamic acid | 0.4 | 0.5 | 0.5 |
| Glutamine | 0.39 | | |
| Glycine | 0.18 | 0.175 | 0.175 |
| Histidine | 0.15 | 0.15 | 0.15 |
| Isoleucine | 0.22 | 0.21 | 0.21 |
| Leucine | 0.48 | 0.475 | 0.475 |
| Lysine | 0.44 | 0.44 | 0.44 |
| Methionine | 0.13 | 0.125 | 0.125 |
| Phenylalanine | 0.28 | 0.275 | 0.275 |
| Proline | 0.68 | 0.675 | 0.675 |
| Serine | 0.34 | 0.34 | 0.34 |
| Threonine | 0.23 | 0.225 | 0.225 |
| Tryptophane | 0.5 | 0.05 | 0.05 |
| Tyrosine | 0.29 | 0.25 | 0.25 |
| Valine | 0.33 | 0.325 | 0.325 |

**Table S2.** Growth of *S. thermophilus* after 24 hours under single amino acid omission in CDM

| Single AA omission<br>Without | OD600 |
|---|---|
| All AA present | 1.64 |
| No AA | 0[a] |
| Alanine | 1.44 |
| Arginine | 1.17 |
| Asparagine | 1.63 |
| Aspartic acid | 1.68 |
| Cysteine | 1.00 |
| Glutamine | 1.49 |
| Glutamic acid | 1.40 |
| Glycine | 1.61 |
| Histidine | 0 |
| Isoleucine | 1.44 |
| Leucine | 1.32 |
| Lysine | 1.63 |
| Methionine | 1.66 |
| Phenylalanine | 1.67 |
| Praline | 1.63 |
| Serine | 1.68 |
| Threonine | 1.69 |
| Tryptophan | 1.28 |
| Tyrosine | 1.48 |
| Valine | 1.57 |

[a]negative control, should be 0

**Table S3.** Identified volatile compounds formed by *L. lactis* MG1363 during growth

| RT | Area | Compound |
|---|---|---|
| 3.67 | 5.62 E+04 | acetaldehyde |
| 4.32 | 2.13 E+06 | ethanol |
| 4.34 | 2.07E+07 | ethanol |
| 4.85 | 1.58E+05 | 2-propanol |
| 4.9 | 3.12E+04 | Nitrous oxide |
| 4.97 | 1.02E+05 | Ethyl ether |
| 5.06 | 1.04E+05 | Formic acid, ethyl ester |
| 5.3 | 2.66E+04 | 2-propanol, 2-methyl- |
| 5.71 | 1.54E+04 | 2-propen-1-ol |
| 5.96 | 1.77E+05 | 1-propanol |
| 6.29 | 1.31E+05 | Silanol, trimethyl |
| 6.45 | 3.88E+04 | 2,3-butanedione |
| 6.89 | 5.43E+04 | propanal |
| 7.11 | 2.40E+04 | 2-butanol |
| 7.29 | 2.01E+04 | Furan, 2-methyl |
| 7.42 | 1.40E+05 | Acetic acid, ethyl ester |
| 9.84 | 1.03E+04 | 4-penten-2-one |
| 11.24 | 1.18E+05 | Methyl thiolacetate |
| 12.15 | 1.60E+04 | Propanoic acid, ethyl ester |
| 12.25 | 3.91E+04 | Furan, 2,5-dimethyl- |
| 13.15 | 2.10E+04 | Pyrazine |
| 13.31 | 1.93E+04 | Thiazole |
| 13.51 | 5.80E+05 | 3-penten-2-one |
| 13.87 | 7.49E+04 | 1-butanol, 3-methyl- |
| 14.13 | 1.97E+05 | 1-butanol, 2-methyl- |
| 14.52 | 1.98E+04 | Ethane, isocyanato |
| 15.18 | 1.65E+04 | Propanoic acid, 2-methyl-, ethyl ester |
| 15.75 | 8.52E+04 | Silane, diethoxydimethyl |
| 17.68 | 1.34E+04 | octane |
| 17.84 | 5.56E+04 | Pyrazine, methyl- |
| 18.4 | 6.29E+04 | Acetyl valeryl |
| 19.23 | 1.46E+04 | Butanoic acid, 3-methyl-,ethyl ester |
| 19.45 | 3.03E+04 | benzenemethanol |
| 20.37 | 1.19E+04 | 2-heptanone |
| 20.59 | 2.56E+04 | 3-hexen-2-one, 5-methyl |
| 20.67 | 1.43E+04 | Acetic acid ethenyl ester |
| 21.07 | 5.69E+04 | Pyrazine, 2,6-dimethyl- |
| 21.24 | 4.53E+04 | Pyrazine, ethyl |
| 22.88 | 1.63E+04 | 2-isopropylpyrazine |
| 23.57 | 2.23E+04 | Pyrazine, 2-ethyl-6-methyl- |
| 23.71 | 3.71E+04 | 3-furancarboxylic acid, 2-methyl-, |
| 24.24 | 3.24E+04 | benzoxazole |
| 24.97 | 2.69E+04 | Pyrazine, 2-methyl-5-(1-methylethyl)- |
| 25.69 | 4.22E+04 | 2-nonanone |

**Table S4.** Identified volatile compounds formed by *Lb. plantarum* WCFS1 during growth

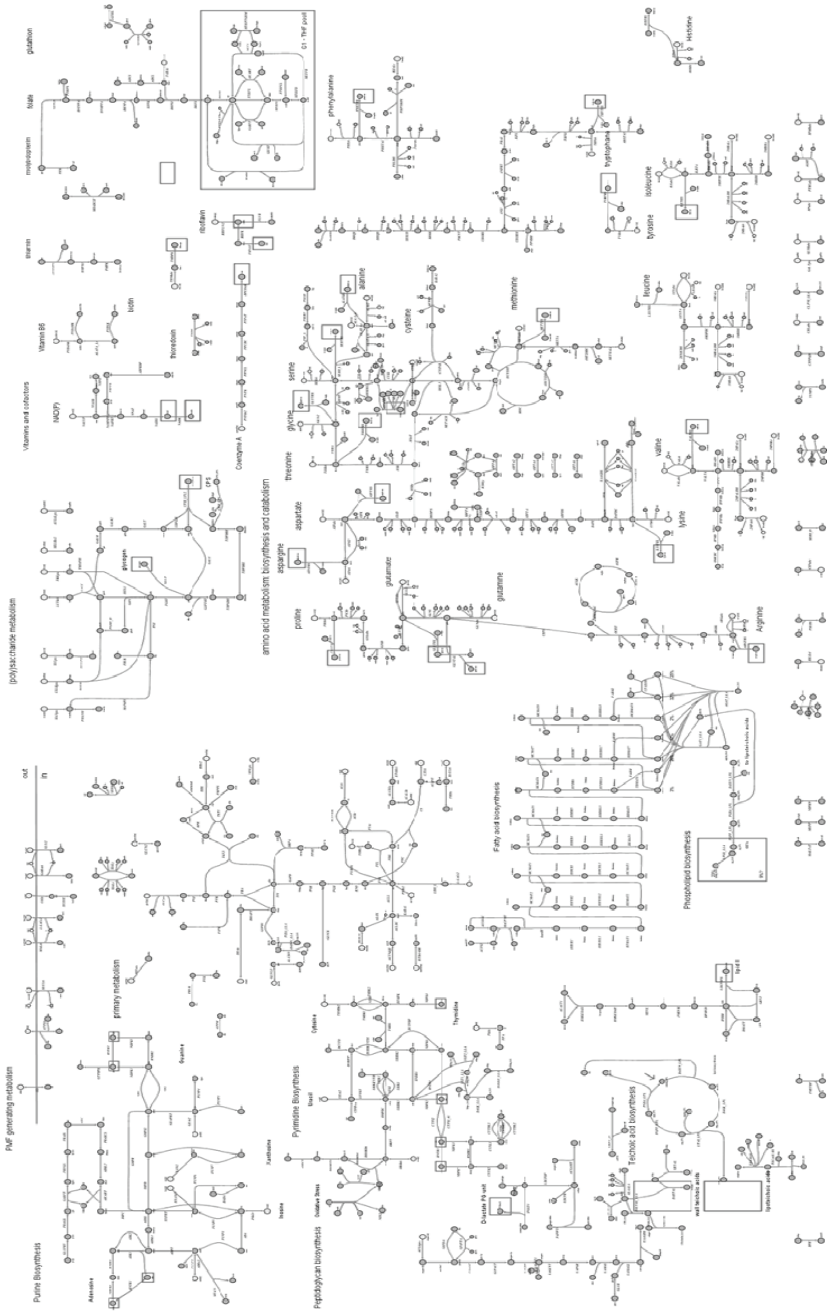
| RT | Area | Compound |
|---|---|---|
| 3.67 | 1.59E+04 | acetaldehyde |
| 4.22 | 1.29E+05 | ethanol |
| 4.24 | 1.90E+04 | ethanol |
| 4.43 | 2.74E+05 | acetonitrile |
| 4.96 | 2.98E+04 | Ethyl ether |
| 5.05 | 8.00E+04 | Formic acid, ethyl ester |
| 5.21 | 2.07E+04 | 2-propanol, 2-methyl |
| 5.68 | 1.17E+04 | Carbon disulfide |
| 5.8 | 2.00E+04 | Propanal, 2-methyl |
| 6.89 | 1.39E+04 | 2,3-butandedione |
| 7.01 | 3.13E+04 | 1-pentene, 2-methyl- |
| 8.03 | 4.22E+04 | Propane, 2-ethoxy-2-methyl |
| 8.52 | 7.11E+04 | 2-butanol, 2-methyl |
| 10.42 | 1.12E+04 | 2-pentanone |
| 12.19 | 1.44E+05 | 2-propenoic acid, 2-methyl-,methyl ester |
| 13.54 | 3.73E+05 | 3-buten-1-ol, 3-methyl |
| 13.83 | 6.09E+04 | 1-butanol, 3-methyl- |
| 14.08 | 7.93E+04 | 1-butanol, 2-methyl- |
| 15.97 | 2.30E+05 | 2-buten-1-ol, 3-methyl |
| 18.11 | 1.79E+04 | Ethanol, 2-chloro-acetate |
| 20.36 | 1.94E+04 | 2-heptanone |
| 24.79 | 4.45E+04 | 2,5-hexanediol, 2,5-dimethyl- |
| 25.67 | 6.33E+04 | 2-nonanone |
| 25.93 | 3.15E+04 | Benzaldehyde, 4-methyl |
| 27.32 | 2.32E+04 | 1-nonanol |
| 28.29 | 5.44E+04 | Pentanoic acid, 2,4-dimethyl-4-nitro, methyl ester |
| 28.87 | 2.95E+04 | Nerol/cis-geraniol |
| 31.54 | 6.27E+04 | Butanoic acid, 2-ethyl-2,3,3-trimethyl-, methylester |

**Table S5.** Identified volatile compounds formed by *S. thermophilus* LMG18311 during growth

| RT | Area | Compound |
|---|---|---|
| 3.67 | 4.31E+05 | acetaldehyde |
| 4.95 | 1.60E+04 | 2-propenenitrile |
| 5.05 | 4.30E+04 | 1,3-pentadiene |
| 6.05 | 1.79E+04 | 2-propenal, 2-methyl- |
| 6.32 | 8.87E+04 | Propane, 2-methoxy-2-methyl- |
| 6.41 | 9.92E+04 | 2,3-butanedione |
| 6.99 | 3.29E+04 | 1-pentene,2-methyl |
| 7.26 | 3.17E+04 | Furan, 2-methyl- |
| 7.42 | 3.06E+04 | 3-buten-2-ol, 2-methyl |
| 8.02 | 4.61E+05 | 1-propanol, 2-methyl- |

| | | |
|---|---|---|
| 8.52 | 2.15E+05 | 2-butanol, 2-methyl |
| 9.12 | 2.40E+04 | 2-butanone, 3-methyl |
| 9.8 | 2.94E+04 | 3-buten-2-one, 3-methyl- |
| 10.43 | 1.23E+05 | 2-pentanone |
| 10.73 | 1.04E+05 | 2,3-pentanedione |
| 11.47 | 4.21E+04 | 2-pentanol |
| 13.11 | 2.15E+04 | pyrazine |
| 13.46 | 3.47E+06 | 3-penten-2-one |
| 13.54 | 2.84E+05 | 3-buten-1-ol, 3-methyl- |
| 13.69 | 9.39E+04 | 2-pentanol, 2-methyl- |
| 13.83 | 1.62E+06 | 1-butanol, 3-methyl- |
| 14.08 | 3.62E+05 | 1-butanol, 2-methyl-, |
| 15.72 | 1.46E+05 | Furan, tetrahydro-2,2,5,5-tetramethyl- |
| 15.83 | 2.64E+04 | 2-butenal, 3-methyl- |
| 15.97 | 7.06E+04 | 2-buten-1-ol, 3-methyl |
| 17.82 | 4.74E+04 | Pyrazine, methyl- |
| 18.55 | 1.73E+04 | 1-pentanol, 2-methyl- |
| 19.23 | 1.05E+04 | 2-propanone |
| 19.96 | 1.46E+04 | Hexane, 2,3-dimethyl- |
| 20.13 | 2.88E+04 | Benzene, 1,2-dimethyl- |
| 20.64 | 1.33E+04 | 2-ethoxyethyl acetate |
| 21.07 | 1.00E+04 | Pyrazine, 2,5-dimethyl- |
| 21.21 | 3.46E+04 | Pyrazine, ethyl- |
| 21.66 | 1.72E+04 | Pyrazine, ethenyl- |
| 21.82 | 1.65E+04 | 3-pentanone |
| 21.93 | 3.31E+05 | 2-heptanone, 4-methyl- |
| 22.86 | 1.62E+04 | 2-isopropylpyrazine |
| 23.39 | 5.58E+04 | 2-heptanone, 4,6-dimethyl- |
| 23.55 | 1.82E+04 | Pyrazine, 2-ethyl-6-methyl- |
| 24.02 | 2.17E+04 | Pyrazine, 2-ethenyl-6-methyl- |
| 24.14 | 1.69E+04 | Hexane, 2,3,4-trimethyl- |
| 24.82 | 3.98E+04 | 2,5-hexanediol, 2,5-dimethyl- |
| 24.87 | 4.28E+04 | Heptane, 2,3-dimethyl- |
| 25.29 | 1.22E+04 | 1-octanol |
| 25.66 | 5.15E+04 | Benzaldehyde, 4-methyl- |
| 25.92 | 2.42E+04 | Benzoic acid, methyl ester |
| 27.49 | 1.51E+04 | 2-heptanone, 4-methyl- |
| 28.3 | 1.32E+04 | Benzoic acid, 4-methyl-, methyl ester |
| 31.68 | 3.21E+04 | Diphenyl ether |

**Figure S1**. Metabolic map of *S. thermophilus* LMG18311, developed with the Simpheny software. For explanation of the different abbreviations, see **Teusink, B., A. Wiersma, D. Molenaar, C. Francke, W. M. de Vos, R. J. Siezen, and E. J. Smid.** 2006. Analysis of growth of *Lactobacillus plantarum* WCFS1 on a complex medium using a genome-scale metabolic model. J. Biol. Chem. **281:**40041-40048. and **Santos, F.** 2008. Vitamin B12 synthesis in *Lactobacillus reuteri*. PhD thesis Wageningen University.



# Chapter 4

## Effect of amino acid quantity on the metabolism of three lactic acid bacteria: a comparative study

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Manuscript in preparation

**Abstract**

To compare amino acid metabolic pathways of three different lactic acid bacteria, metabolic and transcriptional analyses were performed. The growth of three well-known lactic acid bacteria; *Streptococcus thermophilus*, *Lactococcus lactis* and *Lactobacillus plantarum,* on defined media containing all amino acids was compared with that on media with a minimum amount of amino acids, that still allowed growth. The global transcriptional response of each species towards minimal amino acid availability was determined using a species-specific micro array and data were visualized with the use of genome-scale models. Metabolic analyses were performed to study the fermentation behavior and the flavor formation. All three lactic acid bacteria use homolactic fermentation and show a significant response towards low amounts of amino acids in the medium. This response not only affects the amino acid metabolism such as flavor formation, tRNA production and amino acid ABC transporters, but also primary metabolism and overall growth, and stress-related proteins. This study also showed that the different organisms have different strategies to cope with varying amounts of amino acids.

## Introduction

A variety of lactic acid bacteria (LAB) are used for industrial dairy fermentations, such as several species of *Lactococcus, Lactobacillus* and *Streptococcus.* The basis for these fermentations, milk, is a protein-rich environment mainly consisting of caseins, whey proteins and free amino acids; the most abundant amino acid in milk is glutamic acid (25). Most LAB have amino acid auxotrophies, therefore the fermenting LAB depend on the available amino acids in their growth medium. An interesting feature of the amino acid metabolism is the production of flavor compounds. Most amino acids do not directly influence the product flavor, but they will contribute indirectly to the typical flavor because they are precursors of aromatic compounds (39). Flavor formation during fermentation not only depends on the substrate (15, 16) but also on the species and even strains used. It has been described that *S. thermophilus* is less auxotrophic for amino acids than other LAB (7, 29). Strains that require a few amino acids for growth often have a relatively complete set of amino acid converting pathways. This also often results in the production of a large variety of amino acid derived flavors (29).

Several studies have been performed to clear the regulation of the protein metabolism in LAB. CodY, for instance, is a well-studied transcriptional regulator in gram-positive bacteria (10). In *L. lactis,* most of the CodY regulated genes are involved in the proteolytic system, however, CodY also regulates the transcription of genes involved in the metabolism of amino acids (11).

In the last years the genomes of many LAB have been sequenced and have become publicly available (20, 23). These available genomes enable the development of genome-wide tools such as microarrays (24). Microarrays can be used to study the response of an organism to a certain condition and the available genome scale metabolic models (27, 37) are used for visualization of the transcriptional response. Amino acid catabolism and anabolism are complex processes and, thus, metabolic models will be helpful for their understanding. Genome-scale metabolic models provide an overview of all metabolic conversions in an organism, based on its genome sequence, and make it possible to visualize different metabolic pathways, such as amino acid metabolism (36, 37).

In this study we have compared three different LAB with known genome sequences; *Streptococcus (S.) thermophilus* LMG8311 (6), *Lactobacillus (Lb.) plantarum* WCFS1 (21) and *Lactococcus (L.) lactis* MG1363 (38). These strains are all used frequently in the food industry: as yoghurt starter, as vegetable fermenter and flavor enhancer and as cheese starter respectively. With this study, we would like to gain more insights in the regulation of amino acid metabolism. Furthermore, a comparative study can provide extra insights in the differences and similarities between different strains.

**Materials and methods**

**Bacterial strains, media and growth conditions.** The strains used in this study were *S. thermophilus* LMG18311 (6), *L. lactis* MG1363 (38) and *Lb. plantarum* WCFS1 (21). Cells were grown in Chemically Defined Medium (CDM) with an excess of amino acids (complete CDM, Table 1), (22, 28, 30). *S. thermophilus* was grown at 42°C, *L. lactis* MG1363 at 30°C and *Lb. plantarum* at 37°C. The defined media contain 25 mM glucose. When indicated cells were grown in CDM with a minimal amount of amino acids (restricted CDM, Table 1) (18, 29, 37)

**Batch cultivations.** Fermentations were performed in duplicate in 1 L bioreactors (Applikon Biotechnology BV, The Netherlands). The fermentors were controlled by a Bio Controller ADI 1010 and by a Bio Console ADI 1025 (Applikon Biotechnology BV, The Netherlands). Strains were grown overnight in the desired medium and used as an inoculum of 750 ml pH controlled CDM, the medium was 1% inoculated. During growth, pH was kept constant at pH=6.0 by the addition of 2.5M NaOH. Cultures were stirred at a constant speed of 100 rpm. Growth was followed by measuring the cell density at 600 nm. Samples for RNA isolation were taken at mid-exponential phase, samples for GC-MS analysis (3 ml) were taken at mid-exponential phase and at stationary phase and samples for HPLC were taken at end-exponential phase.

**RNA isolation.** RNA was isolated, as described elsewhere (34) with a few modifications. At mid-exponential phase ($OD_{600}$ of *S. thermophilus* ~ 0.6, $OD_{600}$ of *L. lactis* and *Lb. plantarum* ~ 1.0) , 25 ml of culture from each fermentor (duplicate samples for both restricted and complete medium) was immediately pelleted by centrifugation at 14000 rpm for 2 min at room temperature (Herolab, Unicen MR, Germany). The pellet was frozen rapidly in liquid nitrogen and stored at -80°C until further use. The frozen pellet was resuspended in 400 µl TE and transferred to a screw cap tube containing 500 µl phenol-chloroform (5:1), 15 µl 20% sodium dodecyl sulphate, 30 µl 3M sodium acetate pH 4.8 and 0.6 g zirconium glassbeads. Cells were disrupted in a Fastprep (Savant, FP120) for 40 sec at 5.0 and the mixture was centrifuged to remove the beads (13000 rpm, 20 min, 4°C). Subsequently, 500 µl cold chloroform was added to the supernatant followed by a centrifugation step (13000 rpm, 10 min, 4°C). After centrifugation, 0.5 ml of the aqueous phase was used for RNA extraction using the High Pure RNA Isolation kit (Roche Diagnostics, Mannheim, Germany), following the manufacturer's protocol, except for the DNAse I treatment which we changed to 30 min at 37°C. RNA was eluted in 60 µl of elution buffer (supplied in the kit) and samples were stored at -80°C.

The concentration of RNA was checked with a ND-1000 spectrophotometer (NanoDrop Technologies, Inc., USA) and quality was checked using a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Only samples with a 23S/16S ratio higher than 1.6 were used for labeling.

**cDNA synthesis and labeling.** First strand cDNA synthesis from 5 µg RNA was carried out as described previously (31, 32). Synthesized cDNA was purified and labeled with cyanine 3 and cyanine 5 for all samples. The quality of the labeled cDNA and its concentration were measured with the ND-1000 spectrophotometer (NanoDrop Technologies, Inc., USA).

**Hybridization.** Hybridization of the labeled cDNA (0.3 µg per sample) was carried out as described previously (31, 32) (hybridization scheme in supplementary materials figures S1 and S2). The samples were hybridized on custom designed Agilent Technologies oligo microarrays, using the Agilent 60-mer oligo microarray processing protocol version 4.1 (31). The design for the *S. thermophilus* LMG18311 arrays contains 2 arrays per slide (11K slides)

whereas the design for the arrays of *L. lactis* MG1363 and *Lb. plantarum* WCFS1 contain 8 arrays per slide (15K slides).

**Scanning and data analysis.** Slides were scanned with a ScanArray Express scanner at a resolution of 10 μm for the *S. thermophilus* arrays and at 5 μm for the *L. lactis* and *Lb. plantarum* arrays. Data analysis and processing were carried out as described elsewhere (31, 32). Fold change (FC) is defined as $2^M$ where $M = {}_2\log$ (cy5 intensity/cy3 intensity) (32). Significantly regulated genes were defined as genes whose average p-value is less than 5% and whose M-value is equal or higher than 1.5.

**HPLC and GC-MS analysis.** Extracellular metabolites present in the supernatant of fermentation samples were measured as described elsewhere (35). For the identification of volatile components in the samples, purge and trap thermal desorption cold trap gas-chromatography was used as described before (13, 33). The headspace samples were concentrated on a Fisons MFA815 cold trap (CE Instruments, Milan, Italy), followed by separation on a GC-8000 top gas chromatograph (CE Instruments) equipped with a CIP-SIL 5 CB low-bleed column (Chrompack, Middelburg, The Netherlands) and detection by a flame ionization detector.

## Results

**Growth and amino acid utilization.** The impact of variations in amino acid content of the growth medium on the gene expression was studied using global transcriptional profiling. Three different LAB, *S. thermophilus, L. lactis* and *Lb. plantarum* were studied and growth was compared on a complete CDM (surplus of amino acids) with growth on a restricted CDM (minimal amount of amino acids) (Table 1). These three LAB each have their own requirements for amino acids and therefore the restricted media are different (Table 1).

**Table 1.** Composition amino acid mix of CDM for *S. thermophilus* (22) *L. lactis* and *Lb. plantarum* (28, 30). All amino acids listed in this table are present in the complete CDM whereas only the amino acids presented in bold are present in the restricted CDM (18, 37) (in the same amounts as in the complete CDM).

| | Amount (g/l) | | |
|---|---|---|---|
| Amino Acid | *S. thermophilus* | *L. lactis* | *Lb. plantarum* |
| Alanine | 0.24 | 0.24 | 0.24 |
| Arginine | 0.13 | 0.125 | **0.125** |
| Aspartic acid | 0.46 | 0.42 | 0.42 |
| Asparagine | 0.35 | **0.12**[a] | |
| Cysteine | **0.25** | 0.13 | **0.13** |
| Glutamic acid | **0.4** | **0.5** | **0.5** |
| Glutamine | 0.39 | **0.102**[a] | |
| Glycine | 0.18 | 0.175 | 0.175 |
| Histidine | **0.15** | **0.15** | 0.15 |
| Isoleucine | 0.22 | **0.21** | **0.21** |
| Leucine | 0.48 | **0.475** | **0.475** |
| Lysine | 0.44 | 0.44 | 0.44 |
| Methionine | 0.13 | **0.125** | **0.125** |
| Phenylalanine | 0.28 | 0.275 | **0.275** |
| Proline | 0.68 | 0.675 | 0.675 |
| Serine | 0.34 | 0.34 | 0.34 |
| Threonine | 0.23 | 0.225 | **0.225** |
| Tryptophane | 0.5 | 0.05 | **0.05** |
| Tyrosine | 0.29 | 0.25 | **0.25** |
| Valine | 0.33 | **0.325** | **0.325** |

[a]amino acids only present in restricted CDM and not in complete CDM

A summary of growth performance of the three LAB on both restricted and complete medium is also given (Table 2).

**Table 2.** Maximum $OD_{600}$ and growth rate of three LAB - *S. thermophilus, L. lactis* and *Lb. plantarum*- during growth on both complete and restricted medium

| | Complete medium | | Restricted medium | |
|---|---|---|---|---|
| | μmax ($h^{-1}$) | End $OD_{600}$ | μmax ($h^{-1}$) | End $OD_{600}$ |
| *S. thermophilus* | 0.89 | 1.2 | 0.35 | 0.8 |
| *L. lactis* | 0.78 | 2.0 | 0.32 | 1.1 |
| *Lb. plantarum* | 0.78 | 2.4 | 0.42 | 2.0 |

*S. thermophilus* has the highest maximum growth rate, whereas the *L. lactis* and *Lb. plantarum* have a higher final optical density. The growth rate on restricted media is for all three LAB 50% of the growth rate on complete media. *S. thermophilus* utilizes all amino acids partially on complete medium. On restricted medium, all three present amino acids (histidine, cysteine and glutamic acid) are partially utilized by *S. thermophilus*.

*L. lactis* utilizes, partially, all amino acids present on both complete and restricted medium. In both cases, none of the amino acids is utilized completely. *Lb. plantarum* utilizes all amino acids on complete medium in very small amounts. On the restricted defined medium also all amino acids are utilized (a summary of the data is given in Table 3). On restricted medium, *S. thermophilus* and *L. lactis* utilize twice as low amino acids as on complete medium, whereas *Lb. plantarum* does not show this dramatic difference, probably because most amino acids are present.

**Table 3.** HPLC analysis the uptake (medium - sample) of most relevant amino acids in the supernatant of fermentation samples of *S. thermophilus, L. lactis* and *Lb. plantarum* grown under batch conditions on complete and restricted defined medium.

| Consumption of Amino acids (μmol/L) | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | Glu | Val[a] | Leu[a] | Ile[a] | His | Cys |
| *S. thermophilus* | complete medium | 94 | | | | 56 | 157 |
| | restricted medium | 629 | | | | 175 | 574 |
| *L. lactis* | complete medium | 917 | 752 | 903 | 486 | | |
| | restricted medium | 1288 | 995 | 1324 | 651 | | |
| *Lb. plantarum* | complete medium | 563 | 239 | 645 | 240 | | |
| | restricted medium | 1041 | 513 | 795 | 370 | | |

[a] Branched chain amino acids are not present in the restricted medium of *S. thermophilus*

**Comparative Transcriptome Analysis.** The impact of variations in amino acid content of the growth medium on the gene expression was studied using global transcriptional profiling. The response on restricted medium was compared with the response on complete medium.. For differential regulation, we selected only those genes that satisfied the following criteria (i) $M \geq 1.5$ in both duplicates (ii) average p-value $< 0.05$. Within these criteria, we found 61 differentially regulated genes for *S. thermophilus* (constituting about 3% of the genes), 186 genes for *L. lactis* (constituting about 7.3% of the genes) and 81 genes for *Lb. plantarum* (constituting about 2.6% of the genes)

For *S. thermophilus* 61 genes are differentially regulated (Table 4) and are visualized (Figure 1) on the recently developed genome-scale model, developed with the Simpheny$^{TM}$ software package (29, 37). Of these 61 genes, 30 are involved in amino acid metabolism. A striking observation is that the production of 9 tRNA's are down-regulated on restricted medium. It was observed that the biosynthesis of the sulfur amino acids is strongly up-regulated (acetyl homoserine lyase, 5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase, 5,10-methylenetetrahydrofolate reductase). Other regulated genes are predicted to encode proteins involved in growth (e.g. down-regulation of transcriptional regulators), vitamin metabolism (e.g. up-regulation of molybdopterin and tetrahydrofolate) and hypothetical proteins.

**Table 4.** Significant affected genes in *S. thermophilus* growing on restricted CDM compared to complete CDM. Average p-value$< 0.05$ and FC$\geq$1.5 ($2^{1.5}$).

| Locus | Gene | product | FC | p-value |
|---|---|---|---|---|
| stu_t05 | | Leu tRNA | -2.3 | 0.0001 |
| stu_t16 | | Gly tRNA | -2.3 | 0.0000 |
| stu_t06 | | Thr tRNA | -2.2 | 0.0001 |
| stu_t14 | | Met tRNA | -2.1 | 0.0001 |
| stu_t02 | | Val tRNA | -2.0 | 0.0001 |
| stu_t08 | | Leu tRNA | -1.9 | 0.0005 |
| stu_t09 | | Arg tRNA | -1.9 | 0.0002 |
| stu1568 | *rheA* | ATP-dependent RNA helicase | -1.9 | 0.0000 |
| stu0585 | | hypothetical protein stu0585 | -1.8 | 0.0001 |
| stu_t15 | | Phe tRNA | -1.7 | 0.0002 |
| stu_t04 | | Lys tRNA | -1.7 | 0.0000 |
| stu0838 | *cspB* | cold shock protein B | -1.7 | 0.0001 |
| stu1996 | | Conserved hypothetical, predicted membrane protein (TMS3) | -1.6 | 0.0014 |
| stu0586 | | hypothetical protein stu0586 | -1.6 | 0.0001 |

| | | | | |
|---|---|---|---|---|
| stu1338 | | xanthine phosphoribosyltransferase | -1.5 | 0.0007 |
| stu0297 | | ABC transporter substrate binding protein | 1.5 | 0.0001 |
| stu1438 | *amiF1* | oligopeptide ABC uptake transporter ATP-binding protein | 1.5 | 0.0001 |
| stu1268 | *citB* | aconitate hydratase | 1.6 | 0.0004 |
| stu1965 | *amiE* | acetyltransferase, GNAT family | 1.6 | 0.0001 |
| stu1439 | | oligopeptide ABC uptake transporter ATP-binding protein | 1.6 | 0.0001 |
| stu0876 | | polar amino acid ABC uptake transporter ATP-binding protein | 1.6 | 0.0001 |
| stu0291 | | ABC transporter substrate binding protein | 1.7 | 0.0001 |
| stu0304 | | hypothetical protein stu0304 | 1.7 | 0.0001 |
| stu2018 | *trpS* | tryptophanyl-tRNA synthetase | 1.7 | 0.0001 |
| stu1964 | | hypothetical protein stu1964 | 1.7 | 0.0001 |
| stu0296 | | polar amino acid ABC uptake transporter substrate binding protein | 1.7 | 0.0001 |
| stu0987 | *cysD* | O-acetylhomoserine sulfhydrylase | 1.7 | 0.0002 |
| stu1386 | | hypothetical protein stu1386 | 1.7 | 0.0296 |
| stu0452 | | transcriptional regulator, LysR family | 1.8 | 0.0002 |
| stu1389 | | resistance to homoserine/threonine (RhtB) family protein | 1.8 | 0.0002 |
| stu1963 | *nrdD* | anaerobic ribonucleoside triphosphate reductase | 1.8 | 0.0000 |
| stu0353 | | aminotransferase (class II) | 1.8 | 0.0001 |
| stu1492 | | polar amino acid ABC uptake transporter substrate binding protein | 2.0 | 0.0000 |
| stu1838 | *hipO3* | aminoacylase/N-acyl-L-amino acid amidohydrolase/hippurate hydrolase | 2.0 | 0.0001 |
| stu0352 | *metB1* | cystathionine gamma-synthase | 2.2 | 0.0000 |
| stu0605 | | polar amino acid ABC uptake transporter membrane-spanning protein | 2.3 | 0.0000 |
| stu1388 | | hypothetical protein stu1388 | 2.4 | 0.0000 |
| stu1594 | | hypothetical protein stu1594 | 3.0 | 0.0000 |
| stu1493 | | hypothetical protein stu1493 | 3.6 | 0.0000 |
| stu1593 | *trpE* | anthranilate synthase component I | 3.6 | 0.0000 |
| stu1812 | *argH* | argininosuccinate lyase | 3.9 | 0.0000 |
| stu0786 | *metF* | 5,10-methylenetetrahydrofolate reductase | 4.0 | 0.0000 |
| stu0334 | | hypothetical protein stu0334 | 4.5 | 0.0000 |
| stu0603 | *argF* | ornithine carbamoyltransferase | 4.5 | 0.0001 |
| stu0785 | *metE* | 5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase | 4.6 | 0.0000 |
| stu1592 | *trpG* | anthranilate synthase component II | 4.9 | 0.0000 |
| stu1495 | | polar amino acid ABC uptake transporter substrate binding protein | 4.9 | 0.0000 |
| stu1494 | | polar amino acid ABC uptake transporter substrate binding protein | 4.9 | 0.0001 |
| stu1591 | *trpD* | anthranilate phosphoribosyltransferase | 5.0 | 0.0000 |
| stu1590 | *trpC* | indole-3-glycerol-phosphate synthase | 5.1 | 0.0000 |
| stu1589 | *trpF* | N-(5'-phosphoribosyl)anthranilate isomerase | 5.1 | 0.0000 |
| stu1587 | *trpA* | tryptophan synthase subunit alpha | 5.3 | 0.0000 |
| stu1813 | *argG* | argininosuccinate synthase | 5.4 | 0.0000 |
| stu1588 | *trpB* | tryptophan synthase subunit beta | 5.4 | 0.0000 |
| stu0467 | *argD* | acetylornithine aminotransferase | 5.5 | 0.0000 |

| | | | | |
|---|---|---|---|---|
| stu0464 | *argC* | N-acetyl-gamma-glutamyl-phosphate reductase | 5.7 | 0.0000 |
| stu0466 | *argB* | acetylglutamate kinase | 5.9 | 0.0000 |
| stu0465 | *argJ* | bifunctional ornithine acetyltransferase/N-acetylglutamate synthase protein | 6.0 | 0.0000 |
| stu0547 | | ABC transporter membrane-spanning protein | 6.0 | 0.0000 |
| stu0545 | | molybdopterin biosynthesis protein (HesA/MoeB/ThiF family protein), putative | 6.2 | 0.0000 |
| stu0546 | | ABC transporter ATP binding protein | 6.2 | 0.0000 |

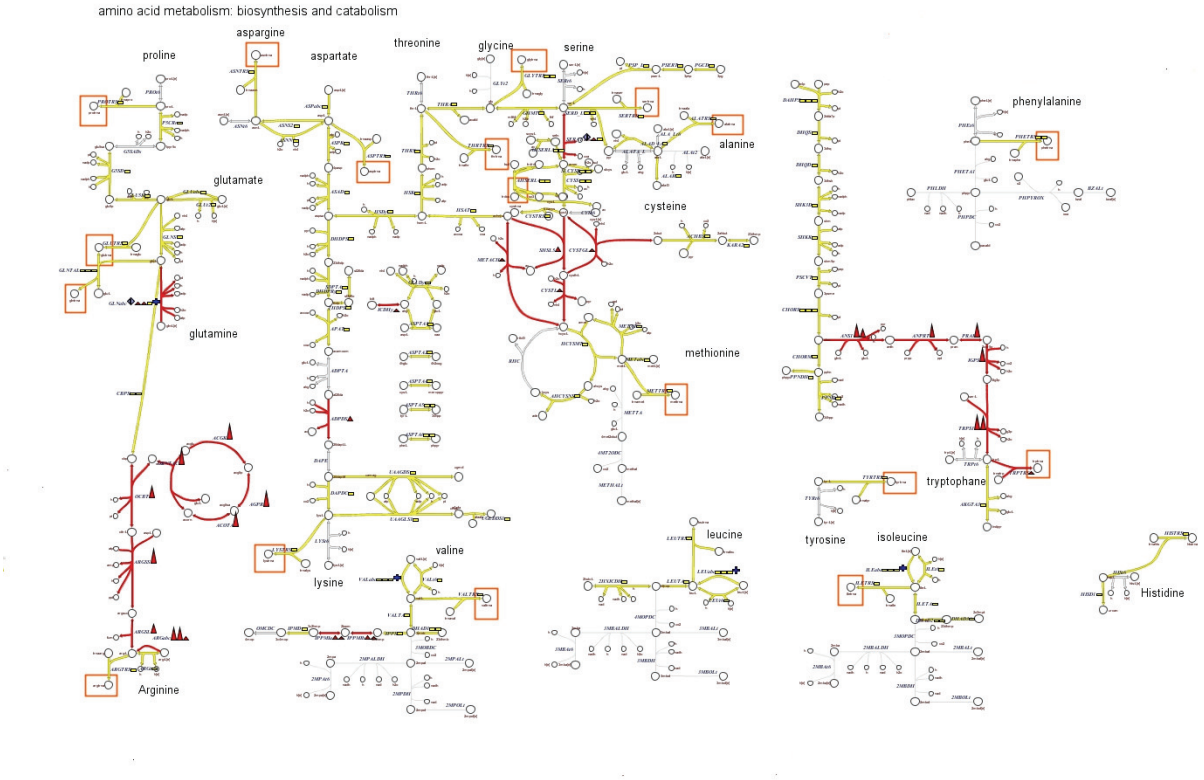


**Figure 1.** Visualization of amino acid biosynthesis in *S. thermophilus* obtained by projecting significantly affected genes (Average p-value< 0.05 and FC≥1.5 ($2^{1.5}$). Red signifies up-regulation (>$2^{1.5}$), green down-regulation (<$2^{1.5}$) and yellow signifies differently expressed by less than $2^{1.5}$ on restricted medium. Blank pathways indicate that those associated genes were not significantly regulated. All abbreviations are explained in the supplementary material of Teusink *et al* (37)

In comparison with *S. thermophilus*, three times more genes (186 versus 61) are differentially regulated in *L. lactis* (Figure 2 and Table 5, end of chapter), in spite of a similar size of their genomes (17, 38) . Some of the regulated genes (20) are predicted to be involved in the amino acid metabolism, like the up-regulated genes involved in arginine metabolism on the restricted medium compared to complete medium. On restricted medium, CodZ is up-regulated, this transcriptional regulator belongs to the CodY family. As mentioned in the introduction, CodY may regulate the transcription of genes involved in amino acid metabolism. Closer analysis of the transcriptome data showed a down-regulation of the transcriptional repressor CodY. This finding was not found with the set criteria and is therefore not included in table 5. Other affected genes on the restricted medium compared to complete medium are predicted to encode proteins involved in growth (e.g. down-regulation of purine and pyrimidine metabolism, up-regulation of transcriptional regulators), primary metabolism (e.g. down-regulation of pyruvate formate lyase) vitamin metabolism (up-regulation of riboflavin biosynthesis, down-regulation of tetrahydrofolate), polysaccharide metabolism (down-regulation of transport of sucrose and mannose) and hypothetical proteins.

**Table 5.** Significant affected genes in *L. lactis* growing on restricted CDM compared to complete CDM. Average p-value< 0.05 and FC≥1.5 ($2^{1.5}$).

| Locus | Gene | Product | FC | p- value |
|---|---|---|---|---|
| llmg_0974 | *purS* | phosphoribosylformylglycinamidine synthetase PurS | -9.4 | 0.0014 |
| llmg_0975 | *purQ* | phosphoribosylformylglycinamidine synthase I | -9.3 | 0.0033 |
| llmg_0976 | *purL* | phosphoribosylformylglycinamidine synthase II | -8.6 | 0.0019 |
| llmg_0977 | *purF* | amidophosphoribosyltransferase | -8.5 | 0.0067 |
| llmg_0973 | *purC* | phosphoribosylaminoimidazole-succinocarboxamide synthase | -8.4 | 0.0021 |
| llmg_0994 | *purH* | bifunctional phosphoribosylaminoimidazolecarboxamide formyltransferase/IMP cyclohydrolase | -7.6 | 0.0006 |
| llmg_0997 | *purD* | phosphoribosylamine--glycine ligase | -7.2 | 0.0022 |
| llmg | - | putative hydrolase | -6.7 | 0.0015 |
| llmg_0999 | *purE* | phosphoribosylaminoimidazole carboxylase catalytic subunit | -6.4 | 0.0007 |
| llmg_0313 | *phnC* | phosphonates import ATP-binding protein phnC | -6.3 | 0.0012 |
| llmg_1000 | *purK* | phosphoribosylaminoimidazole carboxylase ATPase subunit | -6.2 | 0.0005 |
| llmg_0312 | *phnD* | phosphonate ABC transporter, phosphonate-binding protein phnD | -6.0 | 0.0003 |
| llmg_0315 | - | Phosphonate ABC transporter permease protein | -5.4 | 0.0038 |
| llmg_0314 | *phnB* | phosphonate transport system permease protein phnB | -5.4 | 0.0008 |
| llmg_0988 | *purN* | phosphoribosylglycinamide formyltransferase | -5.3 | 0.0019 |

| | | | | |
|---|---|---|---|---|
| llmg_0987 | *purM* | phosphoribosylaminoimidazole synthetase | -5.1 | 0.0003 |
| llmg_0316 | *cpdC* | 2',3'-cyclic-nucleotide 2'-phosphodiesterase | -5.1 | 0.0010 |
| llmg | - | hypothetical protein | -4.7 | 0.0002 |
| llmg_0502 | - | ABC transporter permease protein | -4.1 | 0.0003 |
| llmg_0910 | *amtB* | ammonium transporter AmtB | -4.0 | 0.0001 |
| llmg_0501 | - | ABC transporter ATP-binding protein | -3.9 | 0.0003 |
| llmg | - | putative secreted protein | -3.8 | 0.0003 |
| llmg_0911 | *glnB* | nitrogen regulatory protein P-II | -3.7 | 0.0001 |
| llmg_0643 | *pacL* | cation-transporting ATPase, E1-E2 family | -3.6 | 0.0073 |
| llmg_2485 | *glnR* | glutamine synthetase repressor | -3.6 | 0.0015 |
| llmg_1913 | *pbuO* | Xanthine/uracil/vitamin C permease | -3.6 | 0.0002 |
| llmg | - | hypothetical protein | -3.5 | 0.0004 |
| llmg_0281 | - | anaerobic ribonucleoside triphosphate reductase | -3.3 | 0.0001 |
| llmg_2484 | *glnA* | GlnA protein | -3.1 | 0.0006 |
| llmg_1106 | *pyrDB* | PyrDB protein | -3.0 | 0.0026 |
| llmg_0893 | *pyrB* | aspartate carbamoyltransferase catalytic subunit | -3.0 | 0.0009 |
| llmg_0891 | *pyrP* | uracil permease (Uracil transporter) | -3.0 | 0.0006 |
| llmg_0335 | *plpA* | D-methionine-binding lipoprotein plpA precursor | -2.9 | 0.0004 |
| llmg_1107 | *pyrF* | orotidine 5'-phosphate decarboxylase | -2.8 | 0.0002 |
| llmg_0890 | *pyrR* | pyrimidine regulatory protein PyrR | -2.8 | 0.0008 |
| llmg_0563 | *glyA* | serine hydroxymethyltransferase | -2.8 | 0.0003 |
| llmg_1345 | *pbuX* | Xanthine/uracil permease | -2.8 | 0.0008 |
| llmg_0795 | *ps306* | hypothetical protein | -2.8 | 0.0101 |
| llmg_0336 | *plpB* | D-methionine-binding lipoprotein plpB precursor | -2.8 | 0.0015 |
| llmg_1346 | *xpt* | xanthine phosphoribosyltransferase | -2.8 | 0.0002 |
| llmg | - | hypothetical protein | -2.8 | 0.0341 |
| llmg | - | hypothetical protein | -2.7 | 0.0004 |
| llmg_0894 | *carA* | carbamoyl phosphate synthase small subunit | -2.7 | 0.0015 |
| llmg_0338 | *plpC* | D-methionine-binding lipoprotein plpC precursor | -2.7 | 0.0078 |
| llmg | - | hypothetical protein | -2.6 | 0.0001 |
| llmg_1105 | *pyrK* | dihydroorotate dehydrogenase, electron transfer subunit | -2.6 | 0.0001 |
| llmg_0282 | *nrdG* | anaerobic ribonucleoside-triphosphate reductase activating protein | -2.5 | 0.0002 |
| llmg | - | hypothetical protein | -2.5 | 0.0017 |
| llmg_1089 | *carB* | carbamoyl phosphate synthase large subunit | -2.4 | 0.0010 |
| llmg_1692 | *xseA* | exodeoxyribonuclease VII large subunit | -2.4 | 0.0002 |
| llmg_0796 | *ps307* | hypothetical protein | -2.4 | 0.0187 |
| llmg_0339 | *dar* | acetoin(diacetyl)reductase | -2.4 | 0.0006 |
| llmg_0629 | *pfl* | formate acetyltransferase | -2.3 | 0.0004 |
| llmg_1691 | *xseB* | exodeoxyribonuclease VII, small subunit | -2.2 | 0.0127 |
| llmg | - | hypothetical protein | -2.2 | 0.0003 |
| llmg_0630 | *coaE* | dephospho-CoA kinase | -2.2 | 0.0011 |
| llmg_1155 | - | spx-like protein | -2.2 | 0.0008 |

| | | | | |
|---|---|---|---|---|
| llmg | - | hypothetical protein | -2.1 | 0.0100 |
| llmg_0340 | *plpD* | D-methionine-binding lipoprotein plpD precursor | -2.1 | 0.0014 |
| llmg | - | hypothetical protein | -2.1 | 0.0056 |
| llmg_1541 | *nrdH* | Glutaredoxin-like protein nrdH | -2.1 | 0.0024 |
| llmg | - | hypothetical protein | -2.0 | 0.0016 |
| llmg_1115 | - | XpaC-like protein | -2.0 | 0.0004 |
| llmg | - | transcriptional regulator, LacI family | -2.0 | 0.0097 |
| llmg_0838 | *ps344* | hypothetical protein | -2.0 | 0.0271 |
| llmg_0995 | - | hydrolase, haloacid dehalogenase-like family protein | -2.0 | 0.0006 |
| llmg_2201 | *purA* | adenylosuccinate synthetase | -2.0 | 0.0013 |
| llmg_1693 | *folD* | methylenetetrahydrofolate dehydrogenase (NADP+) / methenyltetrahydrofolate cyclohydrolase | -2.0 | 0.0002 |
| llmg_1412 | *guaC* | guanosine 5'-monophosphate oxidoreductase | -2.0 | 0.0023 |
| llmg_0803 | *ps313* | putative transcription regulator | -2.0 | 0.0391 |
| llmg_1543 | *nrdE* | ribonucleotide-diphosphate reductase subunit alpha | -2.0 | 0.0031 |
| llmg_0839 | *ps345* | hypothetical protein | -1.9 | 0.0331 |
| llmg_2367 | *rpsH* | 30S ribosomal protein S8 | -1.9 | 0.0147 |
| llmg_2310 | *arcC1* | carbamate kinase | -1.9 | 0.0077 |
| llmg_0225 | *rfbX* | Putative O-antigen transporter | -1.8 | 0.0009 |
| llmg | - | putative secreted protein | -1.8 | 0.0132 |
| llmg_1542 | *nrdI* | ribonucleotide reductase stimulatory protein | -1.7 | 0.0022 |
| llmg | - | hypothetical protein | -1.7 | 0.0234 |
| llmg_0447 | *nifJ* | NifJ protein | -1.7 | 0.0013 |
| llmg_1508 | *pyrC* | dihydroorotase | -1.7 | 0.0021 |
| llmg_0341 | - | amino acid ABC transporter ATP binding protein | -1.6 | 0.0048 |
| llmg_2390 | *rpmG* | 50S ribosomal protein L33 | -1.6 | 0.0036 |
| llmg_0332 | - | putative biotin synthesis protein | -1.6 | 0.0014 |
| llmg_0127 | *ptsI* | phosphoenolpyruvate-protein phosphotransferase | -1.6 | 0.0047 |
| llmg_0607 | *apt* | adenine phosphoribosyltransferase | -1.6 | 0.0036 |
| llmg | - | hypothetical protein | -1.6 | 0.0044 |
| llmg_0222 | *wefC* | hypothetical protein | -1.6 | 0.0013 |
| llmg_2277 | *rplK* | 50S ribosomal protein L11 | -1.6 | 0.0033 |
| llmg_2366 | *rplF* | 50S ribosomal protein L6 | -1.6 | 0.0121 |
| llmg_0783 | *purB* | adenylosuccinate lyase | -1.5 | 0.0007 |
| llmg_0456 | *pgmB* | beta-phosphoglucomutase | 1.5 | 0.0053 |
| llmg | - | hypothetical protein | 1.5 | 0.0014 |
| llmg | - | hypothetical protein | 1.5 | 0.0004 |
| llmg_1331 | *thrB* | homoserine kinase | 1.5 | 0.0015 |
| llmg_1297 | *hisZ* | HisZ protein | 1.5 | 0.0010 |
| llmg | - | putative secreted protein | 1.5 | 0.0439 |
| llmg_1298 | *hisC* | histidinol-phosphate aminotransferase | 1.5 | 0.0006 |
| llmg | - | hypothetical protein | 1.6 | 0.0011 |
| llmg | - | putative secreted protein | 1.6 | 0.0008 |

| | | | | |
|---|---|---|---|---|
| llmg | - | hypothetical protein | 1.6 | 0.0009 |
| llmg_1662 | *uspA* | universal stress protein A | 1.6 | 0.0016 |
| llmg | - | hypothetical protein | 1.6 | 0.0107 |
| llmg_2143 | - | putative 20-kDa protein | 1.6 | 0.0002 |
| llmg | - | putative secreted protein | 1.6 | 0.0003 |
| llmg | - | putative secreted protein | 1.6 | 0.0003 |
| llmg | - | hypothetical protein | 1.6 | 0.0017 |
| llmg | - | hypothetical protein | 1.6 | 0.0005 |
| llmg | - | hypothetical protein | 1.6 | 0.0002 |
| llmg | - | putative transcriptional regulator | 1.6 | 0.0192 |
| llmg | - | hypothetical protein | 1.6 | 0.0006 |
| llmg_1594 | - | Gamma-glutamyl-diamino acid-endopeptidase | 1.6 | 0.0275 |
| llmg_0200 | *feoA* | ferrous iron transport protein A | 1.6 | 0.0003 |
| llmg | - | putative secreted protein | 1.6 | 0.0022 |
| llmg | - | putative secreted protein | 1.7 | 0.0497 |
| llmg_0160 | - | Oxygen-insensitive NAD(P)H nitroreductase | 1.7 | 0.0008 |
| llmg_1531 | *ribB* | riboflavin synthase subunit alpha | 1.7 | 0.0034 |
| llmg_1847 | *cspA* | cold shock-like protein cspA | 1.7 | 0.0164 |
| llmg_0080 | *osmC* | osmotically inducible protein C | 1.7 | 0.0201 |
| llmg_1801 | *srtC* | Sortase SrtC | 1.7 | 0.0069 |
| llmg | - | putative secreted protein | 1.8 | 0.0010 |
| llmg | - | hypothetical protein | 1.8 | 0.0004 |
| llmg_2025 | *oppC2* | oligopeptide transport system permease protein oppC2 | 1.8 | 0.0455 |
| llmg_1767 | *rdrA* | transcriptional regulator, DeoR family | 1.8 | 0.0105 |
| llmg | - | putative secreted protein | 1.8 | 0.0052 |
| llmg_0349 | *fhuD* | ferrichrome ABC transporter substrate binding protein | 1.8 | 0.0007 |
| llmg | - | putative methyltransferase | 1.8 | 0.0006 |
| llmg_1449 | *srtA* | Sortase SrtA | 1.8 | 0.0007 |
| llmg | - | Putative ABC transporter ATP-binding protein | 1.8 | 0.0004 |
| llmg_1449 | *thrA* | aspartate kinase | 1.8 | 0.0017 |
| llmg | - | hypothetical protein | 1.8 | 0.0076 |
| llmg_1983 | *codZ* | transcriptional regulator, CodY family | 1.9 | 0.0041 |
| llmg | - | hypothetical protein | 1.9 | 0.0007 |
| llmg_1490 | *mntH* | putative proton-dependent manganese transporter group C beta | 1.9 | 0.0015 |
| llmg_1725 | *trmU* | tRNA (5-methylaminomethyl-2-thiouridylate)-methyltransferase | 1.9 | 0.0003 |
| llmg_0346 | *fhuC* | ferrichrome ABC transporter fhuC | 1.9 | 0.0015 |
| llmg | - | putative transcriptional regulator | 1.9 | 0.0013 |
| llmg | - | hypothetical protein | 1.9 | 0.0252 |
| llmg_1552 | - | putative ABC type transport system permease protein | 1.9 | 0.0007 |
| llmg | - | hypothetical protein | 2.0 | 0.0003 |
| llmg_0874 | *dapA* | dihydrodipicolinate synthase | 2.0 | 0.0002 |
| llmg | - | hypothetical protein | 2.0 | 0.0005 |

| | | | | |
|---|---|---|---|---|
| llmg | - | hypothetical protein | 2.0 | 0.0045 |
| llmg | - | hypothetical protein | 2.0 | 0.0018 |
| llmg | - | transcriptional regulator | 2.0 | 0.0388 |
| llmg_1724 | *rpsA* | 30S ribosomal protein S1 | 2.1 | 0.0015 |
| llmg_2477 | - | lysine specific permease | 2.1 | 0.0005 |
| llmg_1570 | *fruR* | transcriptional regulator of the fructose operon | 2.1 | 0.0053 |
| llmg | - | putative acetyltransferase | 2.1 | 0.0024 |
| llmg | - | hypothetical protein | 2.2 | 0.0341 |
| llmg_2026 | *oppB2* | peptide transport system permease protein oppB2 | 2.2 | 0.0006 |
| llmg_1530 | *ribA* | riboflavin biosynthesis protein ribA | 2.2 | 0.0062 |
| llmg_1661 | *hslB* | HU-like DNA-binding protein | 2.3 | 0.0011 |
| llmg_1514 | *rex* | redox-sensing transcriptional repressor Rex | 2.3 | 0.0008 |
| llmg | - | hypothetical protein | 2.4 | 0.0005 |
| llmg | - | hypothetical protein | 2.5 | 0.0016 |
| llmg_1850 | *qor* | quinone oxidoreductase | 2.5 | 0.0003 |
| llmg_1979 | *gltP* | glutamate ABC transporter permease protein | 2.5 | 0.0002 |
| llmg | - | cell wall surface anchor family protein | 2.5 | 0.0002 |
| llmg_1031 | *trpG* | anthranilate synthase component II | 2.6 | 0.0015 |
| llmg_1019 | - | oxidoreductase, short-chain dehydrogenase/reductase family protein | 2.6 | 0.0001 |
| llmg_2011 | - | putative amino acid permease | 2.7 | 0.0009 |
| llmg_1978 | *gltQ* | glutamate ABC transporter ATP-binding protein | 2.7 | 0.0008 |
| llmg_2024 | *oppA2* | Oligopeptide-binding protein oppA2 precursor | 2.9 | 0.0008 |
| llmg | - | hypothetical protein | 2.9 | 0.0002 |
| llmg | - | Putative Transcriptional regulator | 3.0 | 0.0001 |
| llmg_1045 | *bglP* | similar to PTS system, beta-glucosides specific enzyme IIABC | 3.0 | 0.0467 |
| llmg_0530 | *gapA* | glyceraldehyde 3-phosphate dehydrogenase | 3.1 | 0.0201 |
| llmg | - | cell wall surface anchor family protein | 3.3 | 0.0064 |
| llmg_1032 | *trpD* | anthranilate phosphoribosyltransferase | 3.4 | 0.0006 |
| llmg_1037 | - | putative lyase | 4.2 | 0.0004 |
| llmg | - | hypothetical protein | 4.2 | 0.0005 |
| llmg | - | hypothetical protein | 4.7 | 0.0002 |
| llmg_1038 | *trpC* | indole-3-glycerol-phosphate synthase | 4.7 | 0.0003 |
| llmg | - | acetyltransferase, GNAT family | 4.9 | 0.0003 |
| llmg_0138 | *argG* | ArgG protein | 4.9 | 0.0001 |
| llmg_1039 | *trpF* | N-(5'-phosphoribosyl)-anthranilate isomerase | 5.1 | 0.0001 |
| llmg_0139 | *argH* | ArgH protein | 5.5 | 0.0001 |
| llmg_0535 | *gltS* | Arginine-binding periplasmic protein 1 precursor | 5.6 | 0.0001 |
| llmg_1041 | *trpB* | tryptophan synthase subunit beta | 5.6 | 0.0007 |
| llmg_0536 | *argE* | acetylornithine deacetylase | 5.9 | 0.0001 |
| llmg_1042 | *trpA* | tryptophan synthase subunit alpha | 6.8 | 0.0001 |
| llmg_1755 | *argB* | ArgB protein | 8.3 | 0.0002 |
| llmg_1754 | *argF* | ArgF protein | 8.8 | 0.0001 |

| | | | | |
|---|---|---|---|---|
| llmg_1757 | *argJ* | bifunctional ornithine acetyltransferase/N-acetylglutamate synthase protein | 9.0 | 0.0003 |
| llmg_1756 | *argD* | acetylornithine aminotransferase | 9.2 | 0.0002 |
| llmg_1758 | *argC* | N-acetyl-gamma-glutamyl-phosphate reductase | 9.4 | 0.0002 |

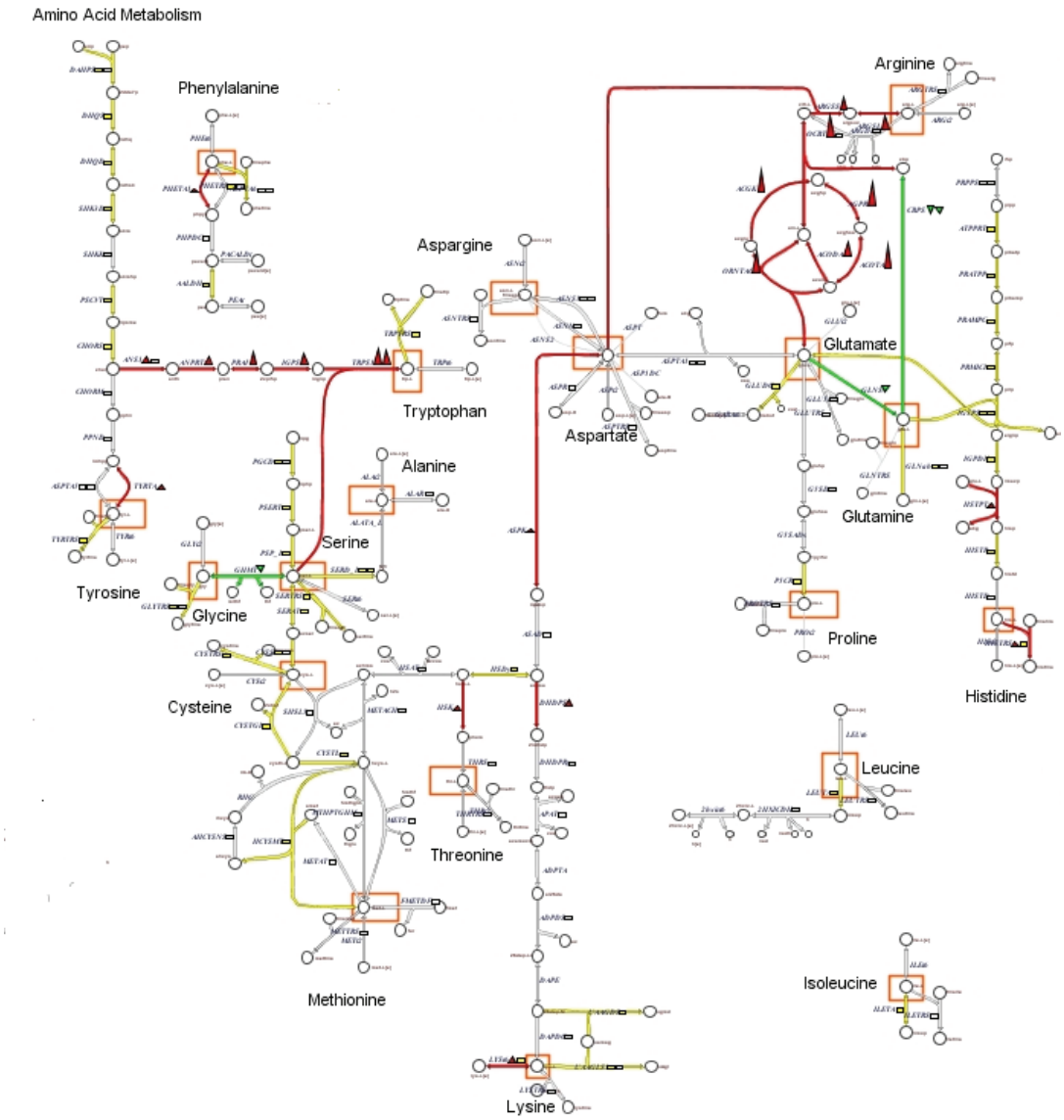


**Figure 2.** Visualization of amino acid biosynthesis in *L. lactis* obtained by projecting significantly affected genes (Average p-value< 0.05 and FC≥1.5 ($2^{1.5}$). Red signifies up-regulation (>$2^{1.5}$), green down-regulation (<$2^{1.5}$) and yellow signifies differently expressed by less than $2^{1.5}$ on restricted medium. Blank pathways indicate that those associated genes were not significantly regulated. All abbreviations are explained in the supplementary material of Teusink *et al* (37).

The transcriptome analysis of *Lb. plantarum* shows that 81 genes are differentially regulated (restricted medium compared to complete medium) of which 10 are predicted to be involved in amino acid metabolism (Figure 3 and Table 6). Some genes involved in the amino acid metabolism are both up-regulated, like those involved in the histidine biosynthesis pathway, and some are down-regulated, like those encoding the glutamate dehydrogenase and different amino acid transporters. Other affected genes are predicted to encode proteins involved, primary metabolism (e.g. up-regulation of pyruvate oxidase and NADH oxidase), vitamin metabolism (down-regulation of tetrahydrofolate metabolism), stress related genes (up-regulation of transport of betaine, glycine and carnitine, prophage) and hypothetical proteins. NADH oxidase and pyruvate oxidase may have a role in maintaining the redox balance (via NADH). In contrast to what was found for *S. thermophilus*, the sulfur amino acids (acetyl homoserine lyase, 5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase, 5,10-methylenetetrahydrofolate reductase) are not differentially regulated in either *L. lactis* or *Lb. plantarum.*

**Table 6.** Significant affected genes in *Lb. plantarum* growing on restricted CDM compared to complete CDM. Average p-value< 0.05 and FC≥1.5 ($2^{1.5}$).

| Locus | Gene | Product | FC | P-value |
|---|---|---|---|---|
| lp_2721 | *purN* | phosphoribosylglycinamide formyltransferase | -3.7 | 0.0006 |
| lp_2723 | *purF* | amidophosphoribosyltransferase precursor | -3.4 | 0.0007 |
| lp_2720 | *purH* | bifunctional protein: phosphoribosylaminoimidazolecarboxamide formyltransferase; IMP cyclohydrolase | -3.4 | 0.0003 |
| lp_2774 | | ABC transporter, ATP-binding protein | -3.0 | 0.0013 |
| lp_2773 | | ABC transporter, permease protein | -2.8 | 0.0010 |
| lp_1118 | *mleS* | malolactic enzyme | -2.2 | 0.0049 |
| lp_0226 | *gnp* | glucosamine-6-phosphate isomerase | -2.2 | 0.0012 |
| lp_3015 | | extracellular protein | -2.2 | 0.0022 |
| lp_1169 | *gdh* | glutamate dehydrogenase (NAD(P)+) | -2.2 | 0.0010 |
| lp_0872 | *gph1* | phosphoglycolate phosphatase (putative) | -2.1 | 0.0028 |
| lp_0928 | | unknown | -2.0 | 0.0003 |
| lp_0513 | | unknown | -2.0 | 0.0005 |
| lp_2375 | *glyA* | glycine hydroxymethyltransferase | -2.0 | 0.0010 |
| lp_0929 | *asp1* | alkaline shock protein | -1.9 | 0.0003 |
| lp_1119 | *mleP2* | malate transport protein | -1.9 | 0.0118 |
| lp_0926 | | integral membrane protein | -1.9 | 0.0007 |
| lp_2475 | | prophage P2b protein 6 | -1.9 | 0.0433 |
| lp_2696 | | integral membrane protein | -1.8 | 0.0019 |

| | | | | |
|---|---|---|---|---|
| lp_3128 | | stress induced DNA binding protein | -1.8 | 0.0005 |
| lp_3548 | *pts35A* | galacitol PTS, EIIA | -1.8 | 0.0013 |
| lp_0242 | *ndk* | nucleoside-diphosphate kinase | -1.7 | 0.0169 |
| lp_0349 | *amtB* | ammonium transport protein | -1.7 | 0.0088 |
| lp_0172 | | transcription regulator | -1.6 | 0.0188 |
| lp_2374 | *upp* | uracil phosphoribosyltransferase | -1.6 | 0.0039 |
| lp_2766 | | unknown | -1.6 | 0.0007 |
| lp_1261 | *oppA* | oligopeptide ABC transporter, substrate binding protein | -1.6 | 0.0008 |
| lp_0737 | | ribosomal protein S30EA | -1.6 | 0.0008 |
| lp_3396 | | unknown | -1.5 | 0.0390 |
| lp_0154 | | unknown | -1.5 | 0.0152 |
| lp_3334 | *adeC* | adenine deaminase | 1.5 | 0.0134 |
| lp_3466 | *brnQ3* | branched-chain amino acid transport protein | 1.5 | 0.0009 |
| lp_3338 | *nha2* | Na(+)/H(+) antiporter | 1.5 | 0.0044 |
| lp_3682 | *thdF* | thiophene and furan oxidation protein ThdF | 1.5 | 0.0005 |
| lp_2216 | *rpsN2* | ribosomal protein S14-2 | 1.5 | 0.0008 |
| lp_1078 | *rpsI* | ribosomal protein S9 | 1.6 | 0.0027 |
| lp_2240 | | amino acid transport protein | 1.6 | 0.0003 |
| lp_2893 | | ABC transporter, ATP-binding and permease protein | 1.6 | 0.0007 |
| lp_3337 | | unknown | 1.6 | 0.0010 |
| lp_0280 | | transport protein | 1.6 | 0.0330 |
| lp_0311 | | acetyltransferase (putative) | 1.6 | 0.0010 |
| lp_2039 | *rbfA* | ribosome-binding factor A | 1.6 | 0.0006 |
| lp_2952 | | unknown | 1.6 | 0.0007 |
| lp_0830 | | transport protein, N-terminal fragment | 1.6 | 0.0003 |
| lp_2789 | | transport protein | 1.6 | 0.0004 |
| lp_0132 | | transport protein | 1.6 | 0.0025 |
| lp_3413 | | cell surface protein precursor | 1.7 | 0.0028 |
| lp_3512 | *pbg8* | 6-phospho-beta-glucosidase | 1.7 | 0.0167 |
| lp_3100 | | oxidoreductase | 1.7 | 0.0013 |
| lp_3279 | *kup2* | potassium uptake protein | 1.7 | 0.0005 |
| lp_3324 | | glycine betaine/carnitine/choline transport protein | 1.8 | 0.0013 |
| lp_2550 | *maa1* | maltose O-acetyltransferase | 1.8 | 0.0011 |
| lp_1637 | | unknown | 1.8 | 0.0002 |
| lp_0783 | | ABC transporter, substrate binding protein | 1.8 | 0.0010 |
| lp_2953 | | esterase (putative) | 1.8 | 0.0004 |
| lp_0831 | | transport protein, C-terminal fragment | 1.9 | 0.0025 |
| lp_3681 | *gidA* | cell division protein GidA | 1.9 | 0.0070 |
| lp_0984 | | unknown | 1.9 | 0.0005 |
| lp_2629 | *pox3* | pyruvate oxidase | 2.0 | 0.0103 |
| lp_3414 | | extracellular protein | 2.0 | 0.0002 |
| lp_1721 | | 4-aminobutyrate aminotransferase | 2.1 | 0.0003 |

| | | | | |
|---|---|---|---|---|
| lp_3666 | | 2-oxo-hept-3-ene-1,7-dioate hydratase; 2-oxo-hept-4-ene-1,7-dioate hydratase | 2.1 | 0.0003 |
| lp_2788 | *panE2* | 2-dehydropantoate 2-reductase | 2.1 | 0.0001 |
| lp_1722 | | amino acid transport protein | 2.1 | 0.0001 |
| lp_0082 | | oxidoreductase | 2.2 | 0.0005 |
| lp_3449 | *nox5* | NADH oxidase | 2.3 | 0.0004 |
| lp_2768 | | transport protein | 2.3 | 0.0019 |
| lp_0200 | | ABC transporter, substrate binding protein | 2.4 | 0.0033 |
| lp_2160 | | unknown | 2.6 | 0.0016 |
| lp_p1_02 | | | 2.6 | 0.0022 |
| lp_0201 | | ABC transporter, substrate binding proteins | 2.8 | 0.0001 |
| lp_1521 | | oxidoreductase | 2.9 | 0.0021 |
| lp_0350 | *hicD1* | L-2-hydroxyisocaproate dehydrogenase | 3.0 | 0.0035 |
| lp_1008 | *lysP* | lysine transport protein | 4.3 | 0.0000 |
| lp_2557 | *hisH* | imidazole glycerol phosphate synthase, amidotransferase sununit | 5.6 | 0.0000 |
| lp_2558 | *hisB* | bifunctional protein: histidinol-phosphatase; imidazoleglycerol-phosphate dehydratase | 5.6 | 0.0001 |
| lp_2559 | *hisD* | histidinol dehydrogenase | 6.0 | 0.0000 |
| lp_2313 | *glnQ4* | glutamine ABC transporter, ATP-binding protein | 6.5 | 0.0000 |
| lp_2312 | *glnH2* | glutamine ABC transporter, substrate binding protein | 6.5 | 0.0000 |
| lp_2314 | *glnP2* | glutamine ABC transporter, permease protein | 6.6 | 0.0000 |
| lp_2560 | *hisG* | ATP phosphoribosyltransferase | 6.7 | 0.0000 |
| lp_2561 | *hisX* | histidine--tRNA ligase (putative) | 7.1 | 0.0001 |

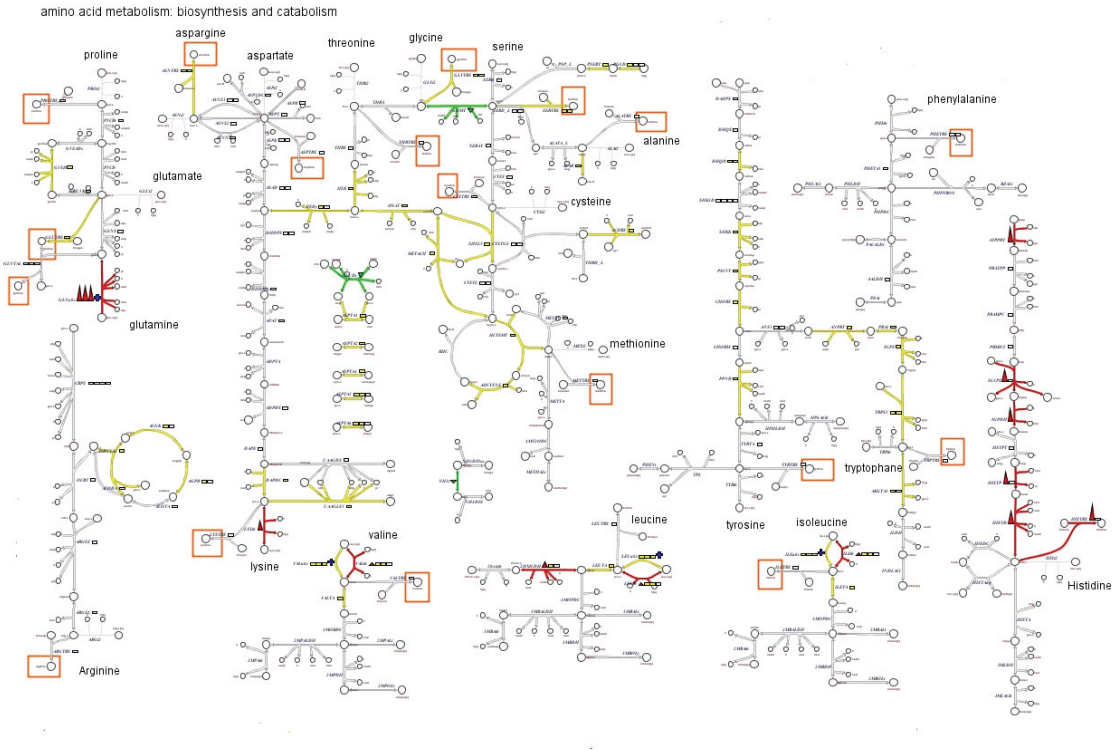


**Figure 3.** Visualization of amino acid biosynthesis in *Lb. plantarum* obtained by projecting significantly affected genes (Average p-value< 0.05 and FC≥1.5 ($2^{1.5}$). Red signifies up-regulation (>$2^{1.5}$), green down-regulation (<$2^{1.5}$) and yellow signifies differently expressed by less than $2^{1.5}$ on restricted medium. Blank pathways indicate that those associated genes were not significantly regulated. All abbreviations are explained in the supplementary material of Teusink *et al* (37).

The transcriptome analysis showed that in all three LAB amino acid metabolism is affected during growth on restricted medium. However, in *S. thermophilus* around 50% of the significantly affected genes is involved in amino acid metabolism, whereas the other two bacteria show a more general response towards amino acid depletion. *S. thermophilus* has a more extensive amino acid metabolism than the other two LAB. The complex amino acid metabolism in *S. thermophilus* faces a simple, non flexible primary metabolism. Therefore it seems logical that the amino acid metabolism, in *S. thermophilus,* is more affected by a different growth environment than the primary metabolism. The metabolism of tetrahydrofolate is also affected in all three LAB, the formation of tetrahydrofolate can play a role in producing precursors for the purine metabolism (12).

**Metabolic analysis.** Supernatant of samples taken at different points during exponential growth of all three LAB (growth characteristics in Table 2) were analyzed by HPLC. Glucose and fermentation products such as lactate, acetate, formate, ethanol, pyruvate, acetoin and succinate were analyzed (Table 7).

**Table 7.** HPLC analysis of supernatant of fermentation samples of *S. thermophilus, L. lactis* and *Lb. plantarum* grown under batch conditions on complete and restricted defined medium. Samples were taken at the end of exponential phase.

| Metabolite mM[a] | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | Citrate | Ethanol | Pyruvate | Lactic acid | Formic acid | Acetic acid | Acetoin | Succinate | Glucose |
| *S. thermophilus* | Blanc | 2.28 | n.d. | n.d. | n.d. | n.d. | 13.73 | n.d. | n.d. | 24.64 |
| complete CDM | Sample | 2.16 | n.d. | n.d. | 31.50 | 1.67 | 14.46 | 0.57 | 0.17 | 6.33 |
| *S. thermophilus* | Blanc | 2.45 | n.d. | n.d. | n.d. | n.d. | 13.01 | n.d. | n.d. | 25.53 |
| restricted CDM | Sample | 1.76 | n.d. | 0.28 | 32.39 | 0.95 | 11.13 | 0.47 | 0.24 | n.d. |
| *L. lactis* | Blanc | 2.18 | n.d. | n.d. | n.d. | n.d. | 13.37 | n.d. | n.d. | 28.66 |
| complete CDM | Sample | 1.69 | 0.30 | 0.15 | 34.75 | 1.43 | 10.81 | n.d. | 0.50 | n.d. |
| *L. lactis* | Blanc | 2.33 | n.d. | n.d. | n.d. | n.d. | 12.93 | n.d. | 0.07 | 25.63 |
| restricted CDM | Sample | 2.14 | n.d. | n.d. | 32.62 | 0.42 | 13.22 | n.d. | 0.78 | 0.90 |
| *Lb. plantarum* | Blanc | 2.28 | n.d. | n.d. | n.d. | n.d. | 12.94 | n.d. | n.d. | 19.83 |
| complete CDM | Sample | 1.39 | n.d. | 0.82 | 35.97 | 0.82 | 10.16 | n.d. | 0.25 | n.d. |
| *Lb. plantarum* | Blanc | 1.85 | n.d. | n.d. | n.d. | n.d. | 12.91 | n.d. | n.d. | 21.82 |
| restricted CDM | Sample | 1.51 | n.d. | 0.31 | 34.11 | n.d. | 9.12 | n.d. | 0.06 | n.d. |

[a] Average of 4 duplicates, N.D. not detected.

Under both complete and restricted conditions, homolactic growth is observed for *S. thermophilus* and *Lb. plantarum*. *L. lactis* also grows using homolactic fermentation, but on complete medium, more formate is produced than on the restricted medium and this corresponds with the down-regulation of pyruvate formate lyase on restricted medium. GC-MS measurements also show the accompanying formation of ethanol and acetaldehyde.

The headspace of the different fermentation samples was analyzed using GC-MS. Samples were taken at mid-exponential phase and at stationary phase, the identified volatiles (of which some of them are also flavors) are summarized (Figures 4 -5).

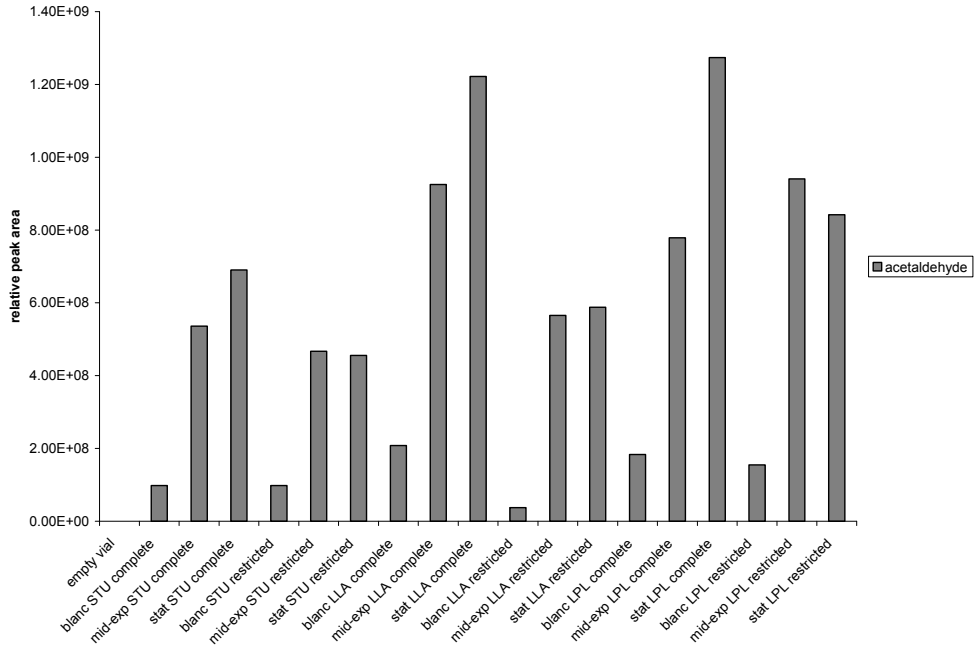


**Figure 4.** Identified acetaldehyde profiles in the different fermentation samples. STU means *S. thermophilus*, LLA means *L. lactis* and LPL means *Lb. plantarum.*

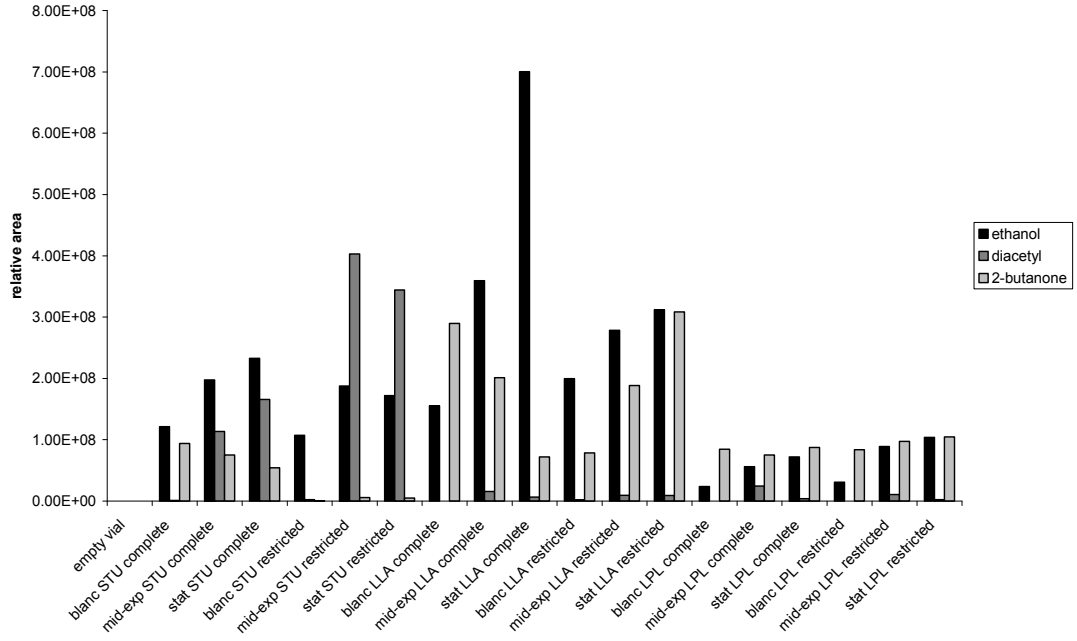


**Figure 5.** Identified volatiles (ethanol, diacetyl, 2-butanone) in the different fermentation samples. STU means *S. thermophilus*, LLA means *L. lactis* and LPL means *Lb. plantarum.*

Acetaldehyde was identified in all samples; all three LAB can produce this flavor compound on both restricted and complete CDM. However, the bacteria produce more acetaldehyde on complete medium than on restricted medium. It was described that *S. thermophilus* can produce acetaldehyde during threonine catabolism (9), the large decrease of the threonine concentration during growth, as measured by HPLC, indeed points to utilization of threonine for other purposes than protein biosynthesis. The expression of the gene encoding threonine aldolase does not differ on restricted and complete CDM, indicating that the gene is constitutively expressed in *S. thermophilus*. *L. lactis* and *Lb. plantarum* probably produce acetaldehyde from pyruvate in primary metabolism; ethanol dehydrogenase can convert acetaldehyde into ethanol.

When *L. lactis* grows on complete medium, large amounts of ethanol are produced. Ethanol is probably produced during primary metabolism, corresponding with the formation of formate and increased production of acetaldehyde under these conditions. Butanone is produced by *L. lactis* growing on restricted medium.

Diacetyl is identified in all samples. Diacetyl is produced by primary metabolism when pyruvate is converted to diacetyl via acetolactate. Especially *S. thermophilus* produces large amounts of diacetyl on the restricted medium. Closer analysis of the transcriptome data showed an up-regulation of acetolactate synthase. This finding was not found with the set criteria and is therefore not included in table 4. But the up-regulation of acetolactate synthase is certainly interesting and may explain the increased diacetyl formation on restricted medium. Acetolactate synthase also plays a role in the synthesis of the branched chain amino acids via feedback inhibition.

**Discussion**

In this study we demonstrated the effect of a restriction of amino acids in the growth medium of different LAB. This amino acid depletion not only influences the growth behavior of these organisms but also the overall cellular response and flavor formation. To study this effect of amino acids in the medium, we compared growth of *S. thermophilus, L. lactis* and *Lb. plantarum* on medium with a surplus of amino acids versus growth on medium with a minimal amount of amino acids. This comparative study provides new insights in amino acid metabolism of different organisms, such as the different strategies to cope with a depletion of amino acids.

Microarrays were used to study the cellular response towards a minimal amount of amino acids. A total of 61 significantly regulated genes were found for *S. thermophilus*. Especially the metabolism of the sulfur-containing amino acids was strongly regulated; this might have important effects on the flavor formation. A striking observation is that the production of 9 tRNA's are down-regulated on restricted medium. It has been described for *E. coli* that the concentration of tRNA's decreases in mid-exponential phase, then stays at a constant level, and decreases as the cell reaches stationary phase. The stagnation of synthesis of tRNA supports theories about tRNA being the rate-limiting factor in the translation (1). Cells growing on the restricted medium have a much longer mid-exponential phase and this can influence the level of tRNA. Furthermore, experimental data showed that the amino acids that these tRNA's transfer (Leu, Gly, Thr, Met, Val, Leu, Arg, Phe, Lys) are not necessary for growth, as was described in our previous paper (29). Also some other groups of genes were affected by the depletion of amino acids, such as those involved in vitamin metabolism. HPLC analysis showed that *S. thermophilus* performs homolactic fermentation on both restricted and complete media. This was observed and described before (17), and is expected because *S. thermophilus* has a simple primary metabolism with no other options than to form lactate.

*S. thermophilus* is able to produce a variety of flavors (29). However, these are mainly produced on complete medium. Most flavors originate from amino acid metabolism (39). Since only three amino acids are present in the restricted medium, less amino acid degradation pathways are active under these conditions. The microarray data indicated a strong regulation of the sulfur amino acids biosynthesis pathways (acetyl homoserine lyase, 5-

methyltetrahydropteroyltriglutamate--homocysteine methyltransferase, 5,10-methylenetetrahydrofolate reductase). The HPLC data suggest that a large amount of the present cysteine (methionine is not present in the restricted medium) is consumed by *S. thermophilus*.

The global transcriptional analysis of *L. lactis* showed that 186 genes were significantly regulated in the restricted medium compared to the complete medium. For *L. lactis*, the response to amino acid depletion is more general and also affects other parts of the metabolism like primary metabolism, purine and pyrimidine pathways and transcriptional factors. Metabolite analysis showed that on restricted medium as well as on complete medium all amino acids present are consumed, although the consumption is higher on complete medium. None of the amino acids is completely consumed. The HPLC measurements showed that *L. lactis* performs homolactic fermentation, but on complete medium *L. lactis* also forms formate, ethanol and acetaldehyde besides lactate. Alanine, (synthesized in one step from pyruvate) allosterically inhibits pyruvate kinase, to signal that building blocks are abundant (5). The restricted medium of *L. lactis* does not contain alanine. Strikingly is the production of 2-butanone by *L. lactis* growing on the restricted medium. Pathways leading to butanone are common among LAB (19), but these pathways are only operated if there is a surplus in the cell relative to the need for $NAD^+$ regeneration (4). Both *L. lactis* and *S. thermophilus* show an up-regulation of the arginine operon during growth on restricted medium. Arginine metabolism plays an important role in LAB, not only for supply of the necessary amount of arginine, but also in energy regeneration and pH regulation (2, 3, 8, 26). In addition, arginine may stimulate growth and may give protection against stress. The restricted media of *L. lactis* and *S. thermophilus* do not contain arginine, whereas the restricted medium of *Lb. plantarum* still contains arginine, so regulation of the arginine genes cannot be expected in this bacterium.

The microarray studies of *Lb. plantarum* showed 81 differentially regulated genes. Most of the affected genes are not involved in amino acid metabolism but in primary metabolism, stress related genes or related to growth. HPLC analyses showed that on complete medium as well as on restricted medium, *Lb. plantarum* performs homolactic fermentation. Both *L. lactis* and *Lb. plantarum* can usually perform homolactic fermentation at high dilution rates and mixed acid fermentation at low dilution rates (14, 37). The

homolactic route is catalytically more efficient and seems to be preferred under these conditions above the energetically more efficient mixed acid fermentation. Of the three LAB studied in this project, *Lb. plantarum* has the highest degree of amino acid auxotrophy, needing at least 11 amino acids. On both complete and restricted medium, all amino acids present are consumed, and these consumptions are similar. Cysteine is also completely consumed during growth on the restricted medium.

When the response of the three LAB towards the depletion of amino acids was compared, we observed that not only the amino acid metabolism is affected but also other parts of the metabolism, growth and in some cases even stress related genes. Obviously, the three LAB have different strategies to cope with a depletion of amino acids. *S. thermophilus* has a simple primary metabolism and a complex amino acid metabolism. A large part of the affected genes is involved in amino acid metabolism, in contrast to what was found for the other two LAB. The primary metabolism is not very flexible due to the presence of pseudogenes and the amino acid metabolism is quite complete and flexible. The other two LAB, *L. lactis* and *Lb. plantarum* have a more flexible primary metabolism in comparison to *S. thermophilus*. They show a more general response than *S. thermophilus*, since not only amino acid metabolism, but also other parts of the metabolism are affected by the depletion of amino acids. *Lb. plantarum* and especially *L. lactis,* show a down-regulation of the purine and pyrimidine metabolism. This response is growth-related and was not found for *S. thermophilus*. Apparently, when *S. thermophilus* grows at a lower growth rate, it responds in another way by f.i. downregulation of tRNA-genes. On restricted medium, less volatile compounds (some volatiles are flavors) are formed than on the complete medium by all three LAB because most flavors are generated during amino acid metabolism. Of the three studied LAB, *L. lactis* is probably most adapted to the protein-rich milk environment and this bacterium also shows the largest response (relatively most differentially regulated genes) towards amino acid depletion.

Overall these data show that not only amino acid metabolism is affected by a depletion (or surplus) of amino acids, but also overall metabolism is affected. This study also showed that different LAB have different strategies to cope with a depletion of amino acids. Even though these organisms are closely related, they have their own unique requirements and their own response.

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**Supplementary materials**

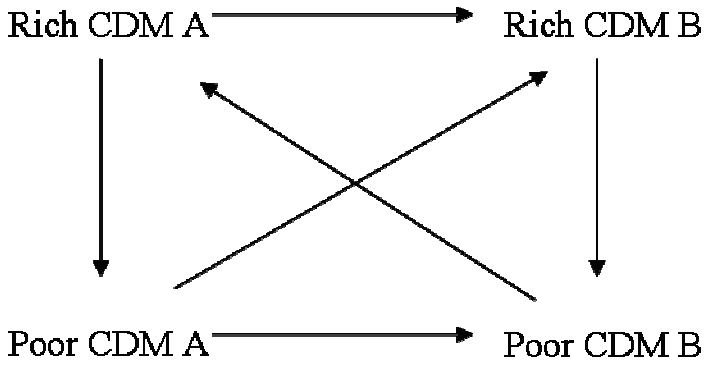


**Figure S1.** Schematic design of the hybridization scheme that was used for the micro array experiments of S. *thermophilus* in this study. Each arrow represents one hybridization; the arrows point from the samples labeled with cy3 to samples labeled with cy5. A and B refer to samples from duplicate fermentations.

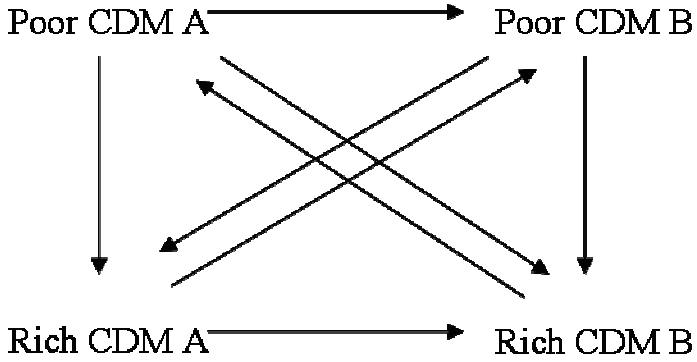


**Figure S2.** Schematic design of the hybridization scheme that was used for the micro array experiments of *L. lactis* and *Lb. plantarum*.in this study. Each arrow represents one hybridization; the arrows point from the samples labeled with cy3 to samples labeled with cy5. A and B refer to samples from duplicate fermentations.

# Chapter 5

## The role of glutamate dehydrogenase in *Streptococcus thermophilus*

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**Abstract**

The lactic acid bacterium *Streptococcus thermophilus* does not have a complete pentose phosphate pathway. Since this pathway is generally important in the generation of NADPH, this bacterium will need to have alternative pathway(s) for the generation of this important reduced co-factor. Comparative genomics showed that most streptococci do not possess a complete pentose phosphate pathway. In this report we describe the search for alternative NADPH producing pathways. One of the alternatives can be glutamate dehydrogenase (encoded by the *gdhA* gene), and a knock-out of the corresponding gene was made. Gene transcription and fermentation behavior of this mutant were compared with the wild-type. The *gdhA* knock-out displayed production of acetaldehyde, consumption of citrate and increased consumption of amino acids. Microarray data showed an up-regulation of the citrate pathway, some amino acid biosynthesis pathways, and the purine metabolism for the *gdhA* knock-out compared to the wild-type. This clear effect was not found for the control organism *Lactobacillus plantarum.*

## Introduction

*Streptococcus thermophilus* is one of the most important Lactic Acid Bacteria (LAB) for the food industry. It is used for the production of Italian and Swiss cheeses, using elevated cookingtemperatures and in co-cultivation with *Lactobacillus delbrueckii* subsp. *bulgaricus* for the production of yoghurt (11).

As was described in our previous paper, *S. thermophilus* LMG18311 (6, 11) has a relatively simple primary metabolism. The number of pyruvate branches is limited; therefore the only possible route under anaerobic conditions is the homolactic pathway. Another striking feature of this strain is the low degree of amino acid auxotrophies; it only needs histidine and cysteine in the presence of citrate or glutamate for (minimal) growth. Furthermore, *S. thermophilus* is able to produce a varied amount of flavors. These findings indicate the presence of most amino acid biosynthesis and converting pathways (19). However, *Streptococcus (S.) thermophilus* LMG18311 (6, 11) is predicted, based on the genome, not to have a complete pentose phosphate pathway. The pentose phosphate pathway meets the need of all organisms for a source of NADPH to use in reductive biosynthesis. Most LAB possess a complete pentose phosphate pathway. Since all living organisms need NADPH, *S. thermophilus* needs alternative pathways to synthesize NADPH.

The goal of this paper is to find out which pathways are used by *S. thermophilus* for NADPH generation. The available genome-scale model of *S. thermophilus* was used (19) and the model initially predicts glutamate dehydrogenase as most likely enzyme. We constructed a knock-out of the *gdhA* gene (encoding for glutamate dehydrogenase) and analyzed the mutant by growth experiments, fermentation behavior and on transcriptional level. As a reference, we used a *gdhA* mutant of the well-studied homofermentative LAB, *Lactobacillus plantarum*, which does contain the pentose phosphate pathway.

## Materials and Methods

**Bacterial strains, media and growth conditions.** The strains used in this study were *Streptococcus (S.) thermophilus* LMG18311 (5), *Lactococcus* (*L.*) *lactis* MG1363(25) and *Lactobacillus* (*Lb.*) *plantarum* WCFS1 (13). Cells were grown anaerobically. *L. lactis* and *S. thermophilus* were grown in M17 broth (Difco, Detroit, MI, USA), supplemented with

glucose (GM17) to a final concentration of 1% (wt/vol) at 30°C and 42°C respectively. *S. thermophilus* Δ*gdhA* was grown in the presence of chloramphenicol (10μg/ml). *Lb. plantarum* was grown in Mann Rogosa Sharpe (MRS) broth (Merck, Whitehouse Station, NY, USA) supplemented with glucose to a final concentration of 1% (wt/vol) at 37°C. *Escherichia coli* DH5α (27) was used for one enzymatic assay and was cultivated aerobically at 37°C on TYB medium (Difco).
For the fermentation experiment, cells were grown on chemically defined medium (CDM) (16).

**Glutamate dehydrogenase (*gdhA*) activity.** Cultures of *Lb. plantarum, L. lactis* MG1363 (negative control) and *S. thermophilus* (both wild-type and *gdhA* knock-out) were grown until OD600~1. Cultures were centrifuged and washed twice (4°C, 5000 rpm, 15 min) in 50mM β-glycerophosphate (pH 7). After the second washing step, pellets were concentrated in the same buffer to OD600~200/ml. Cell disruption by beat-beating (4x30 sec, speed 4.0, Fastprep FP120) was followed by removal of the cell debris by centrifugation (13000 rpm, 10 min, 4°C, Eppendorf Centrifuge 5417R). Because the *gdhA* assay is sensitive for background noise, cell free extracts were purified on a slide-a-lizer (Pierce, Rokcford, IL, USA) and were dialyzed in 50 mM β-glycerophosphate (pH 7) at 4°C overnight. Cell free extracts were removed from the slide-a-lizer using a syringe and were immediately used for measurements of the enzyme activity.
*gdhA* activity was assayed with the colorimetric glutamate assay (Boehringer, Mannheim, Germany, Cat. No. 10 139 092 035). Reaction mixtures were incubated at 37°C and contained 50mM potassium phosphate/TEA buffer pH 9 (solution 1, kit), 1.76 U/ml diaphorase+NAD (solution 2, kit), 2 mM INT (solution 3, kit), 100 mM glutamate, 13.8 mM $NADP^+$ or $NAD^+$ and cell free extract. The formation of NADPH was followed spectrophotometrically by monitoring the increase of absorbance at 492 nm.

**Glucose-6-phosphate dehydrogenase (G6PDH) activity.** Cultures of *Lb. plantarum* (positive control) and *S. thermophilus* were grown until OD600~1. Cultures were centrifuged and washed (5000 rpm, 15 min, 4°C) in a Megafuge 1.0R (Heraeus Instruments, Germany) in 55 mM Tris/HCl buffer (pH 7.8). After the second washing step, pellets were concentrated in

1 ml 55 mM Tris/HCl buffer (pH 7.8) and beat-beated (4x30 sec, speed 4.0, Fastprep FP120) and centrifuged (13000 rpm, 10 min, 4°C, Eppendorf Centrifuge 5417R). G6PDH activity was assayed as described by Honjoh (12). Reaction mixtures were incubated at 25°C and contained 55mM Tris/HCl buffer (pH 7.8), 3.3 mM $MgCl_2$, 0.2 mM $NADP^+$ and 3.3 mM glucose-6-phosphate and cell free extract. The formation of NADPH or NADH was followed spectrophotometrically by monitoring the increase of absorbance at 340 nm.

**Isocitrate dehydrogenase (ICDH) activity.** Cultures of *E. coli* DH5α (used as a positive control) (8) and *S. thermophilus* (wild-type and *gdhA* mutant) were grown until OD600~1. Cells were harvested by centrifugation (5000 rpm, 15 min, 4°C) and washed twice in 35 mM Tris/HCL buffer (pH 7.5). Cell pellet was concentrated in 1 ml 35 mM Tris/HCL buffer (pH 7.5) and beat-beated (4x30 sec, speed 4.0, Fastprep FP120). ICDH activity was assayed as described by Cvitkovitch *et al* (8). Reaction mixtures were incubated at 37°C and contained 35mM Tris/HCl buffer (pH 7.5), 5 mM isocitrate, 3.5 mM $MgCl_2$ or $MnCl_2$, 0.35 mM $NADP^+$ and cell free extract. The formation of NADPH was followed spectrophotometrically by monitoring the increase of absorbance at 340 nm.

**Construction of *gdhA* knock-out for *S. thermophilus*.** Molecular cloning techniques were carried out in accordance with standard laboratory procedures (20). The wild-type strain *S. thermophilus* LMG18311 was used for the construction of a mutant lacking a functional *gdhA* gene (*ΔgdhA*) using natural transformation of an overlapping PCR product as described by Blomqvist *et al* (4).

**Construction of *gdhA / glnA* knock-out for *Lb. plantarum*.** In *Lb. plantarum,* a double knock-out of both *gdhA* and *glnA* (glutamine synthetase) has been constructed following the strategy described before (10)**.** Both genes were inactivated by single cross-over using suicide plasmids (pGIM008 and pJDC9) with insertion of an internal fragment of gene around 600bp. The adjunction of erythromycin and chloramphenicol assure the stability of the insert. This mutant is functionally the same as the *S. thermophilus* gdhA mutant.

**Batch cultivations.** Fermentations were performed in duplicate in 1 L bioreactors (Applikon Biotechnology BV, The Netherlands). The fermentations were controlled by a Bio Controller ADI 1010 and by a Bio Console ADI 1025 (Applikon Biotechnology BV, The Netherlands). *S. thermophilus* wild-type and Δ*gdhA* were grown overnight in CDM and used as an inoculum of 1000 ml pH controlled CDM, the medium was 1% inoculated. The medium for the *gdhA* knock-out was supplemented with chloramphenicol (10µg/ml). Strains were grown at 42°C, during growth pH was kept constant at pH=6.5, by the addition of 2.5M NaOH.
*Lb. plantarum* wild-type and Δ*gdhA* were grown overnight in CDM and used as an inoculum of 1000 ml pH controlled CDM, the medium was 1% inoculated. The medium for the *gdhA* knock-out was supplemented with chloramphenicol (10µg/ml) and erythromycin (10µg/ml). Strains were grown at 37°C, during growth pH was kept constant at pH=5.5, by the addition of 2.5M NaOH.
Cultures were stirred at a constant speed of 100 rpm. Growth was followed by measuring the cell density at 600 nm every 30 min. Samples for HPLC and RNA isolation (2x25 ml) were taken at the end of exponential phase. Samples for GC-MS analysis (3 ml) were taken at mid-exponential phase and at stationary phase.

**Analysis of genome-wide mRNA transcription levels.** All the procedures, from RNA isolation to hybridization were performed as described in our previous paper. The hybridization scheme is shown in the supplementary materials (Figure S1). Selection of scan intensity, normalization, scaling, Cyber-T and False Discovery Rate (FDR) was performed as described elsewhere (3). In order to describe more subtle differential expressions in the microarray data that do not pass the single gene fdr criterion, a geometric mean of the FDRs of multiple genes was calculated provided that they adhered to the following criteria: (i) genes are part of the same operon, (ii) they exhibit similar differential expressions (e.g. all up-expressed in *gdhA* mutant), (iii) they are involved in the subsequent metabolic conversions (thus part of the same metabolic pathway).
With these criteria, we found 2 different pathways consisting of co-expressed genes that for each pathway were organized in an operon (citrate pathway and competence genes).

**Comparative genomics.** The ERGO bioinformatics suite (http://ergo.integratedgenomics.com/ERGO/) was used to compare *S. thermophilus* with other sequenced LAB on genome level. In particular the presence of the pentose phosphate pathway was tested and compared among the available 53 LAB genomes.

**Ammonia measurement.** The concentration of ammonia in the supernatant of fermentation samples was determined using the UV method from an Ammonia kit (R-biopharm AG, Darmstadt, Germany)

**Protein concentration.** The concentration of protein in the cell free extracts was determined using the bicinchoninic acid protein assay reagent (Pierce, Rockford, Il. USA) (22).

**Metabolic analysis.** For the identification of volatile components in the samples, purge and trap thermal desorption cold trap gas-chromatography was used as described before (9, 21). The headspace samples were concentrated on a Fisons MFA815 cold trap (CE Instruments, Milan, Italy), followed by separation on a GC-8000 top gas chromatograph (CE Instruments) equipped with a CIP-SIL 5 CB low-bleed column (Chrompack, Middelburg, The Netherlands) and detection by a flame ionization detector.
Extracellular metabolites present in the supernatant of fermentation samples were measured as described elsewhere (23).

## Results

**Prediction of NADPH generation and construction of a *gdhA* knock-out.** As was described in the introduction, we used the previously developed genome-scale model of *S. thermophilus* (19) to search for NADPH generating pathways. *S. thermophilus* is predicted not to have a complete pentose phosphate pathway and cannot generate NADPH via this pathway. The model predicted that isocitrate dehydrogenase or glutamate dehydrogenase might be possible NADPH producing enzymes. The pathways where these enzymes code for, are connected via α-ketoglutarate, an important biological compound. The model predictions were tested experimentally by assaying enzyme activities. Also, the predicted absence of the PPP was verified by measuring the activity of the first enzyme of the PPP; glucose-6-phosphate dehydrogenase.

Cell free extracts of *Lb. plantarum* and *S. thermophilus* were assayed for G6PDH activity. *Lb. plantarum* was used as positive control, since it is known that this strain has a complete pentose phosphate pathway. The enzymatic assay indeed showed that *Lb. plantarum* has G6PDH activity and *S. thermophilus* does not have G6PDH activity (Table 1).

Cell free extracts of *Lb. plantarum, L. lactis* and *S. thermophilus* wild-type and Δ*gdhA* were assayed for GDH activity. *L. lactis* was used as negative control. *Lb. plantarum* and wild-type *S. thermophilus* possess GDH activity (Table 1). As expected, the *gdhA* knock-out does not have GDH activity.

**Table 1.** Activities of different enzymes tested in this study. Note that not all organisms were included in every assay.

| Strain | GDHA activity[a] | G6PDH activity[a] | ICDH activity [a] |
|---|---|---|---|
| *S. thermophilus* LMG18311 | 5.76 | 0 | 0 |
| *S. thermophilus* Δ*gdhA* | 0 | -[b] | 10.2 |
| *Lb. plantarum* WCFS1 | 0.94 | 9.5 | 0 |
| *Lb. plantarum* Δ*gdhA* | 0 | 7.8 | 0 |
| *E. coli* DH5α | - | - | 371.4 |

[a] Enzyme activity expressed as 1 nmol NADPH (min· mg protein)$^{-1}$, average of two duplicates.

[b] -, not done.

Cell free extracts of *S. thermophilus* wild-type and Δ*gdhA* and *E. coli* DH5α were assayed for isocitrate dehydrogenase activity. *E. coli* was used as positive control and indeed has ICDH activity. The ICDH of *E. coli* is a key regulatory enzyme in the TCA cycle and therefore a high activity of this enzyme is observed. ICDH activity is not observed for the wild-type of *S. thermophilus*), whereas the *gdhA* mutant has ICDH activity (Table 1).

Based on this knowledge and on the observation that the wild-type of *S. thermophilus* can utilize glutamate (24), a glutamate dehydrogenase mutant was constructed, using natural transformation of an overlapping PCR product as described by Blomqvist *et al*. (4). The *gdhA* open reading frame was swapped by the P32-*cat* cassette conferring resistance to chloramphenicol. The primers used in this study are listed in Table 2 and all PCR reactions were performed with the Fhusion polymerase (New England Biolabs Inc, Ipswich, MA, USA).

**Table 2.** Primers used in this study for construction of the *gdhA* knock-out in *S. thermophilus*

| Primer | Name | Sequence[a] |
|---|---|---|
| Primers to amplify the upstream region of *gdhA* on chromosomal DNA | UpDelgdhA1 | ATATATACCTTAGTGACCGTTGAGTTGGTGTCTGC |
| | UpDelgdhA2 | **CCTTATGGGATTTATCTTCCTTA**AGCAACGTATTC TTTACC |
| Primers to amplify theP32-*cat* cassette on pNZ5320 | Upcat | **TAAGGAAGATAAATCCCATAAGG** |
| | Dncat | **TTCACGTTACTAAAGGGAATGTA** |
| Primers to amplify the downstream region of *gdhA* on chromosomal DNA | DnDelgdhA1 | **TACATTCCCTTTAGTAACGTGAA**ATGTCAGCTCA AGGTATT |
| | DnDelgdhA2 | ACGTCTTGAGCCTTCTTTTTGCCTAAGTCTGTCAG |

[a] Bold and underlined sequences are complementary

The upstream and downstream recombination fragments (1.5 kb) of *gdhA* were amplified by PCR using UpDelgdhA1/UpDelgdhA2 and DnDelgdhA1/DnDelgdhA1 primer pairs, respectively. The P32-*cat* cassette from pNZ5320 (a derivative of pNZ5318, removal of lp291 fragment) (14) was amplified by PCR using Upcat and Dncat primers. The 3 overlapping PCR products were mixed in equimolar concentration, joined together by PCR using primers

UpDelgdhA1/ DnDelgdhA2, and the PCR mix was then used for natural transformation. The mutant genotype was confirmed by PCR with primers located upstream and downstream of the recombination regions. As a control, we used a pentose phosphate pathway positive LAB, *Lb. plantarum,* for which a similar *gdhA* mutation was constructed. The primers used for the construction of this mutant are listed in Table 3.

**Table 3.** Primers used in this study for construction of the *gdhA/glnA* knock-out in *Lb. plantarum*

| Primer | Name | Sequence |
|---|---|---|
| Primers to amplify the upstream region of *gdh* on chromosomal DNA | Gdh1up | CCGTTAATCTGAGTATCGTC |
| | moveR | TCAATCAAAGCAACACGTGCT |
| Primers to amplify internal fragment on pGIM008 (cmR) | Gdh_Nco_up | CATGCCATGGCGGTGGTAAAGGGGGCTC |
| | Gdh_Sac_down | CCAGAGCTCTTGGCTTGATCACCGCTG |
| Primers to amplify the downstream region of *gdh* on chromosomal DNA | Gdh2dw | ACCATATAGCAAGCTGGCAG |
| | Move1 | TTTATCGATTACAGCTCCAAG |
| Primers to amplify the upstream region of *glnA* on chromosomal DNA | glnA1up | ATCAACTTGGTTGATCATGC |
| | FP | CGCCCGGGTTTTCCCAGTCAC |
| Primers to amplify internal fragment on pJDC9 (EryR) | gln_Bam_up | CGCGGATCCAACGACCGAATTGAATG |
| | gln_Eco_down | CCGGAATTCAAGAACCGCTGCAAAG |
| Primers to amplify the downstream region of *gdh* on chromosomal DNA | glnA2dw | CATCTTCATCCATAACATAG |
| | RP_24mer | AGCGGATAACAATTTCACACAGGA |

**Metabolic response.** Fermentation samples were analyzed on HPLC (Table 4). The wild-type shows homolactic growth, as was observed and described before (11). The *gdhA* mutant also mainly produces lactate and small amounts of formate, and it consumes citrate. Less glucose is consumed by the mutant than by the wild-type, at the same growth rate, this may indicate a more efficient growth.

**Table 4.** HPLC analyses of fermentation cell supernatants. *S. thermophilus* and *Lb. plantarum* were grown on CDM; samples were taken at the end of the exponential growth phase

| Metabolite (mM)[a] | | Citrate | Lactate | Formate | Acetate | Ethanol | Glucose |
|---|---|---|---|---|---|---|---|
| *S.thermophilus* | CDM (blanc) | 2.08 | ND | ND | 11.07 | ND | 24.31 |
| | Wild-type | 2.13 | 27.62 | 0.66 | 12.26 | ND | 8.74 |
| | *gdhA* knock-out | 1.19 | 23.44 | 1.50 | 12.73 | ND | 12.61 |
| *Lb. plantarum* | CDM (blanc) | 2.20 | ND | 0.65 | 9.61 | ND | 28.33 |
| | Wild-type | 0.82 | 16.21 | 0.00 | 4.25 | ND | 0.00 |
| | *gdhA* knock-out | 0.91 | 17.50 | 0.00 | 4.51 | ND | 0.12 |

[a] Average of 2 duplicates, N.D. not detected.

The volatile profiles are similar for the two strains (Figure 1); however the *gdhA* mutant produces more acetaldehyde than the wild-type.

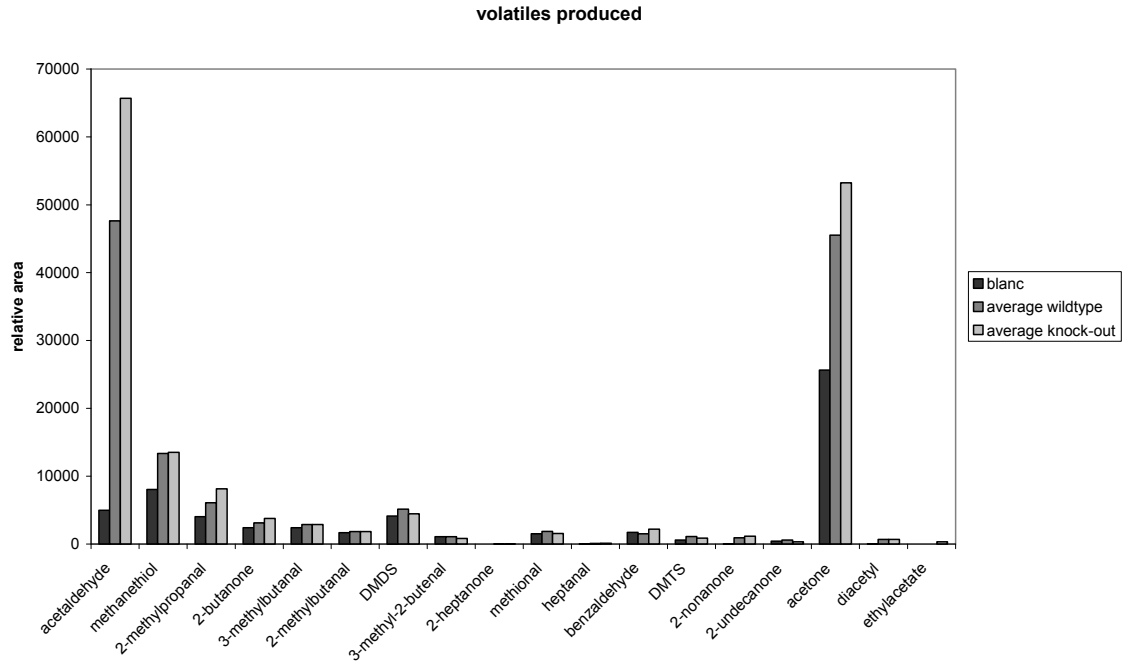


**Figure 1.** GC-MS analyses of the headspace of fermentation samples. *S. thermophilus* was grown on CDM, samples were taken at the end of the exponential growth phase (OD600~1.3).

The *gdhA* also consumes more threonine, and this can point to acetaldehyde production from threonine conversion by threonine aldolase. Also, the *gdhA* mutant produces more propanone than the wild-type does, propanone can be formed as part of glycolysis. Some aldehydes such as 2-methylpropanal and 3-methylbutanal are found in increased concentrations in samples from the *gdhA* mutant. These aldehydes are produced during valine and leucine metabolism respectively and HPLC data indeed show increased consumption of the branched chain amino acids by the mutant compared to the wild-type.

HPLC analyses of amino acids in the same samples (Table 5) shows that all amino acids are more consumed by the *gdhA* mutant than by the wild-type.

**Table 5.** HPLC analyses (amino acids) of fermentation cell supernatants. *S. thermophilus* was grown on CDM; samples were taken at the end of the exponential growth phase (same biomass).

| | Amino acid uptake (medium-sample) μM[a] | | | |
|---|---|---|---|---|
| | *S. thermophilus* | | *Lb. plantarum* | |
| | Wild-type | *gdhA* knock-out | Wild-type | *gdhA* knock-out |
| Alanine | 510.5 | 908.5 | 838.6 | 930.3 |
| Arginine | 112.5 | 194 | N.D. | N.D. |
| Asparagine | -262.5 | 946 | -191.7. | -115.1 |
| Aspartic acid | 93.5 | 280.5 | -2773.3 | 266.4 |
| Cysteine | 1059 | 1135.5 | N.D. | N.D. |
| Glutamine | 481.5 | 838 | 2561.1 | 4309.5 |
| Glutamic acid | 276.5 | 744.5 | 1613.2 | 1042.2 |
| Glycine | 188 | 470 | 970.5 | 1009.1 |
| Histidine | 63 | 220 | 445.9 | 463.4 |
| Isoleucine | 299.5 | 551 | 829.0 | 818.2 |
| Leucine | 475 | 1015 | 1815.5 | 1655.2 |
| Lysine | 219.5 | 520 | 1408.3 | 1650.5 |
| Methionine | 173.5 | 230 | 444.0 | 447.3 |
| Phenylalanine | 266.5 | 506.5 | 1027.5 | 1026.0 |
| Proline | 464.5 | 1190.5 | 1924.7 | 1980.5 |
| Serine | 356 | 891.5 | 2150.6 | 2340.5 |
| Threonine | 153 | 517.5 | 969.6 | 1006 |
| Tryptophan | 215.5 | 495.5 | 33.4 | 83.0 |
| Tyrosine | 758.5 | 826 | 994.6 | 1062.7 |
| Valine | 347.5 | 731 | 1336 | 1349.3 |

[a] Average of 2 duplicates

In addition, the mutant shows an increased production (almost 3x) of ammonia compared to the wild-type (Table 6) and this fits well with the increased amino acid consumption. This probably indicates amino acid degradation.

**Table 6.** Measured ammonia concentrations in fermentation cell supernatants. *S. thermophilus* was grown on CDM; samples were taken at the end of the exponential growth phase (same biomass).

| | $NH_3$ production (mM) |
|---|---|
| Wild-type | 2.2 |
| *gdhA* knock-out | 6.0 |

In the case of *Lb. plantarum*, fermentation samples were analyzed following the same procedure as was used for *S. thermophilus* (Table 4). The *gdh/glnA* mutant and the wild-type do not show a difference in the primary metabolism; lactate, formate and acetate are produced in similar amounts. Furthermore, amino acid measurements show a similar utilisation by the mutant of the different amino acids with an exception for aspartate (Table 5). The volatile profiles of the wild-type and the *gdhA/glnA* mutant were nearly identical (data not shown).

**Transcriptome analysis.** The impact of the absence of the *ghdA* gene on the gene expression was studied with microarrays. For differentially regulation, we only selected those genes that satisfied the following criteria (i) ratio ≥ 1.25 and (ii) FDR-value < 0.05. With these criteria, we found 142 genes to be differentially expressed (Table S1). Logically, glutamate dehydrogenase is heavily down regulated in the *gdhA* mutant. The Δ*gdhA* requires alternative pathways to produce NADPH; a prediction from the recently developed Simpheny model (19) was isocitrate dehydrogenase. The array data showed an up regulation of the citrate metabolism (geometric mean FDR<0.05; average ratio ≥ 1.25); methylcitrate synthase, aconitate synthase and isocitrate dehydrogenase (Figure 2). This up-regulation also corresponds with the consumption of citrate (HPLC analysis), and the increased ICDH activity and may indicate the importance of isocitrate dehydrogenase for NADPH. Furthermore, some parts of the amino acid metabolism are affected in the *gdhA* mutant; some amino acid transporters are up-regulated in the mutant and a branched chain amino acid exporter is down-regulated. Histidine ammonia lyase is down-regulated, this enzyme is part of the nitrogen metabolism. Phosphoserine aminotransferase is highly up-regulated in the *gdhA* mutant; this enzyme catalyzes the formation of glutamate and phosphonooxypyruvate from O-phospho-L-serine and 2-oxoglutarate.

Co-expressed genes (geometric mean FDR<0.05; average ratio ≥ 1.25) involved in competence were down-regulated in the mutant compared to the wild-type.

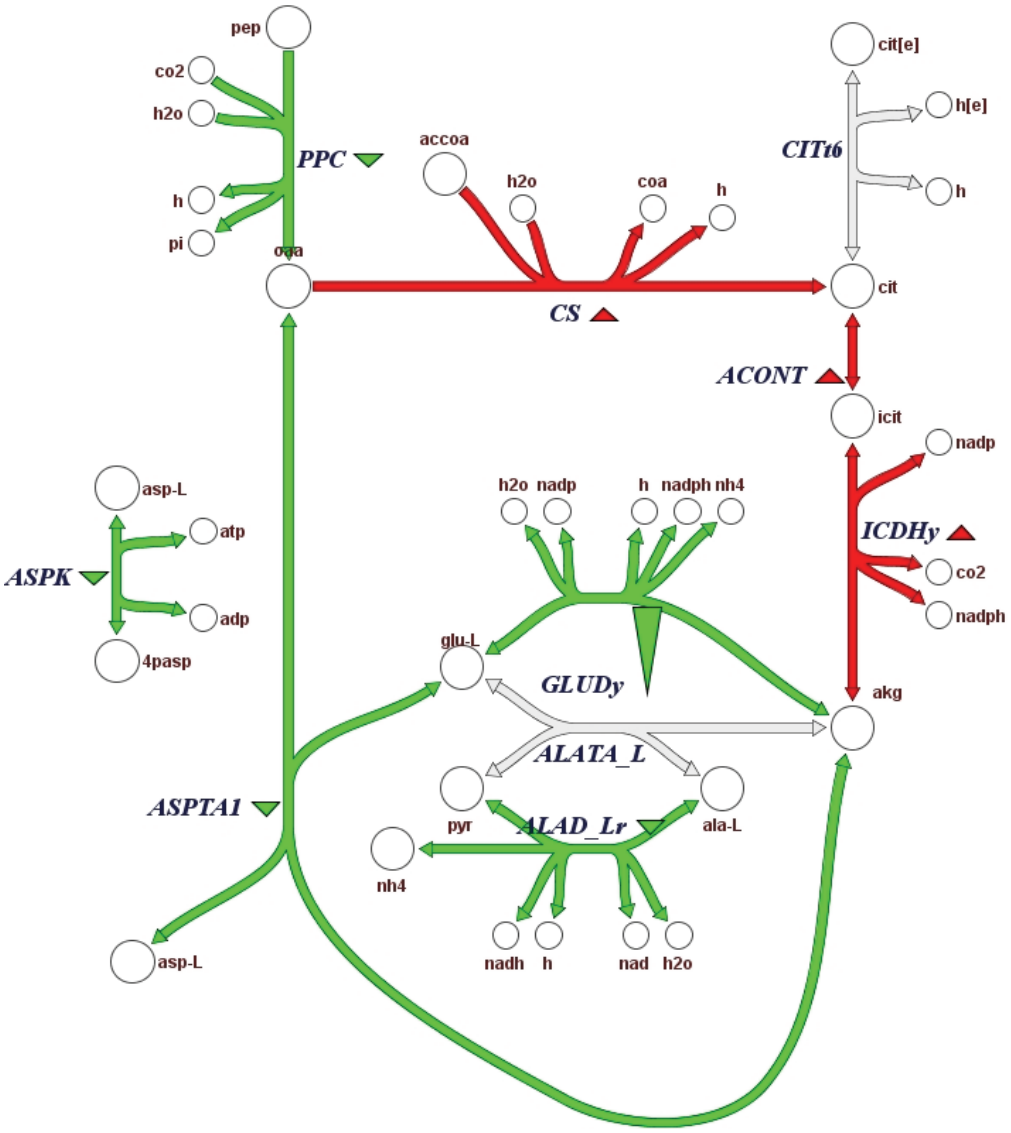


**Figure 2.** Projection of significantly affected genes (geometric mean of the FDRs <0.05 and average FC >1.25) on part of the metabolic map of the genome-scale model of *S. thermophilus*. Red colored reactions are up-regulated in the *gdhA* mutant compared to the wild-type, green colored reactions are down-regulated in the *gdhA* mutant compared to the wild-type. Yellow signifies no differential expression at the above-mentioned criteria. Blank pathways indicate that those associated genes are not present in the microarray data. The size of the triangles signifies the relative up- or down-regulation of the genes associated with the metabolic pathway.

Abbreviations: CS, citrate synthase ; Citt6, citrate transport ; ACONT, aconitase ; ICDHy, isocitrate dehydrogenase ; GLUDy, glutamate dehydrogenase; ALATA_L, L-alanine transaminase; ALA_Lr, alanine dehydrogenase; ASPTA1, aspartate dehydrogenase; ASPK, aspartate kinase; PPC, phosphoenolpyruvate carboxylase.

**Comparative genomics.** The ERGO bioinformatics suite was used to compare the available sequenced LAB on the absence of the pentose phosphate pathway. *S. thermophilus* is not the only LAB with an incomplete PPP (Table7, end of chapter). Actually, almost all Streptococci with the exception of a few *S. suis* and *S. pneumonia* strains, lack the oxidative part of the PPP. All streptococci share the same common ancestor, but this ancestor splits in different branches (17). The event of the gene loss of the PPP genes in most streptococci probably occurred parallel and for a functional reason. *S. thermophilus* is known for its fast growth, and the event of gene loss among different streptococci does not seem unique and does not result in growth delay.

**Table 7.** ERGO assertions table. The presence of the pentose phosphate genes was tested for 54 sequenced lactic acid bacteria. The numbers indicate if the gene is present and how many Open Reading Frames have the assigned function.

| | Enzyme [a] | | | | | | |
|---|---|---|---|---|---|---|---|
| Strain | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| Enterococcus faecium DO (JGI) | 1 | 1 | 1 | | 1 | | 1 |
| Lactobacillus plantarum WCFS1 | 1 | 1 | 2 | 2 | 1 | 5 | 3 |
| Lactobacillus reuteri 100-23 | 1 | 1 | | 1 | 1 | | |
| Lactococcus lactis | | 2 | 1 | | | | |
| Lactococcus lactis str. IL 1403 | 1 | 1 | 2 | 1 | 1 | 1 | |
| Streptococcus mutans UA159 | | | | 1 | 1 | 1 | 2 |
| Streptococcus pneumoniae TIGR4 | 1 | 1 | 1 | 1 | 2 | 4 | 1 |
| Streptococcus agalactiae 2603V/R | | | | 1 | 1 | 3 | 2 |
| Streptococcus thermophilus | | | | | | | |
| Streptococcus pneumoniae 23F | 1 | | 1 | 1 | 1 | 4 | 1 |
| Streptococcus suis | 1 | 1 | 1 | 1 | 1 | 4 | 1 |
| Streptococcus thermophilus LMG18311 | | | | 1 | 1 | 1 | |
| Streptococcus equi | | | | 1 | 1 | 3 | 2 |
| Streptococcus suis 89/1591 (JGI) | 1 | 1 | | 1 | 1 | 3 | 1 |
| Streptococcus uberis 0140J | | 1 | 1 | 1 | 2 | 5 | 3 |
| Streptococcus pyogenes M18 MGAS8232 | | | | 1 | 1 | 1 | 3 |
| Streptococcus pyogenes M3 SSI-1 | | | | 1 | 1 | 1 | 3 |
| Streptococcus pyogenes M5 | | | | 1 | 1 | 1 | 3 |
| Streptococcus pyogenes SF370-M1 | | | | 1 | 1 | 1 | 3 |
| Oenococcus oeni PSU-1 | 1 | 1 | 2 | 2 | 1 | 1 | |
| Enterococcus faecalis V583 | 1 | 1 | 2 | 1 | 1 | | 1 |
| Lactobacillus acidophilus NCFM | 1 | 1 | 1 | 2 | 1 | 2 | |
| Lactobacillus brevis ATCC367 | 1 | 1 | 2 | 1 | 1 | 1 | 1 |
| Lactobacillus delbrueckii bulgaricus ATCCBAA-365 (JGI) | 1 | 1 | 1 | 2 | 1 | | |
| Lactobacillus delbrueckii subsp. bulgaricus ATCC11842 (IG-98) | 1 | 1 | 1 | 3 | 1 | | |
| Lactobacillus gasseri ATCC-33323 (JGI) | 1 | 1 | 1 | 1 | 1 | | |
| Lactobacillus helveticus DPC 4571 (IG-177) | 1 | 1 | 1 | 1 | 1 | | |
| Lactobacillus johnsonii NCC533 | 1 | 1 | 1 | 1 | 1 | 2 | |
| Lactobacillus reuteri JCM 1112 | 1 | 1 | 1 | 1 | 1 | | |
| Lactobacillus reuteri F275 | 1 | 1 | 1 | 1 | 1 | | |
| Lactobacillus casei ATCC334 | 1 | 1 | 2 | 2 | 1 | | 2 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Lactobacillus sakei subsp. sakei 23K | 1 | 1 | 2 | 1 | 1 | | |
| Lactobacillus salivarius subsp. salivarius UCC118 (IG-157) | 1 | 1 | 1 | 2 | 2 | 1 | 2 |
| Pediococcus pentosaceus ATCC25745 | 1 | 1 | 2 | 1 | 1 | | |
| Lactococcus lactis subsp. cremoris SK11 | 1 | 1 | 2 | 1 | 2 | 1 | |
| Lactococcus lactis subsp. cremoris MG1363 | 1 | 1 | 2 | 1 | 2 | 1 | |
| Streptococcus pyogenes M6 MGAS10394 (IG-97) | | | | 1 | 1 | 1 | 3 |
| Streptococcus pyogenes M28 MGAS6180 (IG-100) | | | | 1 | 1 | 1 | 3 |
| Streptococcus pyogenes M1 MGAS5005 (IG-103) | | | | 1 | 1 | 1 | 3 |
| Streptococcus pyogenes M2 MGAS10270 (IG-117) | | | | 1 | 1 | 3 | 3 |
| Streptococcus pyogenes M4 MGAS10750 (IG-124) | | | | 1 | 1 | 3 | 3 |
| Streptococcus agalactiae NEM316 | | | | 1 | 1 | 3 | 2 |
| Streptococcus agalactiae A909 | | | | 1 | 1 | 4 | 3 |
| Streptococcus gordonii str. Challis substr. CH1 | 1 | 1 | 1 | 1 | 1 | | 1 |
| Streptococcus pyogenes str. Manfredo | | | | 1 | 1 | 1 | 3 |
| Streptococcus pneumoniae D39 | 1 | 1 | 1 | 1 | 1 | 3 | 1 |
| Streptococcus pneumoniae Hungary19A-6 | 1 | 1 | 1 | 1 | 2 | 4 | 1 |
| Streptococcus pneumoniae R6 | 1 | 1 | 1 | 1 | 1 | 3 | 1 |
| Streptococcus pyogenes M3 MGAS315 | | | | 1 | 1 | 1 | 3 |
| Streptococcus pyogenes M12 MGAS9429 (IG-115) | | | | 1 | 1 | 1 | 3 |
| Streptococcus suis 05ZYH33 | 1 | 1 | 3 | 1 | 1 | 1 | 1 |
| Streptococcus suis 98HAH33 | 1 | 1 | 1 | 1 | 1 | 1 | 1 |
| Streptococcus thermophilus LMD-9 | | | | 1 | 1 | 1 | |
| Streptococcus thermophilus CNRZ1066 (IG-40) | | | | 1 | 1 | 1 | |

| [a]Nr | Functional description | EC |
|---|---|---|
| 1 | Glucose-6-phosphate 1-dehydrogenase | 1.1.1.49 |
| 2 | 6-phosphogluconolactonase | 3.1.1.31 |
| 3 | Phosphogluconate dehydrogenase | 1.1.1.44 |
| 4 | Ribose-5-phosphate isomerase | 5.3.1.6 |
| 5 | Ribulose-phosphate 3-epimerase | 5.1.3.1 |
| 6 | Transketolase | 2.2.1.1 |
| 7 | Transaldolase | 2.2.1.2 |

**Discussion**

In this paper we discuss the absence of a complete pentose phosphate pathway (PPP) in *S. thermophilus* LMG18311 and the search for other NADPH producing pathways. The pentose phosphate pathway is mainly important for the generation of NADPH that all organisms need for reductive biosynthesis. *S. thermophilus,* however, needs alternative pathways to obtain NADPH. More streptococci do not have a complete PPP and the gene loss seems a parallel event. The available Simpheny model for this strain (19) was used to find these alternative pathways. One predicted alternative NADPH producing enzyme was isocitrate dehydrogenase (encoded by the *icdh* gene). Another prediction was glutamate dehydrogenase (encoded by the *gdhA* gene), *S. thermophilus* indeed has high *gdhA* activity and this strain is known for its glutamate utilization. Moreover, milk, one of the natural habitats of *S. thermophilus,* is glutamate rich (18). Based on this knowledge, a *gdhA* knock-out was constructed and different experiments to compare this mutant with the wild-type were performed. Glutamate dehydrogenase is the major pathway for the formation of α-amino groups directly from ammonia (24). The amino acid glutamate can serve as carbon backbone for many other amino acids (2). A fermentation experiment indicated that the *gdhA* mutant shows the same growth performance as the wild-type. This experiment also showed that the mutant consumes citrate and produces mainly lactate and small amounts of formate and acetaldehyde. The wild-type uses homolactic metabolism for growth optimization. Furthermore, it seems that all the consumed glucose is converted into lactate by the mutant and that for instance acetaldehyde is not produced via mixed acid fermentation but as part of other pathways.

For the control organism *Lb. plantarum*, metabolic responses are not different between the wild-type and the *gdhA* mutant. Furthermore a similar amount of volatiles is produced and the slightly increase in the consumption of the different amino acid tends to show a high ability of *Lb. plantarum* to adapt its metabolism to the double knock-out. In comparison with *S. thermophilus*, this indicates that *Lb. plantarum* does not react in the same way, which could be explained by the presence of a complete pentose phosphate pathway. A transcriptomics study was not performed for *Lb. plantarum.*

Alcohol-acetaldehyde dehydrogenase is probably truncated in *S. thermophilus* as is described elsewhere (11). However, *S. thermophilus* can produce acetaldehyde via threonine aldolase

activity, in this reaction threonine is converted into glycine and acetaldehyde (7). The *gdhA* mutant indeed shows increased threonine consumption.

Amino acid measurements showed that the *gdhA* knock-out consumes more of all amino acids than the wild-type does, this may indicate the importance of the amino acid metabolism for redoxbalance or for NADPH generation. The mutant also shows an increased production of ammonia that fits well with the increased amino acid production indicating amino acid degradation.

In a previous paper, we described that *S. thermophilus* has a simple primary metabolism and a complex amino acid metabolism (19). The transcriptome data also showed that some parts of the amino acid metabolism are affected and this might correspond with the increased amino acid consumption. Still, the increased amino acid consumption is counter-intuitive. Glutamate and especially its α-ketoacid α-ketoglutarate serve as a backbone for most amino acids. When this activity is not present (in the case of the mutant), less amino acids can be synthesized with glutamate as a backbone and less flavors can be formed. However, the *gdhA* mutant of *S. thermophilus* shows a similar flavor profile as the wild-type. Moreover, growth experiments showed that the *gdhA* knock-out needs glutamate for (minimal) growth. Perhaps, glutamate has other (unknown) purposes than just serve as amino acid backbone. The effect of a *gdhA* disruption not only affects NADPH generation, but also pathways that synthesize glutamate or α-ketoglutarate.

As described in our previous paper (19), *S. thermophilus* requires only histidine and cysteine for growth, but when glutamate is added as third amino acid, the growth rate increases significantly. This observation may explain the essential role of glutamate for the *gdhA* mutant.

The transcriptome study of the wild-type and the *gdhA* mutant showed some interesting differences between the two strains. For instance co-expressed genes involved in competence are down-regulated in the *gdhA* mutant compared to the wild-type. In streptococci, competence is not a constant property, it is a transient state regulated by a quorum-sensing mechanism consisting of ComABCDE (4). Spontaneous development of competence in *S. thermophilus* probably requires special, as-yet-undiscovered growth conditions (4). It was observed for *Bacillus subtilis* that the presence of glutamate in the growth medium has adverse effects on the development of competence (26). Our study

showed that the *gdhA* mutant consumes more glutamate than the wild-type does and maybe related to the down-regulation of the competence genes. Also for Group H Streptococci a relationship was found between medium components (glutamate) and development of competent state. Addition of sublethal concentrations of chloramphenicol inhibited competence (15). Chloramphenicol was added to the growth medium of the *gdhA* mutant, although not in sublethal concentrations. These findings suggest an important correlation between glutamate (and probably chloramphenicol) and competence.

Another interesting observation is the up-regulation of the citrate metabolism (methylcitrate synthase, aconitate synthase and isocitrate dehydrogenase) that is observed in the mutant. Isocitrate dehydrogenase was one of the predicted alternatives from our Simpheny model to produce NADPH. Metabolic analysis showed citrate consumption by the mutant and the mutant also possesses ICDH activity. Isocitrate dehydrogenase and glutamate dehydrogenase are connected via α-ketoglutarate (Figure 2). This α-ketoacid plays an important role in the biosynthesis of other amino acids and in the formation of flavors (1, 24). Glutamate dehydrogenase has therefore different important functions in the cell; generation of NADPH and formation of α-ketoglutarate (and thus other amino acids and flavors). α-ketoglutarate can also be generated by the action of isocitrate dehydrogenase.

To test the hypothesis of ICDH as possible NADPH producing enzyme we are currently attempting to construct a knock-out of this gene, both in the wild-type and in the Δ*gdhA* mutant, using the same method as described in the methods section of this paper. Especially the double knock-out will be interesting to study, since this strain probably even has other or more alternative pathways to produce NADPH. Options we can think of are: aspartate semialdehyde dehydrogenase, homoserine dehydrogenase or methylene tetrahydrofolate dehydrogenase. The afore-mentioned hypotheses are currently under investigation.

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## Supplementary material

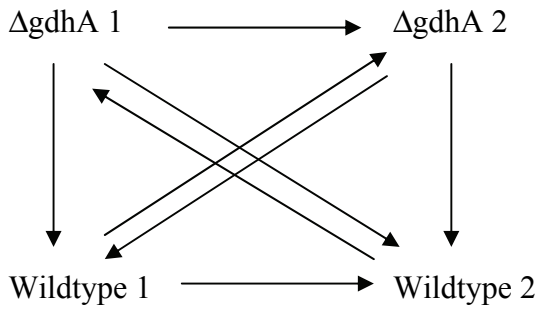


**Figure S1.** Schematic design of the hybridization scheme that was used for the micro array experiment in this study. Each arrow represents one hybridization event; the arrows point from the samples labeled with cy3 to samples labeled with cy5. 1 and 2 refer to samples from duplicate fermentations.

**Table S1**. Transcriptional analysis comparing the response of a *gdhA* mutant compared to the wild-type of *S. thermophilus*. Significant genes have a FDR value< 0.05 and FC>1.25 (142 affected genes)

| locus | gene | Product | FC | fdr |
|---|---|---|---|---|
| stu0430 | *gdhA* | glutamate dehydrogenase | 0.01731 | 0.00000 |
| stu0864 | | hypothetical protein | 0.09308 | 0.00014 |
| stu1017 | | maltose/maltodextrin ABC uptake transporter membrane-spanning protein, truncated | 0.10612 | 0.00673 |
| stu0335 | | hypothetical protein | 0.11401 | 0.00765 |
| stu1391 | | hypothetical protein | 0.12230 | 0.02997 |
| stu1088 | | hypothetical protein | 0.12246 | 0.00000 |
| stu0129 | | truncated IS1193 transposase | 0.14449 | 0.00000 |
| stu0015 | | hypothetical protein | 0.16762 | 0.00337 |
| stu1663 | *int* | integrase/recombinase plasmid associated, putative | 0.20922 | 0.00001 |
| stu0568 | | hypothetical protein | 0.22965 | 0.01278 |
| stu1755 | | hypothetical protein | 0.23072 | 0.01010 |
| stu1603 | | hypothetical protein | 0.23352 | 0.02396 |
| stu1464 | | glutamine amidotransferase (class I), putative | 0.23468 | 0.02446 |
| stu1571 | | hypothetical protein | 0.23734 | 0.00000 |
| stu0051 | | hypothetical protein | 0.24386 | 0.02683 |
| stu1337 | | xanthine phosphoribosyltransferase, truncated | 0.24429 | 0.00539 |
| stu1334 | | peptide-4 ABC transporter ATP binding protein | 0.25118 | 0.04920 |
| stu0169 | | oligopeptide ABC uptake transporter substrate binding protein, truncated | 0.25958 | 0.00776 |
| stu0866 | | hypothetical protein | 0.26318 | 0.00585 |
| stu0016 | | hypothetical protein | 0.26410 | 0.02444 |
| stu0978 | | hypothetical protein | 0.26729 | 0.00411 |
| stu0068 | | hypothetical protein | 0.27980 | 0.01276 |
| stu0155 | | hypothetical protein | 0.28273 | 0.00422 |
| stu1784 | | hypothetical protein | 0.29017 | 0.00348 |
| stu0398 | | hypothetical protein | 0.29028 | 0.02225 |
| stu0650 | *eetB* | exfoliative exotoxin B, putative | 0.29868 | 0.03771 |
| stu0126 | | truncated IS1193 transposase | 0.30208 | 0.00027 |
| stu1302 | | truncated IS1193 transposase | 0.30305 | 0.00164 |
| stu0914 | | hypothetical protein | 0.30463 | 0.00144 |
| stu0935 | | ferrochelatase, truncated | 0.31067 | 0.02433 |
| stu0766 | | hypothetical protein | 0.31399 | 0.02413 |
| stu1432 | *ftsY* | signal recognition particle receptor (docking protein) | 0.31458 | 0.01213 |
| stu0863 | | hypothetical protein | 0.31730 | 0.00048 |
| stu1769 | | branched chain amino acid exporter (LIV-E) family protein | 0.32270 | 0.04971 |
| stu0865 | | hypothetical protein | 0.32566 | 0.00625 |
| stu0974 | | amino acid ABC uptake transporter substrate binding protein, truncated | 0.32635 | 0.01015 |
| stu1300 | *alkD* | DNA alkylation repair protein | 0.32645 | 0.03490 |
| stu1403 | *galR* | galactose operon repressor | 0.32689 | 0.00157 |
| stu0267 | | rRNA methyltransferase | 0.33069 | 0.00106 |
| stu1692 | | hypothetical protein | 0.33108 | 0.02400 |
| stu0295 | | hypothetical protein | 0.33363 | 0.03949 |
| stu0268 | | hypothetical protein | 0.33772 | 0.00050 |
| stu1405 | | hypothetical protein | 0.34276 | 0.03626 |

| | | | | |
|---|---|---|---|---|
| stu1398 | *lacS* | lactose permease | 0.34340 | 0.01167 |
| stu_t31 | | | 0.34351 | 0.01631 |
| stu1670 | | hypothetical protein | 0.34352 | 0.02465 |
| stu1780 | | truncated IS1193 transposase | 0.34468 | 0.00783 |
| stu0426 | | ABC transporter ATP-binding protein | 0.34572 | 0.04071 |
| stu0170 | | oligopeptide ABC uptake transporter substrate binding protein, truncated | 0.34794 | 0.02431 |
| stu0743 | | truncated IS1193 transposase | 0.34797 | 0.02474 |
| stu1086 | *tnp1193* | IS1193 transposase | 0.34887 | 0.04020 |
| stu_t39 | | | 0.35179 | 0.03538 |
| stu0821 | | truncated IS1193 transposase | 0.36150 | 0.02432 |
| stu1303 | | truncated IS1193 transposase | 0.36150 | 0.02458 |
| stu1881 | | alcohol-acetaldehyde dehydrogenase, truncated | 0.36472 | 0.03738 |
| stu0823 | | truncated IS1193 transposase | 0.36502 | 0.01588 |
| stu1313 | *hutH* | histidine ammonia-lyase | 0.36908 | 0.01148 |
| stu0519 | | hypothetical protein | 0.36982 | 0.03521 |
| stu0977 | | amino acid ABC uptake transporter membrane-spanning protein, truncated | 0.37217 | 0.03500 |
| stu1444 | *tnp1193* | IS1193 transposase | 0.37622 | 0.03048 |
| stu0919 | | hypothetical protein | 0.37872 | 0.00756 |
| stu0925 | | truncated IS1193 transposase | 0.37912 | 0.04976 |
| stu0979 | | 5'-nucleotidase, putative | 0.37968 | 0.02567 |
| stu1575 | | hypothetical protein | 0.38025 | 0.00672 |
| stu1414 | | truncated IS1193 transposase | 0.38281 | 0.04476 |
| stu0916 | | transcriptional regulator | 0.38295 | 0.04701 |
| stu1446 | | hypothetical protein | 0.38328 | 0.03157 |
| stu0881 | | alcohol dehydrogenase I, truncated | 0.38806 | 0.04262 |
| stu1272 | | truncated IS1193 transposase | 0.39022 | 0.04002 |
| stu0822 | | truncated IS1193 transposase | 0.39344 | 0.03140 |
| stu0910 | | acetoin reductase, truncated | 0.39416 | 0.03339 |
| stu0025 | | truncated IS1193 transposase | 0.40398 | 0.04868 |
| stu0019 | | hypothetical protein | 0.40949 | 0.03608 |
| stu1339 | | hypothetical protein | 0.41563 | 0.04967 |
| stu1301 | | phosphinothricin acetyltransferase, putative | 0.41627 | 0.02429 |
| stu1664 | | hypothetical protein | 0.42553 | 0.01160 |
| stu1410 | | ABC transporter, putative ATP binding protein, truncated | 0.42671 | 0.00406 |
| stu1433 | | hypothetical protein | 0.44616 | 0.00777 |
| stu1179 | *rpIS* | 50S ribosomal protein L19 | 0.45390 | 0.00022 |
| stu_t11 | | | 0.46014 | 0.00880 |
| stu1451 | | hypothetical protein | 0.46179 | 0.04995 |
| stu_t22 | | | 0.47058 | 0.00017 |
| stu0013 | | hypothetical protein | 0.47079 | 0.01193 |
| stu0586 | | hypothetical protein | 0.47966 | 0.00413 |
| stu0204 | *GroEL* | chaperonin GroEL | 0.49320 | 0.00001 |
| stu_t64 | | | 0.49952 | 0.00681 |
| stu_t12 | | | 0.52039 | 0.03545 |
| stu0796 | *tnpSth1* | IS3 familytransposase orf2 | 0.52789 | 0.04035 |
| stu_t61 | | | 0.53329 | 0.00752 |
| stu_t65 | | | 0.53329 | 0.00769 |
| stu0203 | *GroES* | co-chaperonin GroES | 0.54226 | 0.00024 |

| | | | | |
|---|---|---|---|---|
| stu_t48 | | | 0.54248 | 0.01631 |
| stu_t36 | | | 0.54248 | 0.01654 |
| stu0207 | | hypothetical protein | 0.54408 | 0.04988 |
| stu1564 | | peptidoglycan GlcNAc deacetylase, truncated | 0.54745 | 0.04952 |
| stu1749 | *corA2* | CorA cation transporter (MIT) family protein | 0.54875 | 0.00426 |
| stu1937 | | hypothetical protein | 0.55062 | 0.00325 |
| stu0955 | *pyrDb* | dihydroorotate dehydrogenase 1B | 0.56245 | 0.03767 |
| stu_t10 | | | 0.56365 | 0.01265 |
| stu_t17 | | | 0.57123 | 0.02542 |
| stu_t04 | | | 0.57252 | 0.02417 |
| stu1873 | *ilvB* | acetolactate synthase catalytic subunit | 0.58232 | 0.02465 |
| stu_t15 | | | 0.58982 | 0.02962 |
| stu_t33 | | | 0.60019 | 0.02474 |
| stu_t01 | | | 0.60019 | 0.02505 |
| stu_t02 | | | 0.60019 | 0.02537 |
| stu_t62 | | | 0.60019 | 0.02570 |
| stu_t45 | | | 0.60019 | 0.02604 |
| stu_t43 | | | 0.60019 | 0.02639 |
| stu_t19 | | | 0.60019 | 0.02675 |
| stu1690 | | bacteriocin ABC exporter accessory protein, truncated | 0.60332 | 0.03326 |
| stu0525 | *pyrB* | aspartate carbamoyltransferase catalytic subunit | 0.64831 | 0.03518 |
| stu0120 | *dnaK* | molecular chaperone DnaK | 0.67333 | 0.02538 |
| stu1133 | *rpmI* | 50S ribosomal protein L35 | 0.67939 | 0.01415 |
| stu1733 | *scrK* | fructokinase | 1.37043 | 0.04958 |
| stu1042 | | hypothetical protein | 1.45304 | 0.04075 |
| stu0007 | | hypothetical protein | 1.45693 | 0.02970 |
| stu2006 | | hypothetical protein | 1.47039 | 0.01803 |
| stu1652 | | polar amino acid ABC uptake transporter ATP-binding protein | 1.50981 | 0.01336 |
| stu1672 | *pepXP* | x-prolyl-dipeptidyl aminopeptidase | 1.52573 | 0.02491 |
| stu1033 | | alkaline amylopullulanase, truncated | 1.55237 | 0.03329 |
| stu1043 | *tyrSE* | tyrosyl-tRNA synthetase E | 1.55493 | 0.01321 |
| stu1400 | *galE1* | UDP-glucose 4-epimerase | 1.55641 | 0.01172 |
| stu1032 | | alkaline amylopullulanase, truncated | 1.59069 | 0.02686 |
| stu0630 | *ccpA* | catabolite control protein | 1.61572 | 0.01630 |
| stu1034 | | alkaline amylopullulanase, truncated | 1.64157 | 0.03767 |
| stu1360 | | hypothetical protein | 1.80747 | 0.00756 |
| stu1540 | *folK* | hydroxymethylpterin pyrophosphokinase | 1.90931 | 0.00592 |
| stu1306 | *uvrC* | excinuclease ABC subunit C | 1.95608 | 0.01194 |
| stu1316 | *sdaB* | L-serine dehydratase beta subunit | 1.96373 | 0.04267 |
| stu0301 | | polar amino acid ABC uptake transporter ATP binding protein | 1.97230 | 0.00246 |
| stu0454 | *pepB* | oligopeptidase | 2.05681 | 0.01031 |
| stu0838 | *cspB* | cold shock protein B | 2.08717 | 0.03578 |
| stu0837 | *cspA* | cold shock protein A | 2.18760 | 0.01044 |
| stu1104 | *eps3* | exopolysaccharide biosynthesis protein, glycosyltransferase | 2.21704 | 0.02451 |
| stu0458 | | cell wall protein precursor, choline binding protein, truncated | 2.41362 | 0.02495 |
| stu1027 | *fatC* | iron compound ABC uptake transporter membrane-spanning protein | 2.57056 | 0.00165 |

| | | | | |
|---|---|---|---|---|
| stu0497 | | hypothetical protein | 2.92669 | 0.00018 |
| stu1025 | *fatB* | iron compound ABC uptake transporter substrate-binding protein | 3.31099 | 0.00012 |
| stu1570 | | hypothetical protein | 3.50290 | 0.00001 |
| stu1529 | *serC* | phosphoserine aminotransferase | 6.84862 | 0.00000 |
| stu0496 | *mur1* | peptidoglycan hydrolase | 9.84294 | 0.00018 |

# Chapter 6

## General discussion and future perspectives

## Introduction

The research described in this thesis focuses on the amino acid metabolism of three different, Lactic Acid Bacteria (LAB), of which the genomes are sequenced; *Lactococcus lactis* MG1363 (30)*, Lactobacillus plantarum* WCFS1 (15) and *Streptococcus thermophilus* LMG18311 (4). Several approaches were applied to elucidate the amino acid metabolism in these LAB; (i) fermentations on media with differing composition, (ii) comparison of genome-scale models, (iii) transcriptome studies and (iv) metabolic engineering.

In **Chapter 1** we provided an overview of LAB and highlighted their economic importance as industrial food fermenters. Furthermore, we introduced the amino acid metabolism, functional genomics, and metabolic modeling approaches of LAB. Finally, a brief outline of this thesis was provided.

This chapter will provide an overview of the work described in this thesis. The different sections in this chapter will focus on flavor formation, fermentations on different media, genome-scale modeling, transcriptome studies and metabolic engineering. Finally, future perspectives will be given.

## Flavor formation

The flavor profiles of food products are subject to the metabolic activities of the fermenting LAB (22). For the development of novel or improved dairy and other food products, flavor characteristics are an important selection criterion. In this respect, high-throughput screening systems are important tools to study (and ultimately manipulate) the flavor formed by cultures of LAB. The study of the factors that affect flavor profiles was the primary topic of **Chapter 2**. Since many fermented dairy products are manufactured with mixed cultures (such as yoghurt), we included single cultures but also mixed cultures. As the composition of the growth media also affects the flavor profile, a defined medium was compared with an industrial medium based on milk. GC-MS was used to analyze the flavor profiles and genome-scale models were constructed to link the generated flavor data to metabolic routes.

Flavor analysis of mixed cultures brings additional challenges. The flavor profile of a mixed culture is not simply the sum of the individual cultures, as many metabolic interactions occur. For example, our studies of the mixed yoghurt culture, consisting of *S. thermophilus*

and *Lb. delbrueckii* subsp. *bulgaricus* showed that diacetyl is present at lower levels in the mixed culture than in the pure culture of *S. thermophilus*. In addition, ethanol is only detected in the monoculture of *Lb. bulgaricus*. Moreover, the environmental conditions also appeared to affect the metabolic profiles. Some remarkable differences in metabolic profiles were observed between cultures grown on media of different composition (although many compounds are also produced independently). Acetaldehyde and diacetyl are formed by *S. thermophilus* both on a defined medium (CDM) and an industrial medium (skimmed milk), whereas acetic acid and 2,3-pentanedione are only produced during growth on CDM and not on milk.

A detailed comparison of the metabolites present in the headspace of fermentation samples of *L. lactis, Lb. plantarum* and *S. thermophilus* is described in **Chapter 3.** Comparative analysis of the GC-MS profiles of the three strains, grown on defined medium, revealed that *S. thermophilus* is able to produce a broad variety of flavors, in comparison to *L. lactis* and *Lb. plantarum,* as will be described below.

Genome-scale models can be used, in combination with experimental data, to elucidate the flavor producing pathways. We have used the Simpheny$^{TM}$ software package (Genomatica Inc., San Diego CA, USA) to visualize the similarities and differences between strains or conditions on the metabolic maps. An important result from these types of studies is that the key flavor acetaldehyde that is produced by *L. lactis, Lb. plantarum* and *S. thermophilus,* can be generated via different pathways. As described previously, *L. lactis* and *Lb. plantarum,* among others can produce acetaldehyde during lactose metabolism by pyruvate decarboxylation (6). *S. thermophilus,* which has a truncated acetaldehyde dehydrogenase (13), can convert threonine into acetaldehyde and glycine by the action of threonine aldolase (8), as is shown in Figure 1.

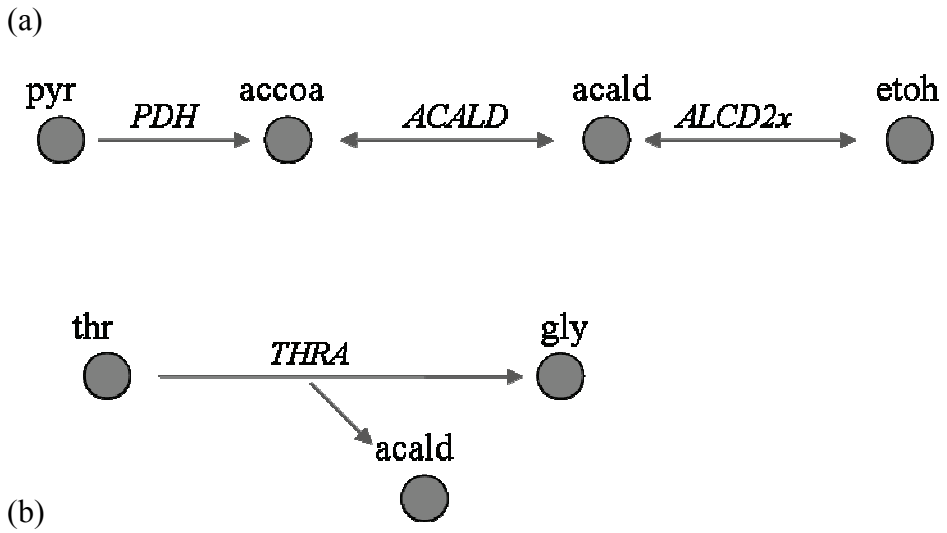


**Figure 1.** (a) Metabolic map (Simpheny) of part of the primary metabolism in *Lactobacillus plantarum* WCFS1, the acetaldehyde pathway is highlighted. pyr = pyruvate, PDH = pyruvate dehydrogenase, accoa = acetyl coenzyme A, ACALD = acetaldehyde dehydrogenase, acald = acetaldehyde, ACALD2x = alcohol dehydrogenase, etoh = ethanol. (b) Metabolic map (Simpheny) of part of the amino acid metabolism in *S. thermophilus* LMG18311, the acetaldehyde pathway is highlighted. thr = threonine, THRA = threonine aldolase, acald = acetaldehyde, gly = glycine.

In **Chapter 4** we studied the effect of environmental conditions on the flavor profiles. Three different LAB (*L. lactis*, *Lb. plantarum* and *S. thermophilus*) were grown on two different defined media; one with a surplus of amino acids (rich medium) and one with a minimal amount of amino acids (poor medium). Volatile analysis of the headspace showed that all three LAB produced less flavors during growth on the poor medium. This observation confirmed that the concentration of present amino acids is important for the flavor profile.

**Development of a minimal medium**

Single amino acid omissions in *S. thermophilus* have shown that the number and type of essential amino acids is strain-dependent and range from 2-5 amino acids (11, 17, 19). However, in general, *S. thermophilus* has a much lower degree of auxotrophy for amino acids than other LAB (7). For example, *L. lactis* MG1363 and *Lb. plantarum* WCFS require respectively 6 and 11 amino acids for (minimal) growth (14, 28).

In **Chapter 3** we describe the development of a medium with a defined amount of amino acids for *S. thermophilus* LMG18311. The amino acid requirements can then be

compared with *in silico* predictions based on the amino acid biosynthesis pathways of *S. thermophilus* LMG18311. The *in silico* predictions, based on the genomic information, suggest a biosynthetic capacity for all amino acids except histidine (13). Indeed our multiple omission experiments showed an auxotrophy for this amino acid. Besides histidine, however, *S. thermophilus* LMG18311 also requires one of the sulfur-containing amino acids (with a preference for cysteine), when grown in the presence of citrate for growth (Table 1) Citrate can play an important role in the generation of NADPH and $CO_2$ . Detailed *in silico* analysis revealed that the *yhcE* gene is truncated. The product of *yhcE* shows similarity to the vitamin B12-independent 5-methyltetrahydropteroyltriglutamate-homocysteine *S*-methyltransferase. Its orthologue in *L. lactis* is involved in the synthesis of cysteine from methionine (13). Therefore, the inactivation of this gene in *S. thermophilus* may explain the auxotrophy for one of the two sulfur amino acids.

**Table 1.** Essential amino acids for three different lactic acid bacteria: *L. lactis* MG1363 (14), *Lb. plantarum* WCFS1 (28) and *S. thermophilus* LMG18311 (**Chapter 3** (23))

| Strain | Essential amino acids |
|---|---|
| *L. lactis* MG1363 | Glutamate, hisitidine, isoleucine, leucine, methionine, valine |
| *Lb. plantarum* WCFS1 | Arginine, cysteine, glutamate, isoleucine, leucine, methionine, phenylalanine, threonine, tryptophan, tyrosine, valine |
| *S. thermophilus* LMG18311 | Cysteine, histidine |

The low degree of amino acid auxotrophies of *S. thermophilus* and the ability to generate a varied amount of flavors, suggests a rather elaborate set of amino acid converting pathways. This is unexpected, because *S. thermophilus,* is used for centuries together with *Lb. bulgaricus* to produce yoghurt resulting in a high degree of gene loss (18). The continued cultivation of *S. thermophilus* in the protein rich milk-environment would make the loss of one or more amino acid biosynthesis pathways a likely scenario. Remarkably, *Lb. bulgaricus* has lost most of its amino acid biosynthetic capacity (29). We can speculate why *S. thermophilus* has retained most of its amino acid biosynthetic capacity. Firstly, amino acid metabolism is not only important for the synthesis of amino acids but also plays a role in maintaining the redox balance and NADPH generation. Secondly, *S. thermophilus* strains are

selected for quick growth and acidification in milk where the availability of amino acids forms the rate-limiting factor for growth in milk. To support such a quick growth, the operation of, apart from histidine and cysteine, all amino acid pathways is required. Thirdly, *S. thermophilus* does not possess an extracellular protease to exploit the abundantly present milk proteins. *S. thermophilus* may have adapted to the protein-rich environment independent or it was co-evolved with the protease positive *Lb. bulgaricus* but maintains an advantage by conserving most of the amino acid biosynthesis pathways (29).

**Development of a genome-scale model**

Genome-scale models are the basis for many developments. They are based on annotated genome sequences and experimental data and have become available for an increasing number of organisms, including various LAB (21, 28). A useful tool for the construction of these *in silico* models is the Simpheny$^{TM}$ software package (Genomatica Inc., San Diego CA, USA). The thus generated *in silico* models are stoichiometric models and are based on a thorough metabolic reconstruction of well-annotated genome sequences (27). In this work we have constructed a metabolic model for *S. thermophilus*. The existing models of *Lb. plantarum* (28) and of *L. lactis,* which was constructed using the Autograph method (20), were used as the initial basis, for the construction and development of the *S. thermophilus* model (**Chapter 3**).

Comparative evaluation of the generated models revealed considerable differences as can be expected as the three LAB are highly diverse. In particular they differ in the size of the genome and thus the number of genes incorporated in the model, as is shown in table 2.

**Table 2.** Some properties about three different lactic acid bacteria: *L. lactis* MG1363 (14), *Lb. plantarum* WCFS1 (28) and *S. thermophilus* LMG18311 (**Chapter 3** (23)) and their respective models.

| | *L. lactis* | *Lb. plantarum* | *S. thermophilus* |
|---|---|---|---|
| Genome size | 2.5 Mb | 3.3 Mb | 1.8 Mb |
| Genes in model | 2563 | 3064 | 1889 |
| Reactions in model | 598 | 598 | 598 |

The metabolic models for these three LAB were used as mapping tools for microarray data, different simulations and a comparative *in silico* analysis. Unique for the *S. thermophilus*

genome and therefore also model, are the absence of a complete pentose phosphate pathway and the simple primary metabolism.

**Homofermentative metabolism**

LAB can be divided into two groups concerning their carbohydrate metabolism (see **Chapter 1**). The LAB belonging to the homofermentative group use the Embden-Meyerhof pathway in which glucose is completely converted into lactate (25). The heterofermentative LAB produce lactate, acetate, $CO_2$ and ethanol using the hexose monophosphate pathway (25). Some homolactic LAB can also have a mixed acid fermentation, producing mainly acetate, formate and ethanol (12, 28).

The metabolic model of *S. thermophilus* predicts that homofermentative lactic acid production is the only primary metabolism operating in *S. thermophilus* (Figure 2) (**Chapter 3**). Our fermentation data indeed showed that *S. thermophilus* consumes (almost) all the glucose and produces mainly lactate, in line with earlier observations (13). The mixed acid fermentation is metabolically the most efficient route for lactic acid bacteria whereas the homolactic route is catalytically more efficient (12). Typically, both *L. lactis* and *Lb. plantarum* grow homolactically at high dilution rates or via mixed acid fermentation at low dilution rates (12, 28). Because *S. thermophilus* has pseudogenes that affect the primary metabolism, the number of pyruvate branches is limited. The presence of these pseudogenes, especially those which are normally important for NAD+ regeneration, limit the number of alternative metabolic routes for lactate dehydrogenase. Thus, when grown anaerobically on glucose, the redox-balance constrains the glucose catabolism by *S. thermophilus* to the production of lactate.

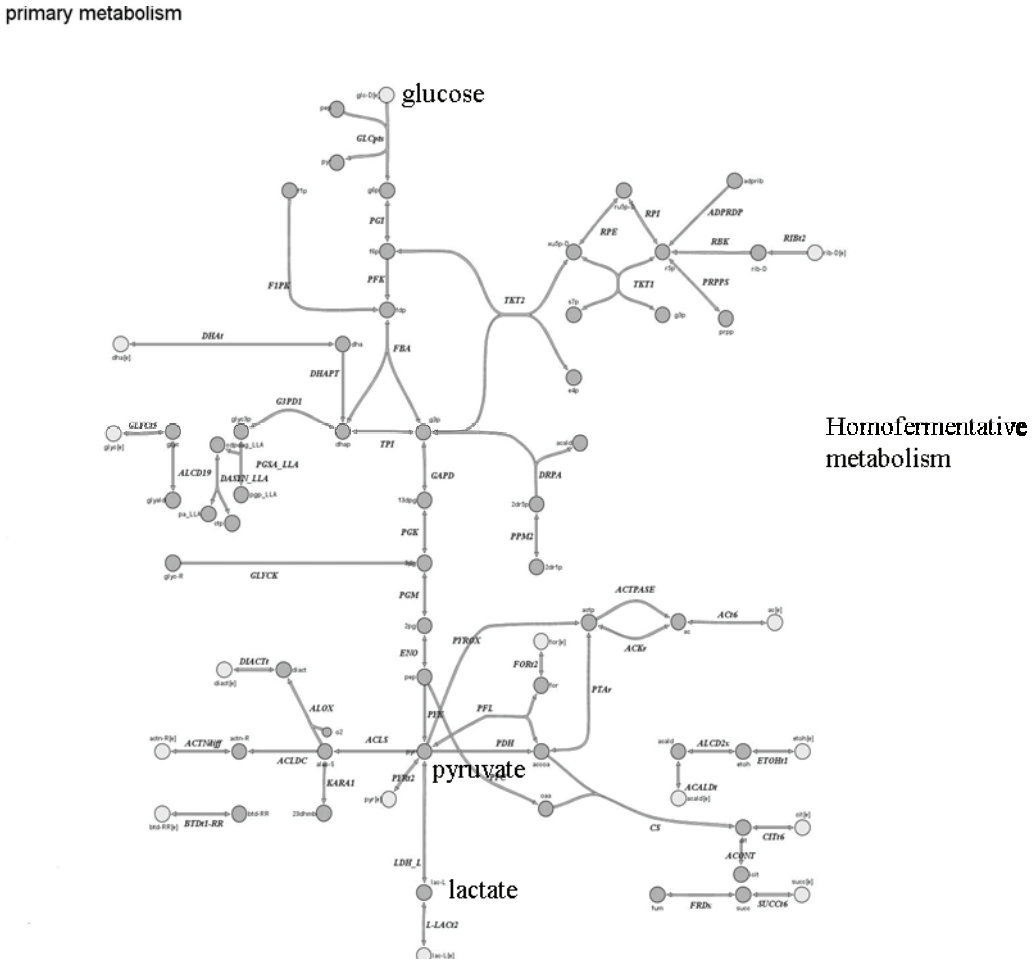


**Figure 2.** Primary metabolism of *Streptococcus thermophilus*. This map is part of the total genome-scale metabolic model developed for *S. thermophilus*. Large bold capital italics indicate the enzymes and normal small italics the metabolites. Taken from **Chapter 3.**

The developed metabolic model of *S. thermophilus* is suitable for Flux Balance Analysis (FBA) (28). FBA can be used to explore the metabolic potential of the metabolic network, while taking into account mass balance equations and other constraints (24). FBA correctly predicted homolactic fermentation in *S. thermophilus*, (growing anaerobically on glucose) in contrast to what was found for *Lb. plantarum* (28) and *L. lactis* (21). The developed model of *S. thermophilus* thus has many different possibilities.

**Transcriptional response towards amino acid depletion**

For the development of novel or improved dairy and other food products, flavor characteristics are an important selection criterion. We elaborated on this observation and in **Chapter 4** we describe a more detailed study of the physiological response of *L. lactis, Lb. plantarum* and *S. thermophilus* to amino acid availability using microarray techniques and volatile analysis. The growth of single cultures of these three different lactic acid bacteria on a defined medium containing all amino acids was compared with that on the same with a minimal amount of amino acids. As expected, growth on medium with a minimum of amino acids triggers a major response in the expression of genes encoding amino acid metabolic pathways.

When the transcriptional response of the three LAB towards the depletion of amino acids was compared, we observed that not only genes involved in the amino acid metabolism are affected but also those involved in other parts of the metabolism. In addition, the specific growth rate and yield is influenced/reduced, and in *Lb. plantarum* the expression of stress-related genes was affected. Obviously, the three LAB have different strategies to cope with a depletion of amino acids. *S. thermophilus* has a simple primary metabolism and a complex amino acid metabolism. A large part of the affected genes is involved in amino acid metabolism, in contrast to what was found for the other two LAB. The primary metabolism is not very flexible due to the presence of pseudogenes and the amino acid metabolism is quite complete and flexible. The other two LAB, *L. lactis* and *Lb. plantarum* have a more flexible primary metabolism in comparison to *S. thermophilus*. They show a more general response than *S. thermophilus*, since not only amino acid metabolism, but also other parts of the metabolism are affected by the depletion of amino acids. On restricted medium, less volatile compounds (some volatiles are flavors) are formed than on the complete medium by all three LAB because most flavors are generated during amino acid metabolism. Of the three studied LAB, *L. lactis* is probably most adapted to the protein-rich milk environment and this bacterium also shows the largest response (relatively most differentially regulated genes) towards amino acid depletion.

Overall these data showed that not only amino acid metabolism is affected by a depletion (or surplus) of amino acids, but also overall metabolism is affected. This study also showed that different LAB have different strategies to cope with a depletion of amino acids.

Even though these organisms are closely related, they have their own unique requirements and their own response.

**Effect of GDH inactivation in *S. thermophilus***

All living organisms need NADPH for reductive biosynthesis. The activity of the pentose phosphate pathway typically generates intracellular NADPH to be used in reductive biosynthesis. This pathway contains two parts: an oxidative part and a nonoxidative part that interconverts phosphorylated sugars (3). Most LAB possess a complete pentose phosphate pathway. However, *S. thermophilus* LMG18311 (5, 13) interestingly, lacks a complete pentose phosphate pathway, as is described in **Chapter 5**. The genome predicts that the oxidative phase (the conversion of glucose-6-phosphate to ribulose-5- phosphate) is absent in *S. thermophilus*. In agreement with this, we have experimentally verified that *S. thermophilus* does not have glucose-6-phosphate dehydrogenase activity. However, *S. thermophilus* must have alternative pathways to synthesize NADPH. To find alternative NADPH-producing pathways, we analyzed the available genome-scale model of *S. thermophilus* (described in **Chapter 3)** and tested the various model-predictions experimentally. One alternative NADPH-generating pathway may involve isocitrate dehydrogenase (encoded by the *icdh* gene), another glutamate dehydrogenase (encoded by the *gdhA* gene). In fact, *S. thermophilus* was found to possess high GdhA activity, which is NADP-dependent (**Chapter 5**). Moreover, wild-type *S. thermophilus* is known for its glutamate consumption. We deleted the *gdhA* gene and characterized the mutant with growth experiments and transcriptomics, to investigate its involvement in NADPH-generation. These experiments indicate that, contrary to expectations, the *gdhA* mutant has a similar growth performance to the wild-type. The mutant produces mainly lactate and small amounts of formate and acetaldehyde, whereas the wild-type uses homolactic metabolism for growth optimization. Amino acid measurements revealed that the *gdhA* mutant has an increased amino acid consumption which may form an alternative pathway to generate NADPH for growth. As a reference, we used the well-studied LAB, *Lb. plantarum*, which does contain the pentose phosphate pathway. In this reference strain, we performed a similar glutamate dehydrogenase mutant and the clear difference between the wild-type and the *gdhA* mutant was not found for this control organism. Comparison of the transcriptome profiles between the wild-type and the *gdhA* mutant of *S.*

*thermophilus*, suggests that metabolic flux through the citrate metabolism (methylcitrate synthase, aconitate synthase and isocitrate dehydrogenase), was upregulated in the *gdhA* mutant (figure 3).

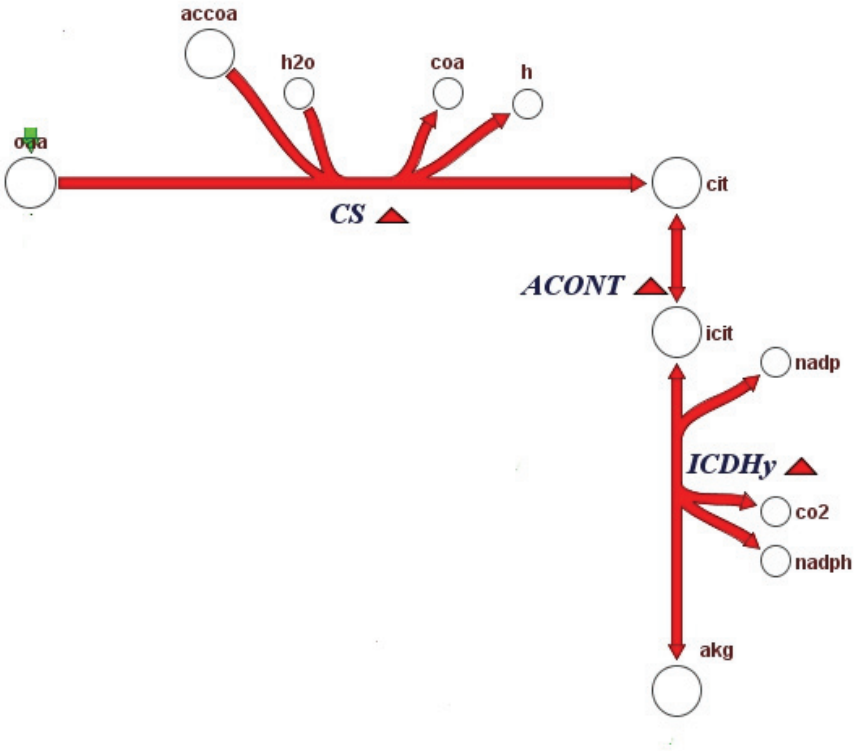


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**Figure 3.** Projection of significantly affected genes (average FDR<0.05 and FC>1.25) on part of the metabolic map of the genome-scale model of *S. thermophilus.* Red colored reactions are up-regulated in the gdhA mutant compared to the wildtype. The size of the triangles signifies the relative up- or down-regulation of the genes associated with the metabolic pathway.

Isocitrate dehydrogenase was the second alternative NADHP generating pathway, as predicted by our metabolic model. In agreement with the observed upregulation of genes encoding the enzymes of this pathway, the *gdhA* knock-out has retained isocitrate dehydrogenase activity (icdh) and increased citrate consumption as well. This shows the importance of citrate as an alternative source of NADPH. Citrate is also present in milk (7-11 mM) and *S. thermophilus* might be adapted to the presence of citrate. The transcriptome data also showed an up-regulation of genes involved in various amino acid converting pathways, which was also in agreement with increased amino acid consumption by the mutant and the

increased ammonia production. As revealed by comparative genomics, the absence of a complete PPP is not unique to *S. thermophilus*, but a common feature among Streptococci.

**Concluding remarks and future perspectives**

This thesis has shed light on the amino acid metabolism of LAB, in particular *L. lactis*, *Lb. plantarum* and *S. thermophilus*. It has shown the importance of the comparative approach to study the differences in amino acid metabolism. Comparative genomics was used for two main reasons: (i) the availability of complete genomes enabled us to identify sets of orthologs involved in amino acid metabolism (10) (ii) comparison of complete genomes not only showed which genes are present, but also which ones are absent (16). The research described in this study deals with the analysis of extensive amino acid metabolism of *S. thermophilus* and its potential to produce a varied amount of flavors. The amino acid metabolism of *S. thermophilus* is rather extensive as compared to that of the other LAB. Since more and more genomes are sequenced, this comparative approach can be expanded to study more strains and species, allowing for the comparison of different features. Comparative metabolomics can also be applied to study mixed cultures of bacteria and eukaryotes. For instance some beer consortia consist of LAB and yeasts. In addition, some type of cheeses such as Brie, Camembert and Roquefort, are fermented by the combined action of LAB and fungi. It would be of interest to study their interaction by comparative ~omics techniques which will ultimately lead to a better understanding of their (combined) metabolism and the flavor profile. Other examples of industrially relevant topics that can be studied and compared include the production of vitamins, flavors or antimicrobials, growth requirements and possible probiotic properties. Nowadays, much attention is given to the contribution of food and dietary habits to public health, especially since the prevalence of welfare related diseases is increasing (31). From this point of view, probiotic properties of LAB can be relevant and important. Strains that are marketed as probiotic, can confer a health benefit in the host (9). As was shown in this study, some important properties can only be revealed and appreciated when a comparative approach is used. This can also be the case when searching for probiotic properties of different LAB with known genomes. This was realized by the genomic comparison of different *Lb. plantarum* strains, revealing the presence of a large number of

genes located in so-called 'lifestyle adaptation islands' predicted to be involved in niche-specific, and possibly probiotic, functions (15).

One of the striking characteristics of *S. thermophilus* is its fast growth. When grown in a rich medium it can have a doubling time of as low as 10 minutes although total biomass production is not very high (23). Based on the insights gained in this study, we can speculate about this typical growth behavior. One possible explanation can be that the high growth rate is a result of excessive loss of unnecessary gene functions. This is supported by the observation that *S. thermophilus* has the highest number of pseudogenes compared to other LAB. Moreover, a clear example of gene loss is the absence of a complete pentose phosphate pathway, the maintenance of which probably costs too much energy and is therefore less efficient than obtaining NADPH via other pathways (**Chapter 5**). Alternatively, fast growth can be considered from the competition point of view as *S. thermophilus* is often co-cultivated with *Lb. bulgaricus* for the production of yoghurt. Transcriptome analysis of this mixed culture showed in *S. thermophilus* a strong up-regulation of peptide transporters. This study of the mixed culture also revealed an up-regulation of the biosynthesis of the sulfur amino acids and up-regulation of the cycling of the folate genes, correlated with the production of purines (26). Our transcriptome data (**Chapter 4**) obtained from *S. thermophilus* during growth on a restricted medium also showed an up-regulation of the biosynthesis of the sulfur amino acids and the biosynthesis of tetrahydrofolate in *S. thermophilus*. Transcriptome analysis of an *S. thermophilus gdhA* mutant also showed an up-regulation of amino acid metabolism and some transporters. *S. thermophilus* has an extensive amino acid metabolism that is combined with a simple, non flexible primary metabolism. Therefore, it seems logical that the amino acid metabolism in *S. thermophilus* is more affected by different growth conditions than the primary metabolism. The consequence of the fast growth is that *S. thermophilus* has to choose a metabolic pathway that is suitable for this growth behavior.

Growth on a medium with only the two restricted amino acids (histidine and cysteine) was also simulated with our genome-scale model. These simulations exposed a redox problem but this could be solved by incorporating additional oxygen. Fermentation experiments also showed that during growth on this minimal medium, it was essential to provide air (nitrogen, oxygen and carbon dioxide) in the headspace. Moreover, the data of Arioli *et al* (1) also suggested an important role of $CO_2$ in *S. thermophilus* physiology and $CO_2$ is also present in

the air headspace. Furthermore, the *in silico* analysis of growth on minimal medium showed the accumulation of α-ketoglutarate and this prevented optimal growth. To solve this problem, an exchange reaction of α-ketoglutarate was added. This solution is artificial but may correspond to the measured increase in succinate production during growth in minimal medium, compared to growth on complete medium. To really solve and understand this problem, more simulations should be performed instead of adding compensating reactions. In addition, α-ketoglutarate concentrations in fermentation samples from growth on both complete and minimal need to be determined in order to validate the model.

We observed a remarkable species-specific distribution of the pentose phosphate pathway and glutamate dehydrogenase. *S. thermophilus* lacks a complete pentose phosphate pathway, but it has a NADP-dependent glutamate dehydrogenase activity. *L. lactis* on the contrary, has a complete pentose phosphate pathway but it does not exhibit glutamate dehydrogenase activity. *Lb. plantarum* as a third partner in the present comparative study has both a complete pentose phosphate pathway and glutamate dehydrogenase activity. In **Chapter 5** we describe the study of a *gdhA* mutant in *S. thermophilus*, which was found to have an increased amino acid uptake and ammonia production in comparison with the wild-type strain. Furthermore, the citrate pathway was up-regulated. A knock-out of *gdhA* was also constructed in *Lb. plantarum* but this mutant did not show a clear phenotype. The effect of the knock-out in this bacterium is expected to be different than for *S. thermophilus* probably because *Lb. plantarum* has a complete pentose phosphate pathway and specifically needs *gdhA* for amino acid and/or flavor biosynthesis, via α-ketoglutarate rather than for NADPH production. It would be of interest to construct a knock-out of the NADP generating part of the pentose phosphate pathway in *L. lactis* and study how this knock-out copes with a defective pentose phosphate pathway and how NADPH is produced. *L. lactis* possesses isocitrate dehydrogenase activity and a pentose phosphate pathway mutant might show an up-regulation of the citrate pathway, since it does not contain a *gdhA* gene. A complementary approach would be to transfer a complete, functional pentose phosphate pathway into *S. thermophilus*. Growth and flavor formation of the resulting strain can be compared with the wild-type. Since the biosynthesis of histidine is coupled to ribose-5-phosphate, the introduction of a functional pentose phosphate pathway is predicted to have an influence on

the amino acid auxotrophy of *S. thermophilus* and it is conceivable that the resulting strain is able to grow without any supplemented amino acid.

Another relevant engineering approach would be to transfer the extensive flavor forming pathways from *S. thermophilus* to other LAB for manipulation of flavor production in fermented (dairy) products. A more direct way, and this is one recommendation coming from this thesis, would be to consider *S. thermophilus* more often as a (thermophilic) cheese or adjunct starter, also under mesophilic conditions. Nowadays, especially Lactobacilli are used as so-called adjunct starters to boost flavor production in several cheese varieties. However, the work described is this thesis showed that *S. thermophilus* has a broad variety of industrially relevant properties (such as an extensive amino acid metabolism and many flavor producing pathways), it is a proper acidifier and it can properly ferment at 30°C, notably, this temperature is also used for yoghurt production in the Netherlands (personal communication J. Hugenholtz). These findings all indicate that *S. thermophilus* could do even better than the frequently used thermophilic lactobacilli for flavor enhancement in cheese.

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# Nederlandse Samenvatting

## Nederlandse samenvatting – in lekentaal

Melkzuurbacteriën worden al eeuwenlang gebruikt voor de productie van diverse levenmiddelen. Voorbeelden hiervan zijn de fermentatie van melk om yoghurt en kaas te maken en de fermentatie van kool om zuurkool te maken. Melkzuurbacteriën zetten de aanwezige suikers om in met name melkzuur (vandaar de naam van deze bacteriën). Tijdens de groei van deze melkzuurbacteriën worden ook andere, interessante voedingsstoffen geproduceerd, zoals vitaminen en smaakstoffen. Door fermentatie worden de organoleptische eigenschappen (smaak, textuur) van het beginproduct beïnvloed. Vergelijk bijvoorbeeld maar melk met kaas; de smaak is anders, het uiterlijk is anders en ook houdbaarheid is verbeterd. De betere houdbaarheid wordt deels veroorzaakt doordat de verzuring (o.a. melkzuur) de groei van andere, mogelijk ziekteverwekkende micro-organismen wordt geremd. Daarnaast produceren veel melkzuurbacteriën antimicrobiële stoffen (zoals nisine) die een verdere conserverende werking hebben.

Momenteel worden een aantal melkzuurbacteriën ook als probioticum op de markt gebracht. Deze stammen zouden na inname, een positieve invloed hebben op de gezondheid van de gastheer, enkele van deze gezondheidsbevorderende eigenschappen zijn: verbetering van het immuunsysteem en het verkorten van de duur van diaree.

Zoals hierboven al is genoemd, kunnen melkzuurbacteriën de smaak van het uiteindelijke fermentatieproduct beïnvloeden. Zo smaakt zuurkool anders dan kool en yoghurt anders dan melk. Er zijn vele verschillende melkzuurbacteriën en zij kunnen, naast de invloed van het productieproces, elk op hun mannier de smaak beïnvloeden van het gefermenteerde voedsel. Goudse kaas smaakt bijvoorbeeld heel anders dan Cheddar kaas en ook weer anders dan een Edammer. Het aminozuurmetabolisme speelt een belangrijke rol in de smaakvorming. Aminozuren zijn de bouwstenen van eiwitten en melkzuurbacteriën kunnen aminozuren omzetten of afbreken tot belangrijke smaakstoffen. Echter, niet alle smaakstoffen zijn gewild, deze kunnen de gefermenteerde producten een vervelende bijsmaak geven. Het is daarom belangrijk om de productie van smaakstoffen te controleren en dit kan gebeuren door de juiste bacteriën toe te voegen of door het juiste groeimedium te gebruiken.

In **hoofdstuk 2** wordt een onderzoek beschreven naar de analyse van smaakvorming door verschillende melkzuurbacteriën en onder verschillende kweekcondities. De onderzochte bacteriën zijn *Streptococcus thermophilus* en *Lactobacillus bulgaricus*, deze twee bacteriën worden gebruikt voor de bereiding van yoghurt. We hebben de bacteriën gekweekt op een industrieel medium, melk, en op een gedefinieerd medium. Tevens hebben we de twee bacteriën samen gekweekt op melk, als zogenaamde mengculture. Tijdens de groei hebben we monsters genomen van de 'headspace', de gaslaag boven de groeiende culture omdat veel smaakstoffen vluchtig zijn en in de gaslaag gaan zitten. De headspace monsters zijn geanalyseerd met een gaschromatograaf. Deze studie liet zien dat tijdens groei op melk andere smaakstoffen worden gevormd dan tijdens groei op een gedefinieerd medium. Daarnaast is de smaakvorming van een mengculture niet simpel de optelsom van de smaakvorming van de twee individuele culturen. Dit laatste waarschijnlijk omdat er allerlei interacties zijn tussen verschillende stammen, die nog niet helemaal ontrafeld zijn.
Als laatste hebben we computermodellen van verschillende bacteriën gebruikt om te zoeken naar smaakvormende routes. Met deze modellen kan groei van bacteriën worden gesimuleerd en kan het gedrag worden bestudeerd, alvorens experimenten te doen.

In **hoofdstuk 3** wordt beschreven hoe een computermodel kan worden gemaakt. Allereerst is de genoomsequentie van een bacterie nodig. Het genoom omvat alle genen van een organisme. Op basis van de basenvolgorde van dit genoom kan worden gezocht naar bepaalde eigenschappen. Wij hebben een model ontwikkeld voor de yoghurtbacterie *Streptococcus thermophilus* en er waren al modellen beschikbaar van twee andere melkzuurbacteriën, *Lactococcus lactis* en *Lactobacillus plantarum.* Hierdoor konden we niet alleen de drie bacteriën experimenteel vergelijken, maar ook via theoretische modellen.
Doordat tijdens een vergelijkende studie verschillen en overeenkomsten tussen organismen aan het licht komen, kan dit soort studies soms meer inzichten geven in het gedrag van een organisme.

Deze studie bracht naar voren dat *Streptococcus thermophilus* al het aanwezige suiker omzet in melkzuur, dat wil zeggen een homofermentatieve groei. Sommige melkzuurbacteriën kunnen naast melkzuur ook nog kleine hoeveelheden ethanol, azijnzuur en mierenzuur maken. Het zelf-ontwikkelde metabole model van *Streptococcus thermophilus*

voorspelt ook inderdaad een homofermentatief metabolisme, dit in tegenstelling tot de bestaande modellen van de andere twee melkzuurbacteriën, die juist een zogenaamde gemengdzuur metabolisme, met azijnzuur, mierenzuur en ethanol als eindproducten, voorspellen. Tevens liet deze studie zien dat *Streptococcus thermophilus* maar twee aminozuren nodig heeft in het groeimedium en de andere achttien aminozuren zelf kan maken. Ook is deze bacterie in staat om veel verschillende smaakstoffen te produceren. *Lactococcus lactis* en *Lactobacillus plantarum* daarentegen, hebben respectievelijk 6 en 11 aminozuren nodig in het medium en produceren minder smaakstoffen dan *Streptococcus thermophilus*.

De vergelijkende studie van de beschikbare modellen bracht tevens de afwezigheid van een complete pentose fosfaat route in *Streptococcus thermophilus* naar voren, dit wordt verder behandeld in **hoofdstuk 5**.

In **hoofdstuk 4** wordt een vergelijkende studie beschreven naar het effect van een tekort aan aminozuren op de groei van *Streptococcus thermophilus, Lactococcus lactis* en *Lactobacillus plantarum*. Elk van deze melkzuurbacteriën heeft specifieke aminozuurbehoeften (zie vorige paragraaf). We hebben de drie bacteriën gekweekt op een gedefinieerd medium met alle aminozuren (rijk medium) en op een zelfde medium maar dan met een minimal hoeveelheid aan aminozuren (arm medium).
De groei van *Streptococcus thermophilus, Lactococcus lactis* en *Lactobacillus plantarum* is vervolgens vergeleken en de respons is bestudeerd op DNA niveau met zogenaamde micro-arrays. Deze studie liet zien dat niet alleen genen betrokken in het aminozuurmetabolisme anders werden gereguleerd op het arme medium, maar ook genen betrokken bij groei en bij stress.
Daarnaast hebben we monsters genomen voor de analyse van smaakstoffen en deze studie liet, zoals verwacht, zien dat op het arme medium minder smaakstoffen worden gevormd dan op het rijke medium. Een laatste resultaat van deze studie was dat de drie melkzuurbacteriën op zowel rijk als arm medium, enkel melkzuur als eindproduct maken.

**Hoofdstuk 5** behandelt de studie van een glutamaat dehydrogenase mutant van *Streptococcus thermophilus*. In hoofdstuk 3 staat beschreven dat deze bacterie geen complete pentose fostaat

route heeft. Deze route is belangrijk voor de productie van NADPH, een co-factor die nodig is voor diverse biosynthese routes. De afwezigheid van een complete pentose fostaat route is ook experimenteel vastgesteld.

Het model van *S. thermophilus* is gebruikt om te zoeken naar alternatieve routes die NADPH kunnen genereren, mogelijke alternatieven waren; glutamaat dehydrogenase en isocitraat dehydrogenase. De activiteit van beide enzymen is in het lab gemeten en met name glutamaat dehydrogenase activiteit was overtuigend aanwezig. Het is ook bekend dat *Streptococcus thermophilus* glutamaat kan consumeren. Gebaseerd op deze kennis, is besloten om het gen dat codeert voor glutamaat dehydrogenase uit het genoom te verwijderen. Deze *gdh* mutant heeft dus geen glutamaat dehydrogenase activiteit meer.

We hebben een fermentatie uitgevoerd om het wild-type en de *gdh* mutant van *Streptococcus thermophilus* te bestuderen. De mutant produceert net als het wildtype voornamelijk melkzuur en daarnaast consumeert de mutant citroenzuur (citraat). Tevens werd er een hogere mate van aminozuur-verbruik gevonden en als gevolg hiervan een toename in ammonia-vorming. De vorming van smaakstoffen was echter vergelijkbaar tussen het wildtype en de *gdh* mutant.

Tijdens de groei van beide stammen, zijn ook monsters genomen voor een micro-array studie. Met de micro-array kan de respons van organismen op DNA niveau worden bekeken. Deze studie liet een aantal verschillen zien tussen het wildtype en de *gdh* mutant. In de *gdh* mutant zijn een aantal routes in het aminozuurmetabolisme anders gereguleerd dan in het wildtype, evenals een aantal routes betrokken bij groei. Tevens is in de *gdh* mutant de citraat route (inclusief het eerder genoemde isocitraat dehydrogenase) anders gereguleerd dan in het wild-type. We hebben ook citraat consumptie gemeten door de *gdh* mutant, en dit zou erop kunnen wijzen dat isocitraat dehydrogenase inderdaad NADPH zou kunnen maken.

In **hoofdstuk 6** tenslotte, wordt een Engelstalige samenvatting van het uitgevoerde werk beschreven evenals de eindconclusies. Het werk dat staat beschreven in dit proefschrift, zou kunnen helpen bij een betere selectie van melkzuurbacteriën voor bestaande (gefermenteerde) producten en voor de ontwikkeling van nieuwe (gefermenteerde) producten. Tevens geeft dit werk een beter inzicht in aminozuurmetabolisme en de vorming van smaakstoffen, belangrijke onderwerpen voor de levensmiddelenindustrie.

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**Gold Elsevier Award, 5$^{th}$ NIZO Dairy Conference, 2007**

## Training and Supervision Plan (VLAG)

### Courses

- Physiology of food associated organisms, VLAG, Wageningen, 2004
- Metabolomics, VLAG, Wageningen, 2005
- Stralingshygiëne 5b, Larenstein, Wageningen, 2005
- Advanced Course Strategic Communication in Biotechnology, Kluyver Centre, Oxford, 2006
- Advanced Course on Applied Genomics of Industrial Fermentation, Kluyver Centre, Wageningen, 2005
- Systems biology, VLAG/CMBI, Nijmegen, 2005
- Food Fermentation, VLAG, Wageningen, 2008
- Probiotics, NIZO food research, Ede, 2007
- Career assesment, Meijer&Meijaard, Wageningen, 2008
- Time planning and project management, WGS, Wageningen, 2006
- Afstudeervak organiseren en begeleiden, WUR, Wageningen, 2006
- Patent course, Kluyver Centre, Wageningen, 2007
- Writing and presenting a scientific paper, WGS, Wageningen, 2007

### Conferences

- Kluyver Centre for Genomics of Industrial Fermentation symposium (oral and poster presentations), 2005-2009
- 8$^{th}$ international LAB symposium, Egmond aan Zee, (poster presentation), 2005
- 9$^{th}$ international LAB symposium, Egmond aan Zee, (poster presentation), 2008
- 5$^{th}$ NIZO dairy conference, Papendal, (oral and poster presentation), 2007
- 13$^{th}$ European Conference on Biotechnology, Barcelona, (poster presentation), 2007

### Optional activities

- Training at Université Catholique de Louvain (2009)
- Participant PhD study excursion California (2006)
- TIFN WE-days (2005-2009, oral presentations)
- Organizing social program of spring WE-days (2006)
- Teaching and supervision Microbial physiology (2005-2006)

## About the Author

Margreet Ineke (Marieke) Pastink was born on the 17th of March 1981 in Amsterdam. After graduating in 1999 from the Ashram College in Alphen a/d Rijn, she moved to Wageningen where she studied food science at Wageningen University and Research Centre. She completed the study with master courses at the departments of Food Microbiology and Food Chemistry. She also performed research internships at the University of Queensland (Brisbane, Australia) and at Kerry Bioscience in Naarden. She obtained her Master's degree in November 2004. From December 2003 until July 2009, she worked on her PhD studies at the department of Microbiology from Wageningen University. The research was performed at NIZO Food Research and was part of TI Food and Nutrition and Kluyver Centre for Genomics of Industrial Fermentation. The research performed during this period is described in this thesis.

From August 2009, she is working at SenterNovem as National Contact Point Food within the European seventh Frame Work Program.

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