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Metabolic comparison of lactic acid bacteria; genome-scale model of *Streptococcus thermophilus* LMG18311

Margreet I. Pastink[a,b,c], Bas Teusink [a,b,d,$], Pascal Hols[e], Sanne Visser[a,b], Willem M. de Vos[c], Jeroen Hugenholtz [a,b,d,*]

[a] *Top Institute Food and Nutrition, Nieuwe Kanaal 9a, 6709 PA Wageningen, the Netherlands*

[b] *Kluyver Centre for Genomics of Industrial Fermentation, Julianalaan 67, 2628 BC Delft, the Netherlands*

[c] *Laboratory of Microbiology, Wageningen University, Dreijenplein 10, 6703 HB Wageningen, the Netherlands*

[d] *NIZO Food Research, Kernhemseweg 2, 6718 ZB Ede, the Netherlands*

[e] *Unité de Génétique, Institut des Sciences de la Vie, Université catholique de Louvain, 5 Place Croix du Sud, 1348 Louvain-La-Neuve, Belgium*

Running title :Comparative metabolic analysis of LAB

*Corresponding author. Mailing address: NIZO food research, P.O. Box 20, 6710 BA Ede, the Netherlands. Tel: +31-318-659540; fax: +31-318-650400

E-mail address: Jeroen.Hugenholtz@nizo.nl

$Present address: Systems Bioinformatics IBIVU, Vrije Universiteit Amsterdam, De Boelelaan 1085, 1081HV Amsterdam, the Netherlands

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™ 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 S5, 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

132 x 50 ml samples were taken and spun down at 4°C in a Unicen MR (Herolab, The
133 Netherlands). Supernatant was used for HPLC analysis of organic compounds (28).
134
135 **GC analyses.** For the identification of volatile components in the samples, purge and
136 trap thermal desorption cold trap gas-chromatography (GC) was used as described
137 before (7, 27). The headspace samples were concentrated on a Fisons MFA815 cold
138 trap (CE Instruments, Milan, Italy), followed by separation on a GC-8000 top gas
139 chromatograph (CE Instruments) equipped with a CIP-SIL 5 CB low-bleed column
140 (Chrompack, Middelburg, The Netherlands) and detection by a flame ionization
141 detector. The GC data were processed in MetAlign, a tool (developed by Plant
142 Research International, The Netherlands) to align spectra and to identify significant
143 differences between the spectra (6, 16).
144
145 **HPLC analyses**. Extracellular metabolites present in the supernatant of fermentation
146 samples were measured using reversed phase HPLC wit a C18-column as described
147 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. 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.

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 S1). 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). 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

198 shows (minimal) growth in the presence of citrate, when both glutamate and
199 glutamine were depleted from the medium. However, *S. thermophilus* possesses a
200 pathway for the synthesis of glutamate from citrate via 2-oxoglutarate involving
201 glutamate dehydrogenase and glutamine synthetase for interconversion between
202 glutamic acid and glutamine
203 Different LAB have different absolute requirements for amino acids; *S. thermophilus*
204 only needs 2 amino acids as described above whereas *L. lactis* and *Lb. plantarum*
205 need 6 and 11 amino acids for minimal growth respectively (Table 3) (12, 30).
206
207 **GC analyses.** In order to get an overview of flavor formation by the three different
208 LAB, we compared fermentation samples using gas chromatography (GC). The
209 headspace of steady state samples of *S. thermophilus* LMG18311, *L. lactis* MG1363
210 and *Lb. plantarum* WCFS1 grown on CDM (containing all amino acids) was
211 compared. The metabolic activities of the fermenting microbes (22) was investigated
212 through flavor profiles in the fermentation fluids, corrected for the medium
213 components at the start of the experiments. An overview of the volatile metabolic
214 products is shown in Figures 2, 3 and 4 and they show multiple differences in the
215 volatile profiles of different strains. Many volatiles or flavors are produced during
216 amino acid metabolism. When the results of the GC analyses of the three LAB are
217 compared, they show that *S. thermophilus* is able to produce a broad variety of
218 flavors. In combination with the low requirements of amino acids (only 2), this
219 reflects a relatively complete set of amino acid biosynthetic and amino acid
220 converting pathways. When *S. thermophilus* grows on CDM, all amino acids are
221 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).

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

284 acids and is able to produce a varied amount of amino acid derived flavors. The
285 genome-scale model, supported by the overall experimental data, suggests a rather
286 complete set of amino acid biosynthesis pathways in *S. thermophilus*. This is
287 unexpected because *S. thermophilus* is used for centuries for the production of
288 yoghurt. The LMG18311 strain is also a yoghurt strain (11). The assumption would be
289 that *S. thermophilus* has evolved in this protein rich environment (milk) and therefore
290 one may have expected loss of some or more amino acid biosynthesis pathways, but
291 this is clearly not the case. It would be interesting to see if all these pathways are
292 operated under all conditions during the different dairy fermentation processes. These
293 studies in which expression data under different interesting conditions are involved,
294 are currently under investigation. Intriguingly, *Lactobacillus delbrueckii* subsp.
295 *bulgaricus*, an organism that is most often co-cultivated with *S. thermophilus* for
296 yoghurt manufacturing, did follow this expected path and lost most of its amino acid
297 biosynthetic capacity (31). An explanation for this unexpected behavior of *S.*
298 *thermophilus* can be that amino acid metabolism is not only important for the
299 synthesis of amino acids but also plays a role in maintaining the redox balance.
300 Another explanation can be that *S. thermophilus* strains are selected for quick growth
301 and acidification in milk, available amino acid are rate-limiting in milk. To support
302 such a quick growth, maintenance of nearly all amino acid pathways is required.
303 In the result section, an *in silico* prediction of the amino acid biosynthesis pathways is
304 described. This analysis showed that *ychE* is truncated by a conserved stop codon. It
305 would be interesting to reconstitute this codon and study the effect of an activated
306 codon. This mutated strain probably only needs one amino acid (histidine) and
307 complete pathways for the sulfur amino acid metabolism may have important effects
308 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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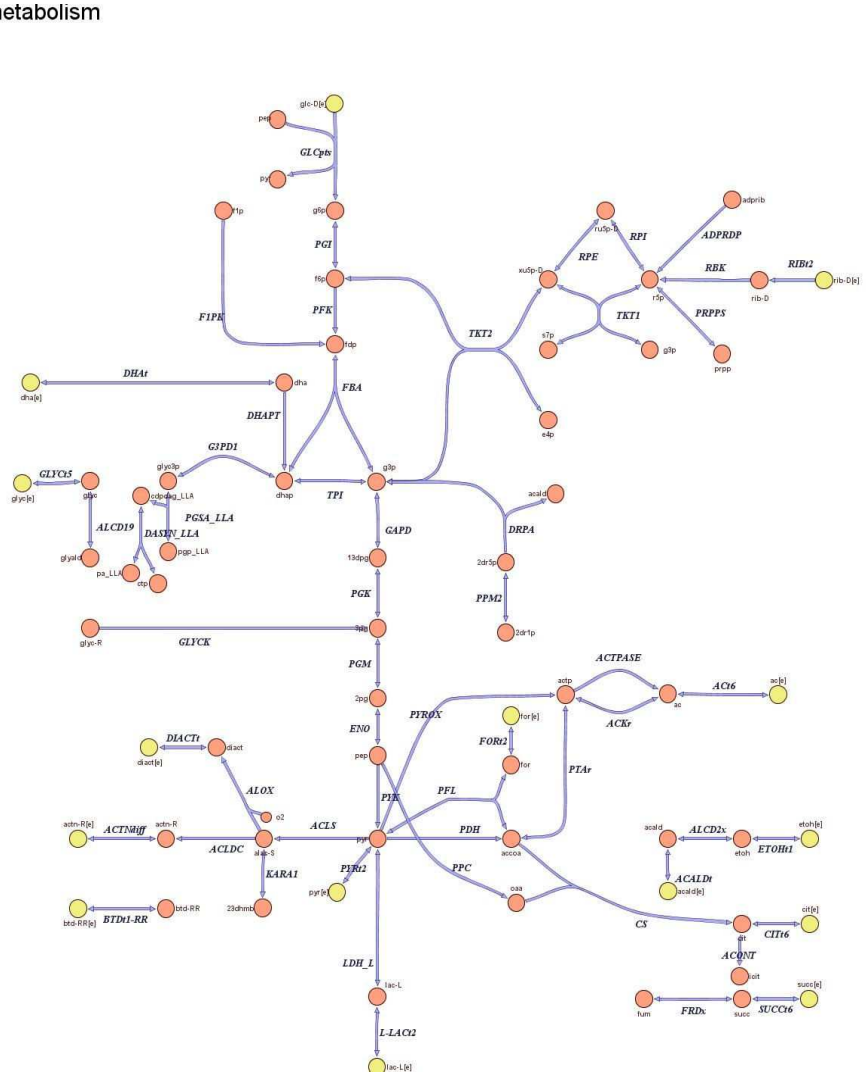
333 **Tables and Figure**

334 FIG. 1. Primary metabolism of *Streptococcus thermophilus*. Part of the total genome-
335 scale metabolic model developed for *S. thermophilus*. Large bold capital italics
336 indicate the enzymes and normal small italics the metabolites. The complete model
337 can be found in the supplementary material (Figure S1).
338
339



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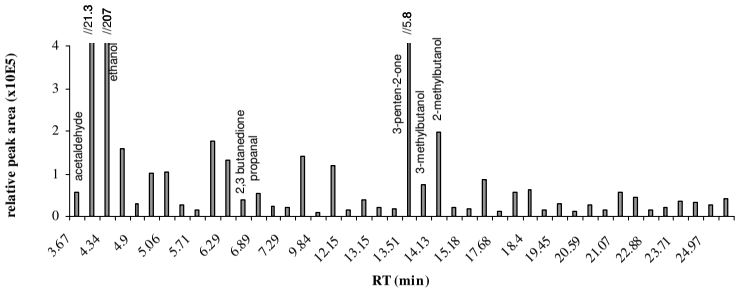

FIG. 2 Major volatiles formed during growth by *L. lactis* on chemically defined medium. Relative peak areas are expressed as arbitrary units, the area of three peaks is indicated since they are beyond the scale. Some important peaks are indicated. Table S2 (Supplemental material) shows all the identified metabolites for *L. lactis*.

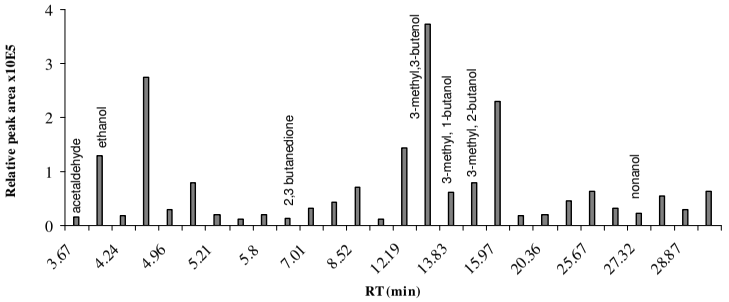

FIG. 3 Major volatiles formed during growth by *Lb. plantarum* on chemically defined medium. Relative peak areas are expressed as arbitrary units. Some important peaks are indicated. Table S3 (Supplementary material) shows all the identified metabolites for *Lb. plantarum*.

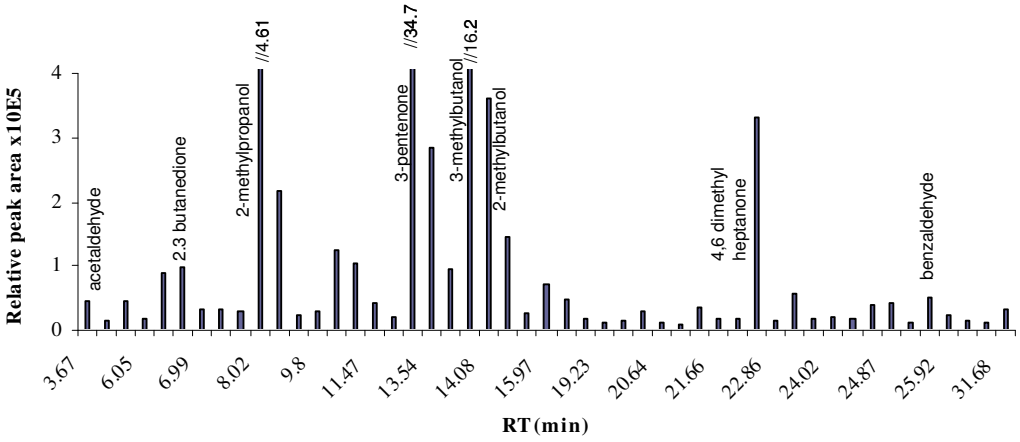

FIG. 4 Major volatiles formed during growth by *S. thermophilus* on chemically defined medium. Relative peak areas are expressed as arbitrary units, the area of three peaks is indicated since they are beyond the scale. Some important peaks are indicated. Table S4 (Supplementary material) shows all the identified metabolites for *S. thermophilus*.

TABLE 1 Biomass composition of three different LAB: *L. lactis* (20), *Lb. plantarum* (30) and *S. thermophilus* LMG18311 (this study; average of 3 fermentations).

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

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 S1)

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

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	

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

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