THESIS ON NATURAL AND EXACT SCIENCES B207

Development and Applications of Chemically Defined Media for Lactic Acid Bacteria

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Declaration:
I hereby declare that this doctoral thesis, submitted for the doctoral degree at Tallinn University of Technology, is my original investigation and achievement and has not been submitted for the defence of any other academic degree elsewhere.

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LOODUS- JA TÄPPISTEADUSED B207

Defineeritud koostisega söötmete arendamine ja kasutusalad piimhappebakteritele

KADRI ALLER

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CONTENTS

ABSTRACT

Lactic acid bacteria are a diverse group of microorganisms that have played an important role in the making of various fermented food products for millennia. Metabolic engineering provides novel applications for these microorganisms, e.g. the production of recombinant proteins or functional compounds. In order to achieve high biomass yields or efficient biosynthesis of value-added metabolites, a suitable growth medium has to be used. Tailor-made chemically defined media for specific strains are an appropriate choice for metabolism studies and other scopes, providing more accurate, easier to explain and better reproducible data than rich media. As lactic acid bacteria are fastidious in nutritional requirements and each species has its own demands, determining nutrient auxotrophies is the first step in developing a suitable synthetic medium. The initial screening of growth demands and proper medium composition is time-consuming and expensive and should be carried out on microtiter plates or test tubes to keep experiments economic. Various types of continuous cultivations, however, are fit for metabolism studies. For instance, examination of amino acid degradation with changestat cultivations offers useful biochemical data for the production of recombinant proteins, aroma compounds or other valuable substances.

This thesis overviews literature data about nutritional requirements and defined media for various lactic acid bacteria, as well as provides newly compiled and balanced synthetic media for the growth of the lactic acid bacterium *Lactococcus lactis* IL1403. The necessity of amino acids, B-group vitamins and metal ions for this strain was experimentally confirmed and the possible reasons behind their essentialness were backed up by literature data. In total, 112 defined media with reduced concentrations of amino acids or metal ions were proposed in this thesis in addition to the initial medium BS7, which are all suitable for the metabolic studies of *Lactococcus lactis*. Amongst these media, the fitness of K40 and V19 was emphasized most as being more balanced in nutrient concentrations and offering higher maximal specific growth rate values than other media for lactic acid bacteria reported in the literature. In the process of developing synthetic media, the tendency of *Lactococcus lactis* to prefer biosynthesis of certain amino acids to their uptake was noticed. Furthermore, in media with reduced concentrations of nutrients, rearrangements in the consumption of arginine, glutamine and pyruvate-directed amino acids and an increased flux of amino acids to cellular proteins were observed. Amino acids were grouped based on whether these were mainly used for the formation of biomass proteins or for other purposes as well. This information is useful for the development of synthetic media for other lactococcal strains as well as for increasing amino acid fluxes for the biosynthesis of recombinant proteins or amino acid degradation products. Lastly, the usefulness of synthetic media for metabolic research was illustrated by determination of the threonine aldolase pathway in *Lactococcus lactis*.

KOKKUVÕTE

Piimhappebakterid on mitmekesine mikroorganismide rühm, kes on aastatuhandeid mänginud tähtsat rolli paljude kääritatud toiduainete valmimisel. Geenitehnoloogia abiga on võimalik piimhappebaktereid kasutada ka rekombinantsete valkude ja funktsionaalsete, s.t inimorganismile kasulike ühendite tootmiseks. Kõrge biomassi saagise ja efektiivse keemiliste ainete tootmise tagamiseks tuleb piimhappebakterite kultiveerimiseks kasutada sobilikku söödet. Defineeritud koostisega söötmed ehk söötmed, mille kõik keemilised komponendid ja nende kontsentratsioonid on teada, on sobilikud bakterite ainevahetusreaktsioonide uuringuteks ja muudeks eesmärkideks, sest nad võimaldavad saada täpsemaid, kergemini tõlgendatavaid ja korratavamaid katsetulemusi kui söötmed, mis sisaldavad pärmiekstrakti või muid keerulise koostisega komponente. Piimhappebakterid on oma kasvukeskkonna suhtes väga nõudlikud ja igal tüvel on erinevad vajadused. Seetõttu on konkreetse tüve jaoks hädavajalike toitainete kindlakstegemine esimene samm uue söötme arendamisel. Kuna kasvuvajaduste väljaselgitamine ja sobiliku söötmekoostise leidmine on aeganõudev ja kallis protsess, on seda soovitav teostada mikrotiiterplaatidel või katseklaasides. Pidevkultivatsioonid bioreaktorites on seevastu sobilikud metaboolsete uuringute läbiviimiseks. Näiteks võib piimhappebakterite aminohapete tarbimisstrateegiate ja lagundamisreaktsioonide jälgimine muutuvstaatseid kultivatsioonitehnikaid kasutades anda väärtuslikku informatsiooni rekombinantsete valkude, aroomiühendite ja teiste hinnaliste produktide tootmiseks.

Käesolev doktoritöö annab ülevaate kirjanduses leiduvatest andmetest erinevate piimhappebakterite kasvuvajaduste ja defineeritud söötmete kohta ning esitab 112 uut tasakaalustatud koostisega söödet piimhappebakteri *Lactococcus lactis* IL1403 kultiveerimiseks, mis sobivad metaboolseteks uuringuteks. Eriti tõsteti esile tasakaalustatud aminohapete koostisega söödet K40 ja vähendatud metalliioonide kontsentratsioonidega söödet V19, mille abil on võimalik saavutada suuremat kasvuerikiirust kui teiste kirjanduses käsitletud minimeeritud söötmetega. Doktoritöös määrati kindlaks mainitud tüve kasvuks hädavajalikud aminohapped, B-rühma vitamiinid ja metalliioonid ning põhjendati nende olulisust kirjandusest leiduvate andmete abil. Täheldati, et *Lactococcus lactis* eelistab mõningaid aminohappeid tarbimise asemel ise biosünteesida. Lisaks märgati, et toitainete kontsentratsioonide vähendamine söötmes tõi kaasa mõnede aminohapete tarbimisstrateegiate muutuse ning suurendas aminohapete voogu biomassi valkudesse. Aminohapped jagati rühmadeks vastavalt sellele, kas neid kasutati peamiselt biomassi valkude tootmiseks või ka teistel eesmärkidel. Saadud andmeid on võimalik kasutada söötmete väljatöötamisel nii teiste piimhappebakterite kultiveerimiseks kui ka rekombinantsete valkude ja aminohapete laguproduktide tootmise suurendamisel. Defineeritud koostisega söötmete sobilikkust aminohapete lagundamisradade uurimiseks tõestati katsetega, mis kinnitasid treoniini aldolaasi katalüüsitava reaktsiooni olemasolu piimhappebakteris *Lactococcus lactis*.

LIST OF PUBLICATIONS

This thesis is based on the following publications which are reproduced in the appendices with permission from the publishers:

I **Aller K**, Adamberg K, Timarova V, Seiman A, Feštšenko D, Vilu R (2014) Nutritional requirements and media development for *Lactococcus lactis* IL1403. Appl Microbiol Biotechnol 98(13):5871-5881.

II **Aller K**, Adamberg K, Reile I, Timarova V, Peebo K, Vilu R (2015) Excess of threonine compared with serine promotes threonine aldolase activity in *Lactococcus lactis* IL1403. Microbiology 161(Pt 5):1073-1080.

III **Aller K**, Adamberg K, Timarova V, Seiman A, Vilu R (2016) Reduction of nutrient concentrations in a chemically defined medium increases amino acid usage efficiency in *Lactococcus lactis* IL1403. The manuscript has been submitted to FEMS Microbiology Letters.

IV Lahtvee PJ, Adamberg K, Arike L, Nahku R, **Aller K**, Vilu R (2011) Multi-omics approach to study the growth efficiency and amino acid metabolism in *Lactococcus lactis* at various specific growth rates. Microb Cell Fact 10(12).

Author's Contribution to the Publications

In publication I, the author designed and coordinated the project and the experiments, performed most of the experiments, performed most of the analyses (excluding partial least square regression and vitamin analysis), analyzed the data and wrote the manuscript.

In publication II, the author designed and coordinated the project and the experiments, performed all the experiments, performed most of the analyses (excluding nuclear magnetic resonance and proteomics), analyzed the data and wrote the manuscript.

In publication III, the author designed and coordinated the project and the experiments, performed most of the experiments, performed most of the analyses (excluding ATP spilling calculations), analyzed the data and wrote the manuscript.

In publication IV, the author contributed to carrying out the experiments and to analyzing the samples and the data.

LIST OF CONFERENCE PRESENTATIONS

Aller K, Adamberg K, Timarova V, Feštšenko D, Vilu R. Comparability of different cultivation methods in media development experiments for *Lactococcus lactis* subsp. *lactis* IL1403.
Poster presentation at the Summer Conference of the *Society for Applied Microbiology (SfAM)*, July 2013, Cardiff, UK.

Aller K, Adamberg K, Timarova V, Vilu R. Effect of L-threonine concentration in the medium on the biosynthesis of glycine in *Lactococcus lactis* ssp. *lactis* IL1403.
Poster presentation at *The 5th Congress of European Microbiologists (FEMS)*, July 2013, Leipzig, Germany.

Aller K, Adamberg K, Timarova V, Vilu R. Media development experiments for lactic acid bacteria: stressful, yet useful.
Poster presentation at the conference of the graduate school *Functional materials and technologies* for PhD students from Tallinn University of Technology and University of Tartu, March 2014, Tartu, Estonia.

Aller K, Adamberg K, Timarova V, Seiman A, Vilu R. Fine-tuning of defined media for chemical production by lactic acid bacteria.
Poster presentation at *The 16th European Congress on Biotechnology (ECB16)*, July 2014, Edinburgh, UK.

Aller K, Adamberg K, Peebo K, Timarova V, Vilu R. L-threonine catabolism to glycine in *Lactococcus lactis* IL1403.
Poster presentation at *The 11th International Symposium on Lactic Acid Bacteria (LAB11)*, September 2014, Egmond aan Zee, The Netherlands.

INTRODUCTION

Metabolic engineering provides the opportunity to exert lactic acid bacteria, a group of procaryotes that have a generally-recognized-as-safe status, as cell reactors for the production of numerous beneficial chemical compounds. In order to understand the mechanisms of biosynthesis of various metabolites and to increase the bioproduction of chemicals, the metabolism of these microorganisms has to be studied in detail. For this purpose, cultivation experiments with chemically defined media are appropriate as these provide more comprehensible and reproducible data than rich, yeast extract containing media. However, distinct species of lactic acid bacteria differ in nutritional requirements and thus, defined media are often tailor-made for a specific species. Nevertheless, once a suitable medium is developed for a certain lactic acid bacterium, it offers miscellaneous applications, e.g. verifying the existence of amino acid catabolic pathways in a particular strain.

This thesis provides information about the nutritional requirements as well as newly composed synthetic media for the cultivation of the lactic acid bacterium *Lactococcus lactis* IL1403. The media developed herein are suitable for studying the catabolic pathways of amino acids in both batch and continuous cultivations. Moreover, these media are more balanced in their nutrient composition and enable higher specific growth rates than media reported earlier in literature. Additionally, the literature overview summarizes data about nutritional requirements and chemically defined media that are appropriate for the cultivation of other lactic acid bacteria. Therefore, this thesis can be used by other researchers as a textbook to gather pertinent information prior to performing experiments with different species of lactic acid bacteria.

ABBREVIATIONS

ADI pathway – arginine deiminase pathway
AGDI pathway – agmatine deiminase pathway
BCAAs – branched-chain amino acids
CDM – chemically defined medium
CMP – cytidine monophosphate
CoA – coenzyme A
dCMP – deoxycytidine monophosphate
dTMP – thymidine monophosphate
E. faecalis – Enterococcus faecalis
FAD – flavin adenine dinucleotide
FMN – flavin mononucleotide
GABA – *gamma*-aminobutyric acid; γ-aminobutyric acid
GRAS – generally recognized as safe
GTP – guanosine 5'-triphosphate
HPLC – high performance liquid chromatography
L. cremoris – Lactococcus lactis subspecies *cremoris*
*L. lactis – Lactococcus lactis*subspecies *lactis*
LAB – lactic acid bacteria
Lb. – Lactobacillus
Lb. bulgaricus – Lactobacillus delbrueckii subspecies *bulgaricus*
LC-MS – liquid chromatography mass spectrometry
Leuc. mesenteroides – Leuconostoc mesenteroides subspecies *mesenteroides*
MOPS – 3-(*N*-morpholino)propanesulfonic acid
$NAD(P)^+$, NAD(P)H – nicotinamide adenine dinucleotide (phosphate)
NMR – nuclear magnetic resonance
O. oeni – Oenococcus oeni
OD – optical density
ORFs – open reading frames
P. pentosaceus – Pedicoccus pentosaceus
*p*ABA – *para*-aminobenzoic acid
PLP – pyridoxal 5'-phosphate
PLSR – partial least square regression analysis
PRPP – 5-phosphoribosyl diphosphate
S. – Streptococcus
SHMT – serine hydroxymethyltransferase
Ssp. – subspecies
TA – threonine aldolase
TDH – threonine dehydrogenase
UMP – uridine monophosphate
UPLC – ultra performance liquid chromatography
W. paramesenteroides – Weissella paramesenteroides

1. LITERATURE OVERVIEW

1.1. Lactic acid bacteria and their applications

Lactic acid bacteria (LAB) are a diverse group of Gram-positive microorganisms that catabolize sugars and other carbon sources into lactic acid. LAB incorporate the following genera: *Carnobacterium*, *Enterococcus*, *Fructobacillus, Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, *Vagococcus* and *Weissella* [1-2]. The cells of LAB are cylindrical (bacilli) or spherical (cocci) and may form pairs or chains [3]. Table 1 and Table 2 show the biomolecular composition of LAB cells and the amino acid content of cellular proteins and peptidoglycan. Lactobacilli and lactococci have a generally-recognized-as-safe (GRAS) status, but some streptococci may cause diseases or tooth deacy [1, 4]. LAB vary in nutritional requirements and stress tolerance and can be found in a wide range of habitats such as grasslands, fruits and vegetables, milk products, fish, gut and mucosal surfaces of mammals [1, 4-5].

Table 1. The biomolecular composition of LAB cells (%).

Species	*L. lactis*	*L. lactis*	*Lb. casei*	*Lb. plantarum*	*S. thermophilus*
Strain	IL1403	NCDO2118	LC2W	WCFS1	LMG18311
Reference	[6][1]	[7]	[8]	[9]	[10]
Composition (%)					
Proteins	46	45	33.5	29.9	43.4
RNA	4	8	29.7	9	8.2
DNA	1	3	6.3	1.9	1
Lipids	7	4.3	7.9	6.3	6.1
Polysaccharides	5	12	8.1	9.9	24.1
Teichoic acid		10			
Wall teichoic acids				13.8	
Lipoteichoic acids			5.9	4.1	
Peptidoglycans			8.7	14.5	
Amino sugars	25	5.5			
Inorganic ions		7			
Other[2]	12	5.2		10.6	17.2

[1] At $\mu = 0.1\ h^{-1}$.

[2] Components not analyzed, e.g. teichoic acid in [6] and [10], peptidoglycans in [10], etc.

The transport systems for nutrient uptake and metabolite secretion are divided into four classes: (i) primary transport systems (electron transport, ABC transporters, ATPases), (ii) secondary transport systems (uniport, symport (proton/solute) and antiport (proton/solute or solute/H^+)), (iii) protein secretion systems and (iv)

phosphoenol-pyruvate phosphotransferase systems [11]. LAB can be divided into homo- and heterofermentative subgroups based on metabolism. Homofermentative LAB (lactococci, pediococci, streptococci, some lactobacilli) degrade hexoses through glycolysis primarily to lactic acid, but mixed acid fermentation, i.e. the production of other compounds from pyruvate, e.g. formate, acetate, ethanol, etc., may occur as well, depending on growth conditions [4]. Heterofermentative LAB (*Oenococcus* and *Leuconostoc* ssp.; some lactobacilli) degrade hexoses through the pentose phosphate route into lactate, acetate, ethanol, CO_2 and other products [4, 12].

Table 2. Amino acid composition (%) of LAB biomass.

Species	*L. lactis*	*L. lactis*	*L. lactis*	*L. lactis*	*L. lactis*	*Lb. plantarum*
Strain	IL1403	IL1403	MG1363	ML3	NCDO 2118	WCFS1
Reference	[13][1]	[14][2]	[15]	[16]	[7]	[9]
Composition (%)						
Ala	7.0	8.3	9.6	12.9	12.8	4.2
Arg	3.7	5.2	4.0	3.6	3.8	1.5
Asn+Asp	10.2 [3]	11.5	9.6 [4]	10.9	9.9	3.3
Cys	0.5	ND	0.5	0.1	2.9	0.1
Gln+Glu	10.7 [5]	15.4	11 [6]	10.6	10.5	5.2
Gly	6.2	4.5	10.7	12.9	8.8	1.2
His	1.8	2.1	1.8	2.2	1.4	0.6
Ile	7.9	5.6	5.6	5.6	5.3	1.2
Leu	10	8.3	8.3	8.3	7.3	2.1
Lys	7.9	10.1	5.9	4.0	8.7	2.2
Met	2.6	2.2	2.0	2.2	2.2	0.7
Phe	4.8	4.9	3.9	3.3	3.2	1.2
Pro	3.0	3.1	4.2	5.1	2.9	0.9
Ser	6.4	4.2	5.6	3.3	4.4	1.2
Thr	5.5	5.4	6.5	5.7	5.0	1.6
Trp	1.1	ND	0.8	ND	2.2	0.3
Tyr	3.6	3.6	2.9	2.0	2.3	1.1
Val	6.5	6.0	7.1	7.1	6.6	1.4

[1] In the CDM used for cultivation the isoleucine concentration was limited.
[2] The CDM used contained no glycine.
[3] 5.1% of asparagine and 5.1% of aspartate.
[4] 4.8% of asparagine and 4.8% of aspartate.
[5] 3.6% of glutamine and 7.1% of glutamate.
[6] 5.5% of glutamine and 5.5% of glutamate.

Due to their ability to contribute to the aroma, texture, healthiness and shelf-life of food, LAB have been used unknowingly (and nowadays knowingly) in the production of various food products for thousands of years. LAB are essential in the manufacturing of milk products, e.g. cheese, buttermilk, yoghurt, kefir, sour cream, etc. [17-18]; meat (e.g. sausages) and fish fermentation [19-20]; fruit and vegetable

fermentation [21]; sourdough bread [22]; alcoholic beverages, i.e. beer and wine [23-24], and cocoa bean fermentation [25].

In addition to contributing to the maturing of certain food products, LAB can be used to produce various health-promoting functional compounds directly into food or in bioreactors to be used in food supplements or pharmaceuticals [26-29]. These compounds include B-group vitamins, especially B_2, B_9 and B_{12} [30-32]; low-calorie sweeteners, e.g. mannitol [33-34]; exopolysaccharides [35-37]; aroma compounds [38, 39]; γ-aminobutyric acid [40-41]; etc. The concentration of toxic metal ions in food and drinking water can be decreased by introducing LAB [42].

In medicine, LAB have emerged as promising new hosts for oral vaccine delivery [43], recombinant protein production [44-45] and biosynthesis of bacteriocins, which are viable alternatives for antibiotics [46-47]. In the chemical industry, LAB can be used to produce commodity chemicals such as lactate [48] or 2,3-butanediol [49]. Proteins extracted from LAB have been shown to modify the growth of minerals [50].

1.2. The species *Lactococcus lactis*

Lactococcus lactis is a catalase-negative, non-spore forming and facultatively anaerobic LAB that can be found in diverse niches from cheese to fermented corn [3, 51]. It is divided into four subspecies: *lactis* (formerly *Streptococcus lactis*), *cremoris* (formerly *S. cremoris*), *hordniae* and *tructae*, whereas the subspecies *lactis* has a biovariant *diacetylactis* which degrades citrate into diacetyl and acetoin [51]. *L. lactis* strains have been shown to be more tolerant to heat and oxidative stress conditions than *L. cremoris* strains [52]. The genome of *L. lactis* IL1043, a strain studied in this thesis, has been sequenced and annotated: it consists of 2.4 Mbp and has 2310 open reading frames (ORFs) [53]. The subspecies *lactis* and *cremoris* have 85% overall identity based on comparative genome analysis [51].

The subspecies of *L. lactis* are homofermentative and can be distinguished by the following phenotypic traits: *tructae* and *cremoris* (excluding the MG1363 strain) cannot deaminate arginine, *hordniae* is unable to ferment lactose, *cremoris* is incapable of growing at 40°C or in 4% (w/v) NaCl and *lactis* can produce γ-aminobutyric acid [51]. *L. cremoris* has shown to have homolactic fermentation at higher growth rates, whereas mixed-acid fermentation, i.e. production of formate, acetate and ethanol in addition to lactate, was observed at lower μ values due to higher pyruvate formate-lyase activity [54].

Lactococcus lactis has a wide variety of applications, including the making of cheese [55], vaccine delivery [56] and production of various compounds either naturally or by metabolic engineering: recombinant proteins [45, 57], ethanol [58], exopolysaccharides [59-60], B-group vitamins – folates and riboflavin [61-63], aroma compounds – acetaldehyde, diacetyl and acetoin [64-66], alanine [67], glucose [68], 2,3-butanediol and mannitol [69], biopolyesters [70], etc.

1.3. Types and applications of cultivation media for lactic acid bacteria

The growth media used for the cultivation of microorganisms can be divided into [71]:

(i) **Rich** or **complex media**, which contain components that are rich in nutrients, e.g. yeast extract, meat extract, peptone, tryptone. Thus, the exact chemical composition of the medium is unknown. Examples are MRS [72] and M17 [73].
(ii) **Semi-defined media** consist of defined components with known concentrations (e.g. amino acids) and a few more complex compounds such as peptides [74-76].
(iii) **Chemically defined** or **synthetic media**, which consist only of defined components with known concentrations, e.g. sugars, amino acids, metal ions, vitamins, nucleotides, fatty acids etc. Examples are presented in Supplementary Tables S6, S7 and S8 in the Electronic Appendix.

The main differences between rich and chemically defined media (CDMs) are presented in Table 3. A CDM, which has been minimized both in the number of components and their concentrations, can be classified as a **minimal medium**.

If a high yield of biomass (e.g. that to be used as starter in the food industry) or high concentrations of microbial metabolites are desired, a rich medium should be used. Examples for metabolite production with yeast extract based rich media include GABA [77] and mannitol [78]. However, lots for inexpensive carbon and nitrogen sources may vary and thus rich media may not provide reproducible results. In addition, as the composition of a rich medium is not fully defined, data about substrate consumption and the biosynthesis of metabolites may be misinterpreted. CDM is an appropriate option for metabolism studies (i.e. gene regulation, protein expression, metabolic fluxes) as it reduces the inconstancy of the process, affords reproducible results and more explainable data [71, 79]. Note that the composition of a CDM used for metabolic studies has to be **balanced**, i.e. match the requirements of cells and not contain an excess of nutrients. The cost of the growth medium is lower, analytical measurements are more precise and a CDM is more appropriate for metabolic screening if the residual concentrations of nutrients are as low as possible after the growth of cells [80, 88].

Table 3. The comparison of rich and chemically defined media.

	Rich medium	**CDM**
Scope	Industrial production of biomass and microbial metabolites	Production of microbial metabolites; metabolism studies
Composition	An environment rich in nutrients. Exact composition and concentrations undefined	Simple compounds with known concentrations
Biomass yield	High	Low
Price	Low	High
Preparation	Fast and simple	Time-consuming
Reproducibility	Inconsistent results	High
Data interpretation	Aggravated	Explainable data

In addition to metabolism studies, CDMs are applicable in the following cases:

(i) the production of several functional compounds, e.g. exopolysaccharides or bacteriocins, because of the complexity of downstream processing and purification of these products in rich media [81-83];
(ii) incorporation of amino acid analogues, e.g. tryptophan analogues or selenomethionine, into proteins during heterologous protein production [84, 85].

Tailor-made CDMs for several LAB, e. g. for exopolysaccharide production, have been introduced in the literature and can be modified to match the growth demands of specific strains (Supplementary Tables S7 and S8 in the Electronic Appendix). Some CDMs are more abundant in nutrients and are thus suitable for a wide variety of LAB species (Supplementary Table S6 in Electronic Appendix). These CDMs can be used as basal media for further medium optimization for individual strains. Lowering the concentrations of nutrients can be a trade-off between the growth of cells and medium cost [86]. Nevertheless, if a proper ratio between nutrient concentrations is achieved, the efficiency of metabolism is increased at lower nutrient availability [87-88]. Response surface methodology (RSM) [89] and the nonlinear design of experiment (*n*-DOE) [90] approaches reduce the cost and time of medium optimization experiments. The simultaneous omission of several nonessential nutrients commonly results in low biomass yield and maximal specific growth rate [86, 91-92]. Thus, the concentrations of nonessential nutrients should be reduced rather than discarded entirely [80]. Tips and tricks for assessing the appropriateness of medium composition can be found in the work of Egli and coworkers [93]. Commonly, one compound of the medium (usually the carbon source) is kept in limited concentration and this substance determines the requirements for all other nutrients [93].

1.4. Growth requirements of lactic acid bacteria

In the course of evolution, LAB have developed symbiont/parasite relationships with plants and animals that provide them with plentiful nutrients. Thus, LAB lack the ability to biosynthesize several vitamins, amino acids and other compounds and are fastidious in their growth requirements [4]. Rich media supply all nutrients required for the growth of cells due to the complex nature of yeast extracts. To a CDM, however, amino acids, vitamins, mineral salts and other compounds have to be added individually. The genome of *L. lactis* IL1403, for example, contains biosynthetic genes of 20 amino acids and at least four cofactors (folic acid, menaquinone, riboflavin, thioredoxin), but some of these genes are probably not functional as IL1403 is auxotrophic for several nutrients [53, 79-80, 91].

Nutritional requirements of numerous LAB have been determined by the single omission (leave-one-out) technology, where one compound of CDM is omitted from the medium and the effect on the growth of cells is observed [10, 15, 79-81, 91, 94-108]. However, not all authors use the same criteria for assessing the significance of nutrients. For example, some reseachers define a nutrient as essential or nonessential if the optical density (OD) in a CDM lacking this compound is respectively less than 15-50% or more than 70-90% of that obtained in a complete medium [80, 98-100, 103]. Note that the nutritional requirements of strains may depend on their isolation source, storage time in culture collection and the medium used for strain depositing [109]. Supplementary Tables S1-S5 (Appendix 1) contain literature data about the nutritional requirements of LAB and were used for compiling Table 4. The necessity of nutrients for the growth of LAB were assessed and the amino acids arginine, histidine, isoleucine, leucine, methionine and valine, and Mg^{2+} and vitamins B_2, B_3, B_5 were revealed to be the top 10 most required compounds (Table 4).

Table 4. Importance of nutrients for LAB and the percentage of investigated LAB species requiring the indicated nutrients for growth. Data was taken from Supplementary Tables S1-S5, Appendix 1. [1]

Group	Nutrient	%	Group	Nutrient	%
Vitamin	B_5	94	Amino acid	Ala	19
Amino acid	Leu	91	Amino acid	Asn	18
Amino acid	Val	91	Vitamin	B_7	16
Vitamin	B_3	86	Nucleotide	Guanine	14
Amino acid	Ile	83	Vitamin	B_1	11
Amino acid	Arg	76	Metal ion	Ca	10
Amino acid	Met	72	Metal ion	Cu	10
Metal ion	Mg	66	Vitamin	B_{12}	9
Amino acid	His	66	Fatty acid	Lipoic acid	9
Vitamin	B_2	59	Metal ion	B	8
Amino acid	Trp	52	Metal ion	Co	8
Amino acid	Cys	47	Metal ion	Fe	8
Vitamin	B_6	45	Metal ion	Mo	8
Amino acid	Phe	44	Metal ion	Zn	8
Amino acid	Thr	43	Amino acid	Asp	6
Metal ion	Mn	43	Vitamin	*p* ABA	5
Nucleotide	Uracil	39	Nucleotide	Inosine	4
Amino acid	Tyr	38	Metal ion	Na	3
Fatty acid	Tween 80	37	Vitamin	Inositol	2
Amino acid	Pro	35	Vitamin	Vitamin C	2
Amino acid	Glu	30	Nucleotide	Deoxythymidine	1
Amino acid	Lys	30	Nucleotide	Cytosine	0
Amino acid	Ser	30	Nucleotide	Deoxyguanosine	0
Nucleotide	Adenine	28	Nucleotide	Hypoxanthine	0
Amino acid	Gln	27	Metal ion	Li	0
Vitamin	B_9	25	Metal ion	Ni	0
Amino acid	Gly	22	Fatty acid	Oleic acid	0
Fatty acid	Acetate	22	Nucleotide	Orotic acid	0
Nucleotide	Xanthine	20	Nucleotide	Thymine	0

[1] The necessity of nutrients was calculated by comparing individual scores of nutrients to the respective number of species examined. Individual scores for nutrients for each species were calculated as follows: essential – 1 point, essential/stimulatory – 0.75 points, stimulatory or essential/nonessential – 0.5 points, stimulatory/nonessential – 0.25 points, nonessential – 0 points. The number of species investigated for nutrient auxotrophies was 27 for amino acids, 22 for vitamins, 20 for metal ions and nucleotides, 17 for fatty acids.

1.4.1. Aerobic *versus* anaerobic environment

Several LAB possess a double metabolic life: they can switch from fermentation (anaerobic metabolism) to aerobic respiration metabolism, but only in the presence of exogenous heme (an indispensable component of cytochromes), as they lack the ability to biosynthesize it [4, 110]. Whilst several streptococci and lactobacilli can respire in the presence of heme (e.g. *E. faecalis*, *L. lactis*, *Leuc. mesenteroides*, *W. paramesenteroides*) or heme+menaquinone (e.g. *Lb. brevis*, *Lb. casei*, *Lb. plantarum*, *O. oeni*), there are still numerous LAB incapable of respiration (e.g. *Lb. acidophilus*, *Lb. sakei*, *S. pyogenes*, *S. thermophilus*) [110]. Nevertheless, LAB can tolerate oxygen and several species can be defined as microaerophilic (for example *L. cremoris*, [111]), i.e. growth in the presence of low oxygen concentrations is improved in comparison with the growth in a completely anaerobic environment [4].

In case of *L. cremoris* MG1363, addition of acetate into the medium during microaerobic cultivations was found to be necessary to prevent a decrease in biomass yield [112]. The optical density of *Leuc. mesenteroides* can be increased 10-20% with constant stirring, i.e. by higher O_2 availability [95]. The availability of cysteine and pyridoxamine in the culture medium increases the oxygen tolerance of *Lb. plantarum* [113].

1.4.2. Temperature and pH

In general, LAB are mesophilic microorganisms with high acid tolerance: the optimal growth temperature ranges from 20 to 45°C, depending on the genera, and cells can survive at pH < 5 [114]. Species used as yogurt starter cultures, i.e. *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*, prefer temperatures above 40°C, whereas the optimum for a species of *Lactococcus* genera is around 30°C [114-115]. *L. lactis* ATCC 19435 has the highest lactic acid productivity between 33 and 35°C and at pH 6 [116], whereas the optimum growth conditions for the strain IL1403 are 34°C and pH 6.4 [117].

1.4.3. Buffers

A number of CDMs reported in the literature for the cultivation of LAB contain phosphate buffers (K_2HPO_4/KH_2PO_4) for the maintenance of optimal pH during cultivations (Supplementary Tables S6, S7 and S8 in the Electronic Appendix). In addition to phosphates, 3-(*N*-morpholino)propanesulfonic acid (MOPS) and tricine have been included in some CDMs [79-80, 92]. MOPS provides a higher initial pH which has been shown to be beneficial for lactococci, enterococci and streptococci [79]. Phosphates may cause partial dissipation of the proton gradient across the cytoplasmic membrane, whereas MOPS may be used for osmoregulation in the cells [92]. Also, in a CDM for meat lactobacilli [102], phosphate buffers inflicted precipitation and 2-(*N*-morpholino)ethanesulfonic acid had insufficient buffering capacity. However, for *Leuc. mesenteroides* phosphates provided higher biomass yield than MOPS [95].

1.4.4. Sugars

LAB have the ability to metabolize a wide variety of sugars and other carbon sources. Species from genera *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Streptococcus* and *Weissella* have been shown to utilize glucose, fructose, lactose, galactose, maltose, mannose, ribose, trehalose, cellobiose, arabinose, xylose, raffinose and N-acetylglucosamine, to name a few [3, 118-121]. Most CDMs reported in the literature for the cultivation of LAB contain glucose as the main carbon source (Supplementary Tables S6, S7 and S8 in the Electronic Appendix).

1.4.5. Amino acids

The amino acids requirements of several species and strains of LAB have been presented in Supplementary Table S1 (Appendix 1). In general, lactic acid bacteria are fastidious in their requirements for amino acids, with the exception of *S. thermophilus,* which is able to synthesize the majority of amino acids itself. Six amino acids – arginine, histidine, methionine and the branched-chain amino acids isoleucine, leucine and valine – are essential for most of the species examined. Moreover, several lactobacilli are auxotrophic for the aromatic amino acids phenylalanine, tyrosine and tryptophan whereas lactococci are not.

In addition, some amino acids become indispensable, when the availability of other amino acids is restricted. For example, cysteine and glutamine become essential for *L. lactis* IL1403 in a medium where the concentrations of their precursors (serine and glutamate) are low [80]. Similarly, omitting an entire group of amino acids at once (e.g. asparagine/aspartate, glutamine/glutamate, pyruvate-directed amino acids) may severely hamper the growth of cells even if these amino acids are not essential upon individual exclusion [91, 97-98, 100, 102]. This derives from the fact that some amino acids are used as substrates for the biosynthesis of other amino acids. The amino acid composition of biomass indicated in Table 2 gives a tentative idea of the ratio of amino acids or their precursors to be used in a growth medium.

1.4.6. Metal ions

The effect of metal ions on the growth of LABs is described in Supplementary Table S2 (Appendix 1). Note that metal ions may be present in media as impurities from other medium components and thus aggravate the determination of the essentiality of these elements [109]. Magnesium is essential for the species from genera *Lactococcus*, *Lactobacillus* and *Streptococcus*, stimulatory for *Leuconostoc* ssp. and nonessential for *O. oeni*. Manganese(II) is essential for *O. oeni* and a few *Lactobacillus* ssp., and stimulatory for the majority of LAB presented in Supplementary Table S2 (Appendix 1). Copper is stimulatory for *L. lactis* IL1403 and iron for *E. faecalis* and *Lb. sake*. Other metal ions are nonessential.

1.4.7. Vitamins

The B-group vitamins B_5 (pantothenic acid), B_3 (nicotinic acid, nicotinamide) and B_2 (riboflavin) are essential for the majority of species shown in Supplementary Table S3 (Appendix 1). B_6 (pyridoxine, pyridoxamine, pyridoxal) is essential for *E. faecalis*, *L. lactis* and *Lb. bulgaricus*. The latter also needs B_9 (folic acid) and B_{12} (cobalamin). Other B-group vitamins and vitamin C (ascorbic acid) are stimulatory or nonessential. Some LAB (e.g. *Lb. plantarum, L. lactis*) can biosynthesize folates from *para*-aminobenzoate (*p*ABA, B_x) and thus the poorly soluble, unstable and expensive folic acid can be replaced with *p*ABA in a CDM [80, 113, 122].

1.4.8. Other compounds

Adenine, uracil and xanthine have been shown to enhance the growth of certain LAB species, whereas other purines, pyrimidines, nucleosides and their precursors are nonessential altogether (Supplementary Table S4 in Appendix 1). Uracil is classified as essential for some lactobacilli strains (Supplementary Table S4 in Appendix 1).

Compounds related to fatty acids, i.e. acetate salts (fatty acid biosynthesis), lipoic acid (acetate biosynthesis) and Tween 80 (source of oleic acid), promote the growth of strains from genera *Lactococcus*, *Lactobacillus* and *Leuconostoc* (Supplementary Table S5 in Appendix 1). Lipoic acid is needed to sustain the growth of *L. lactis*, while sodium or potassium acetate and Tween 80 are essential for a few lactobacilli species (Supplementary Table S5 in Appendix 1).

Other nutrients such as choline chloride, ammonium salts and citrate salts are nonessential for LAB in CDMs that contain sugars, amino acids, metal ions and vitamins [80-81, 91, 94, 97-98, 102, 105].

1.5. Selection of an appropriate cultivation method

Numerous vessels can be used for the growth of microbial cells, such as microtiter plates (MTPs), test tubes, flasks and bioreactors, whereas the latter provide the best opportunities for manipulating the growth environment. Nutrient and gas supply, pH of the environment and produced metabolites can be altered or kept static in bioreactors, and constant stirring of the biomass guarantees a homogenous culture and prevents the occurrence of chemical gradients upon compound addition [123].

In conventional batch cultivation, the growth rate of cells is defined by the nutrient availability of the growth medium and cannot be altered without changing the composition or complexity of the medium [93]. In a continuous cultivation, however, the specific growth rate is set by the experimenter and the steady state is time-independent, i.e. analyses can be repeated frequently [93, 124]. Furthermore, continuous cultivations provide defined, constant and controllable growth conditions which lead to reproducible and decipherable data, whereas a batch culture is dynamic and heterogeneous [123]. On the other hand, it is easier to prepare and conduct a batch experiment than a continuous cultivation, since complicated

equipment is not required. Thus, the initial screening of nutritional requirements and medium composition for *L. lactis* IL1403 in this thesis was conducted using batch experiments in MTPs and test tubes, to save time and resources. The metabolism of *L. lactis* IL1403 on selected CDMs was studied using the continuous cultivation method A-stat, due to the possibility to explore cellular growth at numerous specific growth rates. As glycine production has been noticed to occur at lower μ values (0.1-0.2 h^{-1}) [6], D-stat was the best choice for studying threonine catabolism to glycine, due to the opportunity to maintain a constant μ in the course of altering other growth parameters (e.g. threonine concentration).

1.5.1. Types of continuous cultivation

In this thesis, A-stat and D-stat as continuous cultivation methods were used to study cellular growth in synthetic media. A brief overview of the most common continuous cultivation technologies is given herein in order to demonstrate the scope of different methods.

Chemostat is a conventional type of continuous cultivation in which fresh medium is constantly pumped into the growth environment and biomass is removed from the bioreactor with a fixed speed which is called the dilution rate D [124-126]. When the culture reaches a steady state, the specific growth rate μ is equal to the dilution rate [124]. Chemostats can be implemented to study strain evolution, single-cell protein production and the effects of environmental parameters on cells at different μ values [123-124].

Auxostat is a continuous cultivation method in case of which a growth-associated parameter (e.g. pH in a pH auxostat) is kept stable by altering the dilution rate [124, 127]. An auxostat where culture density is controlled with photoelectric or laser devices and held constant throughout the experiment is called a **turbidostat** [124, 127]. Nutrient limitation is avoided in a turbidostat and the dilution rate is close to μ_{max} [125].

Retentostat is used to study cell physiology at zero growth, while cells maintain metabolic activity and metabolite biosynthesis but do not grow – a condition that occurs with LAB during cheese ripening and dry sausage production [128-129].

The term **changestat** applies to a cultivation method in which an environmental parameter is constantly altered during the experiment with a fixed speed, while the physiology of the culture is comparable to a steady state in chemostats [130]. **Accelerostat** (A-stat) is a changestat method in case of which the dilution rate is smoothly increased with a constant rate [131]. In order to maintain steady-state-like conditions, the acceleration of the dilution rate should be in the range of 0.01-0.005 h^{-2} [6, 132]. **D-stat** is a changestat method in which the dilution rate is kept constant, while other parameters such as pH, temperature or nutrient concentration are altered [14, 117, 133]. Auxostat-based changestats (experiments that begin as auxostats) are called **auxo-accelerostats**: pH-, pO_2-, CO_2-auxo-accelerostat, etc. [133].

There are several other methods of continuous cultivation, e.g. gradostat [134], morbidostat [135], but these are not discussed herein.

1.5.2. Advantages of A-stat over a conventional chemostat

In contrast with a chemostat, the dilution rate in A-stat is smoothly increased throughout the experiment, which reduces the time and cost of cultivation. The advantages of A-stat over the chemostat method include the ability to determine metabolic switch points (e.g. start of overflow metabolism, determination of exact μ_{max} value) and the possibility to obtain numerous parallel samples from steady state with just one experiment [6, 132]. The μ-dependent simultaneous consumption of several nutrients and various physiological responses of cells can be followed with ease by the A-stat method [132].

1.6. Why to study amino acid metabolism?

The importance of studying the metabolic pathways of amino acids in LAB cells is mostly derived from two issues:

(i) LAB can be used to produce recombinant proteins.
(ii) LAB degrade amino acids into chemical compounds that may affect the quality, healthiness, taste and aroma of food products in a positive or negative way.

Amongst prokaryotic cell factories, *Escherichia coli* (see on mitmuses) affords the highest protein yields, but the process is restricted by aggravated downstream purification. Due to their GRAS status and facilitated protein secretion and purification in Gram-positive bacteria, LAB have emerged as promising hosts for recombinant protein production [44-45]. Hence, researchers have developed key tools for this purpose (e.g. optimal promoters, improved secretion systems, etc.) and studied the effect of amino acid availability on protein expression levels [44, 57].

Several aroma compounds such as α-keto acids, aldehydes, alcohols, carboxylic acids and other chemicals (like methanethiol) are degradation products of amino acids and contribute to the aroma of milk products (cheese, yoghurt, butter, buttermilk), wine and fermented sausages [136-137]. Also, several strains from genera *Lactobacillus* and *Leuconostoc* degrade phenylalanine to phenyllactic acids and tyrosine to hydroxyl-phenyllactic acids, which have antifungal activity and prolong the shelf-life of bread [138-139]. Moreover, strains from genera *Lactobacillus* and *Lactococcus* catabolize glutamate to γ-aminobutyric acid in acidic conditions, while the latter compound has multiple physiological functions such as neurotransmission, and diuretic and tranquilizing effects [41]. Furthermore, amino acids are precursors to biogenic amines, e.g. histidine to histamine, tyrosine to tyramine and arginine to putrescine [140]. These compounds may induce foodborne intoxications upon high consumption, e.g. tyramine in wine and beer [141-142].

1.6.1. Threonine catabolism in lactic acid bacteria

The catabolism of threonine (Figure 1) has evoked interest because LAB degrade this amino acid primarily to glycine and acetaldehyde – an important aroma compound in yoghurt [140, 143]. This reaction is catalyzed by threonine aldolase (TA) and in *S. thermophilus* serine hydroxymethyltransferase (SHMT) has secondary activity as TA [144]. The latter case is not widespread amongst LAB [145], as SHMT (encoded by *glyA*) primarily degrades serine to glycine and 5,10-methylenetetrahydrofolate [140]. A low-specificity TA (encoded by *ltaE*) has been found in *E. coli* [146], but no homologs exist in LAB [140]. Yoghurt starter cultures, i.e. *Lb. bulgaricus* and *S. thermophilus*, are acetaldehyde producers [144], whereas the yield of acetaldehyde in *L. lactis* is low and originates from other substrates, e.g. glucose [64]. Nevertheless, the work of Aller and coworkers [14] showed that TA is present in *L. lactis* IL1403 and may be associated with *glyA*. Note that threonine was used for glycine biosynthesis if threonine concentration in CDM was in excess compared with serine [14]. This may be due to a competitive inhibition of amino acid transporters as SerP1 has been shown to transport cysteine, serine and threonine [147].

Furthermore, threonine can be degraded to 2-oxobutanoate (the precursor to isoleucine, leucine and valine) by threonine hydratase (encoded by *ilvA*) and two spontaneous reactions [140]. However, this pathway is probably not functional in *L. lactis* IL1403 as this strain is auxotrophic for branched-chained amino acids (BCAAs) [79-80, 91] and no BCAAs production from threonine was observed in the work of Aller and coworkers [14].

In *E.coli*, threonine is catabolized to 2-amino-3-ketobutyrate by threonine dehydrogenase (TDH) and subsequently to acetyl-CoA and glycine (catalyzed by 2-amino-3-ketobutyrate CoA ligase) or aminoacetone and CO_2 (spontaneous decarboxylation) [148]. Genomic data suggests that TDH may exist in *O. oeni* [149], *S. suis* [150] and *S. thermophilus* [151].

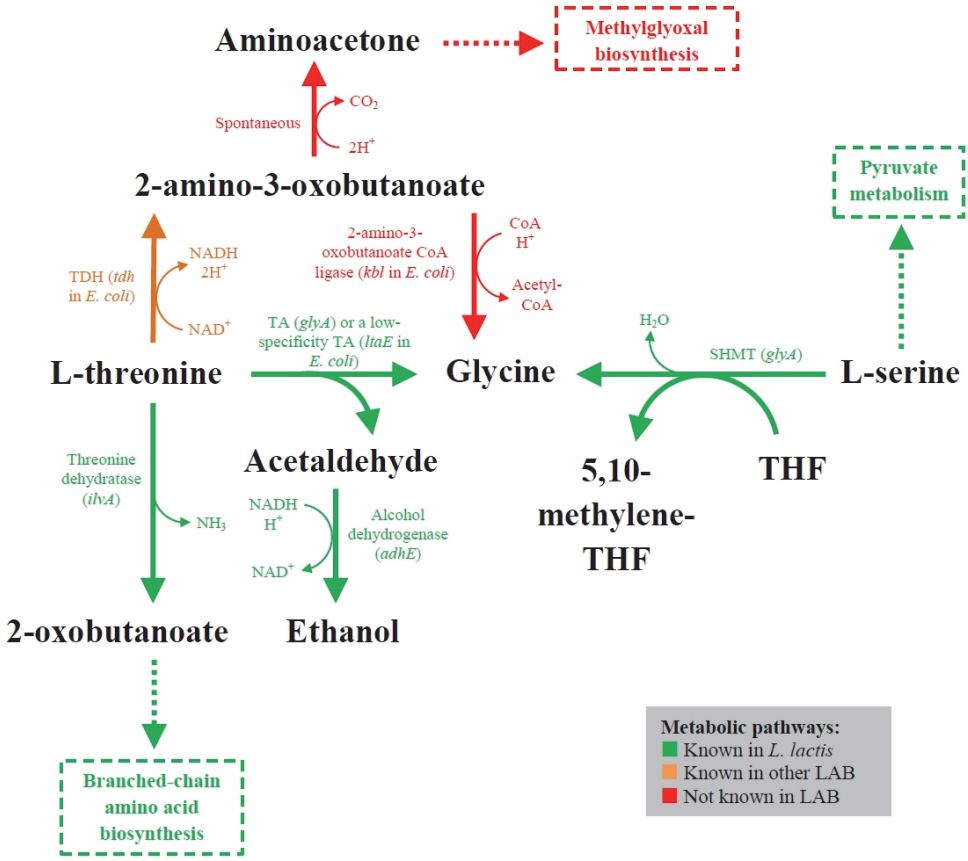


Figure 1. Threonine catabolism. Pathways in procaryotes for L-threonine and L-serine degradation and glycine biosynthesis. Green lines represent pathways that are known in *L. lactis* IL1403 based on genome sequence or experimental data. Orange lines represent pathways that are known in other LAB, but not in *L. lactis* IL1403. Red lines represent pathways that are known in other micro-organisms (e.g. *E. coli*), but not in LAB. The pathways leading to methylglyoxal, pyruvate and BCAAs biosynthesis have not been given in detail. Threonine dehydratase converts L-threonine into 2-aminobut-2-enoate and two following spontaneous reactions turn the latter compound into 2-oxobutanoate. (TA – threonine aldolase, TDH – threonine dehydrogenase, THF – tetrahydrofolate, SHMT – serine hydroxymethyltransferase.)

2. AIMS OF THE THESIS

The aims of this thesis are the following:

I. to develop a balanced chemically defined medium for *L. lactis* IL1403 with minimal residual concentrations of nutrients. (Publications I and III);

II. to apply chemically defined media to examine the effect of medium minimization on amino acid consumption strategies in batch and continuous cultivations (Publications I, III and IV);

III. to apply a chemically defined medium to ascertaining the degradation pathways of L-threonine in *L. lactis* IL1403. (Publication II).

3. MATERIALS AND METHODS

3.1. Microorganisms

Lactococcus lactis subspecies *lactis* IL1403 was provided by the National Institute for Agricultural Research (INRA) (Jouy-en-Josas, France). Inoculum was prepared from the lyophilized stock culture stored at -80°C, which was pregrown twice in test tubes at 34°C (i) on the commercially available complex medium M.R.S. Broth (Lab M, Lancashire, UK) in media development experiments with microtiter plates, (ii) on chemically defined medium (CDM) BS7 (Table 5) for media development experiments with test tubes and for A-stat and D-stat cultivations with BS7, K40 and V19 media, (iii) on a CDM called HAMmod (see the next chapter) for A-stat experiments with the HAMmod medium. In order to prevent the carryover of nutrients, the pregrown culture was washed three times with a physiological solution before inoculation to other media.

The strain used in experiments for ascertaining the threonine aldolase activity in milk was *Lactococcus lactis* subspecies *lactis* 1387, which was provided by Chr. Hansen (Denmark). Inoculum was prepared from the stock culture stored at -80°C, which was pre-grown once on M.R.S. Broth (Lab M, Lancashire, UK) and twice on reconstituted skim milk (Valio Ltd., Helsinki, Finland) in test tubes at 30°C.

3.2. Media

The CDMs developed in this thesis are listed in Table 5 and Supplementary Tables S9, S10 and S11 (Electronic Appendix). The basis for the newly constructed media, BS7, is a self-weighed and slightly modified alternative to the HAMmod medium previously used in our laboratory [6], which was expensive due to containing the commercial GIBCO™ F-12 Nutrient Mixture (Invitrogen Corporation, Carlsbad, CA, USA). In the process of minimalizing nutrient concentrations in a synthetic medium (BS7), data about amino acid auxotrophies and residual concentrations were taken into account simultaneously to obtain a medium with balanced composition.

The HAMmod medium is composed of 70% of GIBCO™ F-12 Nutrient Mixture and 30% of a CDM in [100]. The exact composition of HAMmod is as follows ($mg \cdot L^{-1}$): D-Glucose – 3500; L-Alanine – 78; L-Arginine – 185; L-Asparagine – 74; L-Aspartic acid – 72; L-Cysteine – 64; L-Glutamic acid – 70; L-Glutamine – 132; Glycine – 58; L-Histidine – 60; L-Isoleucine – 102; L-Leucine – 207; L-Lysine – 158; L-Methionine – 41; L-Phenylalanine – 86; L-Proline – 92; L-Serine – 163; L-Threonine – 76; L-Trypthophan – 16; L-Tyrosine – 29; L-Valine – 107; Biotin – 0.305; Choline chloride – 9.8; D-Pantothenate – 0.65; Folic acid – 1.21; Niacinamide – 0.325; Pyridoxine hydrochloride – 0.642; Riboflavin – 0.326; Thiamine hydrochloride – 0.51; Vitamin B_{12} – 0.98; i-Inositol – 12.6; $CaCl_2$ – 28; $CuSO_4 \cdot 5H_2O$ – 0.272; $FeSO_4 \cdot 7H_2O$ – 0.71; $MgCl_2$ – 58; KCl – 157; NaCl – 5580;

Na_2PO_4 – 99; $ZnSO_4 \cdot 7H_2O$ – 1; Hypoxanthine-Na – 3; Linoleic acid – 0.1; Lipoic acid – 0.1; Phenol Red – 0.8; Putrescine·2HCl – 0.1; Na-Pyruvate – 77; Thymidine – 0.5.

The components were obtained from Serva Electrophoresis GmbH (Heidelberg, Germany) and Sigma-Aldrich (St. Louis, Missouri, USA) and were mixed together in the following order (to avoid precipitation): sugar, buffers, amino acids, mineral salts, vitamins, additional components. All media were sterilized by filtration, using 0.2 µm cellulose acetate filters (Whatman, Pittsburgh, PA, USA) in MTP and test tube scale experiments or stainless steel filter holders and 0.22 µm nitrocellulose membrane filters in continuous cultivations (Merck Millipore, Darmstadt, Germany).

Table 5. Chemically defined media BS7, K40 and V19. The table shows (i) the maximal specific growth rate (μ_{max}, h^{-1}) obtained in A-stat cultivations[1], (ii) the c-molar ratio of amino acids to glucose (AA:Glc), (iii) the total price of the medium components per liter (prices of Sigma-Aldrich on July 8, 2015), (iv) the chemical composition of media (grams per liter) with the reduction rates of nutrient concentrations in K40 and V19 in comparison to BS7, (v) the proportion (%) of each amino acid (AA) in the total amount of amino acids. Unlike other media in Supplementary Tables S9-11 (Electronic Appendix), adenine and hypoxanthine were added and the amount of glucose was increased.

Medium	BS7		K40		V19	
μ_{max}; h^{-1}, n=2	0.57±0.04		0.42±0.07		0.49±0.07	
AA:Glc (c-mol·c-mol^{-1})	0.44		0.20		0.44	
Price (EUR·L^{-1})	3.21		2.22		2.97	
COMPOSITION (g·L^{-1})						
SUGAR						
Glucose	4.5		4.5		4.5	
BUFFERS						
K_2HPO_4	0.9 (3)[1]		0.9 (3)[1]		0.9 (3)[1]	
KH_2PO_4	0.75 (2.5)[1]		0.75 (2.5)[1]		0.75 (2.5)[1]	
MOPS	7.5 (0)[1]		7.5 (0)[1]		7.5 (0)[1]	
AMINO ACIDS		**AA ratio (%)**			**AA ratio (%)**	
L-Alanine (Ala)	0.0782	4.2	0.0391	2x↓	4.5	0.0782
L-Arginine (Arg)	0.1852	9.9	0.0926	2x↓	10.7	0.1852
L-Asparagine (Asn)	0.0735	3.9	0.0735		8.5	0.0735
L-Aspartate (Asp)	0.0723	3.9	0.0723		8.4	0.0723
L-Cysteine (Cys)	0.0636	3.4	0.0079	8x↓	0.9	0.0636

(Table 5 continued)

L-Glutamate (Glu)	0.0703	3.8	0.0703		8.1	0.0703	
L-Glutamine (Gln)	0.1322	7.1	0.1322		15.3	0.1322	
Glycine (Gly)	0.0578	3.1	0.0072	8x↓	0.8	0.0578	
L-Histidine (His)	0.0597	3.2	0.0075	8x↓	0.9	0.0597	
L-Isoleucine (Ile)	0.1018	5.4	0.0255	4x↓	3.0	0.1018	
L-Leucine (Leu)	0.2072	11.1	0.0518	4x↓	6.0	0.2072	
L-Lysine-HCl (Lys)	0.1576	8.4	0.0394	4x↓	4.6	0.1576	
L-Methionine (Met)	0.0407	2.2	0.0203	2x↓	2.4	0.0407	
L-Phenylalanine (Phe)	0.0860	4.6	0.0430	2x↓	5.0	0.0860	
L-Proline (Pro)	0.0917	4.9	0.0115	8x↓	1.3	0.0917	
L-Serine (Ser)	0.1634	8.7	0.0817	2x↓	9.5	0.1634	
L-Threonine (Thr)	0.0758	4.1	0.0379	2x↓	4.4	0.0758	
L-Tryptophan (Trp)	0.0164	0.9	0.0082	2x↓	0.9	0.0164	
L-Tyrosine (Tyr)	0.0295	1.6	0.0147	2x↓	1.7	0.0295	
L-Valine (Val)	0.1072	5.7	0.0268	4x↓	3.1	0.1072	
MINERAL SALTS							
$CaCl_2$	0.0500		0.0500			0.0125	4x↓
$CoSO_4 \cdot 7H_2O$	0.0030		0.0030			0.0008	4x↓
$CuSO_4 \cdot 5H_2O$	0.0030		0.0030			0.0015	2x↓
$FeSO_4 \cdot 7H_2O$	0.0014		0.0014			0.0007	2x↓
$MgSO_4 \cdot 7H_2O$	0.2000		0.2000			0.1000	2x↓
$MnSO_4 \cdot H_2O$	0.0160		0.0160			0.0080	2x↓
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.0030		0.0030			0.0008	4x↓
NaCl	2.9000		2.9000			0.7250	4x↓
$ZnSO_4 \cdot 7H_2O$	0.0050		0.0050			0.0013	4x↓
VITAMINS							
Thiamine-HCl (B_1)	0.00051		0.00051			0.00051	
Riboflavin (B_2)	0.00033		0.00033			0.00033	
Nicotinamide (B_3)	0.00033		0.00033			0.00033	
Ca-pantothenate (B_5)	0.00065		0.00065			0.00065	
Pyridoxine (B_6)	0.00064		0.00064			0.00064	
Biotin (B_7)	0.00031		0.00031			0.00031	
Folic acid (B_9)	0.00121		0.00121			0.00121	
OTHER COMPOUNDS							
Adenine	0.0250		0.0250			0.0250	
Hypoxanthine	0.0250		0.0250			0.0250	
Lipoic acid	0.00018		0.00018			0.00018	

[1] The growth results (n = 3) for **BS7** medium were OD_{max} = 0.516 ± 0.002 (at 600 nm and $V_{cultivation}$ = 0.3 mL) and μ_{max} = 0.59 ± 0.01 h^{-1} on **microtiter plates** and OD_{max} = 1.38 ±

0.03 (at 600 nm), μ_{max} = 0.72 ± 0.06 h^{-1}, lag phase 4.5 hours, exponential phase 5.5 hours in **test tube** scale experiments. The growth results (n = 3) for **K40** medium in **test tube** scale experiments were OD_{max} = 1.33 ± 0.04 (at 600 nm), μ_{max} = 0.59 ± 0.02 h^{-1} and lag-phase 6.0 hours. The growth results (n = 3) for **V19** medium on **microtiter plates** were OD_{max} = 0.449 ± 0.012 (at 600 nm and $V_{cultivation}$ = 0.3 mL) and μ_{max} = 0.60 ± 0.05 h^{-1}. In case of test tube or microtiter plate experiments the glucose concentration was 3.5 $g \cdot L^{-1}$ and adenine and hypoxanthine were not added to the media. However, to enhance the growth of cells the use of 4.5 $g \cdot L^{-1}$ of glucose and addition of adenine and hypoxanthine were recommended as indicated in the table.

[2] Concentrations in brackets were used in pH-regulated bioreactors.

3.3. Cultivation conditions

3.3.1. Microtiter plate and test tube experiments

Microtiter plate (MTP) experiments were carried out using plates with 100 wells and the Bioscreen C automated microtiter plate reader (Oy Growth Curves Ab Ltd., Helsinki, Finland). OD was measured every 15 minutes at 600 nm, preceded by shaking the plate for 10 seconds. In test tube experiments, OD was measured once an hour (see the Analytical Methods section) and a vortex was used before each OD measurement to homogenize the biomass. The cultivation volume was 0.24 (Table S9 in the Electronic Appendix) or 0.3 (Table S11 in the Electronic Appendix) mL for MTP and 5 mL for test tube experiments (Table S10 in the Electronic Appendix). The cultivation temperature was 34°C and no control of pH or gas environment was applied. Each medium had three replicates. Amino acid consumption was measured from supernatants collected after 24 h of growth (the stationary phase) by removing the cells with centrifugation (14800 rpm, 5 min, 4°C; MicroCL 21R centrifuge; Thermo Fisher Scientific Inc., Waltham, MA, USA).

In auxotrophy experiments, one component was omitted at once from the medium BS7 (Table 5) in MTPs and the nutrient was considered essential (E), growth stimulating (S) or nonessential (NE) if the final OD was respectively < 40, 40-80 or > 80% of that obtained in the complete medium.

3.3.2. Continuous cultivations

The continuous cultivations were carried out in 1.25 L Biobundle bioreactors with ez-Control biocontrollers and the BioXpert Pharma 3.80M XP control program, all provided by Applikon Biotechnology B.V. (Delft, The Netherlands). The system was equipped with pH, O_2 and temperature sensors. A detailed description of the fermentation equipment has been given in [117] and [152]. The cultivation temperature in all experiments was 34°C, agitation speed 300 rpm and cultivation volume 300 mL. pH was kept at 6.4 by addition of 2M NaOH. The gas environment was kept anaerobic by flushing the bioreactors with N_2.

Experiments with BS7, K40 and V19 media were A-stats in two parallels, where the culture was stabilized at 0.1 h^{-1} by pumping through five bioreactor volumes. After stabilization the dilution rate was increased with an acceleration of 0.006 h^{-2}.

Experiments with the HAMmod medium were A-stats in five parallels, where the culture was stabilized at 0.1 h^{-1} and the dilution rate was increased with an acceleration of 0.01 h^{-2}. The catabolic pathways of threonine were studied using D-stat cultivations in three parallels at 0.2 h^{-1}, where the concentration of threonine in the feed (BS7 without glycine) was increased from 1.24 to 4.13 mM. The experiment with labelled threonine was a D-stat at 0.2 h^{-1} with BS7 containing 2.5 mM of threonine. After stabilization of culture the medium was switched to a labelled BS7 medium, which contained 2.5 mM of $^{13}C_4$,^{15}N-labelled L-threonine (CortecNet, Voisins-le-Bretonneux, France).

Samples were collected every 1-3 hours and the supernatant was obtained by removing the cells with centrifugation (14800 rpm, 5 min, 4°C; MicroCL 21R centrifuge; Thermo Fisher Scientific, Waltham, MA, USA). Biomass samples for measuring intracellular amino acids were immediately freezed in liquid N_2 and used for analyses afterwards.

3.3.3. Threonine aldolase activity experiment in milk

Low heat skim milk powder (Valio Ltd., Helsinki, Finland) was reconstituted in dH_2O to 9% (w/v) of milk solids. The reconstituted milk was stirred for 30 min and autoclaved at 96°C for 30 min. 1, 2.5, 5, 10 or 25 mM of L-threonine was added to the milk, using a presterilized L-threonine stock solution (0.2 µm cellulose acetate filters; Whatman, Pittsburgh, PA, USA). Growth in milk in three replicates and with an inoculum of 1% (v/v) was monitored by measuring acidification speed (changes in pH) with an iCinac^{TM} system (Unity Scientific, Brookfield, CT, USA). After reaching stable pH values, samples for amino acid analysis were mixed 1:1 with isopropanol (v/v) and the cells were removed by centrifugation (5 min, 4°C, 14800 rpm; MicroCL 21R centrifuge; Thermo Fisher Scientific, Waltham, MA, USA) and filtration (0.2 µm cellulose acetate filters; Whatman, Pittsburgh, PA, USA).

3.4. Analytical methods

Optical density (OD) in test tube experiments and continuous cultivations was measured with the WPA CO 8000 Biowave Cell Density Meter (Biochrom GmbH, Berlin, Germany) at 600 nm.

The dry weight of biomass in continuous cultivations was determined gravimetrically by washing 10 mL of biomass in three replicates with dH_2O (n = 3, 11500 rpm, 5 min, 4°C; Rotanta 460R centrifuge, Hettich, Tuttlingen, Germany). The pellets were heated at 105°C for 24 h on a piece of aluminum foil and weighed thereafter. The k value was 0.30 ± 0.03, 0.31 ± 0.02 and 0.30 ± 0.02 in A-stat experiments with BS7, K40 and V19 media, respectively, and 0.32 ± 0.01 in D-stat experiments for determining threonine catabolic pathways.

Glucose and main fermentation end products (lactate, formate, acetate, ethanol) were measured with an Alliance 2795 system liquid chromatograph , a Model 2414 refractive index detector and the Empower software, all provided by Waters Corp. (Milford, MA, USA). The column used was BioRad HPX-87H

(Hercules, CA, USA) at 35°C with an isocratic elution of 5 mM H_2SO_4 at a flow rate of 0.6 mL·min^{-1}. **Acetaldehyde** was measured with a specific assay kit provided by Megazyme (Bray, Ireland).

Free amino acids were detected with an amino acid analyzer Acquity UPLC. The samples were derivatized beforehand for 10 min at 55°C, using the AccQ-Fluor reagent (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate) and then loaded on the AccQ-Tag Ultra column at 55°C. Amino acids were separated using a gradient of AccQ-Tag Ultra eluents A and B. The photodiode array detector was used for detection and data was processed with the Empower software. In order to measure the labelled L-threonine or glycine in the labeling experiment, an LCT Premier™ XE ESI TOF MS was used, where amino acids were separated as described above and sprayed directly into the mass-spectrometer operated in a positive ionisation mode (solvatation temperature 300°C, source temperature 120°C, capillary voltage 2.5 kV). The MassLynx V4.1 software was used for data processing. All the equipment, reagents and eluents were provided by Waters Corp. (Milford, MA, USA). For **intracellular amino acid** measurements, biomass was hydrolysed with 6M HCl for 24 h at 105ºC. From hydrolyte, amino acids were measured as described above. L-tryptophan and L-cysteine could not be detected with this method because they were degraded during hydrolysis. Also, L-asparagine was converted to L-aspartate and L-glutamine to L-glutamate during hydrolysis.

B-group vitamins were measured by Kristel Hälvin as described in [153].

The concentration of Mg^{2+} ions was detected with an ion chromatography system that consisted of an isocratic HPLC pump (model 1515), a conductivity detector (model 432), an IC Pak Cation M/D column and the Breeze™ software, all provided by Waters Corp. (Milford, MA, USA). A solution of 0.1 mM EDTA / 3 mM HNO_3 was used for elution at a flow rate of 1 mL·min^{-1}. A Certipur® magnesium standard solution was used for quantification (Merck Millipore, Darmstadt, Germany).

NMR spectra were measured by Indrek Reile on a Bruker Avance III 800 MHz spectometer equipped with a He cryoprobe. Samples were prepared by adding 60 µL of D_2O to 540 µL of supernatant (10% D_2O concentration). ^{13}C spectra were measured at a 15°C sample temperature with 30 degree flip angle pulses and proton decoupling. 10k scans with a 2 s relaxation delay were collected for each sample. All spectra were frequency referenced according to the lactate signal and intensities were scaled to constant lactate signal intensity. Spectral assignment was based on chemical shifts, integrals and ^{13}C-^{13}C couplings and was confirmed with a separate gated decoupling ^{13}C experiment.

3.5. Proteome analysis

Sample selection: (i) Samples from one, two or three individual A-stat experiments with the HAMmod medium were analyzed in single runs. Samples were taken at μ = 0.1 (n = 1), 0.2 (n = 1), 0.3 (n = 3), 0.4 (n = 3) and 0.5 h^{-1} (n = 2) and compared to 0.1 h^{-1}. Proteome analysis was conducted by Liisa Arike as described in [6]. (ii) Samples from two individual D-stat experiments for clarification of threonine catabolic pathways were analyzed in single runs. Samples were taken at $C_{Threonine}$ =

1.24; 2.86 mM; 3.99 mM and compared to $C_{Threonine}$ = 1.24. Proteome analysis was conducted by Karl Peebo as described below.

Sample preparation and analysis: 100 µg of the cell pellet was mixed in a 1:1 ratio with heavy SILAC-labelled [154] *L. lactis* biomass, suspended in 100 µL SDS lysis buffer (4% SDS/100 mM TRIS-HCl, pH 8/100 mM DTT) and heated at 95°C for 15 min. Cell lysates were sonicated with ultrasound for a few pulses and pelleted by centrifugation. The cell lysates were digested with trypsin according to the Filter Aided Sample Preparation protocol (FASP) [155] and purified with C-18 StageTips [156]. Peptides were separated on an Ultimate 3000 RSLCnano system (Dionex; Thermo Fisher Scientific, Waltham, MA, USA) using a cartridge trap-column in backflush configuration and an analytical 50 cm Easy-spray column (Thermo Fisher Scientific, Waltham, MA, USA). Peptides were eluted at 200 nL/min with an 8-40% B gradient (buffer B: 80% acetonitrile + 0.1% formic acid, buffer A: 0.1% formic acid) to a Q Exactive MS/MS operating with a top-10 strategy and a maximum cycle time of 1 s. Briefly, one 350-1400 m/z MS scan at a resolution setting of R = 70000 was followed by fragmentation of 10 most intense ions > + 1 charge state at R = 17500. The dynamic exclusion was set to 70 s.

Data analysis: Raw MS files were analysed by the MaxQuant software package version 1.3.0.5 [157]. Peak lists were searched against *L. lactis* database (downloaded in 2013 from http://www.uniprot.org/) which was supplemented with common contaminants (e.g. human keratin, trypsin). Full tryptic specificity, a maximum of two missed cleavages and a mass tolerance of 0.5 Da for fragment ions, was specified in the MaxQuant search. Carbamidomethylation of cysteine was set as a fixed modification, and methionine oxidation and protein N-terminal acetylation were set as variable modifications. The required false discovery rate was set to 1% for both peptide and protein levels and the minimum required peptide length was set to seven amino acids. "Match between runs" option with a time window of 2 min was allowed.

3.6. Statistical analysis

Partial least squares regression (PLSR) analysis (Figure 4) was done by Andrus Seiman in Matlab (Mathworks, Natick, MA, USA) using self-written scripts as well as algorithms from Statistics Toolbox. OD and specific growth rate were chosen as dependent parameters. Amino acid concentrations in the media were used as independent variables. Mean centering and scaling were used in data preparation. This way each amino acid had an equal weight in regression analysis. The number of components for the PLSR analysis was estimated using the leave-one-out cross-correlation and residual sum of squares.

3.7. Flux calculations

ATP spilling is defined as non-growth related ATP consumption and is calculated by subtracting the total ATP spent for the synthesis of biomonomers from the total

ATP produced. The total ATP production was calculated by Andrus Seiman with a stoichiometric metabolic model by summing the values of all reactions producing ATP. An in-house metabolic model of *L. lactis* subsp. *lactis* IL1403 was constructed based on metabolic network information in BioCyc (www.biocyc.org) and KEGG databases (www.genome.jp/kegg/) [158-159]. The model consists of 247 network reactions and involves equations for calculating biomass composition according to the need for enzymes that are required for achieving a particular flux pattern. Model calculations were done in Wolfram Mathematica 8.0 environment (Wolfram Research, Champaign, IL, US).

4. RESULTS AND DISCUSSION

4.1. Development of chemically defined media for *L. lactis* IL1403 (Publications I, III)

4.1.1. Nutrient auxotrophies of *L. lactis* IL1403 (Publication I)

4.1.1.1. Amino acids, vitamins and metal ions

Similarly to other LAB, *L. lactis* IL1403 is a mesophilic microorganism. The optimal temperature and pH for this strain are 34°C and 6.4, respectively [117], and were used herein. The nutrient auxotrophies for *L. lactis* IL1403 have been determined previously [79, 91], but were confirmed in Publication I by the leave-one-out strategy in the BS7 medium (Table 5) by using microtiter plates (MTPs). Arginine, asparagine, histidine, methionine, serine and the branched-chain amino acids (BCAAs) isoleucine, leucine and valine were essential and alanine, cysteine, glutamine, lysine and threonine were stimulatory for this strain (Table 6). Other amino acids were nonessential. Aspartate and glutamate could be simultaneously omitted from the medium without affecting OD, whereas the concurrent exclusion of aspartate and glutamine led to zero growth (data not shown). In addition, in amino acid limitation conditions, i.e. when concentrations of all amino acids were lowered four times, cysteine and glutamine became essential for IL1403 (Table 6).

In MTP experiments, the only essential B-group vitamin was riboflavin (B_2) (Table 6) and IL1403 was able to grow in a medium with riboflavin if other vitamins – thiamine (B_1), nicotinamide (B_3), pantothenic acid (B_5), pyridoxine (B_6), biotin (B_7), folic acid (B_9) and cobalamin (B_{12}) – were omitted at once from the medium (data not shown). However, when the amino acid concentrations were lowered four times, folic acid was stimulatory for cell growth (OD was 74.2 ± 1.7% of the initial medium). Furthermore, in test-tube scale experiments, only cobalamin (B_{12}) could be excluded from the medium, whereas the omission of folic acid (B_9) provided poor growth (OD = 33.2 ± 0.7%). In addition, the omission of pantothenate (B_3) or pyridoxine (B_6) from media with concentrations of amino acid reduced 2–8 times (compared with the BS7 medium) led to zero growth (Table 6).

With regard to metal ions, Mg^{2+} was shown to be essential, while Mn^{2+} and Cu^{2+} were growth-stimulating for IL1403 in MTP experiments (Table 6). Furthermore, the effect of some additional compounds, which were present in a CDM previously used in our laboratory [6], i.e. choline chloride, hypoxanthine, lipoic acid, *myo*-inositol, sodium pyruvate and thymidine, was observed. None of those compounds were needed by the cells upon first inoculation. Nevertheless, when reinoculations were made, the omission of lipoic acid inflicted zero growth and the compound was classified as essential for *L. lactis* IL1403 (Table 6).

Table 6. Hypotheses about the essential or stimulatory nature of nutrients for *L. lactis* IL1403.[1] B-group vitamins are thiamine (B_1), riboflavin (B_2), nicotinamide (B_3), pantothenate (B_5), pyridoxine (B_6), biotin (B_7), folic acid (B_9) and cobalamin (B_{12}).

Nutrient requirement	Group	Compound	Hypothesis about (in)essentiality
Essential	Amino acids	Arg	Inactive biosynthesis genes or insufficient CO_2 availability
		Asn	Repression of CodY regulon by Ile
		Cys[1], Gln[1], Ser	Insufficient supply of substrates for biosynthesis or repression of CodY regulon by Ile (for Cys and Ser)
		His	Inactive biosynthetic genes or repression of CodY regulon by Ile
		Ile, Leu, Val	Inactive biosynthetic genes
		Met	Low activity of biosynthetic enzymes
	Vitamins	B_2	Low activity of biosynthetic enzymes or insufficient supply of substrates for biosynthesis
		B_5[1]	Biosynthetic genes are related to amino acid supply in the medium
		B_6[1]	Necessary for amino acid biosynthesis in CDMs with low amino acid concentrations
	Metal ions	Mg^{2+}	Cofactor in various cellular reactions
	Others	Lipoic acid	Needed in microaerobic experiments due to absence of biosynthetic genes
Stimulatory	Amino acids	Ala	Insufficient supply of substrates for biosynthesis
		Lys	Repression of CodY regulon by Ile
		Thr	Low activity of biosynthetic genes or repression of CodY regulon by Ile
	Vitamins	B_9	Low activity of enzymes for precursor biosynthesis (*p*ABA)
	Metal ions	Cu^{2+}, Mn^{2+}	Microaerobic experiments
Nonessential	Amino acids	Asp, Glu, Gly, Phe, Pro, Trp, Tyr	Biosynthetic genes exist and are functional
	Vitamins	B_1, B_3, B_7, B_{12}	Biosynthetic genes exist
	Metal ions	Ca^{2+}, Co^{2+}, Fe^{2+}, Mo^{6+}, Na^{+}, Zn^{2+}	Can be replaced by other ions or are not used by LAB at all

[1] In auxotrophy experiments, one component was omitted at once from the medium BS7 (Table 5) in MTPs and the nutrient was considered essential, growth stimulating or nonessential, if the final OD was respectively < 40, 40-80 or > 80% of that obtained in the complete medium.

[2] Compound essential in media with low concentrations of amino acids (amounts were reduced 2-8 times compared with the BS7 medium). Cysteine and glutamine were stimulatory in case of adequate amino acid concentrations (BS7 medium), whereas pantothenate (B_5) and pyridoxine (B_6) were nonessential.

4.1.1.2. Experimental *versus* theoretical determination of nutritional requirements

As more and more microbial genomes are being sequenced and systems biology is used extensively, a question arises whether it is still necessary to carry out experiments for determination of auxotrophies. Metabolic models that are based on genome data provide the possibility to theoretically calculate the nutritional requirements of cells. However, theoretical data does not always coincide with information about the actual metabolic capability of bacteria. For instance, based solely on genome sequence, *L. lactis* IL1403 does not require any amino acids for growth as it can biosynthesize all of them itself [53]. However, calculations with a metabolic model determined that this strain requires at least four amino acids (arginine, methionine, valine and glutamate or glutamine) for *in silico* growth [160], whereas *in vivo* experiments demonstrated the need for nine amino acids [91]. Similarly, calculations with a metabolic model or the presence of biosynthetic genes did not match the actual nutritional requirements of *L. cremoris* MG1363 [15] or *Lb. plantarum* WCFS1 [106]. Therefore, predictions about auxotrophies can be made theoretically, but experimental data is still needed for correction of metabolic models. However, with the constant improvement in understanding the relationships between proteins, genes and reactions and the construction of more precise metabolic models, the need for *in vivo* experiments for determination of auxotrophies may be rendered futile in the future.

4.1.2. Genetic and metabolic background for nutrient auxotrophies (Publication I)

The following subchapters are dedicated to searching possible reasons from the literature for the essential or stimulatory nature of nutrients for *L. lactis* IL1403 (the summary of hypotheses is given in Table 6 of chapter 4.1.1.1.). The biosynthetic pathways of amino acids and vitamins are represented in Figure 2.

4.1.2.1. Loss of amino acid biosynthetic capability due to adaptation to milk

The genome sequence of *L. lactis* IL1403 contains biosynthetic genes for 20 proteinogenic amino acids [53]. The decrease in the biosynthetic capability of LAB and auxotrophy for various nutrients arises from genome reduction and inactivation of certain biosynthetic genes, which was evoked by adaptation of these microorganisms to nutrient-rich environments (e.g. milk, gastrointestinal tract) in the course of evolution [1]. The common ancestor of *Lactobacillales* has lost 600-1200 genes and gained less than 100 genes in comparison with the ancestor of all *Bacilli* during adaptation to nutritionally rich medium [161]. Also, LAB possess niche-specific genes. For example, dairy and gut strains revealed respectively six and three genes, which are characteristic of these groups [162]. Similarly, 23% of the genome of sourdough isolate *L. lactis* A12 is niche-specific in comparison with the IL1403 strain [163]. All LAB genomes contain pseudogenes and genome degradation seems to be an ongoing process [160]. Note that milk has a low content of free amino acids

and peptides and thus, lactococcal strains have gained a proteolytic system for the breakdown of casein into amino acids [164].

The biosynthetic genes of BCAAs (*leuA, leuB*) and histidine (*hisCGDBH*) are dysfunctional in several *L. lactis* dairy strains, including IL1403 [165-167]. The non-functionality of the BCAAs genomic locus specifically occurs in the adaptation of plant-derived *L. lactis* strains to the dairy environment [168]. In *Lb. plantarum*, the auxotrophy of dairy strains for arginine has also been associated with adaptation to milk, as isolates from fermented vegetable products or humans did not require arginine for growth [169]. In several arginine-auxotrophic strains of *Lb. plantarum*, only one gene of *argCJBDF* biosynthetic operon had a mutation [169]. Another explanation for the requirement of *L. lactis* IL1403 for arginine is that the latter amino acid cannot be produced from glutamate and carbamoylphosphate due to the lack of CO_2 for the biosynthesis of carbamoylphosphate (Figure 2).

The reductive evolution of *L. lactis* strains used in the dairy industry is extensive enough to render them incapable of growth in other environments [170]. However, *S. thermophilus*, which is used as a yoghurt starter culture, has maintained the ability to biosynthesize the majority of amino acids (Supplementary Table S1 in Appendix 1). This may be related to strain selection: the dairy industry uses *S. thermophilus* strains that are capable of fast growth and acidification, while these features derive from conservation of virtually all amino acid pathways, as amino acid availability in milk is rate limiting [10].

4.1.2.2. Low activity of amino acid biosynthetic enzymes

Methionine auxotrophy may arise from the low activity of biosynthetic enzymes [15], which may be related to the deficiency of the cofactor flavin adenine dinucleotide (FAD) [160]. The impaired growth of *L. lactis* IL1403 in a medium lacking threonine suggests low activity of biosynthetic enzymes as well. For example, the transcription of *hom-thrB* genes in *L. cremoris* MG1363, which are involved in degrading aspartate into threonine, is not influenced by the presence or absence of threonine in the growth environment [171].

High isoleucine concentration in a CDM (C = 0.16 $g \cdot L^{-1}$) may result in growth inhibition due to the blockage of CodY-dependent amino acid biosynthetic pathways, e.g. the biosynthesis of asparagine (from aspartate; *asnB*), histidine (from PRPP; *hisCZD, hisB, hisAFIK*), lysine (from aspartate; *dapB, lysA, thrA, ychH*) and threonine (from aspartate; *hom, thrA, thrB, thrC*) (Figure 2) [172]. CodY is a pleiotropic transcriptional regulator in Gram-positive bacteria [172]. In this thesis, the CDM for determination of auxotrophies contained 0.10 $g \cdot L^{-1}$ isoleucine. Therefore, isoleucine excess might be the reason behind inhibiting the biosynthesis of the aforementioned amino acids in this thesis as well.

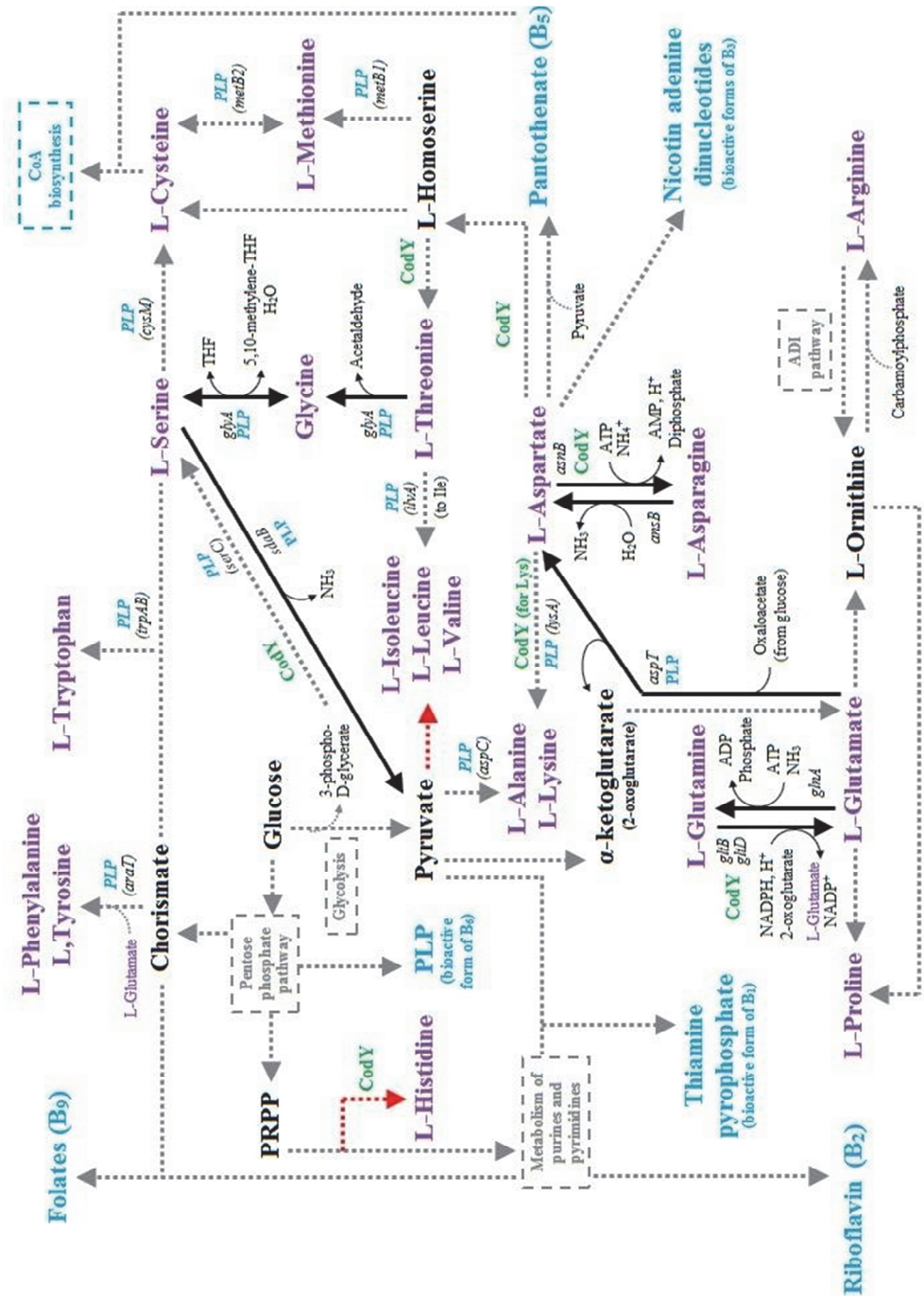


Figure 2. Biosynthetic pathways of amino acids (purple) and vitamins (light blue) from glucose or other amino acids in LAB with the respective genes encoding enzymes for these reactions. The dotted grey arrows represent pathways with multiple reactions which are not given in detail herein. Red arrows represent the presence of pseudogenes in the corresponding biosynthetic pathways. Pathways that are regulated by CodY regulon are marked with CodY. Enzymes containing the cofactor pyridoxal 5'-phosphate (bioactive form of vitamin B_6) as a cofactor are marked with the abbreviation PLP.

In contrast to *L. lactis* IL1403, serine is stimulatory or nonessential for the majority of LAB species (Supplementary Table S1 in Appendix 1). This may be due to the fact that other authors used media with higher concentrations of cysteine, glycine, threonine and glucose (Supplementary Tables S6 and S7 in the Electronic Appendix) and thus, serine could be obtained from sugar or amino acids (Figure 2).

Therefore, the requirement of *L. lactis* IL1403 for serine in this thesis may be inflicted by the low availability of precursors. In addition, the genes needed for the synthesis of serine from glucose (*serA, serB, serC*) are regulated by CodY which may be repressed by high isoleucine concentration, as mentioned previously [172]. The stimulatory effect of alanine (Table 6), which can be biosynthesized from glucose [7], is probably inflicted by the low concentration of glucose (3.5 $g \cdot L^{-1}$) in our leave-one-out CDM BS7, in comparison with CDMs from the literature, in which the concentration of glucose, fructose, lactose or ribose was 10-20 $g \cdot L^{-1}$ (Supplementary Tables S1 in Appendix 1 and S7 in the Electronic Appendix). Similarly, cysteine and glutamine were essential for IL1403 due to the insufficient supply of precursors serine and glutamate, respectively (Table 6, Figure 2). Also, the biosynthesis of cysteine from other substrates (e.g. aspartate) is regulated by CodY, which may have been repressed by isoleucine (Figure2).

4.1.2.3. Riboflavin biosynthesis

Riboflavin (B_2) biosynthesis genes (*ribGBAHC*) exist in *L. lactis* IL1403 [53] and are functional in *L. cremoris* NZ9000 [61]. According to gene homology, riboflavin biosynthetic genes are present in *Leuc. mesenteroides, Lb. brevis* and *P. pentosaceus* and absent in *Lb. casei*, *Lb. bulgaricus*, *O. oeni* and *E. faecalis* [61]. It has been determined previously that riboflavin is specifically required for the growth of *L. lactis* dairy strain (IL1403), but not for the vegetable strain (NCDO2118) [91]. The inability of the IL1403 strain to grow without riboflavin has also been noticed by other researchers [173]. *L. lactis* has an efficient riboflavin transporter RibU [174], which suggests that the cells are more dedicated to the consumption of exogenous riboflavin than biosynthesis. The requirement of *L. lactis* IL1403 for riboflavin may thus derive from the low activity of biosynthetic enzymes, which in turn leads to the inefficient supply of flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD). These cofactors are needed in various enzymes, including NADH oxidases and pyruvate dehydrogenase in aerobic conditions [173]. The biosynthesis of riboflavin consists of seven enzymatic steps and requires guanosine 5'-triphosphate (GTP) and ribulose-5'phosphate as precursors [30]. Metabolites in histidine biosynthetic pathways are directed to purine biosynthesis (Figure 2), but as *L. lactis* IL1403 is auxotrophic for histidine (Table 6), the biosynthesis of this amino acid is probably inactive and thus, there might be an insufficient supply of purines for compounds other than nucleic acids (e.g. for riboflavin biosynthesis).

Lactococcal cells have been shown to need riboflavin upon growth at superoptimal temperatures to alleviate oxidative stress [173]. However, the growth temperature used in this thesis (34°C) was optimal for IL1403 [117] and cannot be the reason for riboflavin auxotrophy, although the gas environment in MTP experiments was uncontrolled and thus microaerobic.

4.1.2.4. Other B-group vitamins

In leave-one-out experiments of [79], the omission of pantothenic (B_5) or nicotinic acid (B_3) decreased OD, but did not inflict zero growth. Thus, biosynthetic pathways are probably functional for these vitamins or their bioactive forms, although not all relevant genes have been characterized [53, 159]. The requirement of *L. lactis* IL1403 for pantothenic acid in CDMs with low amino acid concentrations (Table 6) suggests the involvement of some amino acid in the biosynthetic pathway. Indeed, in procaryotes aspartate can be degraded to pantothenate through β-alanine and cysteine is required for coenzyme A (CoA) production from pantothenic acid [175]. The biosynthesis of nicotine adenine dinucleotides (NAD^+, NADH, $NADP^+$, NADPH) in bacteria occurs through aspartate catabolism [176].

Pyridoxine (B_6) became essential in CDMs with low amino acid concentrations (Table 6). *Lb. plantarum* has also been shown to require this vitamin only in media with limited amino acid availability [106]. Pyridoxal 5'-phosphate (PLP), a bioactive form of B_6, is the cofactor in the biosynthesis of several amino acids (Figure 2) and is thus not needed in complete CDMs, where interconversions between amino acids are not required [106].

The genome of *L. lactis* IL1403 contains biosynthetic genes for folic acid [53] and its precursor *para*-aminobenzoic acid (*p*ABA) [122]. When folic acid (B_9) was replaced by *p*ABA in the medium (see chapter 4.1.3.2.), growth was unaffected, which proved that the biosynthetic enzymes for folates are functional. However, without the supplementation of *p*ABA, folic acid omission impaired the growth of cells (Table 6), which hints at its incapability to biosynthesize sufficient amounts of folates for nucleotide biosynthesis [177]. Therefore, enzymes for the production of folic acid precursor *p*ABA in *L. lactis* IL1403 may have limited activity.

The inessentiality of thiamine (B_1), biotin (B_7) and cobalamin (B_{12}) suggests the capability of *L. lactis* IL1403 to biosynthesize these vitamins or the cofactors obtained from them. Thiamine biosynthesis pathway has been partially determined in *L. lactis* (*thiMDE*) [178].

4.1.2.5. Metal ions and lipoic acid

Inorganic ions cannot be biosynthesized by bacteria and must be obtained from the growth environment. Mg^{2+} is the most important metal ion for LAB (Supplementary Table S2 in Appendix 1) as it is the cofactor in various enzymes, including DNA topoisomerases [179]. Mg^{2+} binds to teichoic acids in the cell wall, wherefrom the ions can be directed to intracellular enzymes [180]. The essential or stimulatory effect of lipoic acid, Mn^{2+} and Cu^{2+} for *L. lactis* IL1403 noticed in this thesis probably derives from the microaerobic nature of MTP experiments. LAB are known to accumulate Mn^{2+} into the cells as a defense mechanism against oxidative stress [181] and Cu^{2+} induces lactate oxidase (LctO), which may be useful in the scavenging of molecular oxygen [182]. Lipoic acid is the cofactor for the pyruvate dehydrogenase complex, which is mainly active in aerobic growth conditions [183]. The genome of *L. lactis* IL1403 does not contain the genes *lipA* and *lipB*, which are required for biosynthesis of lipoic acid [53, 184].

Na^+ and divalent ions (Ca^{2+}, Co^{2+}, Fe^{2+}, Zn^{2+}) might be replaced by K^+ (derived from phosphate buffers) or Mg^{2+}, Mn^{2+}, Cu^{2+}, respectively, in cellular reactions. Another explanation is that nonessential metal ions enter the medium as impurities from other compounds and thus no effect on growth was seen upon their individual omission. Molybdenum is probably not used by LAB as cells are able to grow in media lacking this compound (Supplementary Tables S6, S7 and S8 in the Electronic Appendix). The growth of several LAB strains from genera *Carnobacterium, Lactobacillus, Lactococcus, Leuconostoc* and *Pediococcus* is unaffected by iron supplementation or depletion [185-186], although the requirement of this element by lactobacilli in the metabolism of purines and pyrimidines has been noticed [187].

4.1.3. Development of defined media with balanced composition (Publication I)

4.1.3.1. Modifications in amino acid content

The evaluation of nutrient auxotrophies was followed by construction of a CDM that would be appropriate for the metabolic studies of *L. lactis* IL1403. The objective was to develop a medium with minimal residual concentrations of amino acids. For starters, as the beneficial effect of the MOPS buffer on the growth of lactococci has been shown [79, 92], several combinations of phosphate buffers and MOPS were tested for our CDM [80]. A mixture containing 35.8 mM of MOPS, 5.2 mM of K_2HPO_4 and 5.5 mM of KH_2PO_4 (7.5, 0.9 and 0.75 $g \cdot L^{-1}$, respectively) was chosen as an optimal combination.

In the process of minimalizing nutrient concentrations in a synthetic medium (BS7) data about amino acid auxotrophies and residual concentrations were taken into account simultaneously. Reduction of amino acid concentrations was preferred to omission of nutrients to maintain the high OD and μ. In MTP experiments, amino acid concentrations could be lowered 4-16 times (J series; Supplementary Table S9 in the Electronic Appendix), compared to our initial medium BS7, whereas in test tube scale experiments the reduction rate was limited to 2-8 times (K, M, N, O and P series; Supplementary Table S10 in the Electronic Appendix). Thus, these series of defined media are suitable for batch cultivations on MTPs or in test tubes, respectively.

In test tube experiments, the concentrations of cysteine, glycine, histidine and tryptophan could be lowered most compared with our start-off medium BS7 (Table 5 and Supplementary Table S10 in the Electronic Appendix). Histidine is an essential amino acid, but its content in cellular proteins, similarly to tryptophan, is low compared to other amino acids (Table 2) and thus, even minute concentrations of histidine in a CDM permit the growth of LAB (Supplementary Table S10 in the Electronic Appendix). The different extent of reducing amino acid concentrations in MTP and test tube experiments was probably inflicted by dissimilar oxygen availability [188-189]. The activity of various enzymes, e.g. those involved in pyruvate metabolism, is affected by O_2 supply [111]. The maximal OD in media from series K, M, N, O and P varied from 0.68 to 1.38, the maximal specific growth

rate ranged from 0.29 to 0.59 h^{-1}, the length of the lag phase varied from 5 to 10 hours and the duration of the exponential phase was from 5 to 6.5 hours (Figure 3 and Supplementary Table S10 in the Electronic Appendix). The concentrations of all amino acids were reduced at least two times in the M series and at least four times in the N series. The media from K, O and P series were different combinations with amino acid concentrations decreased 2–8 times. O and P series contain no aspartate and glutamate.

Partial least square regression (PLSR) analysis of these media showed that asparagine and glutamine affected OD most and aromatic amino acids (phenylalanine, tryptophan, tyrosine) least (Figure 4). BCAAs, threonine (MTP experiments) and aspartate/glutamate (test tube experiments) were needed for the fast growth of cells, i.e. these amino acids had the highest influence on specific growth rate (Figure 4). The amount of most of the amino acids utilized in these media was higher than the measurement error (see Table 7, chapter 4.2.1.1.). Vitamin utilization measurements in one medium from the O series showed the full consumption of riboflavin (B_2) and nicotinamide (B_3) and no utilization of pyridoxine (B_6) (see Table 9 in chapter 4.2.1.2.). The K40 medium (Table 5) provided the best trade-off between OD, μ_{max} and amino acid reduction rate and was thus investigated further in continuous cultivations (see the following chapters).

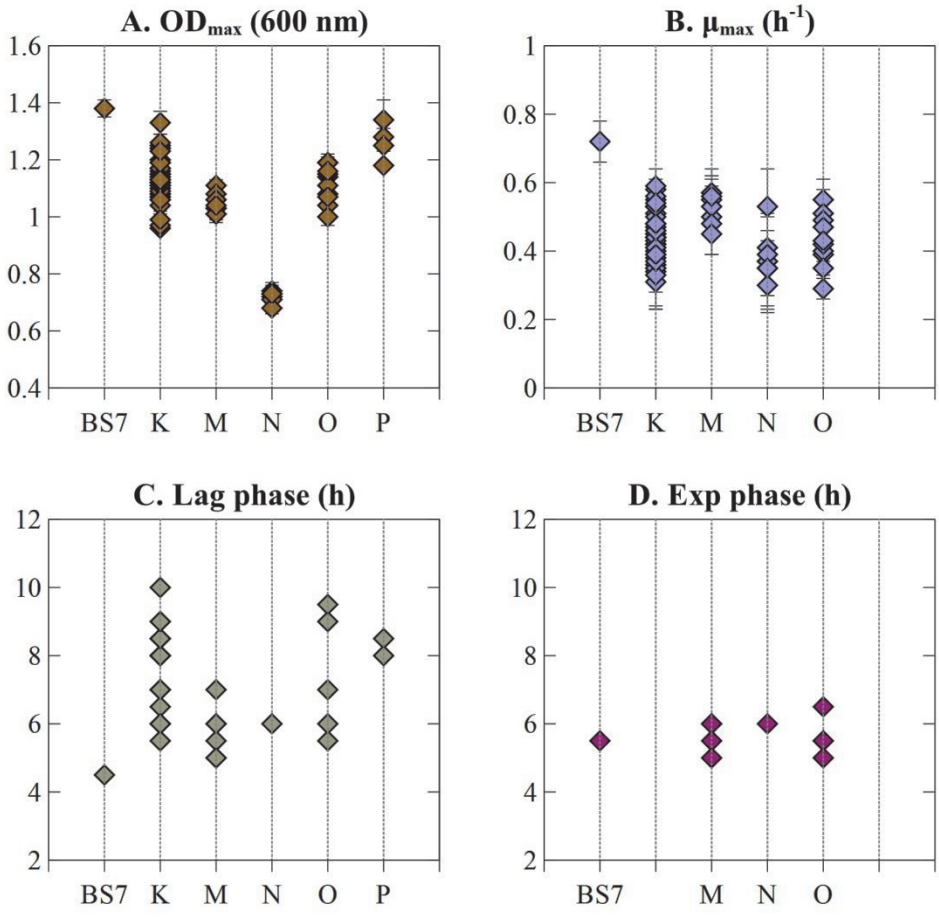


Figure 3. Characteristics of *L. lactis* IL1403 growth on synthetic media in batch experiments. The maximal optical density (OD_{max}) at 600 nm, the maximal specific growth rate (μ_{max}, h^{-1}) and the duration of lag and exponential phases (h) in three replicates in the initial medium BS7 and the newly developed synthetic media from K (44 different media), M (8 different media), N (8 different media), O (10 different media) and P (4 different media) series (Supplementary Table S10 in the Electronic Appendix). μ_{max} was not measured in the P series and the exponential phase was not estimated in K and P media.

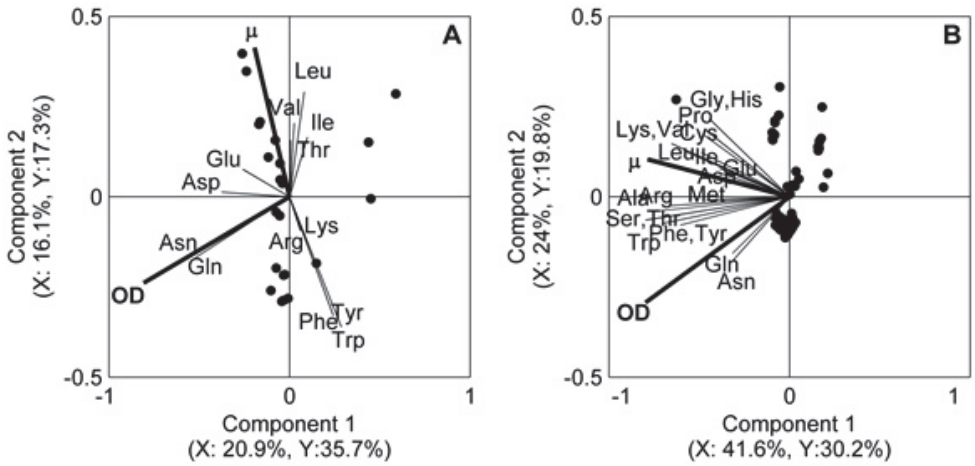


Figure 4. Influence of amino acids on the growth of cells (OD and specific growth rate) in batch experiments: A – microtiter plate experiments; **B** – test tube experiments. Black dots (●) represent experiments on media with various amino acid concentrations. Thin black lines represent loadings of independent variables in PLSR analysis, i.e. amino acid composition. Thick black lines represent loadings of dependent parameters, i.e. OD and specific growth rate. The media used for PLSR analysis were as follows: J series (20 different media in Supplementary Table S9 in Electronic Appendix) for experiments on microtiter plates and BS7 (Table 5), K, M, N and O series (70 different media in Supplementary Table S10 in the Electronic Appendix) for experiments in test tubes.

4.1.3.2. Changes in metal ion and vitamin concentrations

The concentrations of metal ions could be lowered 2-4 times in both MTP and test tube cultivations without a significant loss in biomass concentration or growth speed (Supplementary Table S11 in the Electronic Appendix). Thus, these CDMs with low amounts of metal ions (V series) are suitable to be used in both MTP and test tube scale experiments (Supplementary Table S11 in the Electronic Appendix). A medium called V19 (Table 5) provided the best trade-off between OD, μ_{max} and metal ion reduction rate and was thus investigated further in continuous cultivations (see the following chapters).

The simultaneous reduction of the concentrations of all B-group vitamins two or ten times decreased OD 56 or 97%, respectively, compared with the initial medium BS7. When the amounts of vitamins were raised 1.5 or 2.5 times, OD was increased only 1.1 or 1.2 times and thus, changing vitamin concentrations was regarded unreasonable. However, 2.74 μM of folic acid (B_9), which is an unstable, poorly soluble and expensive compound, could be replaced with its precursor, *para*-aminobenzoic acid (*p*ABA), in concentrations of 1.10-70.58 μM without affecting OD or μ (data not shown).

The concurrent decrease in amino acid and metal ion concentrations in test tube batch experiments resulted in long lag phases (more than 9 hours) and the development of these types of media was not pursued any further.

4.1.4. Comparison of growth in three media in continuous cultivations (Publication III)

The μ-dependent growth of *L. lactis* IL1403 was studied in continuous cultivations by using the A-stat method and three CDMs: BS7 (initial medium), K40 (medium with low concentrations of amino acids) and V19 (medium with low concentrations of metal ions) (Table 5). Maximal specific growth rates (μ_{max}) obtained using these media were 0.57 ± 0.04, 0.42 ± 0.07 and 0.49 ± 0.07 h^{-1} and the biomass yield (Y_{XS}; in the range from $\mu = 0.1$ h^{-1} to μ_{max}) was from 0.103 ± 0.001 to 0.162 ± 0, from 0.107 ± 0.003 to 0.138 ± 0, and from 0.096 ± 0.013 to 0.160 ± 0 $g \cdot g_{glucose}^{-1}$ for BS7, K40 and V19, respectively. Specific biomass and lactate productivity, ATP spilling (the difference between total ATP produced and total ATP spent for the synthesis of biomonomers), channeling of glucose to lactate and the production of unidentified metabolites from glucose were similar between the media, as seen from Figure 5.

The production of formate (Figure 5) and acetate (data not shown) was decreased in V19. The change towards a more homolactic fermentation is a way of increasing metabolism efficiency [6]. The minimization of nutrient concentrations induced rearrangements in amino acid consumption strategies and increased the flux of amino acids to biomass proteins and peptidoglycans (see subsequent chapters).

The amino acid concentration in K40 and the metal ion content in the V19 medium were more balanced and satisfied cell requirements more than in BS7 (Figures 6 and 7). In K40, more than 50% of 14 amino acids were utilized from the medium at $\mu = 0.1$ h^{-1} and the consumption of eight amino acids exceeded 80% at $\mu = 0.4$ h^{-1} (Figure 6). The utilization of metal ions (namely magnesium) could be detected more accurately in V19 than in BS7 (Figure 7). In BS7, less than 5% of magnesium was consumed and the results were fluctuating and incorrect. Therefore, it seems as if magnesium was produced in minute amounts instead of utilization in BS7. This misinterpretation was bypassed in V19 due to the higher percentual consumption of Mg^{2+} (Figure 7). The consumption of four B-group vitamins was measured in a continuous cultivation (A-stat) with the BS7 medium. The results indicate a balanced composition for these nutrients (Figure 8). K40 and V19 have the same vitamin concentration as BS7 (Table 5), which suggests similar vitamin consumption in these media.

However, the BS7 medium had a more similar composition to cellular amino acid ratios than K40 (see cellular amino acid ratios in Table 2 and ratios in media in Table 5). Thus, data about amino acid ratios in biomass is not always reliable to enable compilation of synthetic media as there exist biosynthetic pathways and interconversions between amino acids (Figure 2).

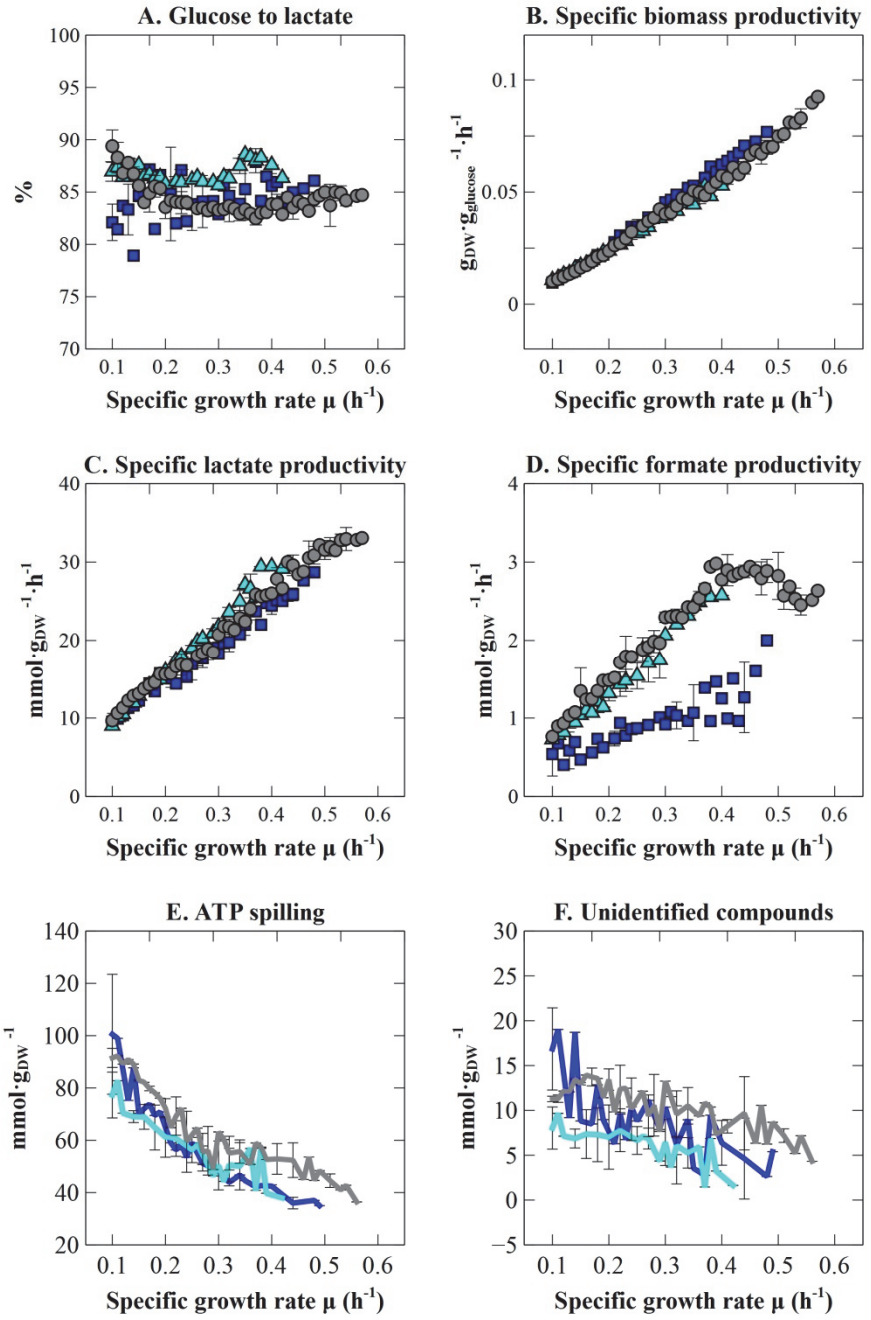


Figure 5. Biomass yield and pyruvate metabolism in A-stat cultivations: A – Conversion of glucose to lactate (percentage of the total amount of consumed glucose); **B** – Specific biomass productivity (gram of dry weight per gram of consumed glucose per hour); **C** – Specific lactate productivity (millimoles per gram cell dry weight per hour); **D** – Specific formate productivity (millimoles per gram cell dry weight per hour); **E** – Flux calculations: ATP spilling (millimoles per gram cell dry weight); **F** – Flux calculations: production of

unidentified compounds (metabolites other than lactate, formate, acetate, ethanol) from glucose (millimoles per gram cell dry weight. (Average results of two fermentations per each medium: BS7 – grey circles (●) or grey line; K40 – blue triangles (▲) or blue line; V19 – dark blue squares (■) or dark blue line.)

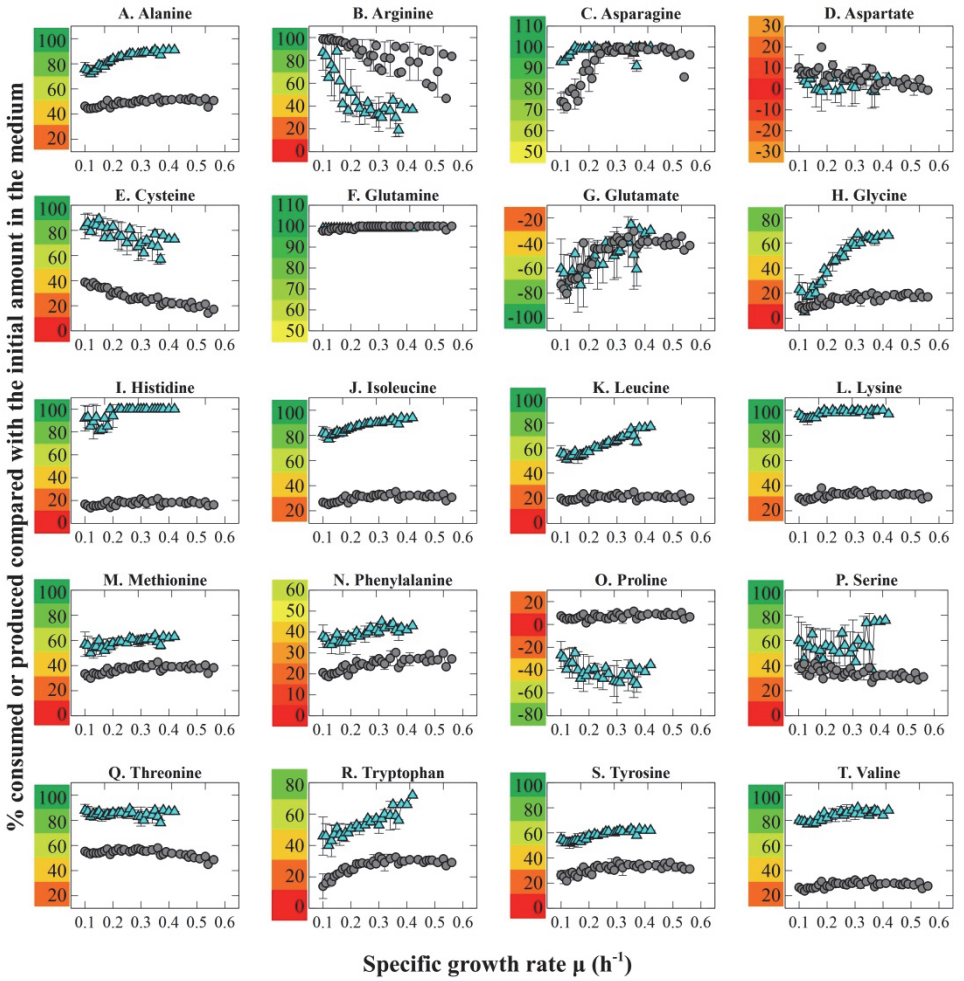


Figure 6. Amino acid consumed or produced in A-stat cultivations, % of the initial amount in the medium. (The red-green scale on the left indicates the magnitude of consumption. Average results of two fermentations per each medium: BS7 – grey (●) circles; K40 – blue triangles (▲). Negative values represent production.)

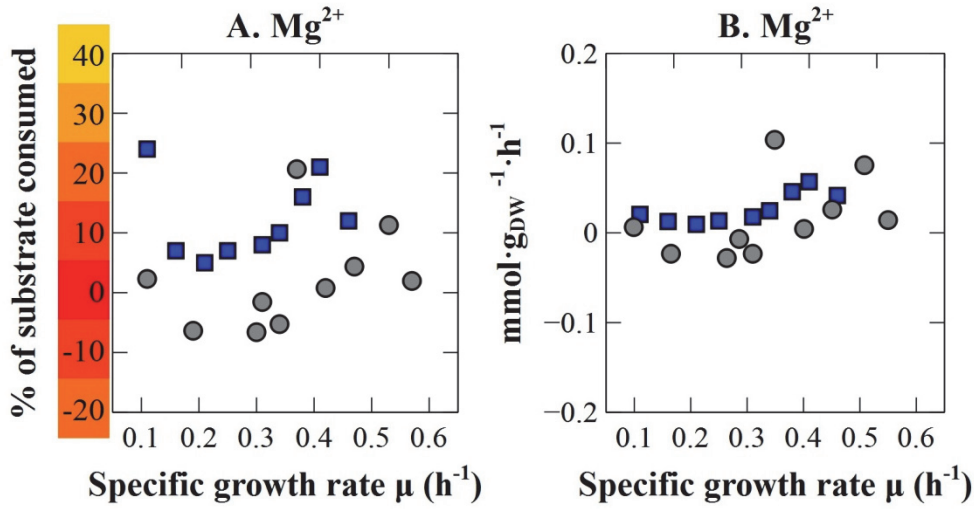


Figure 7. Mg^{2+} utilization in A-stat cultivations: A – Mg^{2+} consumption, % of the intial amount in the medium; **B** – Mg^{2+} specific utilization (millimoles per gram cell dry weight per hour). (Results of one fermentation per each medium: BS7 – grey circles (●) and V19 – dark blue squares (■). Negative values represent production. The concentration of Mg^{2+} ions in V19 is two times lower than in BS7.)

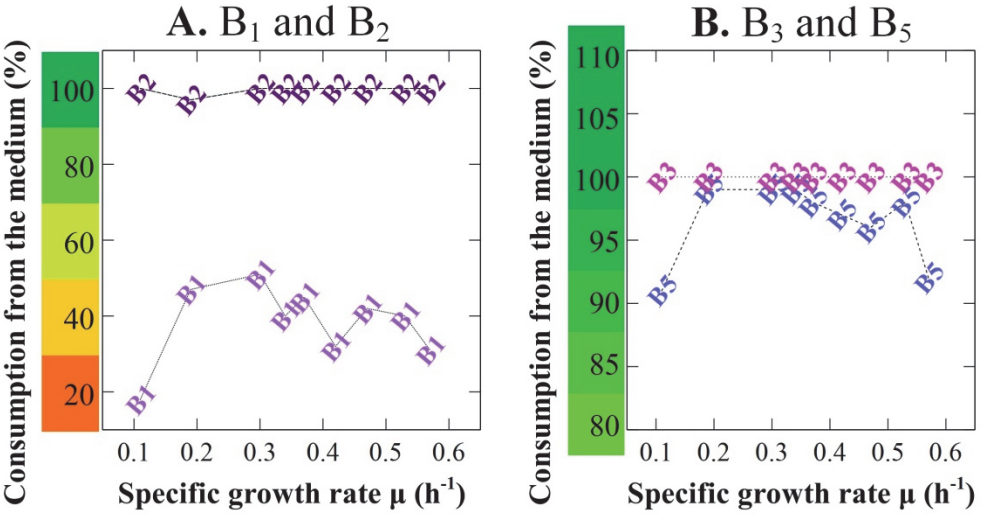


Figure 8. Percentual B-group vitamin consumption in A-stat cultivations: Utilization of thiamine (B_1) and riboflavin (B_2) (**A**), and nicotinamide (B_3) and pantothenate (B_5) (**B**) in one A-stat cultivation with the BS7 medium. (The red-green scale on the left indicates the magnitude of consumption (green colour represents high utilization).

4.1.5. Comparison of results with literature data (Publication III)

The CDMs presented in this thesis, especially BS7, K40 and V19, are suitable for metabolic studies of *L. lactis*. BS7 offers the possibility to conduct experiments at higher specific growth rates, whereas K40 and V19 have been balanced in amino acid and metal ion concentrations, respectively, i.e. nutrient concentrations are close to cell requirements. In comparison, biomass concentration and amino acid consumption data presented in [9] indicates that *Lb. plantarum* consumes less than 17% of 16 amino acids from a synthetic medium (see composition in [16] and [190] in Supplementary Table S6 in the Electronic Appendix). Thus, many CDMs reported in the literature for LAB contain an excess of nutrients, which may influence the precision of analytical measurements and increase the price of the medium. On the other hand, not all CDMs have been designed for metabolic research, which justifies their abundance of nutrients. For example, the media reported in [79] (Supplementary Table S6 in the Electronic Appendix) were compiled for high-cell-density fed-batch experiments, whereas some other CDMs have been constructed for the synthesis of certain bioproducts like lactate or exopolysaccharides [82, 94, 191] (Supplementary Table S7 in the Electronic Appendix).

According to the 8 July 2015 prices of chemical products from Sigma-Aldrich, the cost for BS7, K40 and V19 were 3.21, 2.22 and 2.97 $EUR \cdot L^{-1}$, respectively (Table 5). In comparison, the price of the CDM used in [192] was 7.48 $EUR \cdot L^{-1}$ and that of the minimal medium MS15 [91] (Supplementary Table S8 in the Electronic Appendix) was 3.16 $EUR \cdot L^{-1}$, as these media contain higher amino acid concentrations than the CDMs presented herein. Thus, the only advantage of MS15 over K40 is shorter preparation time, due to the smaller number of components. K40 is cheaper and offers higher maximal specific growth rate than M15: 0.42 *versus* 0.37 h^{-1} (Table 5 and Supplementary Table S8 in the Electronic Appendix). The CDMs of this thesis do not compete in price with low-molecular media derived from yeast extract by membrane filtration (e.g. the medium reported in [193]). Nevertheless, a CDM obtained by yeast extract filtration contains hundreds of peptides, nucleosides and other compounds, which makes their individual determination problematic. Hence, self-weighed synthetic media still have an advantage of facilitating analytical measurements and data interpretation.

4.2. Applications of chemically defined media (Publications I, III, IV)

4.2.1. Effect of medium minimization on amino acid consumption strategies (Publications I, III, IV)

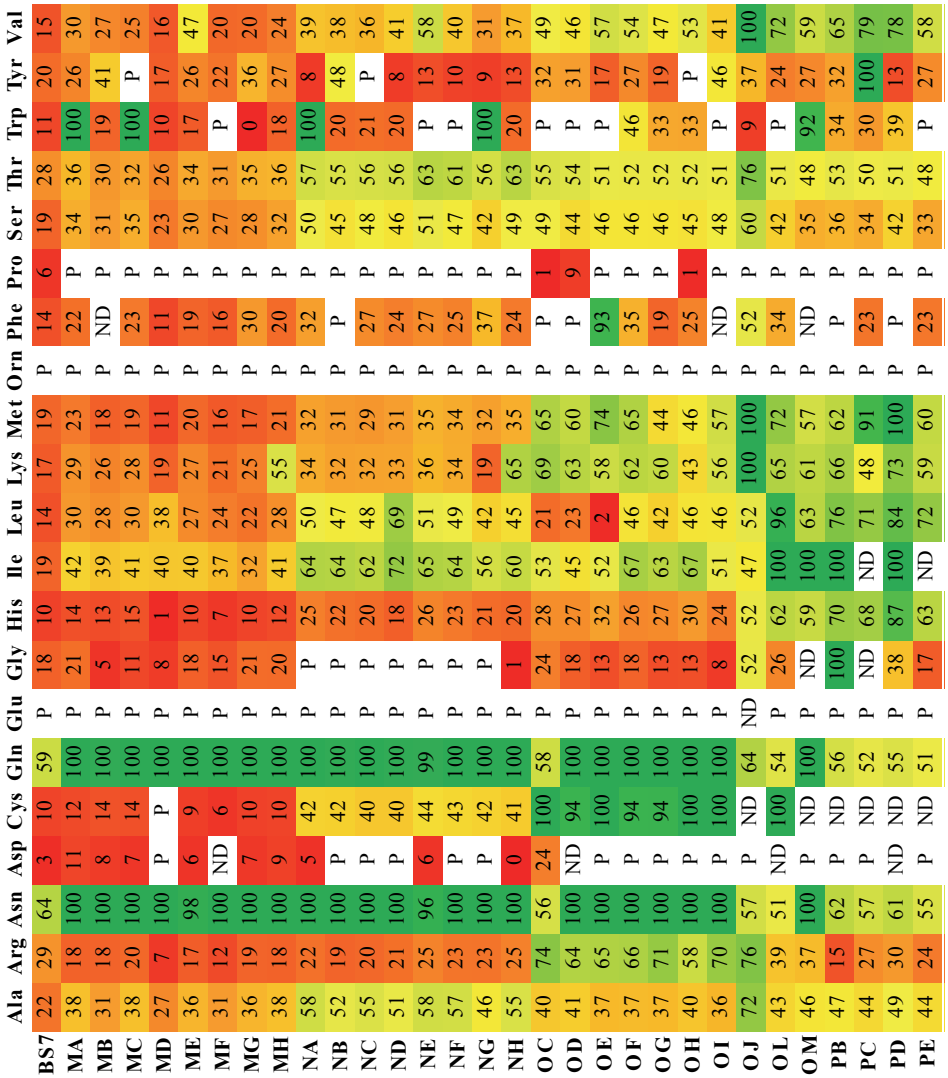
CDMs are an efficient tool for metabolic research and bioproduction of functional compounds. Studying amino acid consumption in synthetic media provides practical information to be made use of in the manufacturing of recombinant proteins and amino acid catabolism products. For example, it was noticed that some amino acids were biosynthesized rather than taken up from the medium. In addition, the flux of certain amino acids through known catabolic pathways changed with specific growth rate.

4.2.1.1. Amino acid consumption in batch experiments

4.2.1.1.1. Biosynthesis of certain amino acids preferable to their uptake

L. lactis IL1403 was able to biosynthesize aspartate, glutamate, glycine, proline and the aromatic amino acids phenylalanine, tryptophan and tyrosine if these compounds were omitted individually or were available in low concentrations (Table 7). Also, earlier some researchers noted that *L. lactis* strains preferred the biosynthesis of alanine, aspartate, glycine, phenylalanine and threonine to their uptake from the growth environment, and the production of these amino acids was associated with 2-oxoglutarate and subsequent glutamate formation [160, 194]. The transcription initation of tryptophan biosynthetic operon *trpEGDCFBA* in *L. lactis* IL1403 is known to be increased four-fold if any amino acid is depleted [195]. In *L. lactis* NCDO2118 and MG1363, alanine, arginine, aspartate, glycine, histidine, lysine, phenylalanine, threonine and tyrosine in intracellular proteins have been shown to partially or completely originate from glucose [7, 194]. Also, aromatic amino acids can be converted to one another. For example, the omission of phenylalanine from the medium and its concurrent biosynthesis resulted in an increase in the utilization of tyrosine (the NB medium in Table 7). Similarly, the omission of tyrosine from the medium led to its biosynthesis and depletion of tryptophan from the medium (the MC medium in Table 7). Also, the production of glycine in N series media (Table 7) occurred due to the degradation of serine via serine hydroxymethyltransferase or threonine via threonine aldolase (Figure 2). Glutamate was probably obtained from glutamine (Figure 2), whereas aspartate and proline apparently originated from asparagine (Figure 2) and glutamate [196], respectively, in addition to glucose.

Table 7. Amino acid consumption or production in batch cultivations with 31 different media, % of the initial amount in the medium. Numbers in green represent high and in red low consumption. P – amino acid was produced. ND – not determined.



Alanine, lysine and methionine were mainly consumed for the formation of biomass proteins in all media, whereas the utilization of histidine and valine met cell requirements only in the media of O and P series (Table 8). In fact, in O-media 4-52% of cellular methionine originated from sources other than extracellular methionine, e.g. from degradation of other amino acids (data not shown). The latter corresponds to the drastic increase in cysteine consumption in O-media (Table 7). Thus, the assumption that the auxotrophy of *L. lactis* IL1403 for methionine would derive from the low activity of biosynthetic enzymes rather than inactivation of genes made in previous chapters has to be valid, as this strain has the ability to

biosynthesize methione (e.g. from cysteine), but its biosynthetic capability is insufficient to sustain growth if methionine is depleted.

4.2.1.1.2. Overconsumption of amino acids

Comparison of the amount of amino acid utilized by *L. lactis* IL1403 in the stationary phase and their concentration in the biomass shows that some amino acids, most notably arginine and serine, were overconsumed, i.e. they were used for other purposes in addition to biomass formation (Table 8). Hence, in the leave-one-out experiments for determination of auxotrophies, serine was presumably the precursor of cysteine biosynthesis (Figure 2), although *L. lactis* IL1403 can convert methionine to cysteine as well [197]. Thus, the low availability of serine made IL1403 incapable of growth without an extracellular supply of cysteine (Table 6). A portion of serine might have been directed to the production of pyruvate [7, 17].

Furthermore, approximately half of the arginine consumed was converted to unidentified compounds, whereas the rest was used for the biosynthesis of biomass proteins and ornithine (Table 8). In the N series media, the production of ornithine was low (Table 8), which suggests that the cells avoided ATP generation through the arginine deiminase (ADI) pathway in the nutritionally poor environment. In addition, the overconsumption of arginine in optimized media was lower than in the initial medium BS7 (Table 8).

Asparagine was fully consumed in several CDMs, whereas aspartate was utilized in minute amounts or even produced into the environment (Table 7). *L. lactis* transports asparagine into the cells more effectively than aspartate [198]. The overconsumed asparagine (Table 8) may have been used for the biosynthesis of aspartate, which in turn could have been directed through oxaloacetate to pyruvate, acetoin and diacetyl [199]. However, as organic acid production was not measured in batch experiments, this hypothesis cannot be verified.

Also, as glutamine and glutamate can be converted to one another (Figure 2), at least one of these amino acids should be available in adequate amounts to provide the cells with amino groups and precursors for nucleotide synthesis [140, 177]. Consequently, the omission of glutamine with concurrent reduction of glutamate concentration prevented the growth of IL1403 completely (Table 6). Roughly half of the glutamine consumed was channeled to unknown products, whilst the other half was used as glutamine or glutamate by biomass proteins or for the production of additional glutamate, which was secreted into the growth environment (Table 8). Glutamate biosynthesis was diminished in N series media (Table 8), presumably to cut back energetic costs in case of low nutrient availability: the concentrations of all amino acids were reduced at least four times in these media.

The overconsumption of leucine (Table 8) indicates lactate-mediated stress and energy depletion: acidic conditions increase leucine catabolism to 2-methylbutanoic acid and 3 ATP molecules in *Lb. sanfranciscensis* [200].

Glycine, phenylalanine and tyrosine present in cellular proteins and peptidoglycan were partially obtained by biosynthesis, i.e. from other amino acids or glucose (data not shown).

Table 8. The percentage of amino acids used in batch cultivations for the formation of biomass proteins and peptidoglycans or other compounds (arginine for ornithine and glutamine for glutamate). Analysis involved 31 different media in single paralles. The amino acid consumption data was compared to intracellular amino acid concentrations. Numbers in green indicate utilization of amino acids mainly for biomass proteins and in red represent overconsumption. ND – not determined, NOC – not overconsumed.

	Amino acids to biomass (%)												Arg to Orn (%)	Gln to Glu (%)
	Ala	Arg	Asn	Gln	His	Ile	Leu	Lys	Met	Ser	Thr	Val		
BS7	72	11	29	27	48	41	30	66	49	19	36	47	52	33
MA	80	36	36	32	68	35	28	72	90	22	55	46	17	27
MB	84	31	32	27	62	33	25	70	98	21	58	45	19	25
MC	70	29	33	28	56	33	25	67	92	19	55	50	14	27
MD	NOC	ND	38	33	NOC	39	45	NOC	NOC	33	79	90	ND	35
ME	86	39	38	32	90	38	31	80	NOC	25	61	59	25	30
MF	NOC	56	38	33	NOC	42	36	NOC	NOC	28	68	73	24	30
MG	75	32	33	28	79	42	34	75	NOC	23	51	62	29	33
MH	77	36	36	31	74	37	29	73	97	23	55	57	22	27
NA	70	41	49	43	47	31	23	82	86	20	47	49	6	15
NB	78	47	50	43	55	32	24	89	88	22	49	50	5	11
NC	75	46	50	44	60	33	25	89	93	21	49	55	5	11
ND	82	43	50	44	71	29	35	89	89	22	49	48	18	10
NE	65	32	47	40	43	29	21	72	71	18	40	61	17	8
NF	68	37	47	41	50	30	22	79	75	20	42	46	9	16
NG	93	37	47	41	54	34	27	NOC	77	23	47	62	12	21
NH	73	35	48	42	60	33	26	86	74	20	42	51	20	12
OC	100	23	41	32	87	46	65	83	NOC	20	47	96	43	31
OD	84	22	39	32	79	45	49	74	NOC	19	43	89	41	27
OE	NOC	24	41	33	73	42	NOC	90	NOC	19	47	76	47	27
OF	NOC	25	44	35	91	35	54	88	NOC	20	49	86	46	24
OG	NOC	23	44	36	88	38	58	90	NOC	20	50	NOC	50	29
OH	96	27	43	34	78	34	52	NOC	NOC	20	47	88	38	25
OI	99	22	41	33	96	42	49	88	NOC	18	46	NOC	50	26
OJ	90	36	34	24	95	NOC	42	NOC	NOC	28	57	NOC	9	ND
OL	93	22	46	34	98	63	27	88	NOC	23	51	67	42	27
OM	84	19	44	35	92	NOC	40	88	NOC	27	52	79	6	25
PB	90	ND	39	34	76	66	22	91	NOC	29	53	77	ND	23
PC	91	26	40	35	76	ND	25	NOC	73	29	52	62	29	24
PD	83	32	41	37	58	61	20	82	100	25	55	60	30	19
PE	94	38	42	37	85	ND	25	98	NOC	31	57	88	23	24

4.2.1.2. Vitamin consumption in batch experiments

Table 9 shows the percentual utilization of five B-group vitamins by *L. lactis* IL1403 in a batch experiment with the OL medium, in which riboflavin (B_2) and nicotinamide (B_3) as precursors of cofactors FAD/FMN and NADH, respectively,

were fully consumed. At the same time, 47% of thiamine (B_1) and 67% of pantothenate (B_5) were taken up from the medium. Thiamine is a precursor of thiamine pyrophosphate (also called thiamine phosphate) which is a cofactor in various enzymes, including KdcA, which is involved in BCAAs degradation [201].

The zero consumption of pyridoxine (B_6) in the OL medium (Table 9) suggests that *L. lactis* IL1403 is unable to transport this vitamer of B_6 into the cells. Other media for the cultivation of the IL1403 strain contain pyridoxamine or pyridoxal-HCl as B_6 vitamers (see MCD/ZBM1/ZBM2, MS10 and MS15 media in Supplementary Tables S6, S7 and S8, respectively, in the Electronic Appendix). *Lb. casei* can transport only pyridoxal into the cells, whereas *Lb. bulgaricus* can transport the phosphorylated forms of pyridoxal, pyridoxine and pyridoxamine [202]. Nevertheless, as this thesis contains data about vitamin utilization for only one medium (OL), it cannot be excluded that pyridoxine utilization occured in other CDMs or even in the OL medium in the amounts below the detection limit. Moreover, *L. lactis* IL1403 was unable to grow in CDMs with reduced amino acid concentrations if pyridoxine was omitted (Table 6). Thus, this vitamer of B_6 has to be involved in the biosynthesis of pyridoxal 5'-phosphate (PLP) in *L. lactis* IL1403, which is a bioactive form of B_6. PLP is a cofactor of AraT, which is needed for the catabolism of aromatic amino acids, methionine and leucine and the biosynthesis of phenylalanine and tyrosine [203-204]. Serine hydroxymethyltransferase (responsible for interconversion between serine and glycine) and threonine aldolase (degradation of threonine to glycine and acetaldehyde) are also PLP-dependent [145].

Table 9. Vitamin consumed, % of the initial amount in batch cultivations with the OL medium. Green represents high and red low consumption. B_1 – thiamine, B_2 – riboflavin, B_3 – nicotinamide, B_5 – pantothenate, B_6 – pyridoxine.

	B_1	**B_2**	**B_3**	**B_5**	**B_6**
OL	42	100	100	67	0

4.2.1.3. Amino acid consumption in continuous cultivations

Consumption of amino acids by *L. lactis* IL1403 in A-stat cultivations in three media described in previous chapters – BS7, K40 (media with reduced amino acid concentrations) and V19 (media with reduced metal ion concentrations) was examined (Table 5). Amino acid utilization profiles in these media were generally similar, with some exceptions. The flux of amino acids into biomass proteins and peptidoglycans increased when the amino acid availability in the medium was limited and the specific growth rate was increased.

The next chapters will provide information that is useful for the development of synthetic media for other lactococcal strains, indicating which amino acids can be mainly used for the formation of biomass proteins and which for other purposes. Furthermore, the data presented in this thesis could be taken advantage of for increasing amino acid fluxes for the biosynthesis of recombinant proteins or amino acid degradation products by changing the composition of the medium.

4.2.1.3.1. Rerouting of arginine catabolism

In comparison with BS7 and V19, arginine consumption in K40 was reduced up to 74% (Figure 9). Moreover, in BS7 and V19 media, respectively 66 and 54% or 77 and 59% of arginine was converted to ornithine at $\mu = 0.1$ or 0.4 h^{-1} (Figure 10). In K40, however, 41% of arginine was converted to ornithine at $\mu = 0.1$ h^{-1} and only 2% at $\mu = 0.4$ h^{-1} (Figure 10). If ornithine was partially degraded to proline, then 47% of arginine in the K40 medium was directed to the ADI pathway at $\mu = 0.1$ h^{-1} and 23% at $\mu = 0.4$ h^{-1}. However, the genes for proline biosynthesis from ornithine have not been fully determined in the IL1403 strain [159] and glutamate was more likely used as a substrate for proline production. Thus, in case of limited amino acid availability, the cells avoided additional ATP production through the ADI pathway, where one mole of ATP, ornithine and carbamoylphosphate was biosynthesized per one mole of arginine. The same trend was noticed in batch experiments with media of N series (Table 8 in chapter 4.2.1.1.2.). Rearranging amino acid metabolism is one option for the cells to increase the efficiency of ATP usage [87].

The minute production of ornithine in the K40 medium at higher specific growth rates raises a question whether cells had a sufficient supply of carbamoylphosphate for pyrimidine biosynthesis. The estimated sum of intracellular CMP, UMP, dCMP and dTMP is 0.11-0.15 $mmol \cdot g_{DW}^{-1}$ at specific growth rates of 0.1-0.6 h^{-1} [6]. We proposed that in K40 at $\mu > 0.2$ h^{-1} the cells biosynthesized the necessary carbamoylphosphate from CO_2, which in turn was retrieved from glycolysis or by catabolism of other amino acids. Note that no putrescine production was observed (data not shown), which suggests that carbamoylphosphate was not obtained from arginine degradation through the agmatine deiminase (AGDI) pathway, although the AGDI gene cluster exists in *L. lactis* IL1403 [205].

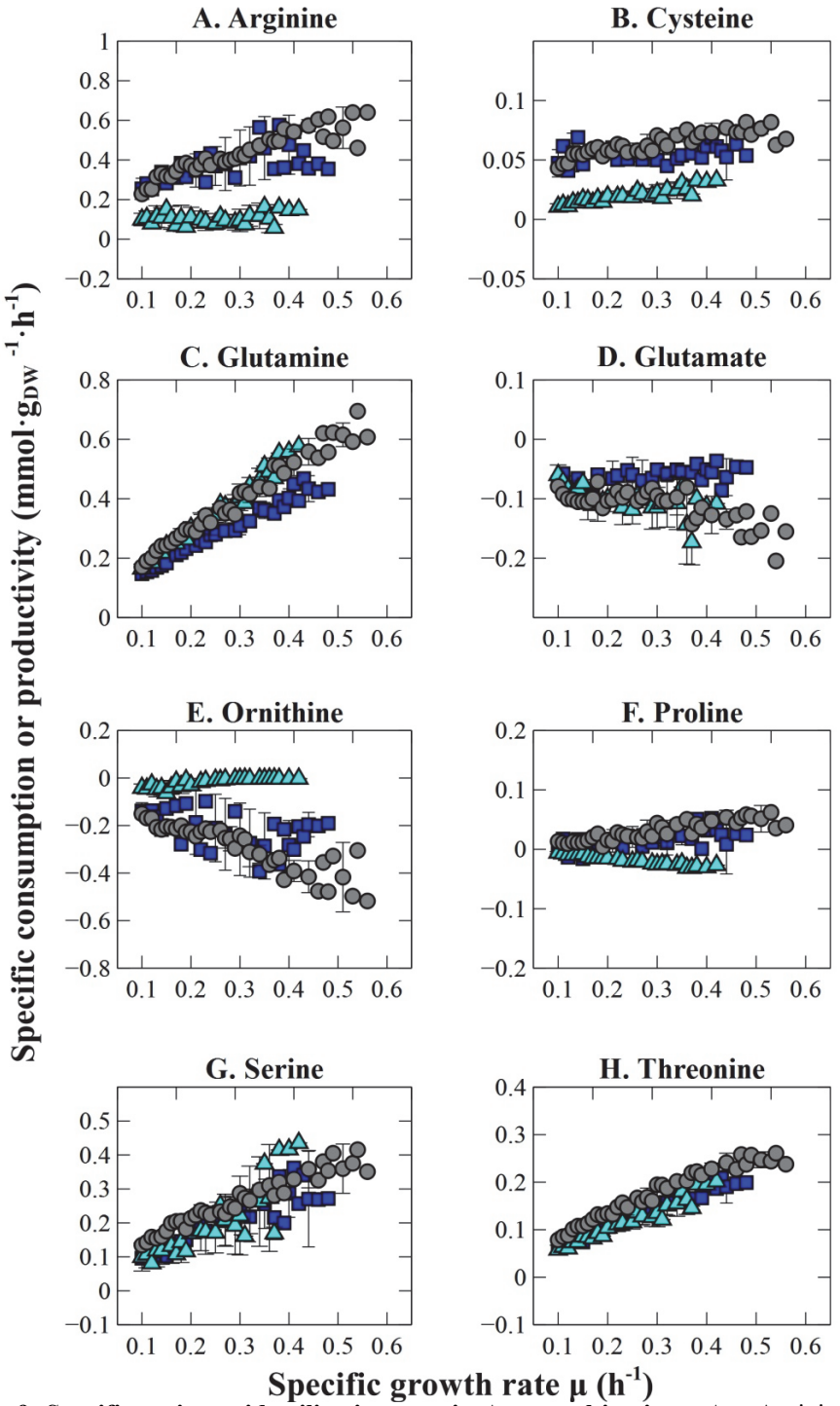


Figure 9. Specific amino acid utilization rate in A-stat cultivations: A – Arginine; **B** – Cysteine; **C** – Glutamine; **D** – Glutamate; **E** – Ornithine; **F** – Proline; **G** – Serine; **H** – Threonine. (Average results of two fermentations per each medium (millimoles per gram cell dry weight per hour): BS7 – grey circles (●); K40 – blue triangles (▲); V19 – dark blue squares (■). Negative values represent the production of amino acids.).

4.2.1.3.2. Reduction in glutamate biosynthesis

In the V19 medium, the consumption of glutamine was 13-33% lower and glutamate production was decreased 49%, compared with the BS7 medium (Figure 9). In addition, the conversion of glutamine to glutamate was respectively 46, 36 and 49% in BS7, K40 and V19 at $\mu = 0.1\ h^{-1}$, and 24, 20 and 13% at $\mu = 0.4\ h^{-1}$ (Figure 10). The reduced glutamine consumption and glutamate biosynthesis in the V19 medium may be explained as follows: (i) glutamine overconsumption is avoided for energy maintenance, while the GlnPQ complex, which transports glutamine/glutamate into the cells, requires ATP; (ii) the V19 medium has a lower Na^+ content than K40 and BS7, whereas glutamine/glutamate are more efficiently taken up by GlnPQ if osmolyte concentrations are increased [206]; (iii) the produced amount of formate, which hampers the growth of cells more than lactate, acetate and ethanol [207], is lower in V19 (see Figure 5 in chapter 4.1.4.), which indicates a decreased demand for acid stress alleviation by production of ammonia (and glutamate) from glutamine.

In addition, glutamate was probably the precursor to proline production in the K40 medium (Figure 9) as it provides an opportunity for $NAD(P)^+$ regeneration [7, 158]. Proline production in media with reduced amino acid content was noticed in batch experiments as well (Table 7 in chapter 4.2.1.1.1.).

4.2.1.3.3. Overconsumption of pyruvate-directed amino acids

In the K40 medium, cysteine consumption was up to 77% lower than in BS7 and K40, whereas the utilization of threonine (and serine at lower specific growth rates) was up to 26% lower (Figure 9). Less than 40 and 60% of consumed serine and threonine, respectively, can be found in cellular proteins (Figure 10). Therefore, the overconsumed portions of these amino acids must have been converted into other amino acids in biomonomers or into pyruvate and unidentified byproducts. In the K40 medium, more than 50% of glycine in biomonomers was obtained from biosynthesis (Figure 10). Serine and threonine are the potential substrates for glycine production. The concentration of serine in K40 was higher than that of threonine, which renders the former the main precursor for glycine production due to a competitive inhibition of serine and threonine for the SerP1 transporter [147].

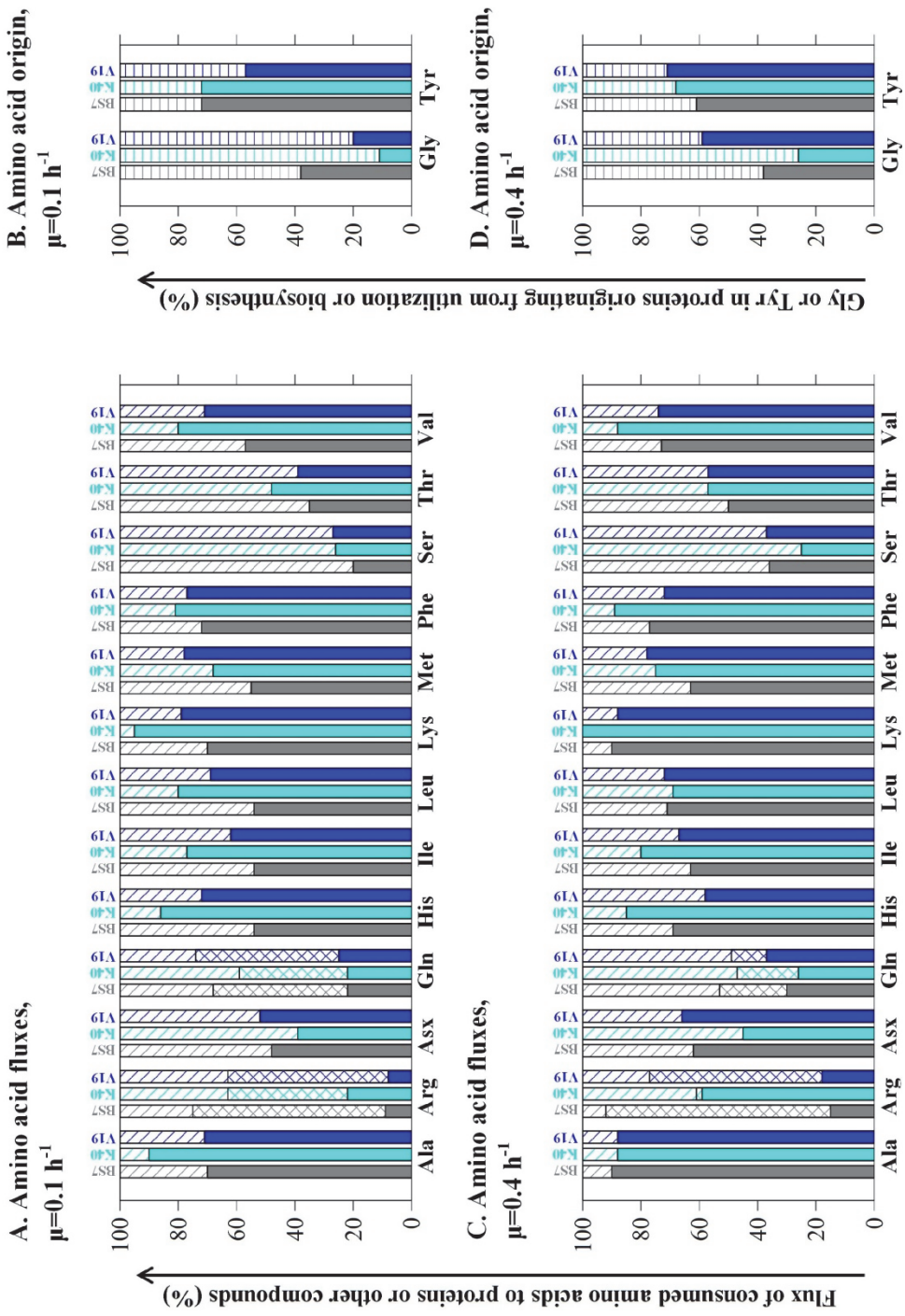


Figure 10. Amino acid degradation products in A-stat cultivations: A, C – The percentage of utilized amino acid used for the formation of cellular proteins (solid bars); other compounds – arginine for ornithine and glutamine for glutamate (criss-crossed bars); and unidentified compounds (diagonally striped bars) at μ = 0.1 and 0.4 h^{-1}; **B, D** – The

percentage of glycine and tyrosine in cellular proteins originating from glycine or tyrosine utilization (solid bars) and biosynthesis from other compounds (vertically striped bars). (Calculations were based on comparison of amino acid utilization data for BS7 (grey), K40 (blue) and V19 (dark blue) with their concentrations in cellular proteins and peptidoglycan obtained from [3].).

4.2.1.3.4. Consumption strategies for other amino acids

Figure 10 shows that in addition to arginine, glutamine, serine and threonine, asparagine was also heavily overconsumed, i.e. utilized for the production of unidentified byproducts in addition to biomass protein and peptidoglycan biosynthesis, especially at $\mu = 0.1$ h^{-1}. Isoleucine, leucine and methionine were overconsumed as well (Figure 10), but their flux to unidentified products was lower than in case of amino acids mentioned in the previous sections. The consumption of alanine, histidine, lysine, phenylalanine and valine was close to the requirements for protein formation, especially in the case of K40. The flux of consumed amino acids to biomass proteins (excluding arginine, asparagine and glutamine) was more intense in K40 and V19 (simplified media) than in BS7 (initial medium), demonstrating a decrease in the formation of amino acid degradation byproducts and a more efficient usage of amino acids in simplified media. Furthermore, the flux of most amino acids to proteins increased with the rise of specific growth rate (0.1 versus 0.4 h^{-1}), which emphasizes the role of μ in enhancing the amino acid metabolism efficiency.

Glycine and tyrosine in biomass proteins were partially biosynthesized: more than 50% of glycine and more than 20% of tyrosine in cellular proteins originated from biosynthesis (Figure 10), whereas the remaining portions were obtained by consuming these amino acids from the medium.

4.2.1.3.5. Grouping of amino acids based on cell requirements

The data about amino acid consumption in glucose-limited conditions presented above enables the distribution of the acids into five groups: (i) amino acids that are largely overconsumed, i.e. mainly utilized for objectives other than protein formation (arginine, asparagine, glutamine, serine, threonine); (ii) amino acids that are slightly overconsumed, i.e. mostly utilized for protein formation, but byproduct synthesis occurs as well (isoleucine, leucine, methionine); (iii) amino acids that are mainly used for protein formation as these are consumed in amounts comparable with the concentrations in cellular proteins and peptidoglycan (alanine, histidine, lysine, phenylalanine, valine); (iv) amino acids which are partially biosynthesized despite their presence in the medium (glycine, tyrosine); (v) amino acids which are completely synthesized from other amino acids or compounds rather than taken up from the medium (glutamate, proline).

Hence, when compiling new defined media for other species of lactococci, the concentrations of amino acids in group (iii) should correspond to the amounts needed for the formation of biomass proteins and peptidoglycan, i.e. their concentrations in the medium should be in the same range than their amounts in the biomass. The concentrations of amino acids in groups (i) and (ii), however, should

be in excess, i.e. higher than in biomass proteins, while these are needed for other biosynthetic pathways (e.g. nucleotide synthesis), additional energy costs, regeneration of redox compounds or biosynthesis of ammonia for balancing acid production. The concentrations of amino acids of groups (iv) and (v) may be relatively low compared with the amounts in biomass proteins, or the acids may be even omitted from the medium. Note that the requirements for some amino acids may increase at higher μ and amino acid concentartion limitation may lead to an earlier growth halt as seen by comparing μ_{max} values of K40 and BS7 (Table 5).

4.2.1.3.6. Comparison of the BS7 and HAMmod media

Prior to the development of BS7, K40, V19 and other defined media presented in this thesis a medium called HAMmod was used in our laboratory for the cultivation of *Lactococcus lactis*. HAMmod is a defined medium that consists of self-weighed compounds and the commercial GIBCO^{TM} F-12 Nutrient Mixture (see the Materials and Methods section). The BS7 medium is a completely self-weighed analogue of the HAMmod medium and was developed in order to dispose of the need for buying an expensive commercial nutrient mixture and thus reduce the cost of fermentation.

The amino acid concentration of the HAMmod medium as well as the maximal specific growth rate (0.59 ± 0.02 h^{-1}) and biomass yield (from 0.13 ± 0 to 0.17 ± 0.01 $g \cdot g_{DW}$ from $\mu = 0.1$ h^{-1} to μ_{max}) obtained with *L. lactis* IL1403 were similar to those of BS7 (Table 5). Similarly, the groups of amino acids based on cell requirements were the same as indicated in the previous chapter.

In addition, a thorough examination of the amino acid and energy metabolism of *L. lactis* IL1403 on the HAMmod medium by using continuous cultivations and various omics methods has been conducted in Publication IV. The results demonstrated that the efficiency of the metabolism and incorporation of amino acids into the biomass of IL1403 increased with specific growth rate. Thus, changing the specific growth rate is another factor, in addition to medium optimization, for altering the flux of consumed amino acids to protein and peptidoglycan formation.

The A-stat cultivations of *L. lactis* IL1403 on the HAMmod medium demonstrated that several proteins involved in amino acid metabolism were downregulated with increasing μ (Figure 11). Examples include proteins from the ADI pathway (Figure 11A), glutamine and BCAAs degradation reactions (Figures 11B and 11C) and biosynthesis of several amino acids (Figure 11D). Thus, similarly to growth in the conditions of limited nutrient supply, additional energy generation through arginine degradation and the production of amino acid degradation byproducts were decreased at higher μ values and the cells preferred to take up amino acids from the environment instead of biosynthesizing them.

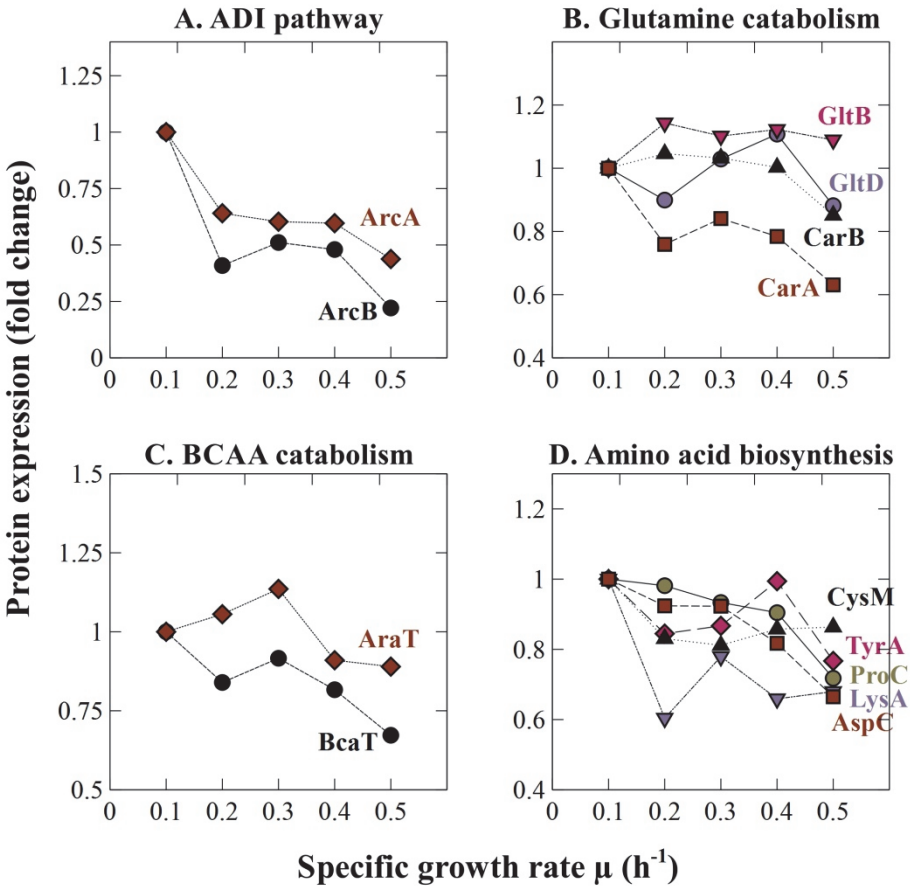


Figure 11. Changes in protein expressions of *L. lactis* IL1403 during A-stat cultivations with the HAMmod medium: A – Proteins involved in arginine degradation to ornithine (ADI pathway). Arginine deiminase ArcA (◆); ornithine carbamoyltransferase ArcB (●); **B** – Proteins involved in glutamine degradation to carbamoyl-phosphate or glutamate. Carbamoyl phosphate synthases CarA (■) and CarB (▲); glutamate synthases GltB (▼) and GltD (●); **C** – Proteins involved in the biosynthesis of aroma compounds from branched-chain amino acids (BCAAs) isoleucine, leucine and valine. Aromatic amino acid aminotransferase AraT (◆); branched-chain amino acid aminotransferase BcaT (●); **D** – Proteins involved in the biosynthesis of amino acids. Aminotransferase AspC (■, biosynthesis of alanine from pyruvate); cysteine synthase CysM (▲, biosynthesis of cysteine from serine); diaminopimelate decarboxylase LysA (▼, biosynthesis of lysine from aspartate); pyrroline-5-carboxylate reductase ProC (●, biosynthesis of proline from glutamate); prephenate dehydrogenase TyrA (◆, biosynthesis of tyrosine from glucose). Samples were taken at μ = 0.1, 0.2, 0.3, 0.4 and 0.5 h^{-1} and compared to 0.1 h^{-1}.

4.2.2. Determination of threonine catabolic pathways in *L. lactis* IL1403 (Publication II)

4.2.2.1. Effect of L-threonine concentration on glycine production

The synthetic medium developed in this thesis, BS7, was used to ascertain whether the threonine aldolase (TA) pathway leading to the biosynthesis of glycine and acetaldehyde, an important aroma compound in yoghurt, exists in *L. lactis* IL1403. It was noticed in previous experiments that glycine in biomass proteins was partially obtained by biosynthesis rather than taken up from the medium at low μ values [6]. Potential precursors for glycine biosynthesis in the IL1403 strain are glucose and L-serine [7], but the existence of a TA has not been verified. Nevertheless, preliminary experiments in test tubes demonstrated that glycine production increased when the concentration of L-serine or L-threonine was raised (data not shown). Glycine biosynthesis was at maximum and consumption of L-threonine was higher than that of L-serine if the concentration of the former in the medium exceeded that of L-serine (data not shown). The transport protein SerP1 is responsible for the uptake of both L-serine and L-threonine, which leads to a competitive inhibition [147].

In order to prove the presence of TA in *L. lactis* IL1403, D-stats at $\mu = 0.2\ h^{-1}$ were carried out in three parallels where the concentration of L-threonine was smoothly changed from 1.24 to 4.13 mM and glycine was omitted from the medium. As we used a CDM with known compounds, measuring the amounts of nutrients utilized and produced in the medium gave easily interpretable results. The obtained data indicated a correlation between glycine production and L-threonine utilization (Figure 12). If the concentration of L-threonine in the medium was higher, then more L-threonine was consumed and more glycine biosynthesized, whereas L-serine utilization decreased (Figure 12). L-threonine was not fully consumed from the medium: about 57 ± 2% of the amount in the medium was utilized in the beginning and 30 ± 1% at the end of the experiment. Also, L-threonine and glycine in intracellular proteins were most likely obtained from the consumed L-threonine and about 14% of this amino acid was degraded to unknown products (Figure 13). However, acetaldehyde was produced in minute amounts and seemingly not converted to ethanol, as the production of the latter compound was constant and not dependent on the utilization of L-threonine or its concentration in the medium (Figure 12). The existence of TA in *L. lactis* IL1403 was thus rendered dubious and an experiment with the labelled L-threonine was carried out to shed light on this issue.

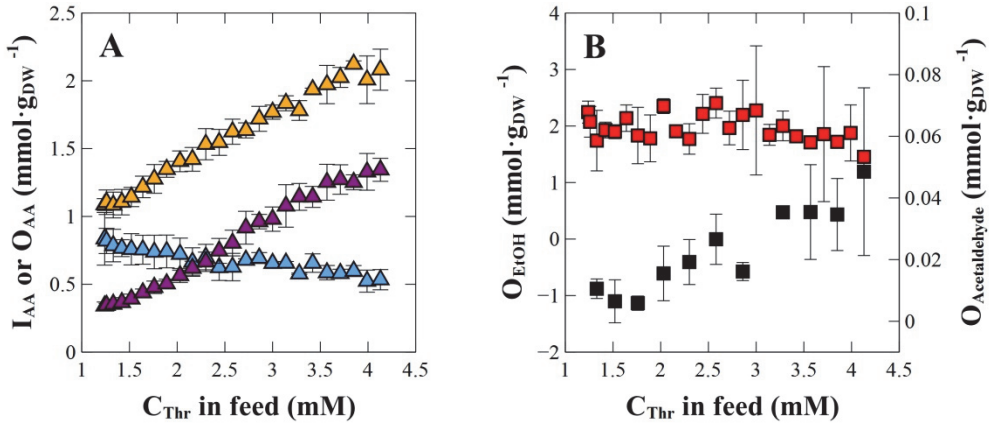


Figure 12. Threonine aldolase experiments: A – Production of glycine (purple triangles, ▲) and utilization of L-serine (blue triangles, ▲) and L-threonine (yellow triangles, ▲); **B –** Production of ethanol (red squares, ■) and acetaldehyde (black squares, ■). (Average results of three D-stat fermentations: millimoles per gram cell dry weight. I – utilization, O – production, AA – amino acid.).

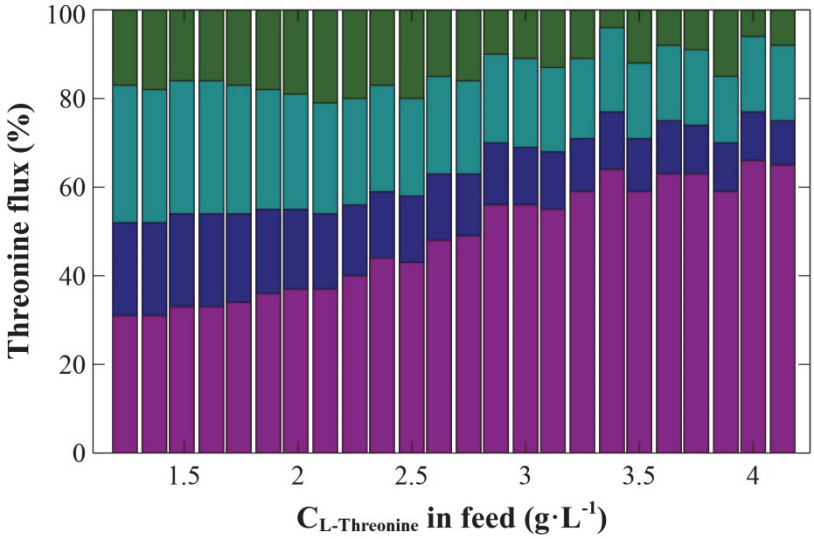


Figure 13. Threonine degradation products. Calculations were based on comparing the amount of threonine utilized with the amount of glycine produced and amino acid concentrations in cellular proteins. (Purple – produced glycine secreted into the growth environment, dark blue – threonine in biomass, light blue – glycine in biomass, green – unknown degradation products.)

4.2.2.2. Experiment with $^{13}C_4$,^{15}N-labelled L-threonine

In order to confirm or disprove the occurrence of TA, a continuous cultivation was carried out where *L. lactis* IL1403 was first stabilized at D = 0.2 h^{-1} with 2.5 mM of L-threonine in CDM (BS7). After reaching the stationary phase, the feed was switched to a medium that contained 2.5 mM of $^{13}C_4$,^{15}N-labelled L-threonine. The samples were collected at 60 min intervals during ten hours and measurements by LC-MS showed a clear incorporation of $^{13}C_4$,^{15}N-threonine into the cells, its conversion to glycine and secretion of the latter compound into the extracellular environment (Figures 14a and 14b).

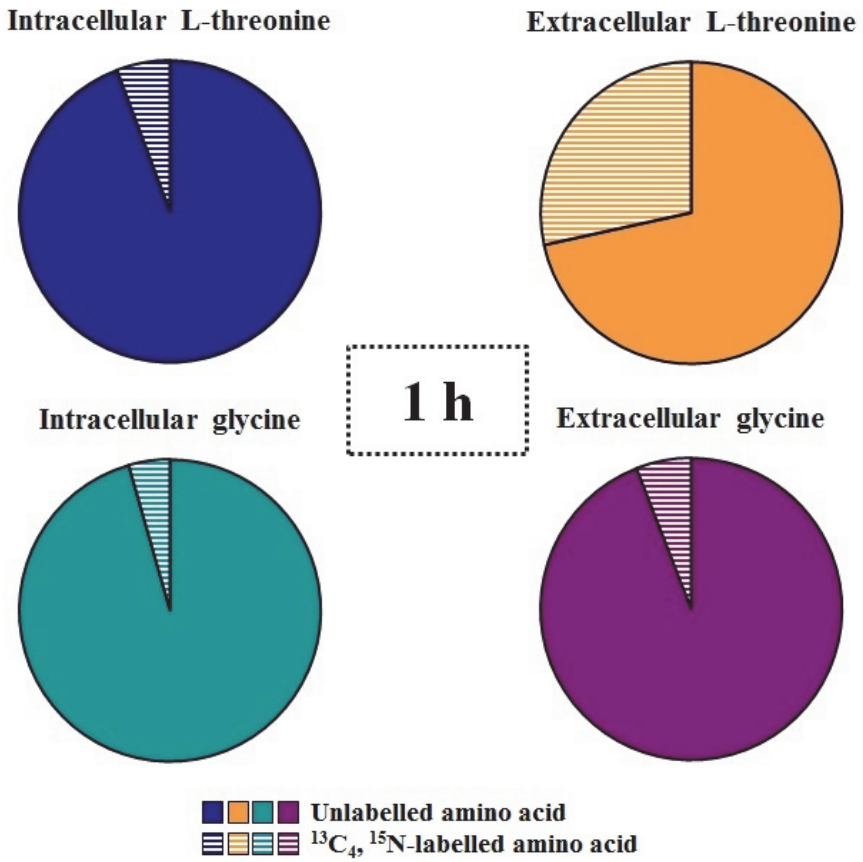


Figure 14a. Incorporation of labelled L-threonine. Ratios of extra- and intracellular labelled and unlabelled glycine to threonine after one hour of growth on the medium containing the $^{13}C_4$,^{15}N-labelled L-threonine.

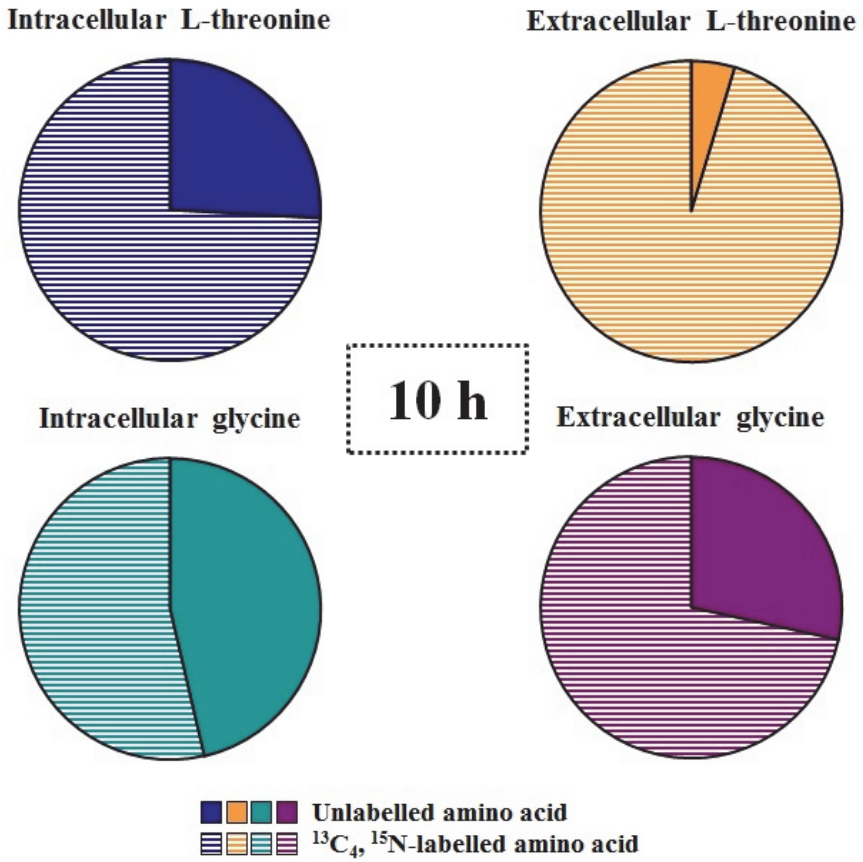


Figure 14b. Incorporation of labelled L-threonine. Ratios of extra- and intracellular labelled and unlabelled glycine to threonine after 10 hours of growth on the medium containing the $^{13}C_4$,^{15}N-labelled L-threonine.

NMR measurements determined that the labelled L-threonine was degraded into glycine, ethanol, acetaldehyde and acetaldehyde hydrate and the ratio of glycine to the other three compounds was almost 1:1 (Figure 15). Therefore, TA was indeed present in the genome of *L. lactis* IL1403 and was activated if no extracellular glycine was available in the growth environment and when the L-threonine concentration in the medium exceeded that of L-serine.

In addition to the previously mentioned degradation products of L-threonine, peaks of other labelled compounds were seen in NMR spectra as well, but their concentrations were too low for accurate identification. *E. coli* is known to catabolize L-threonine via threonine dehydrogenase to 2-amino-3-ketobutyrate, which is followed by degradation to acetyl-CoA and glycine or aminoacetone and CO_2 [148]. The comparison of unidentified peaks to aminoacetone standard showed no match. Furthermore, according to NMR, L-threonine was degraded neither to lactate nor BCAAs. The ability to biosynthesize BCAAs would lead to a decreased consumption of these amino acids at higher L-threonine availability, but this did not happen (data not shown). The production of BCAAs from L-threonine could not

have taken place, since the IL1403 strain is auxotrophic for these amino acids (see Table 6 and [79, 91]) and the enzyme involved in the first step of the biosynthetic pathway was downregulated (see the next chapter). The unknown labelled peak in NMR disappeared in lyophilized samples, which hints that the unidentified degradation product of L-threonine was volatile and/or unstable.

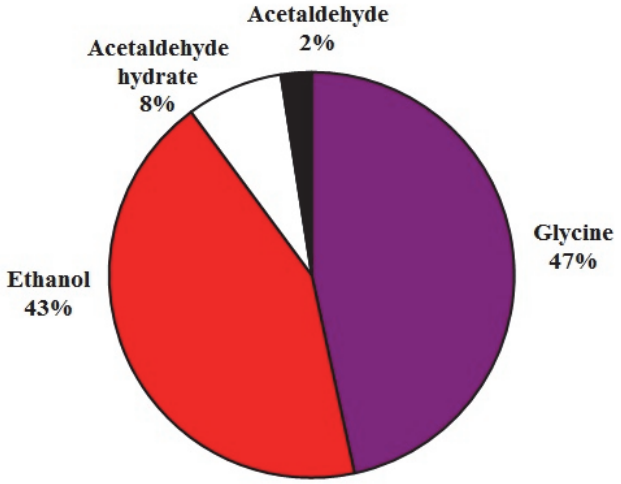


Figure 15. NMR results for threonine degradation. The ratio between four products biosynthesised from the $^{13}C_4$,^{15}N-labelled L-threonine. The sample was taken from the labelling experiment 10 hours after the regular feed was switched to the medium containing the $^{13}C_4$,^{15}N-labelled L-threonine. The concentration of L-threonine in the feed was 2.5 mM and 37% of it was consumed by *L. lactis* IL1403.

4.2.2.3. Changes in protein expressions

Changes in protein expressions were measured in two D-stat experiments in which the concentration of the unlabeled L-threonine was increased from 1.24 to 4.13 mM. The samples were collected at three L-threonine concentrations (1.24, 2.86 and 3.99 mM) and these were compared with the initial sample (1.24 mM). The expression of serine hydroxymethyltransferase (SHMT) GlyA was upregulated at a higher L-threonine availability (Figure 16), which hints that similarly to *S. thermophilus* [144], GlyA may possess a secondary activity as TA in *L. lactis* IL1403. In addition, the expression of four hypothetical proteins was increased when there was more L-threonine in the feed (Figure 16). Thus, these proteins might be associated with threonine catabolism as well.

The expressions of threonine dehydratase (IlvA) involved in the biosynthesis of BCAAs and alcohol dehydrogenase (AdhE), which is responsible for the conversion of acetaldehyde to ethanol, were both downregulated (Figure 16). The decrease in the expression of AdhE explains the constant production of ethanol (Figure 12 in chapter 4.2.2.1.) upon increasing the L-threonine concentration in the feed. The

conversion of acetaldehyde to ethanol is apparently a bottle-neck reaction: at higher L-threonine concentrations the cells were incapable of increasing ethanol production from acetaldehyde and the excess of acetaldehyde probably evaporated. Furthermore, the betaine ABC transporter permease (BusAB), which is involved in osmotic stress alleviation, was upregulated (Figure 16). This suggests an increase in the intracellular pools of acetaldehyde, which in turn, like lactate, might induce osmotic pressure [207-208]. The hypothesis of osmotic stress is supported by a 10% decrease in biomass yield and a 1.1–1.5-fold increase in the consumption of additional substrates – arginine, asparagine and cysteine – during the experiments (data not shown).

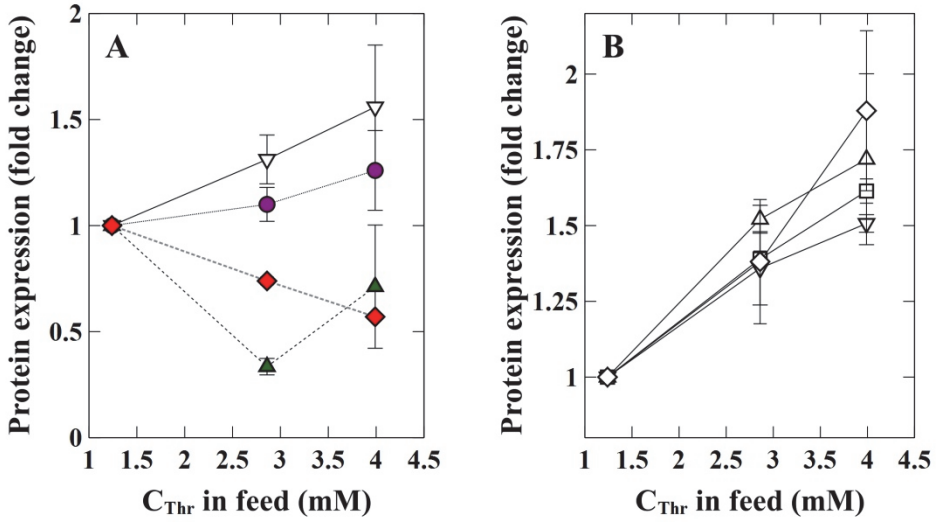


Figure 16. Changes in protein expressions during D-stat experiments at various L-threonine concentrations in the feed (average results of two fermentations): **A** – Red diamonds (◆) – alcohol dehydrogenase (AdhE), purple circles (●) – serine hydroxymethyltansferase (GlyA) green triangles (▲) – threonine dehydratase (IlvA), white inverted triangles (▽) – betaine ABC transporter permease (BusAB); **B** – Putative uncharacterized proteins whose average expression changed at least 1.5-fold at different L-threonine concentrations and the standard error between the two analyzed fermentations was less than 20%. (White diamonds – YrgH; white squares – YsfA; white triangles – YsjD; white inverted triangles – YuaA. Samples were taken at C_{Thr} = 1.24, 2.86 and 3.99 mM and were compared to C_{Thr} = 1.24.).

Lastly, RibAB, which is part of the riboflavin biosynthetic pathway, was formidably upregulated. However, there was a great variability between parallel measurements: the fold change of RibAB was 2.01 during one and 6.18 during the other experiment. Nevertheless, as threonine supplementation and TA overexpression increase riboflavin production in *Ashbya gossypii* [209], speculations can be made that a small portion of glycine was used for purine and subsequent riboflavin biosynthesis. The facilitated biosynthesis of purines is supported by a 1.3-fold increase in the utilization of glutamine, which is also involved in nucleotide

biosynthesis [177]. Note that the produced riboflavin was apparently promptly directed to intracellular coenzyme biosynthesis, as no secretion of this vitamin was observed (data not shown).

4.2.2.4. Threonine aldolase activity in milk

Glycine is a sweet amino acid. In order to assess the possibility to develop fermented milk products which are sweetened with *in situ* produced glycine, experiments were carried out in which 1, 2.5, 5, 10 or 25 mM of L-threonine was added to milk. As *L. lactis* IL1403 is unable to metabolize lactose, another strain (1387) was used. The concentration of free glycine in pure milk was 0.063 mM. The addition of L-threonine had no effect on acidification speed and only minute amounts of glycine were produced: addition of 25 mM of L-threonine resulted in the biosynthesis of 0.124 mM of glycine. Based on these results three assumptions were made: (i) in contrast with the IL1403 strain, *L. lactis* 1387 might not have TA activity; (ii) TA is activated only if glycine is completely absent from the growth environment; (iii) TA activity in milk is low. The second hypothesis was supported by data obtained in experiments with yoghurt LAB *S. thermophilus* and *Lb. bulgaricus* in which inhibition of TA activity by glycine was observed [210]. The third claim was based on reference [211] reporting that 90-100% of acetaldehyde in milk was biosynthesized from glucose by *S. thermophilus* and *Lb. bulgaricus*.

5. CONCLUSIONS

I Several new chemically defined media were developed for *Lactococcus lactis* IL1403, which are more balanced in composition, suit better for studying amino acid metabolism, provide higher μ values and are cheaper than the media reported in the literature.

i. Low substrate supply limits interconversions between amino acids. Thus, decreasing nutrient concentrations in the medium increases the number of essential amino acids for growth. In addition to using different strains, the application of different media may be one reason behind the variations in literature data on nutritional requirements of a particular species.

II Amino acids can be divided into groups based on whether these are solely used for the biosynthesis of cellular proteins and peptidoglycans or for other purposes as well.

i. The biosynthesis of glycine and proline is energetically favorable to consumption as the production of these amino acids is preferred to their uptake even if these are fully available in the growth medium.

ii. Balancing the medium composition and raising specific growth rate induce the cells to save resources by decreasing the production of amino acid byproducts and increasing the flux of amino acids to proteins.

iii. The division of amino acids into groups enables one to better design the development of defined media for other lactic acid bacteria by indicating which amino acids should be present in the medium in amounts lower than, equal to or higher than their respective concentrations in the biomass.

III The use of synthetic media in combination with advanced cultivation methods and omics data is advantageous for confirming the presence of anabolic or catabolic pathways of nutrients in microbes, as was proved in this thesis by affirming the presence of the threonine aldolase route in *Lactococcus lactis* IL1403.

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APPENDIX 1. NUTRITIONAL REQUIREMENTS OF LACTIC ACID BACTERIA

The following abbreviations have been used in Tables S1-S5 (Appendix 1) and Tables S6-S11 (Electronic Appendix):

E – essential
E. faecalis – Enterococcus faecalis
EDTA – ethylenediaminetetraacetic acid
EPS – exopolysaccharides
L. cremoris – Lactococcus lactis subspecies *cremoris*
L. lactis – Lactococcus lactis subspecies *lactis*
Lb. – Lactobacillus
Lb. bulgaricus – Lactobacillus delbrueckii subspecies *bulgaricus*
Lb. lactis – Lactobacillus delbrueckii subspecies *lactis*
Leuc. dextranicum – Leuconostoc mesenteroides subspecies *dextranicum*
Leuc. mesenteroides – Leuconostoc mesenteroides subspecies *mesenteroides*
MOPS – 3-(*N*-morpholino)propanesulfonic acid
NE – nonessential
O. oeni – Oenococcus oeni
OD – optical density
P. – Pediococcus
S – stimulatory
S. thermophilus – Streptococcus thermophilus

Table S1. Amino acid requirements for the growth of different LAB species.

Species	Strain (and origin)	Reference	Ala	Arg	Asn	Asp	Cys
E. faecalis	B9510 (silage)	[81]	NE	E	NE	NE	S
E. faecalis	V583	[107]	NE	E	NE	NE	NE
L. cremoris	MG1363	[15]	NE	NE	NE	NE	NE
L. lactis	IL1403 (dairy)	[91]	NE	E	S		S
L. lactis	IL1403	[79][1]	S	E	S	NE	NE
L. lactis	IL1403	[80]	S	E	E	NE	E[11]
L. lactis	IO-1	[101][2]	S	S	S	NE	S
L. lactis	NCDO2118 (vegetable)	[91]	S	S	S	NE[3]	S
Lb. buchneri	CUC-3 (wine)	[105]	NE	E	NE	NE	S
Lb. bulgaricus	NCFB2772	[96]	E	E	S	S	E
Lb. casei	ATCC334 (cheese), 12A (corn silage)	[108]	NE	E	NE	S	NE
Lb. curvatus	NCFB2739 (meat products)	[102]	NE	NE	NE	NE	NE
Lb. helveticus	CRL974, 1062 (Argentinian hard cheeses)	[97]	S/NE[3]	E	NE	NE	NE
Lb. hilgardii	MHP (wine)	[105]	NE	E	NE	NE	E
Lb. lactis	CRL581, CRL654 (Argentinian hard cheeses)	[98]	S	E	NE	NE	E
Lb. pentosus	NCFB363 (meat products)	[102]	NE	NE	NE	NE	NE
Lb. plantarum	N4, N8 (orange peels)	[103]	S	S	NE	NE	E
Lb. plantarum	NC8, NCFB1752 (meat products)	[102]	NE	E	NE	NE	NE
Lb. plantarum	WCFS1	[106]	NE	E		NE	S/NE[12]
Lb. reuteri	JCM1112	[104]	NE	E		NE	NE
Lb. sake	NCFB2714, Lb16 (meat products)	[102]	S	E	E/S[6]	NE	S
Leuc. dextranicum	CNRZ1694, CNRZ1742, CNRZ1744 (French raw milk cheeses)	[95]	NE	E	NE	NE	E
Leuc. mesenteroides	ATCC8293 (olive fermentation brines)	[99]	NE	S	NE	NE	S
Leuc. mesenteroides	CNRZ1463, CNRZ1697, CNRZ1750 (French raw milk cheeses)	[95]	NE	E	NE	NE	E
O. oeni	R1034, R1054 (wine)	[105]	S	E	E/NE[7]	S/NE[9]	E
S. thermophilus	LMG18311	[10]	NE	S/NE[5]	NE	NE	S
S. thermophilus	ST1, ST7, ST8, ST11, ST18, ST21	[100]	NE	S/NE[4]	NE	S/NE[10]	S

(Table S1 continued)

Species	Gln	Glu	Gly	His	Ile	Leu	Lys	Met	Phe	Pro	Ser	Thr	Trp	Tyr	Val
E. faecalis	NE	NE	E	E	E	E	S	E	S	NE	S	S	E	NE	E
E. faecalis	NE	NE	S	E	E	E	NE	E	NE	NE	NE	NE	E	NE	E
L. cremoris	NE	E	NE	E	NE	E	NE	E	NE	NE	NE	NE	NE	NE	NE
L. lactis	E		NE	E	E	E	NE	E	NE	NE	S	S	NE	NE	E
L. lactis	NE	NE	S	E	E	E	S	E	NE	S	S	S	NE	S	E
L. lactis	E[11]	NE	NE	E	E	E	S	E	NE	NE	E	S	NE	NE	E
L. lactis	NE	NE	NE	E	S	E	S	E	NE	NE	NE	S	NE	NE	E
L. lactis	S	NE[8]	S	S	S	S	S	S	NE	S	S	S	NE	NE	E
Lb. buchneri	NE	NE	NE	S	E	E	NE	NE	NE	E	S	NE	NE	NE	E
Lb. bulgaricus	S	S	S	E	E	E	E	E	E	E	E	S	E	E	E
Lb. casei	NE	E/S[14]	NE	NE	E	E	NE	NE	E	NE	NE	NE	E	E	E
Lb. curvatus	NE	NE	NE	NE	E	E	E	E	NE	NE	NE	E	E	E	E
Lb. helveticus	NE	E	S/NE[3]	E	E	E	E/S[17]	E	E	E	E/S[17]	E	E	E	E
Lb. hilgardii	S	E	S	NE	E	E	NE	NE	E	E	NE	NE	NE	NE	E
Lb. lactis	NE	NE	NE	E	E	E	E/NE[16]	E	E	NE	E	NE	E	E	E
Lb. pentosus	NE	NE	NE	NE	E	E	NE	E	N	NE	NE	NE	NE	NE	E
Lb. plantarum		E	NE	NE	E	E	E/S[18]	NE	S	NE	NE	E	E/S[18]	NE	E
Lb. plantarum	NE	NE	NE	NE	E	E	NE	NE	NE	NE	NE	NE	NE	E	E
Lb. plantarum		E	S	NE	E	E	NE	S	S	NE	NE	NE	E	S	E
Lb. reuteri		E	NE	E	S	E	NE	E	E	NE	S	E	E	E	E
Lb. sake	S	NE	E/S[6]	E/S[6]	E	E	E	E	S	S	S	S	S	E	E
Leuc. dextranicum	E		NE	E	E	E	NE	E	E	E	NE	E	E	NE	E
Leuc. mesenteroides	E	NE	NE	S	E	S	NE	E	S	S	NE	S	S	NE	E
Leuc. mesenteroides	E		NE	E	E	E	NE	E	E	E	NE	E	E	NE	E
O. oeni	NE	E/NE[7]	E	E	E	E	S/NE[9]	E	E	E	E/S[20]	E	E	E	E
S. thermophilus	NE	NE	NE	E	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE	NE
S. thermophilus	S/NE[13]	S/NE[10]	NE	E/NE[15]	NE	S	S/NE[13]	S	S/NE[19]	E/NE[15]	NE	NE	S/NE[21]	S/NE[22]	S

[1] The following classification for nutrients was used in the reference: essential component (OD < 0.1); important component (0.1 ≤ OD < 0.4); somewhat important component (0.4 ≤ OD < 0.6); least important or detrimental component (OD ≥ 0.6). In this table the least important components have been reclassified as nonessential (NE) and important or somewhat important components have been reclassified as stimulatory (S).

[2] This reference did not classify amino acids as stimulatory or nonessential. Thus, the amino acids have been listed as "stimulatory" in this table, if the omission of these lead to a growth rate < 80% of that obtained in the complete medium.

[3] Stimulatory for the strain CRL1062 and nonessential for CRL974.

[4] Stimulatory for strains ST1 and ST8 and nonessential for others.

[5] The optical density of this strain in the medium lacking arginine was 71% of the value obtained on the complete medium, but the authors defined this amino acid as nonessential.

[6] Essential for the strain Lb16 and stimulatory for NCFB2714.

[7] Essential for the strain R1054 and nonessential for R1034.

[8] Experiments were conducted on the MCD medium, which contained no aspartate or glutamate. The addition of these compounds to the complete MCD did not enhance growth rate.

[9] Stimulatory for the strain R1054 and nonessential for R1034.

[10] Stimulatory for the strain ST8 and nonessential for others.

[11] Cysteine and glutamine become essential in media where the concentrations of other amino acids are low.

[12] Growth rate of WCFS1 in a medium without cysteine was 82% and optical density 92% of the values obtained on the complete medium.

[13] Stimulatory for strains ST1, ST7, ST8, ST11 and nonessential for others.

[14] Essential for the strain 12A and stimulatory for ATCC334.

[15] Essential for the strain ST8 and nonessential for others.

[16] Essential for the strain CRL654 and nonessential for CRL581.

[17] Essential for the strain CRL974 and stimulatory for CRL1062.

[18] Essential for the strain N4 and stimulatory for N8.

[19] Stimulatory for strains ST7, ST8, ST11 and nonessential for others.

[20] Essential for the strain R1054 and stimulatory for R1034.

[21] Nonessential for the strain ST7 and stimulatory for others.

[22] Stimulatory for strains ST8 and ST21 and nonessential for others.

Table S2. Metal ion requirements for the growth of different LAB species.

Species	Strain	Reference	B^{3+} [2]	Ca^{2+}	Co^{2+}	Cu^{2+}	Fe^{2+}	Li^{1+}	Mg^{2+}	Mn^{2+}	Mo^{6+}	Na^{1+}	Ni^{2+}	Zn^{2+}
E. faecalis	B9510	[81]					S		E					
L. lactis	IL1403	[91]	NE[3]	NE	NE	NE[3]	NE	NE[3]	E	NE[3]	NE[3]			NE
L. lactis	IL1403	[79][1]	S	S	S	S	S		E	S	S	S		S
L. lactis	IL1403	[80]		NE	NE	S	NE		E	S	NE			NE
L. lactis	NCDO2118	[91]	NE[3]	NE	NE	NE[3]	NE	NE[3]	E	NE[3]	NE[3]			NE
Lb. buchneri	CUC-3	[105]		NE		NE	NE		NE	E				NE
Lb. bulgaricus	CNRZ397	[94]	E[4]	E	E[4]	E[4]			E		E[4]			E[4]
Lb. bulgaricus	NCFB2772	[96]	NE	NE[5]	NE	NE	NE		E	E	NE		NE	NE
Lb. curvatus	NCFB2739	[102]					NE		S	S		NE[8]		
Lb. helveticus	CRL1062	[97]		NE			NE		E	NE				NE
Lb. hilgardii	MHP	[105]		NE		NE	NE		S	E				NE
Lb. lactis	CRL581, CRL654	[98]					NE		E	NE				
Lb. pentosus	NCFB363	[102]					NE		NE	S		NE		
Lb. plantarum	NC8, NCFB1752	[102]					NE[6]		NE	S		NE		
Lb. sake	NCFB2714, Lb16	[102]					S		E/S[7]	S		NE[8]		
Leuc. dextranicum	CNRZ1694, CNRZ1742, CNRZ1744	[95]		NE	NE	NE	NE		S	S				NE
Leuc. mesenteroides	ATCC8293	[99]		NE	NE	NE	NE		S	S				NE
Leuc. mesenteroides	CNRZ1463, CNRZ1697, CNRZ1750	[95]		NE	NE	NE	NE		S	S				NE
O. oeni	R1034, R1054	[105]		NE		NE	NE		NE	E				NE
S. thermophilus	ST1, ST7, ST8, ST11, ST18, ST21	[100]		S	NE	NE	NE		E	NE				NE

[1] The following classification for nutrients was used in the reference: essential component (OD < 0.1); important component (0.1 ≤ OD < 0.4); somewhat important component (0.4 ≤ OD < 0.6); least important or detrimental component (OD ≥ 0.6). In this table the least important components have been reclassified as nonessential and important or somewhat important components have been reclassified as stimulatory.

[2] Boron is classified as a metalloid: a chemical element with properties in between those of metals and nonmetals.

[3] This reference used the MCD medium which contained none of these metal ions. The addition of these compounds to the complete MCD did not increase growth rate. The composition of the MCD medium can be seen in Supplementary Table S6 (Electronic Appendix).

[4] Boron, cobalt, copper, molybdenum and zinc were essential when all of these were omitted from the medium. Single omissions were not tested.

[5] This reference used a medium that contained no calcium, but the addition of $CaCl_2$ to the medium had no effect on growth.

[6] Iron was nonessential for the strain NC8 and inhibited the cell growth of NCFB1752.

[7] Essential for the strain Lb16 and stimulatory for NCFB2714.

[8] NaCl was found to be inhibitory for cell growth.

Table S3. Vitamin requirements for the growth of different LAB species. Thiamine (B_1), riboflavin (B_2), nicotinamide (B_3), pantothenate (B_5), pyridoxine (B_6), biotin (B_7), inositol (formerly B_8), folic acid (B_9), *para*-aminobenzoic acid (*p*ABA, formerly B_x), cobalamin (B_{12}) and ascorbic acid (C).

Species	Strain	Reference	B_1	B_2	B_3[3]	B_5	B_6[8]	B_7	Inositol (B_8)[15]	B_9	B_{12}	*p*ABA (B_x)[18]	C
E. faecalis	B9510	[81]	NE	S	E	E	E	NE		S	NE	NE	
L. lactis	IL1403	[91]	NE	E	E	E	S[9]	S		NE	NE		
L. lactis	IL1403	[79][1]	S	S	S	S	S	S	S	S		S	
L. lactis	IL1403	[80]	NE	E	NE	E[7]	E[7]	NE	NE	S[7]	NE	S[7]	
L. lactis	IO-1	[101]	NE	NE	E	E	NE	S[14]		NE			NE
L. lactis	NCDO2118	[91]	NE	NE	E	E	S[9]	S		NE	NE		
Lb. buchneri	CUC-3	[105]	NE	E	E	E	S	NE		NE	NE	NE	
Lb. bulgaricus	CNRZ397	[94]	NE	E	E	E	E/NE[10]	NE		E	E	NE	NE
Lb. bulgaricus	NCFB2772	[96]	NE	E	E	E	NE	NE		NE	S	NE	
Lb. curvatus	NCFB2739	[102]	NE	E	E	E	S	NE		S	NE	NE	
Lb. helveticus	CRL1062	[97]	NE	E	E/NE[4]	E	E	NE		NE	NE	NE	
Lb. hilgardii	MHP	[105]	NE	E	E	E	S	NE		NE	NE	NE	
Lb. lactis	CRL581, CRL654	[98]	NE	S	E	E	E/S[11]	NE		NE	S	NE	
Lb. pentosus	NCFB363	[102]	NE	NE	E	E	S	S		S	NE	NE	
Lb. plantarum	NC8, NCFB1752	[102]	NE	NE	S	E	S	S		S	NE	NE	
Lb. plantarum	WCFS1	[106]	NE	E	E	E	S/NE[12]			NE		NE	
Lb. sake	NCFB2714, Lb16	[102]	S	E	E	E	S	NE		S	NE	NE	
Leuc. dextranicum	CNRZ1694, CNRZ1742, CNRZ1744	[95]	S	S/NE[2]	E/S[5]	E	S/NE[13]	NE		NE			NE
Leuc. mesenteroides	ATCC8293	[99]	S	NE	E	S	NE	NE		S	NE	NE	S
Leuc. mesenteroides	CNRZ1463, CNRZ1697, CNRZ1750	[95]	S	S/NE[2]	E	E	S/NE[13]	NE		S/NE[16]			NE
O. oeni	R1034, R1054	[105]	NE	NE	E	E	S	S		S/NE[17]	NE	NE	
S. thermophilus	ST1, ST7, ST8, ST11, ST18, ST21	[100]	NE	E	E/S[6]	E/S[6]	NE	NE		NE	NE		NE[19]

APPENDIX 1 CONTINUED

[1] The following classification for nutrients was used in this reference: important component ($0.1 \leq OD < 0.4$); somewhat important component ($0.4 \leq OD < 0.6$). In this table these two groups have been reclassified as stimulatory.

[2] Stimulatory for strains CNRZ1463, CNRZ1694, CNRZ1742 and nonessential for others.

[3] This column does not distinguish between B_3 vitamers, nicotinic acid (nicotinate, niacin) and nicotinamide (niacinamide), unless stated otherwise.

[4] Nicotinic acid was essential and niacinamide nonessential.

[5] Stimulatory for the strain CNRZ1742 and essential for others.

[6] Stimulatory for strains ST7 and ST11 and essential for others.

[7] Pantothenate, pyridoxine and folic acid were needed to sustain growth in media with low amino acid concentrations and folic acid could be replaced with its precursor *p*ABA.

[8] This column does not distinguish between B_6 vitamers – pyridoxal and pyridoxine or pyridoxamine – unless stated otherwise.

[9] The strain needed this vitamin when nucleotide bases were removed from the medium.

[10] Pyridoxal was essential and pyridoxamine nonessential.

[11] Stimulatory for the strain CRL654 and essential for CRL581.

[12] Pyridoxamine was stimulatory and pyridoxine was nonessential.

[13] Stimulatory for strains CNRZ1750 and CNRZ1742 and nonessential for others.

[14] Cell growth in case of biotin omission was < 80% of that obtained in the complete medium.

[15] Inositol was once considered a member of the vitamin B complex (B_8).

[16] Stimulatory for the strain CNRZ1750 and nonessential for others.

[17] Stimulatory for the strain R1054 and nonessential for R1034.

[18] *Para*-aminobenzoic acid is not considered a vitamin anymore, but is used by lactic acid bacteria as a precursor for the biosynthesis of folates.

[19] The removal of ascorbic acid was possible only if replaced by a protective agent against oxygen such as dithiotreitol.

Table S4. Purine, pyrimidine and nucleoside requirements for the growth of different LAB species.

Species	Strain	Reference	Adenine (A)	Cytosine (C)	Guanine (G)	Thymine (T)	Uracil (U)	Hypoxanthine	Xanthine	Deoxyguanosine (dG)	Deoxythymidine (dT)	Inosine (I)	Orotic acid[6]
E. faecalis	B9510	[81]	NE		NE		S						
L. lactis	IL1403	[91]	S		S		S		S				
L. lactis	IL1403	[79][1]	S		S		S		S				
L. lactis	IL1403	[80]						NE			NE		
L. lactis	NCDO2118	[91]	S		S		S		S				
Lb. buchneri	CUC-3	[105]	NE	NE	NE	NE	NE		NE				
Lb. curvatus	NCFB2739	[102]	NE		NE		S		S				
Lb. bulgaricus	NCFB2772	[96]	S		NE		E		NE				
Lb. bulgaricus	CNRZ397	[94]	E[2]		E[2]		E[2]	NE	E[2]	NE			NE
Lb. helveticus	CRL1062	[97]	NE		NE		E	NE	NE	NE	NE	NE	NE
Lb. lactis	CRL581, CRL654	[98]	NE		NE	NE	E		NE			NE	NE
Lb. hilgardii	MHP	[105]	S	NE	NE	NE	NE		NE				
Lb. pentosus	NCFB363	[102]	S		NE		NE		S				
Lb. plantarum	NC8, NCFB1752	[102]	S/NE[3]		NE		S/NE[3]		NE				
Lb. sake	NCFB2714, Lb16	[102]	S		NE		S		S				
Leuc. dextranicum	CNRZ1694, CNRZ1742, CNRZ1744	[95]	NE		NE		NE		NE		NE	NE	
Leuc. mesenteroides	CNRZ1463, CNRZ1697, CNRZ1750	[95]	NE		NE		NE		NE		S/NE[5]	S/NE[5]	
Leuc. mesenteroides	ATCC8293	[99]	S				S				NE	S	
O. oeni	R1034, R1054	[105]	S/NE[4]	NE	S/NE[4]	NE	NE		NE				
S. thermophilus	ST1, ST7, ST8, ST11, ST18, ST21	[100]	NE		NE		NE		NE		NE	NE	NE

APPENDIX 1 CONTINUED

[1] The following classification for nutrients was used in this reference: important component ($0.1 \leq OD < 0.4$); somewhat important component ($0.4 \leq OD < 0.6$). In this table these two groups have been reclassified as stimulatory.

[2] Nitrogen bases were essential when all four were omitted. Single omissions were not tested.

[3] Stimulatory for the strain NC8 and nonessential for NCFB1752.

[4] Stimulatory for the strain R1054 and nonessential for R1034.

[5] Stimulatory for the strain CNRZ1750 and nonessential for others.

[6] Orotic acid is a precursor of pyrimidine biosynthesis.

Table S5. Fatty acid precursor requirements for the growth of different LAB species.

Species	Strain	Reference	K- or Na-acetate	Lipoic acid	Oleic acid	Tween 80
L. lactis	IL1403	[79][1]	S	S		S
L. lactis	IL1403	[80]		E		
L. lactis	NCDO2118	[91]	NE			NE
Lb. curvatus	NCFB2739	[102]	NE[2]			E
Lb. bulgaricus	NCFB2772	[96]	E	NE		S
Lb. bulgaricus	CNRZ397	[94]	E		NE	E
Lb. helveticus	CRL1062	[97]	NE			S
Lb. lactis	CRL581, CRL654	[98]	S/NE[3]			NE
Lb. pentosus	NCFB363	[102]	S			S
Lb. plantarum	WCFS1	[106]		NE		
Lb. plantarum	NC8, NCFB1752	[102]	S/NE[4]			S
Lb. sake	NCFB2714, Lb16	[102]	S/NE[5]			E/NE[6]
Leuc. dextranicum	CNRZ1694, CNRZ1742, CNRZ1744	[95]				S
Leuc. mesenteroides	CNRZ1463, CNRZ1697, CNRZ1750	[95]				S/NE[7]
Leuc. mesenteroides	ATCC8293	[99]				S
S. thermophilus	ST1, ST7, ST8, ST11, ST18, ST21	[100]		NE		

1 The following classification for nutrients was used in this reference: important component ($0.1 \leq OD < 0.4$); somewhat important component ($0.4 \leq OD < 0.6$). In this table these two groups have been reclassified as stimulatory.

2 Stimulatory for all strains except CNRZ1750.

3 Omission of acetate did not affect growth rate and optical density was reduced only 8%.

4 Tween 80 was essential for the strain NC8 and inhibited the cell growth of NCFB1752.

5 Sodium acetate was nonessential for the strain Lb16. In case of NCFB2714, sodium acetate had a negative effect on specific growth rate and a positive effect on optical density.

6 Sodium acetate was found to be inhibitory for cell growth.

7 Stimulatory for the strain NC8 and nonessential for NCFB1752.

ORIGINAL PUBLICATIONS

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Nutritional requirements and media development for *Lactococcus lactis* IL1403

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Abstract Lactic acid bacteria are extensively used in food technology and for the production of various compounds, but they are fastidious in nutrient requirements. In order to elucidate the role of each component precisely, defined multicomponent media are required. This study focuses on determining nutrient auxotrophies and minimizing media components (amino acids, vitamins, metal ions, buffers and additional compounds) for the cultivation of *Lactococcus lactis* subsp. *lactis* IL1403, using microtitre plates and test tubes. It was shown that glutamine and asparagine were the most important media components for achieving higher biomass yields while the branched-chain amino acids were necessary to increase specific growth rate. The amino acid and glucose ratio was reduced to achieve minimal residual concentration of amino acids in the medium after the growth of cells, whereas the specific growth rate and biomass yield of cells were not considerably affected. As the percentage of each consumed amino acid compared to initial amount is larger than measurement error, these optimized media are important for achieving more precise data about amino acid utilization and metabolism.

Keywords Chemically defined media · *Lactococcus lactis* · Media development · Nutrient requirements

Introduction

Lactic acid bacteria (LAB) play an important role in food industry, especially in the production of dairy products due to their ability to contribute to the aroma and texture of products and to prevent spoilage of food (Price et al. 2012). In addition, LAB can enhance the consumer's health by stimulating the immune system, counteracting pathogens and producing a wide range of beneficial compounds such us vitamins and bioactive peptides (Sybesma et al. 2003; Hébert et al. 2004; Santos et al. 2009).

In order to increase the production of bioactive substances by LAB, the bacterial metabolism has to be studied in detail. Complex media such as MRS (de Man et al. 1960) are often used for the cultivation of LAB, but these types of media are not suitable for determining nutritional requirements due to the fact that they contain complex mixture of compounds (e.g. peptides), which are difficult to quantify by analytical methods (van Niel and Hahn-Hägerdal 1999; Kim et al. 2012). Also, as nutrients are abundant in complex media, the consumption of media components is low compared to the initial amounts present in the media, rendering analytical measurements imprecise. Thus, development and use of chemically defined media (CDM), i.e. synthetic media containing only simple molecules (sugars, amino acids, mineral salts, vitamins), is necessary for studying the quantitative peculiarities of growth physiology of LAB. Synthetic media that are used for studying bacterial metabolism have to contain nutrients in amounts comparable to their consumption, whereby the growth rate and yield of biomass should not be compromised. Chemically defined media have been developed for the cultivation of various species of LAB (Jensen and

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Hammer 1993; Cocaign-Bousquet et al. 1995; Hébert et al. 2004; Wegkamp et al. 2009; Zhang et al. 2009; Kim et al. 2012). However, in these media, amino acids and glucose ratio is not balanced to obtain maximal efficiency of consumption of amino acids (minimal residual concentrations after the growth of cells) which is required for comprehensive quantitative studies of amino acid metabolism as well as for optimization of high-density cultivation of biomass. Also, none of these publications have data about measuring the utilization of amino acids during growth in the media developed therein.

The aim of the current study was development of CDMs that can be used for exploring the amino acid metabolism of *Lactococcus lactis* subsp. IL1403. Our focus was specifically on developing a CDM, which has optimized carbohydrate to amino acid ratio supporting the highest possible biomass yields and specific growth rates. This is important for studying amino acid metabolism of cells in continuous cultivation experiments and for working out media, which can be used to produce starter cultures with high productivity. It is desirable that media for ensuring high productivity are also with balanced nutrient concentrations which would allow the nutrients to be fully consumed keeping their residual concentrations in the cultivations low. The process of media development involves extensive screening of nutrient auxotrophies and optimal concentrations of the components, which makes it necessary to carry out numerous cultivation experiments. Batch cultivations in microtitre plates (MTP) are feasible and affordable for such research and have been effectively used for the media development of lactic acid bacteria (Wegkamp et al. 2009; Zhang et al. 2009). Therefore, this type of cultivation was also implemented in the current study.

The organism used in this research, *L. lactis* IL1403, possesses the genes necessary for the biosynthesis of 20 standard amino acids and some cofactors (i.e. folic acid, riboflavin) (Bolotin et al. 2001). However, some of these genes are probably not functional, since the synthetic medium for *L. lactis* has to contain several amino acids, vitamins, metal ions and also some additional compounds like fatty acids or nitrogen bases to enable full maximal specific growth rate and yield of the cells (Jensen and Hammer 1993; Cocaign-Bousquet et al. 1995; Zhang et al. 2009). The metabolism of this organism is homofermentative, i.e. the main product derived from glucose is lactic acid, although small amounts of formate, acetate and ethanol are synthesized as well (Loubiere et al. 1997).

Materials and methods

Microorganism

The strain used in all the experiments was *L. lactis* subsp. *lactis* IL1403, which was provided by INRA (Jouy-en-Josas, France). Inoculum was prepared from lyophilized stock culture stored at −80 °C which was pre-grown twice in test tubes at 34 °C on the commercially available complex medium M.R.S. Broth (Lab M; Lancashire, UK) for microtitre plate experiments or on a chemically defined medium BS7 (developed in our laboratory) for test tube experiments. In order to prevent carryover of nutrients, the pre-grown culture was washed three times with autoclaved (121 °C, 3 min) physiological solution (0.85 % NaCl (*w*/*v*)) before inoculation to other media, using Rotanta 460R centrifuge (Hettich, Tuttlingen, Germany; 11,500 rpm, 5 min, 4 °C) and sterile polypropylene centrifuge tubes (15 mL; VWR International, Radnor, PA, USA).

Media

The basis of our media development experiments was a chemically defined medium BS1 which was designed in our laboratory specifically for the better detection of amino acids. BS1 is a slight alteration of the CDM that has been previously used in our laboratory (Lahtvee et al. 2011). Composition of BS1 is as follows (mg L^{-1}): D-glucose—3,500, K_2HPO_4—900, KH_2PO_4—750, L-alanine—78, L-arginine—185, L-asparagine—74, L-aspartic acid—72, L-cysteine—64, L-glutamic acid—70, L-glutamine—132, glycine—58, L-histidine—60, L-isoleucine—102, L-leucine—207, L-lysine—158, L-methionine—41, L-phenylalanine—86, L-proline—92, L-serine—163, L-threonine—76, L-tryptophan—16, L-tyrosine—29, L-valine—107, $CaCl_2$—50, $CoSO_4{\cdot}7H_2O$—3, $CuSO_4{\cdot}5H_2O$—3, $FeSO_4{\cdot}7H_2O$—1.4, $MnSO_4{\cdot}H_2O$—16, $MgSO_4{\cdot}7H_2O$—200, NaCl—2,900, $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$—3, $ZnSO_4{\cdot}7H_2O$—5, thiamine hydrochloride (B_1)—0.51, riboflavin (B_2)—0.326, nicotinamide (B_3)—0.325, D-pantothenate (B_5)—0.65, pyridoxine (B_6)—0.642, biotin (B_7)—0.305, folic acid (B_9)—1.21, cobalamin (B_{12})—0.98, choline chloride—9.8, *myo*-inositol—12.6. Chemicals were obtained from Serva Electrophoresis GmbH (Heidelberg, Germany) and Sigma-Aldrich (St. Louis, Missouri, USA).

All the media that were used for determining auxotrophies or in the media development experiments were variations of BS1. Exact compositions with optical densities and specific growth rate values that were obtained with each of those media are given in Supplementary material 1. This data is also complemented with respective short descriptions about the purpose of each series of media, e.g. A—determination of amino acid auxotrophies, B—determination of vitamin auxotrophies, etc. Media were mixed together from stock solutions which were sterilized by filtration (cellulose acetate filters, 0.2 μm; Whatman, Pittsburgh, PA, USA). Separate stock solutions were made for each component and the solutions were mixed together in the following order (to avoid precipitation): sugar, buffers, amino acids, minerals, vitamins, additional components.

Cultivation conditions

Microtitre plate experiments were carried out using plates with 100 wells and Bioscreen C automated microtitre plate reader (Oy Growth Curves Ab Ltd.; Helsinki, Finland). In case of test tube experiments, WPA CO 8000 Biowave Cell Density Meter (Biochrom GmbH; Berlin, Germany) was used to measure optical density. The cultivation volume was 0.24 or 0.3 in microtitre plate and 5 mL in test tube experiments. In case of microtitre plates, OD was measured after every 15 min, preceded by shaking the plate for 10 s. In test tube experiments, OD was measured once per hour and vortex was used before each OD measurement to homogenize the biomass. In both type of experiments, OD was measured at 600 nm and the cultivation temperature was 34 °C. pH and gas environment were not controlled during the cultivations. Specific growth rate (μ) for each medium was calculated separately per every hour during growth and the highest of these values was considered the maximum specific growth rate (μ_{max}) for the given medium.

In auxotrophy experiments (leave-one-out media), a component was considered essential if the final OD was less than 40 % of the OD achieved in the complete medium. When the optical density was between 40 and 80 % of that obtained in the complete medium, the omitted substance was considered growth stimulating. A compound was classified non-essential if the final OD was more than 80 % of the OD in the complete medium.

Amino acid analysis

In order to determine amino acid consumption by *L. lactis* IL1403, cells from stationary phase were removed by centrifugation (14,800 rpm, 5 min, 4 °C) and the supernatant was used for free amino acid detection with an amino acid analyzer (Acquity UPLC; Waters Corp., Milford, MA, USA), according to the manufacturer's instructions. The samples were derivatized beforehand for 10 min at 55 °C, using AccQ-Fluor reagent (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate; Waters Corp.). Data was processed with Empower software (Waters Corp.).

Amino acids were also determined in the biomass proteins of *L. lactis* IL1403 grown on OL medium (variation of BS7; composition is given in Supplementary material 1). For that purpose, biomass was hydrolysed with 6 M HCl for 24 h at 105 °C. Contents of amino acids in hydrolysates (derived from proteins) were measured as described above. L-Tryptophan and L-cysteine could not be detected with this method because these are degraded during hydrolysis. Also, L-asparagine is converted to L-aspartate and L-glutamine to L-glutamate during hydrolysis.

Statistical analysis

Partial least squares regression analysis (PLSR) was performed to analyse the content of different media and its influence on OD or specific growth rate. Data analysis was done in Matlab (Mathworks, Natick, MA, USA) using self-written scripts as well as algorithms from Statistics Toolbox. For PLSR analysis, OD and specific growth rate were chosen as dependant parameters. Amino acid and other components' concentrations in the media were used as independent variables. Mean centering and scaling was used in data preparation. This way each amino acid or other media components have equal weight in regression analysis. Number of components for PLSR analysis was estimated using leave-one-out cross-correlation and residual sum of squares. The media used for PLSR analysis were as follows: JA-JW for experiments on microtitre plates and MA-MF, NA-NF, OA-OM, K1-K44 and BS7 for experiments in test tubes.

Results

In this study, nutrient requirements were ascertained for *L. lactis* IL1403 and defined media developed. First of all, the buffer composition of the CDM was optimized. Secondly, amino acid auxotrophies for *L. lactis* IL1403 were determined and nutrient concentrations in the media were minimized in MTP and test tube experiments using batch cultivations. Thirdly, auxotrophies for B-group vitamins and metal ions were determined. The final outcome of this research was the proposal of several chemically defined media, which are appropriate for studying the amino acid metabolism of *L. lactis* IL1403, due to the fact that the percentage of each consumed amino acid compared to initial amount in the media was larger than measurement error (>5 %). The compositions of all media and consumption patterns of amino acids can be seen in Supplementary materials 1 and 2, respectively.

Optimization of the buffer content

Initially, the start-off medium used in this study (BS1) contained only phosphate buffers. However, it was observed in Jensen and Hammer 1993 article that 3-(N-morpholino)propanesulfonic acid (MOPS) was a more suitable buffer for the cultivation of LAB than phosphate buffers. Thus, we tested different combinations of MOPS and phosphate buffers for our medium in MTP experiments and results showed that higher biomass yield can be obtained with MOPS (Fig. 1).

We considered the medium that contained 35.8 mM of MOPS, 5.2 mM of K_2HPO_4 and 5.5 mM of KH_2PO_4 (7.5, 0.9 and 0.75 g L^{-1}, respectively; DC medium) the most cost-effective and used this combination in media development

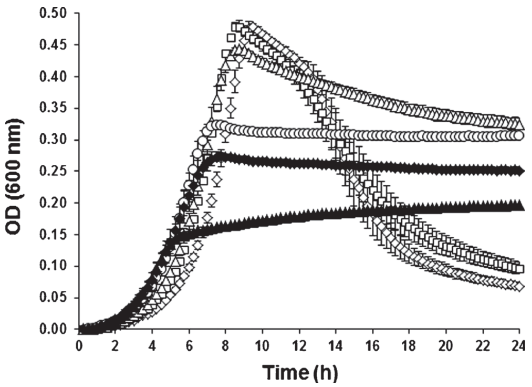

Fig. 1 Growth curves of *L. lactis* IL1403 with standard deviations in media with different buffer content. Each symbol is the average of three replicas. Buffer composition of these media was as follows (mM): DA (*diamond*)—MOPS 71.7, K_2HPO_4 5.2, KH_2PO_4 5.5; DB (*empty square*)—MOPS 53.8, K_2HPO_4 5.2, KH_2PO_4 5.5; DC (*empty triangle*)—MOPS 35.8, K_2HPO_4 5.2, KH_2PO_4 5.5; DD (*empty circle*)—MOPS 17.9, K_2HPO_4 5.2, KH_2PO_4 5.5; BS1 (*filled triangle*)—K_2HPO_4 5.2, KH_2PO_4 5.5; BS2 (*filled diamond*)—K_2HPO_4 17.2, KH_2PO_4 18.4

experiments. Incidentally, a drop in OD value at the end of exponential phase can be seen in the growth curves of three media that contained higher amounts of MOPS (Fig. 1), hinting that cell lysis might have occurred, but this was not checked. A drop in OD has never been observed with the buffer combination of DC medium in test tube experiments (data not shown).

Amino acid auxotrophies

As observed previously by other authors (Cocaign-Bousquet et al. 1995; Zhang et al. 2009) and confirmed in this study, L-arginine (Arg), L-asparagine (Asn), L-histidine (His), L-methionine (Met), L-serine (Ser) and the branched-chain amino acids L-isoleucine (Ile), L-leucine (Leu) and L-valine (Val) are essential amino acids for this strain (Fig. 2a). Additionally, L-alanine (Ala), L-cysteine (Cys), L-glutamine (Gln), L-lysine (Lys) and L-threonine (Thr) were shown to stimulate or promote growth. L-Aspartate (Asp), L-glutamate (Glu), glycine (Gly), L-proline (Pro) and aromatic amino acids L-phenylalanine (Phe), L-tryptophan (Trp) and L-tyrosine (Tyr) were considered non-essential.

Furthermore, it was checked whether any amino acid that is non-essential in normal conditions becomes indispensable in amino acid limitation conditions, i.e. if the concentrations of other amino acids (that might be used as substrates in the biosynthesis of non-essential amino acids) are low. Thus, 20 media were prepared, where the concentrations of 19 amino acids were decreased four times (compared to the original medium) and one amino acid was omitted. In such conditions, *L. lactis* IL1403 could not grow without L-cysteine and L-glutamine (Fig. 2a). These two amino acids were growth stimulating in normal conditions, but became essential in amino acid limitation conditions.

Moreover, an experiment with 20 media was carried out where the concentrations of 19 amino acids were lowered four times, whereas the concentration of one amino acid was left unaltered. The most noticeable observation from this experiment was that the final OD of *L. lactis* IL1403 is higher, if the concentration of L-asparagine is not decreased in a medium with low amino acid amounts (see Supplementary material 1 for media EA-EU).

Growth on media with reduced amino acid concentrations

Determination of amino acid auxotrophies was followed by experiments on media with reduced amino acid concentrations. The aim was to test the growth of bacteria in media containing amino acids as much as required for biomass synthesis. Concentrations of all amino acid in MTP scale could be lowered at least four times compared to the initial medium. Additionally, the concentrations of Leu, Lys and Val could be decreased eight times, and the amounts of Arg, Ile, Phe, Thr, Trp and Tyr even 16 times without a loss in biomass formation. It was seen that Asp and Glu could be simultaneously omitted from the medium (see CI or JI medium in Supplementary material 1), but not Asp and Gln (CL and JL in Supplementary material 1). PLSR analysis showed that Asn and Gln have the best correlation with optical density, i.e. these amino acids influence OD the most (Fig. 3a). Also, the aromatic amino acids Phe, Trp and Tyr had the smallest effect on OD. The correlation of specific growth rate (μ) was the highest with the branched-chain amino acids Ile, Leu and Val and also Thr, i.e. these amino acids were needed the most for the fast growth of *L. lactis* IL1403.

The amino acid combinations that provided the highest OD and μ values in MTP experiments were chosen for scale-up experiments in test tubes. However, the optical densities with these media in test tube experiments were lower than 40 % of that obtained in the reference medium. Further experiments confirmed that concentrations of amino acids could be lowered 2–8 times in test tubes not 4–16 times as shown in MTP experiments. Nevertheless, the amino acids that had the most noteworthy effect on biomass yield and specific growth rate were the same as in MTP experiments (Fig. 3b): Asn and Gln were the most influential amino acids for OD and the branched-chain amino acids Ile, Leu and Val for specific growth rate values. Also, amino acid utilization measurements (Supplementary material 2) carried out for a number of media in stationary phase showed that although Asp and Glu were present in the medium, they were additionally produced and secreted into the environment by *L. lactis* IL1403. These amino acids were apparently required to achieve faster specific growth rate (see correlation in Fig. 3b).

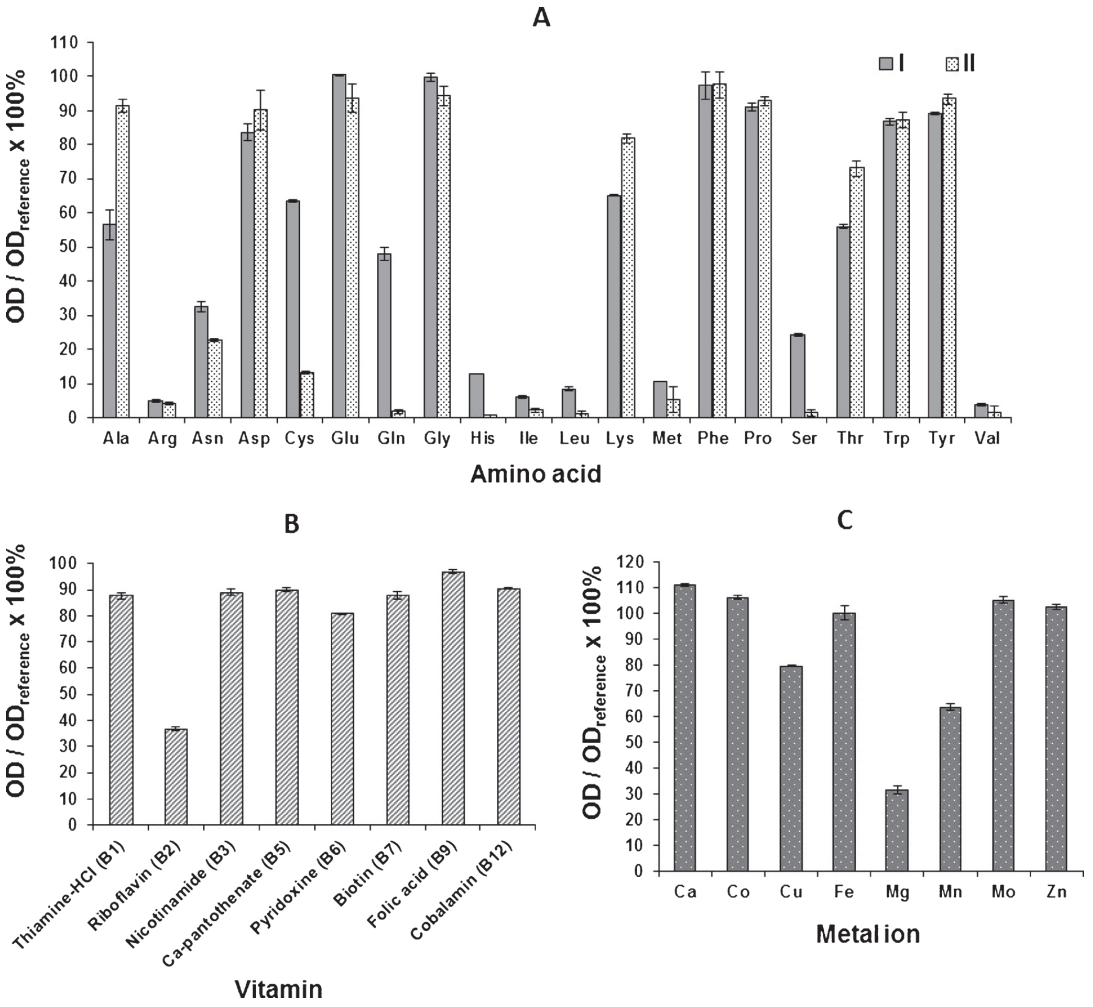

Fig. 2 Determination of nutrient auxotrophies (leave-one-out experiments) in MTP scale experiments. Results are given as percent of OD obtained compared to the reference medium. Each column is the average of three replicas. The omitted or altered component is indicated by the title of the column. **a** Amino acids. *I*—One amino acid has been omitted from the medium. *II*—Amino acid auxotrophies in amino acid limitation conditions. One amino acid has been omitted from the medium and the amounts of all other amino acids have been reduced four times compared to the original medium. **b** B-group vitamin auxotrophies—one vitamin has been omitted from the medium. **c** Metal ion (Mo^{6+}; other ions $^{2+}$) auxotrophies—one metal ion has been omitted from the medium

It was confirmed in the test tube experiments that aromatic amino acids (Phe, Tyr, Trp) and Pro can be removed from the media to maintain the same biomass yield but not to obtain the same specific growth rate compared to the reference medium. *L. lactis* IL1403 was able to biosynthesize Phe and Tyr, if these compounds were omitted from the media and Pro was produced even if the media contained the latter component (see NB for Phe and MC, NC, OH for Tyr in Supplementary material 2). The consumption of Cys, Gly and His was low in all cases, which allowed us to conclude that the concentrations of these three amino acids can be lowered at least eight times, compared to the initial medium. The majority of Asn and Gln, however, were consumed even in the initial medium (BS7). Thus, the concentrations of these two amino acids cannot be reduced without affecting biomass yield and specific growth rate.

B-group vitamins

Similarly to determining amino acid auxotrophies, we identified essential vitamins for *L. lactis* IL1403 in MTP

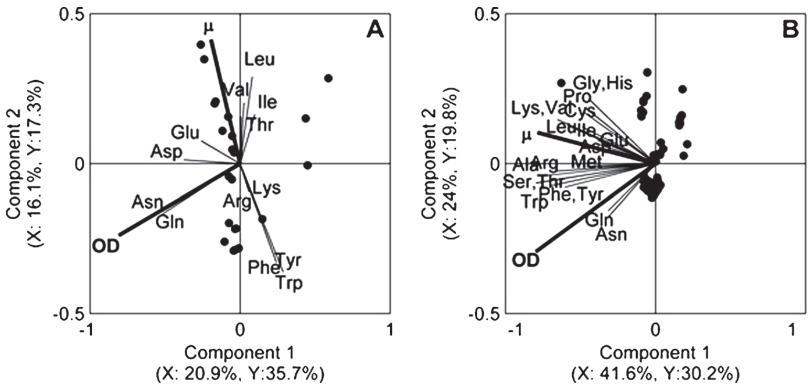

Fig. 3 Combined scores and loadings plot of PLSR analysis of growth media composition and growth related parameters (OD and specific growth rate). **a** Microtitre plate experiments. **b**—Test tube experiments. *Black dots* represent experiments on media with various amino acid composition. *Thin black lines* represent loadings of independent variables in PLSR analysis, i.e. media composition. *Thick black lines* represent loadings of dependent parameters, i.e. OD and specific growth rate

experiments studying eight CDMs lacking one of the vitamins. Results (Fig. 2b) indicated that only riboflavin was essential for this strain and IL1403 could grow in media supplemented solely with this vitamin (FA compared to BS2 in Supplementary material 1). However, growth was hampered in a medium with reduced concentrations of amino acids (four times) that did not contain folic acid (B_9) and cobalamin (B_{12}) (OD 74.2±1.7 %; IA compared to BS2 in Supplementary material 1) or if the medium contained only riboflavin (in three times larger amounts than in the reference medium; OD 60.3±0.2 %; IB compared to BS2 in Supplementary material 1). Moreover, in test tube experiments, *L. lactis* IL1403 was unable to grow in a medium with reduced amino acid concentrations, if the medium contained less than seven B-group vitamins (only cobalamin/vitamin B_{12} could be omitted). The exclusion of pantothenate or pyridoxine led to zero growth (see OA, OB, PA, PF-PJ in Supplementary material 1), whilst the omission of folic acid permitted only poor growth (OD 33.2± 0.7 %; BS4 compared to BS7 in Supplementary material 1). Also, we ascertained whether folic acid can be replaced with its precursor *para*-aminobenzoic acid (*p*ABA), since the latter compound is cheaper, more soluble and stable than folic acid. Results in Fig. 4a show that 2.74 μM of folic acid can be replaced by *p*ABA in concentrations of 1.10–70.58 μM.

One additional aim of this work was to optimize vitamin concentrations in the media by reducing (2 or 10 times) or increasing (1.5 to 2.5 times) amounts of vitamins. Results obtained (Fig. 5a) indicated that an increase in vitamin concentrations did not have any effect on the growth of cells but concentrations could not be reduced without affecting biomass yield.

Metal ions and additional compounds

Our initial CDM contained eight trace minerals from which magnesium proved to be essential, manganese strongly and copper slightly growth-promoting in a leave-one-out experiment in MTP scale (Fig. 2c). The optimization of mineral salt concentrations in our experiments demonstrated that neither two times increase nor four times decrease of amounts in the medium had significant effect on the growth of cells (Fig. 5b).

In addition to amino acids, vitamins and metal ions, the essentiality of several other compounds (choline chloride, hypoxanthine, lipoic acid, *myo*-inositol, sodium pyruvate and thymidine) was tested. These substances were present in a CDM previously used in our laboratory (Lahtvee et al. 2011). Results obtained (Fig. 4b) showed that none of those compounds were necessary upon first inoculation. However, when reinoculations were made from stationary phase of each culture to the respective media, no growth occurred without lipoic acid.

Discussion

The aim of this work was to study the effects of media components on the specific growth rate and biomass yield of *L. lactis* subsp. *lactis* IL1403. A wide variety of CDMs with reduced nutrient concentrations was developed for detailed metabolic research of lactic acid bacteria:

1. Media with reduced amino acid concentrations: JA-JW (excluding JK and JL) for microtitre plates and MA-MH,

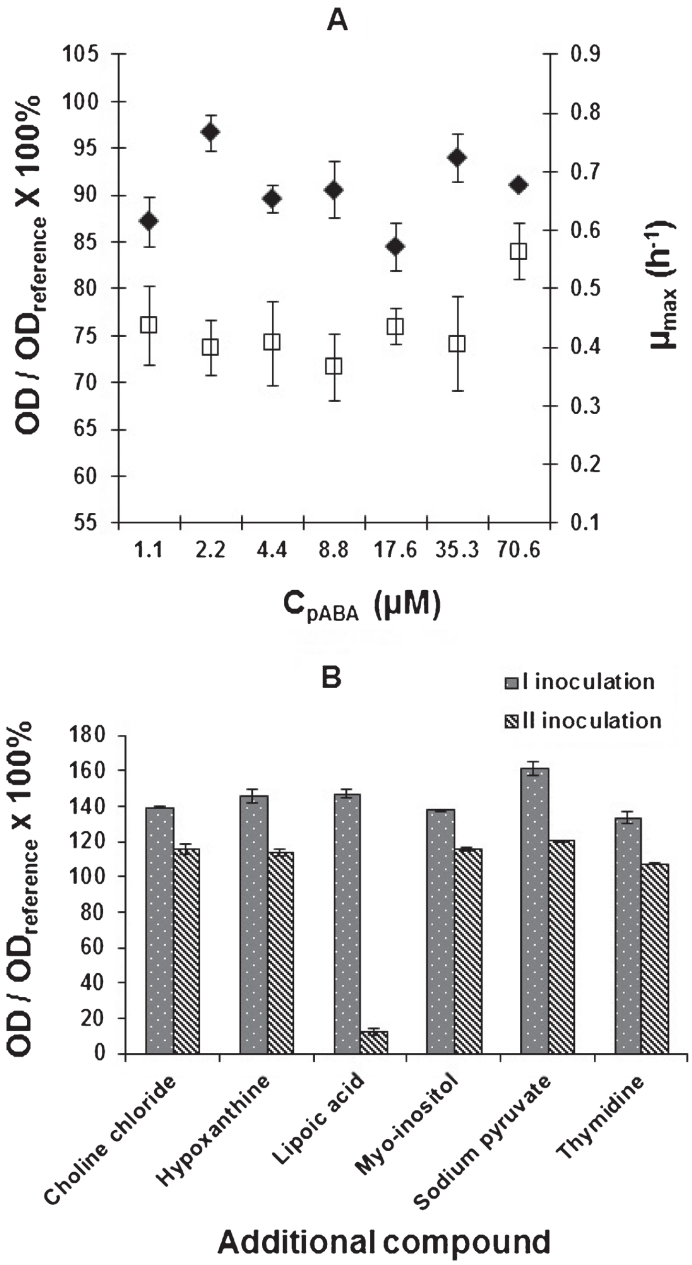

Fig. 4 Effect of additional compounds on growth. Results are given as percent of OD obtained compared to the reference. All values are averages of three replicas. **a** Substitution of folic acid with its precursor *para*-aminobenzoic acid at various concentrations (μM). Effect on OD (*filled diamond*) and specific growth rate μ (*empty square*; h^{-1}) values. The initial medium containing folic acid was used as reference. **b** Omission of choline chloride, hypoxanthine, lipoic acid, *myo*-inositol, sodium pyruvate and thymidine (indicated by the title of the column). A medium containing all of those additional compounds has been used as reference

NA-NH, OC-OM (excluding OK), PB-PE and K1-K44 for test tube scale experiments.

2. Media with reduced mineral salts concentrations: V2, V3, V12-V14, V17-V20 and V24-V32 for MTPs, as well as test tubes.

These media (compositions are given in Supplementary material 1) allow more precise determination of nutrient utilization as the percentage of each consumed compound compared to initial amount in the media was larger than measurement error (>5 %). Unlike previous publications about media

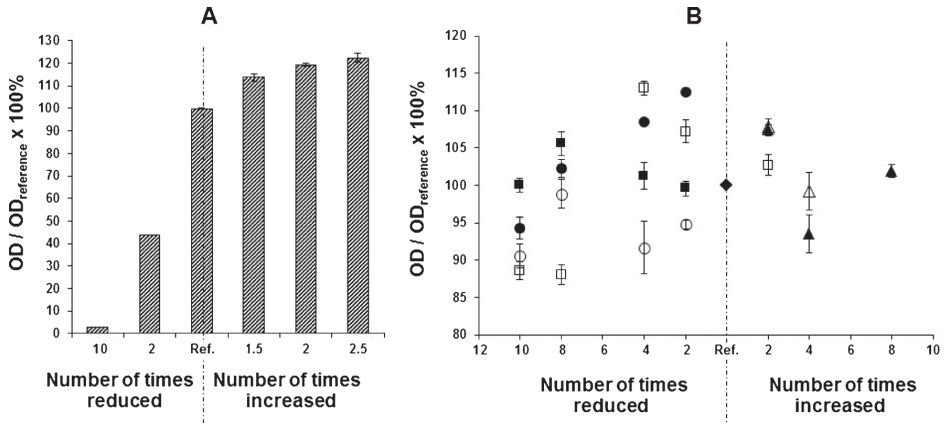

Fig. 5 Media with altered vitamin (**a**) or mineral salts (**b**) concentrations. Results are given as percent of OD obtained compared to the reference (=Ref.) medium. Each value is the average of three replicas. The *x*-axis indicates how many times the concentrations of compounds have been reduced or increased in comparison to the initial medium. *Filled circle*, NaCl; *empty circle*, non-essential metal ions (Ca^{2+}, Co^{2+}, Fe^{2+}, Mo^{6+}, Zn^{2+}); *filled square*, non-essential metal ions (Ca^{2+}, Co^{2+}, Fe^{2+}, Mo^{6+}, Zn^{2+}) and NaCl; *empty square*, all metal ions (Ca^{2+}, Co^{2+}, Cu^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, Mo^{6+}, Zn^{2+}) and NaCl; *filled triangle*, Mg^{2+}; *empty triangle*, Mn^{2+}; *filled diamond*, reference

development for *L. lactis* IL1403 (Jensen and Hammer 1993; Cocaign-Bousquet et al. 1995; Zhang et al. 2009), the current research provides data about amino acid utilization during growth on media that were developed herein (Supplementary material 2). CDMs for *L. lactis* IL1403 that have been published by other authors, e.g. MS10 (Cocaign-Bousquet et al. 1995), SA (Jensen and Hammer 1993), ZBM1 and ZBM2 (Zhang et al. 2009) contain excess of components. Hence, these media are unsuitable for precise investigation of lactococcal amino acid metabolism due to low consumption of amino acids. As for the minimal media BL that contains only eight amino acids (Jensen and Hammer 1993) and MS15 that contains nine amino acids (Cocaign-Bousquet et al. 1995), these media provide lower maximum specific growth rate values (0.30 and 0.37 h^{-1}, respectively) than the media in this study and thus cannot be used for studying the metabolism of LAB in continuous cultivations at higher dilution rates.

In regard to nutrient auxotrophies of *L. lactis* IL1403, the results were generally comparable to those obtained in earlier publications (Cocaign-Bousquet et al. 1995; Zhang et al. 2009). Similarly to earlier publications, it was observed that MOPS facilitated higher biomass yields than phosphate buffers. Jensen and Hammer (1993) speculated that MOPS might be transported into the cells, where it may play a role in the osmoregulation of *L. lactis* IL1403. Also, it was mentioned that high concentration of phosphate (over 0.1 M) reduces the specific growth rate of cells probably due to the partial dissipation of proton gradient across the membrane.

An important result in this research was that L-cysteine and L-glutamine became indispensable when the availability of other amino acids in the environment was low. *L. lactis* IL1403 is able to produce L-cysteine from L-methionine, whereby the former amino acid is needed for protein folding and stability (due to forming disulfide bonds) and the latter for translation initiation control and in numerous methyltransferase reactions (Sperandio et al. 2005). Comparison of amino acid utilization and intracellular amino acid concentrations (Table 1) showed that in media with four times reduced amino acid concentrations (NB), the utilization of L-methionine was similar to the quantity in biomass, i.e. there was not enough L-methionine for the production of additional compounds, e.g. L-cysteine. Concurrently, the utilization of L-cysteine in NB medium increased two times compared to BS7 (medium with higher amino acid amounts), showing that if L-methionine was scarce in the environment, *L. lactis* preferred to take L-cysteine up from the media rather than biosynthesize this compound. Thus, if L-cysteine was omitted from a medium with four times reduced amino acid concentrations, *L. lactis* would not grow due to low availability of L-methionine needed for the biosynthesis of L-cysteine. Also, it might be that in microaerobic growth conditions the cells have to resist oxidative stress and thus need high amounts of L-cysteine, as glutathione and thioredoxin, which help to protect the cell from oxidative stress can be derived from L-cysteine (Sperandio et al. 2005).

It can be seen from Table 1 that L-glutamine has been consumed in excess, i.e. in more than three times larger amounts than needed for biomass synthesis. This prodigious utilization occurs because L-glutamine and L-glutamate are important for the regulation of nitrogen metabolism, as these are amino group donors for amino sugars and nucleotide synthesis (Larsen et al. 2006). In addition, L-glutamine and α-ketoglutarate are the precursors for L-glutamate production

Table 1 Comparison of amino acid utilization (BS7, NB media) and amino acid content of biomass proteins. Amino acid concentrations in NB medium were reduced four times (compared to BS7) and L-phenylalanine was omitted. Negative utilization values represent production of amino acids. Intracellular amino acid concentrations were measured as described in materials and methods section

Amino acid	BS7	NB (low amino acid content)	In biomass proteins
	mmol g_{DW}^{-1}		
Ala	0.567	0.522	0.409
Arg	1.045	0.253	0.118
Asn	1.230	0.720	0.357[a]
Asp	0.045	−0.020	
Cys	0.142	0.215	ND
Glu	−0.435	−0.089	0.362[b]
Gln	1.333	1.147	
Gly	0.430	−0.078	0.276
His	0.110	0.096	0.052
Ile	0.424	0.543	0.172
Leu	0.810	1.012	0.247
Lys	0.428	0.313	0.280
Met	0.138	0.077	0.068
Orn	−0.541	−0.013	ND
Phe	0.191	−0.072	0.119
Pro	0.161	−0.140	0.109
Ser	0.917	0.787	0.175
Thr	0.548	0.400	0.195
Trp	0.026	0.021	ND
Tyr	0.107	0.098	0.090
Val	0.427	0.403	0.202

ND not determined

[a] Sum of L-asparagine and L-aspartate

[b] Sum of L-glutamine and L-glutamate

(Lapujade et al. 1998) and glutamine synthetase enzymatically converts L-glutamate to L-glutamine (Larsen et al. 2006). Moreover, L-glutamate is the precursor for L-proline biosynthesis (Sleator et al. 2001) and decarboxylation of L-glutamate to γ-aminobutyric acid (GABA) is needed for energy generation and protecting the cells against low external pH (Nomura et al. 1999; Fernández and Zúñiga 2006). Therefore, as interconversion between L-glutamine and L-glutamate is possible, at least one of those amino acids should be abundant in the medium to sustain important cellular functions. This was supported by the fact that if amino acid concentrations in the medium were reduced four times and L-glutamine was omitted, no growth occurred.

PLSR analysis specified that the branched-chain amino acids—L-isoleucine, L-leucine and L-valine—were the most important compounds for supporting faster growth (higher specific growth rate) of *L. lactis*. These amino acids are precursors for branched-chain fatty acids and the most abundant amino acids in membrane proteins (den Hengst et al. 2006). Also, degradation of branched-chain amino acids to carboxylic acids provides the cells with additional ATP, since *L. lactis* can generate 1 mol of ATP per 1 mol of branched-chain amino acid in these metabolic routes (Chambellon et al. 2009). It can be seen from Table 1 that the branched-chain amino acids were utilized in excess and were thus probably used for the biosynthesis of fatty acids and carboxylic acids.

In addition to branched-chain amino acids, the concentration of L-threonine influenced specific growth rate in MTP experiments. The data presented in Table 1 demonstrated that in case of low available amino acid amounts, glycine was in deficit and thus was biosynthesized. L-Threonine can be degraded to acetaldehyde and glycine via threonine aldolase pathway (Fernández and Zúñiga 2006). However, some of glycine was probably obtained from L-serine in a reaction catalysed by serine hydroxymethyltransferase (Ogawa et al. 2000). In addition, L-serine is involved in the production of main fermentation end-products (lactate, formate, acetate) via biosynthesis of pyruvate (Novak and Loubiere 2000). The data in Table 1 affirms that L-serine was utilized in excess, e.g. consumed not only for biomass formation but also for the production of other compounds, e.g. glycine and pyruvate.

Regarding the results of PLSR analysis, the most remarkable impact on optical density was observed in case of L-asparagine and L-glutamine. L-Asparagine can be converted to L-aspartate and decarboxylation of the latter amino acid to L-alanine is needed for energy generation and protecting the cells against low external pH. Also, *L. lactis* transports L-asparagine into cells much more effectively than L-aspartate (Fernández and Zúñiga 2006). The data in Table 1 indicates that L-asparagine was utilized in excess and thus must have been used for the production of other amino acids, whereas L-aspartate was consumed in minute amounts or even produced.

L. lactis showed capability of biosynthesizing L-phenylalanine and L-tyrosine, if these substances were absent from the media, whereby L-proline was produced even if the media contained L-proline (see Supplementary material 2). The production of L-proline can be explained by high salt concentration in the medium (2.9 g L^{-1}), since L-proline accumulation in LAB is generally associated with response to osmotic stress (Fernández and Zúñiga 2006).

Riboflavin (B_2), the only essential B-group vitamin in MTP scale experiments, is indispensible for cellular metabolism since it is the precursor of coenzymes flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD), which are hydrogen carriers in redox reactions (LeBlanc et al. 2011). Folic acid in the media could be replaced with its precursor *p*ABA and decrease in the concentration of the precursor did not affect biomass yield or specific growth rate, indicating that the cells needed folic acid in lower amounts than were used in the initial media. Tetrahydrofolate (the biologically active

form of folate) is needed for the biosynthesis of purines and pyrimidines (Wegkamp et al. 2007). Increasing the concentration of *p*ABA in the medium leads to higher folate production yields (Sybesma et al. 2003; Wegkamp et al. 2007).

As for other components, *L. lactis* was auxotrophic for one metal ion (Mg^{2+}), while Mn^{2+}, Cu^{2+} and lipoic acid were growth stimulating. Magnesium is a significant compound in many enzymatical reactions (van Niel and Hahn-Hägerdal 1999) and binds to cell wall teichoic acids and intracellularly to nucleic acids and adenine nucleotides (Goel et al. 2012). Manganese and copper are required for activation of several enzymes, e.g. superoxide dismutase (van Niel and Hahn-Hägerdal 1999; Magnani et al. 2008). In addition, *Lactobacillus plantarum* is able to accumulate Mn^{2+} inside the cells in order to protect them from the toxic effect of oxygen (Nierop Groot and de Bont 1999). As our experiments were microaerobic, Mn^{2+} might have the same role in *L. lactis*. With regard to other metal ions, it has been noticed that results obtained by different authors and with a number of strains vary a great deal and that it has been difficult to evaluate metal ion requirements due to the fact that these compounds may enter the media also as additives from other components (Van Niel and Hahn-Hägerdal 1999). The functions of lipoic acid in the cell are acyl-generation, acyl-transfer and oxidative reactions of 2-oxo acids (van Niel and Hahn-Hägerdal 1999). This compound is also the cofactor in pyruvate dehydrogenase and is thus linked to the production of acetate and acetoin (van Niel and Hahn-Hägerdal 1999; Nordkvist et al. 2003).

Lastly, *L. lactis* IL1403 was shown to require larger amounts of amino acids and B-group vitamins in test tubes than in MTPs. This effect could be explained by the fact that gas environment was uncontrolled and thus the growth environment was more aerobic in MTP experiments than that in test tube experiments due to higher surface to volume ratio and frequent shaking in MTPs. Variations in the amounts of nutrients required for growth can be explained by changes in the activities of various enzymes, influenced by O_2 availability. For instance, it has been shown that pyruvate metabolism is strongly influenced by oxygen supply (Jensen et al. 2001). In order to obtain similar growth results in various cultivation vessels and volumes, such factors as mixing, oxygen transfer rate to the cells and volumetric mass transfer coefficient have to be tuned comparable (Duetz 2007; Kensy et al. 2009).

In conclusion, multiple chemically defined media, which can be used in quantitative analysis of the physiological role of media components in the metabolism of amino acids, vitamins, etc. of *L. lactis* IL1403, were developed. These media can also be used to study other species of lactic acid bacteria. In the future perspective, these chemically defined media will be applied for the elucidation of factors involved in overconsumption of amino acids in *L. lactis* IL1403 using continuous cultivation. This approach enables to determine the ratios of amino acids in the medium required for production of biomass or certain compound at different specific growth rates.

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Excess of threonine compared with serine promotes threonine aldolase activity in *Lactococcus lactis* IL1403

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Lactococcus lactis is an important lactic acid starter for food production as well as a cell factory for production of food grade additives, among which natural flavour production is one of the main interests of food producers. Flavour production is associated with the degradation of amino acids and comprehensive studies are required to elucidate mechanisms behind these pathways. In this study using chemically defined medium, labelled substrate and steady-state cultivation, new data for the catabolism of threonine in *Lc. lactis* have been obtained. The biosynthesis of glycine in this organism is associated with the catabolic pathways of glucose and serine. Nevertheless, if threonine concentration in the growth environment exceeds that of serine, threonine becomes the main source for glycine biosynthesis and the utilization of serine decreases. Also, the conversion of threonine to glycine was initiated by a threonine aldolase and this was the principal pathway used for threonine degradation. As in *Streptococcus thermophilus*, serine hydroxymethyltransferase in *Lc. lactis* may possess a secondary activity as threonine aldolase. Other catabolic pathways of threonine (e.g. threonine dehydrogenase and threonine dehydratase) were not detected.

INTRODUCTION

Lactic acid bacteria (LAB) are essential in the food industry, particularly in the production of dairy products, as they contribute to the aroma and texture of products and prevent spoilage of food (Price *et al.*, 2012). Moreover, the technological advances in the last decade have created the opportunity to exploit LAB for the bioproduction of value-added chemicals, e.g. vitamins, polysaccharides, low-calorie sweeteners (polyols), flavour compounds and ethanol (Gaspar *et al.*, 2013).

The catabolism of threonine in LAB (Fig. 1) has attracted interest owing to the fact that this amino acid can be degraded to acetaldehyde and glycine by threonine aldolase (TA) (Fernández & Zúñiga, 2006). Acetaldehyde, as well as acetone and diacetyl, are important flavour components in yogurt. In fact, acetaldehyde can be produced by several other biochemical pathways in LAB (e.g. by pyruvate decarboxylation), which can operate simultaneously (Ott *et al.*, 2000; Chaves *et al.*, 2002). However, the yield of acetaldehyde during fermentation of *Lactococcus lactis* is low (Bongers *et al.*, 2005), as opposed to species used in yogurt starter culture, *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* (Chaves *et al.*, 2002). Acetaldehyde can be further metabolized into ethanol by alcohol dehydrogenase, and the gene encoding this enzyme (*adhE*) is present in *Lc. lactis* (Christensen *et al.*, 1999; Bolotin *et al.*, 2001).

TA, which catalyses the conversion of threonine to acetaldehyde and glycine, has been characterized as the basic catabolic pathway of threonine in LAB (Christensen *et al.*, 1999; Fernández & Zúñiga, 2006). TA activity in bacteria can be associated with two enzymes: serine hydroxymethyltransferase (SHMT), encoded by *glyA*, and

Abbreviations: BCAA, branched-chain amino acid; CDM, chemically defined medium; DW, cell dry weight; LAB, lactic acid bacteria; SHMT, serine hydroxymethyltransferase; TA, threonine aldolase; TDH, threonine dehydrogenase; THF, tetrahydrofolate.

Supplementary methods, four supplementary figures and a supplementary movie are available with the online Supplementary Material.

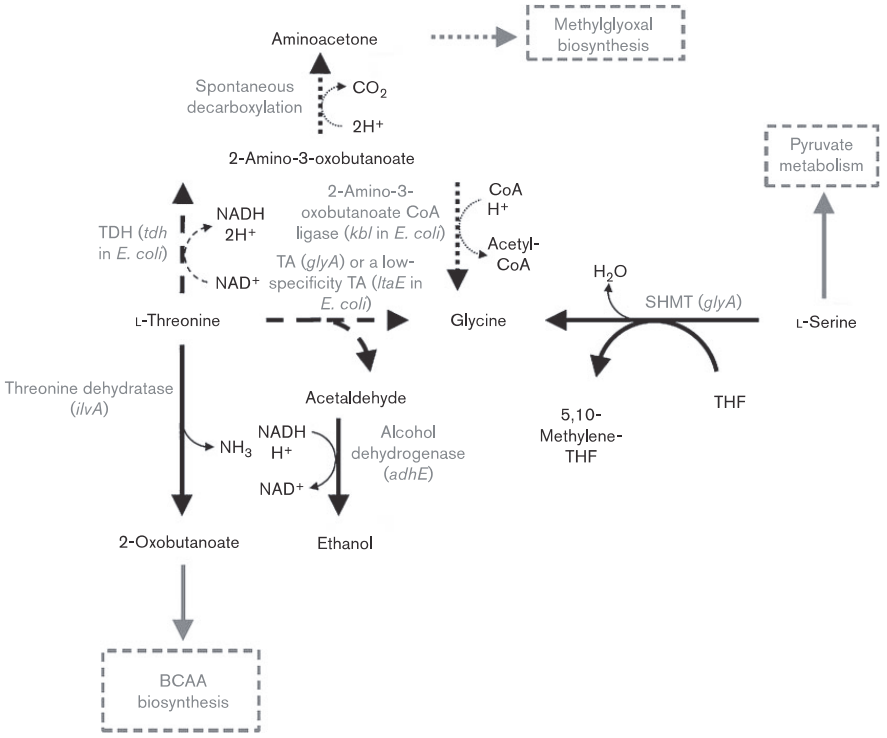

Fig. 1. Potential pathways in LAB for L-threonine and L-serine degradation and glycine biosynthesis. Solid lines represent pathways that are known in *Lc. lactis* IL1403 based on genome sequence or experimental data. Dashed lines represent pathways that are known in other LAB, but not in *Lc. lactis* IL1403. Dotted lines represent pathways that are known in other micro-organisms (e.g. *E. coli*), but not in LAB. The pathways leading to methylglyoxal, pyruvate and BCAA biosynthesis have not been given in detail. Threonine dehydratase converts L-threonine into 2-aminobut-2-enoate and two following spontaneous reactions turn the latter compound into 2-oxobutanoate.

a low-specificity TA (Liu *et al.*, 1998; Chaves *et al.*, 2002; Simic *et al.*, 2002; Fernández & Zúñiga, 2006). The principal role of SHMT is catalysing the reversible formation of glycine and 5,10-methylenetetrahydrofolate from serine (Fernández & Zúñiga, 2006). Novak & Loubiere (2000) proved with labelled substrates that, if *Lc. lactis* is cultivated in a simple synthetic medium, 68 % of the glycine in cellular proteins is derived from glucose and 32 % from serine. Interestingly, some SHMTs have a secondary activity as TA, e.g. in *S. thermophilus* (Chaves *et al.*, 2002), although this is not a ubiquitous property amongst LAB (Ogawa *et al.*, 2000). A low-specificity TA (encoded by *ltaE*), which catalyses the cleavage of L-threonine or L-*allo*-threonine to glycine and acetaldehyde, has been found in *Escherichia coli* (Liu *et al.*, 1998). Yet, no homologues of *ltaE* have been found in LAB (Fernández & Zúñiga, 2006).

A well-described route for threonine catabolism in both prokaryotes and eukaryotes is the conversion of threonine to 2-amino-3-ketobutyrate by threonine dehydrogenase (TDH), followed by the formation of acetyl-CoA and glycine (initiated by 2-amino-3-ketobutyrate-CoA ligase) or aminoacetone and CO_2 (spontaneous decarboxylation) (Marcus & Dekker, 1993). This pathway has been investigated the most in *E. coli*, where the genes associated with this route are *tdh* and *kbl* (Epperly & Dekker, 1991; Marcus & Dekker, 1993; Schmidt *et al.*, 2001). Based on annotated genomes, the first reaction in this pathway (conversion of threonine to 2-amino-3-ketobutyrate) may exist in such LAB as *Oenococcus oeni* (Borneman *et al.*, 2012), *Streptococcus suis* (Hu *et al.*, 2011) and *S. thermophilus* (Sun *et al.*, 2011).

In addition to glycine production, threonine can be converted to 2-oxobutanoate by threonine dehydratase (and two spontaneous reactions) and the gene encoding this enzyme (*ilvA*) exists in *Lc. lactis* (Bolotin *et al.*, 2001; Fernández & Zúñiga, 2006). 2-Oxobutanoate is the precursor in the biosynthetic pathway of branched-chain

amino acids (BCAAs) isoleucine, leucine and valine (Fernández & Zúñiga, 2006). However, the strain used in the current study, *Lc. lactis* IL1403, is known to be auxotrophic for BCAAs (Cocaign-Bousquet *et al.*, 1995; Zhang *et al.*, 2009; Aller *et al.*, 2014), and so this pathway might not be functional in IL1403, although the genes associated with the reactions have been characterized (Bolotin *et al.*, 2001). Moreover, neither of the previously mentioned catabolic reactions of threonine (catalysed by TA and TDH) has been characterized in *Lc. lactis* IL1403.

A chemically defined medium (CDM) is indispensable in studying the metabolic pathways and physiology of micro-organisms, because unlike in rich media, all components in a CDM are defined (Zhang *et al.*, 2009). Thus, experiments with CDM produce more comprehensible data, as the substrates and bioproducts can be easily quantified by analytical methods. Therefore, this article focuses on determining the degradation routes of threonine in *Lc. lactis* IL1403 using a CDM (BS7) developed in-house.

METHODS

Methods are given in full detail in the Supplementary Methods, available in the online Supplementary Material.

Micro-organism. The strain used in all experiments is *Lc. lactis* subsp. *lactis* IL1403, which was provided by INRA. Inoculum was prepared from lyophilized stock culture stored at −80 °C, which was pre-grown twice in test tubes at 34 °C on CDM BS7.

Media. CDM BS7, which has been designed specifically for the better detection of amino acids and quantification of full amino acid composition, was used for cultivation. The composition of BS7, which did not contain glycine, was as follows (mg l^{-1}): D-glucose, 4500; K_2HPO_4, 900; KH_2PO_4, 750; MOPS, 7500; L-alanine, 78; L-arginine, 185; L-asparagine, 74; L-aspartic acid, 72; L-cysteine, 64; L-glutamic acid, 70; L-glutamine, 132; L-histidine, 60; L-isoleucine, 102; L-leucine, 207; L-lysine, 158; L-methionine, 41; L-phenylalanine, 86; L-proline, 92; L-serine, 163; L-threonine, 76; L-tryptophan, 16; L-tyrosine, 29; L-valine, 107; $CaCl_2$, 50; $CoSO_4.7H_2O$, 3; $CuSO_4.5H_2O$, 3; $FeSO_4.7H_2O$, 1.4; $MnSO_4.H_2O$, 16; $MgSO_4.7H_2O$, 200; NaCl, 2900; $(NH_4)_6Mo_7O_{24}.4H_2O$, 3; $ZnSO_4.7H_2O$, 5; thiamine hydrochloride (B_1), 0.51; riboflavin (B_2), 0.326; nicotinamide (B_3), 0.325; D-pantothenate (B_5), 0.65; pyridoxine (B_6), 0.642; biotin (B_7), 0.305; folic acid (B_9), 1.21; adenine, 25; hypoxanthine, 25; lipoic acid, 0.176. These numbers indicate the concentrations that were used in preliminary test tube experiments and for growing inoculum for continuous cultivations. In the case of continuous cultivation, a different buffer composition was used (mg l^{-1}): K_2HPO_4, 3000; KH_2PO_4, 2500; no MOPS.

Chemicals were obtained from Serva Electrophoresis and Sigma-Aldrich and were mixed together in the following order (to avoid precipitation): sugar, buffers, amino acids, minerals, vitamins, additional components. All media were sterilized by filtration, using stainless steel filter holders and nitrocellulose membrane filters (0.22 µm), provided by Merck Millipore.

Continuous cultivation. Continuous cultivation was carried out using the D-stat method and 1.25 l Biobundle bioreactors with EZ-Control biocontrollers and BioXpert Pharma 3.80 M XP control program, all provided by Applikon Biotechnology. The system was equipped with pH, O_2 and temperature sensors. Two BS7 media with different threonine concentrations (0.3 and 5 mM) were simultaneously pumped into the fermenter, whereas after stabilization was complete, the ratio of these media was changed over time (using computer algorithms), leading to the constant increase of threonine concentration in the feed. Three parallel experiments with a dilution rate of 0.2 h^{-1} were carried out, in which the concentration of threonine in the fermenter inflow was changed from 1.24 to 2.58 mM (one experiment) or 4.13 mM (two experiments). The cultivation temperature in all experiments was 34 °C, agitation speed 300 r.p.m. and cultivation volume 300 ml. The pH was kept at 6.4 by addition of 2 M NaOH. The gas enviroment was kept anaerobic by flushing the bioreactors with N_2. Sampling is described in the Supplementary Methods.

Labelling experiment. In order to correctly determine L-threonine degradation products, we carried out a D-stat experiment at 0.2 h^{-1} with BS7 medium, where the concentration of L-threonine was 2.5 mM. The equipment and environmental conditions were the same as in previous experiments (see Continuous cultivation, above). After stabilization of the culture, the medium was switched to labelled BS7 medium, which contained 2.5 mM ^{13}C,^{15}N-labelled L-threonine (CortecNet). Sampling is described in the Supplementary Methods.

Analytical methods. Glucose and main fermentation end products (lactate, formate, acetate, ethanol) were measured with LC (Alliance 2795 system), a refractive index detector (model 2414) and Empower software, all provided by Waters. The column used was Bio-Rad HPX-87H at 35 °C with isocratic elution of 5 mM H_2SO_4 at a flow rate of 0.6 ml min^{-1}. Acetaldehyde was measured with a specific assay kit provided by Megazyme.

Free amino acids were detected with an amino acid analyser Acquity UPLC. The samples were derivatized beforehand for 10 min at 55 °C, using AccQ-Fluor reagent (6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate) and then loaded on an AccQ-Tag Ultra column at 55 °C. Amino acids were separated using a gradient of AccQ-Tag Ultra eluents A and B. They were detected with a photodiode array detector, and data were processed with Empower software. In order to measure labelled L-threonine or glycine in the labelling experiment, an LCT Premier^{TM} XE ESI TOF MS was used, where amino acids were separated as described above and then sprayed directly into an MS operated in positive ionization mode (solvation temperature 300 °C, source temperature 120 °C, capillary voltage 2.5 kV). MassLynx V4.1 software was used for data processing. All the equipment, reagents and eluents were provided by Waters.

For intracellular amino acid measurements, biomass was hydrolysed with 6 M HCl for 24 h at 105 °C. Amino acids were measured as described above. L-Tryptophan and L-cysteine could not be detected with this method, because they are degraded during hydrolysis. Also, L-asparagine is converted to L-aspartate and L-glutamine to L-glutamate during hydrolysis.

NMR spectra were measured on a Bruker Avance III 800 MHz spectrometer equipped with a He cryoprobe. Samples were prepared by adding 60 µl D_2O to 540 µl supernatant (10 % D_2O concentration). ^{13}C spectra were measured at 15 °C sample temperature with 30° flip angle pulses and proton decoupling. Ten thousand scans with 2 s relaxation delay were collected for each sample. All spectra were frequency-referenced according to the lactate signal and intensities were scaled to constant lactate signal intensity. Spectral assignment is based on chemical shifts, integrals and ^{13}C–^{13}C couplings and was confirmed with a separate gated decoupling ^{13}C experiment.

Proteome analysis. Samples from two D-stat experiments were analysed in single runs. Peptides were separated on an Ultimate 3000 RSLCnano system (Dionex; Fisher Scientific) using a cartridge trap-column in backflush configuration and an analytical 50 cm Easy-Spray column (Thermo Fisher Scientific). Peptides were eluted at

200 nl min^{-1} with an 8–40 % buffer B gradient (buffer B, 80 % acetonitrile + 0.1 % formic acid; buffer A, 0.1 % FA) to a Q Exactive MS/MS operating with a top-10 strategy and a maximum cycle time of 1 s. The MS proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository (Vizcaíno *et al.*, 2013) with the dataset identifier PXD001602. Raw MS files were analysed by the MaxQuant software package version 1.3.0.5 (Cox & Mann, 2008). Proteome analysis is described in full detail in the Supplementary Methods.

RESULTS

Biomass and end-product yields at various L-threonine concentrations

We have observed previously that in *Lc. lactis* IL1403 some of the glycine in biomass proteins is derived via biosynthesis (Lahtvee *et al.*, 2011) and that biosynthesis of glycine is preferred over utilization mainly at lower specific growth rates, i.e. at 0.1–0.2 h^{-1}. Preliminary test tube experiments (Fig. S1a and S1b) with varied concentrations of L-serine and L-threonine indicated that the sum of glycine in cellular proteins and in extracellular secretion was up to 31 % higher in media with additional L-threonine (Fig. S1c). The utilization of L-serine was decreased when the concentration of L-threonine was higher than that of L-serine, and vice versa (Fig. S1d). In order to study this effect in detail, continuous cultivations were carried out in bioreactors.

Three parallel D-stats with dilution rate 0.2 h^{-1} were carried out where the concentration of L-threonine in the feed was gradually raised from 1.24 to 4.13 mM. Biomass yield, Y_{XS} (Fig. 2a), was constant in the first half of the experiment (0.144 ± 0.003 g $g_{glucose}^{-1}$) and not dependent on the concentration of L-threonine in the medium. However, Y_{XS} started to drop slightly when L-threonine concentration in the medium was higher than 3 mM, and reached 0.129 ± 0.008 g $g_{glucose}^{-1}$ by the end of experiment.

The production of lactate (Fig. 2a) was constant in the first half of the experiment [64.88 ± 1.73 mmol (g cell dry weight, g_{DW})$^{-1}$] and not dependent on the concentration of L-threonine in the medium. Yet, lactate production increased slightly at higher L-threonine concentrations and was 74.15 ± 5.77 mmol g_{DW}^{-1} by the end of the experiment. Production of acetate, formate and ethanol (Fig. 2b) was constant throughout the experiments. The production of acetaldehyde was never higher than 0.05 mmol g_{DW}^{-1}.

Amino acid utilization and biosynthesis

The production of extracellular glycine was linked to the concentration of L-threonine in the medium and increased

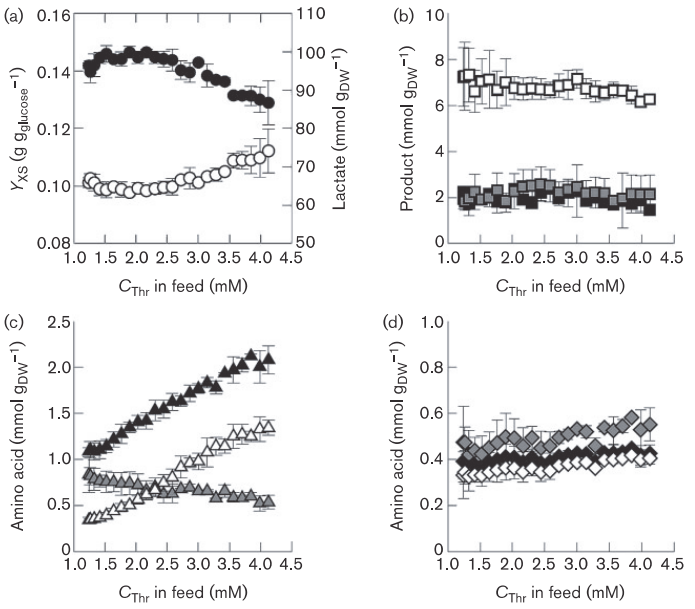

Fig. 2. Growth characteristics in D-stat experiments (mean results of three fermentations). (a) ●, Biomass yield Y_{XS} [g (g glucose consumed)$^{-1}$]; ○, production of lactate. (b) Production of formate (□), acetate (■) and ethanol (■). (c) Production of glycine (△) and utilization of L-serine (▲) and L-threonine (▲). (d) Utilization of L-isoleucine (◇), L-leucine (◆) and L-valine (◆). C_{Thr}, concentration of threonine.

from 0.34±0.03 to 1.34±0.08 mmol g_{DW}^{-1} (Fig. 2c; mean of three experiments), whereas the intracellular concentrations of glycine and all other amino acids in biomass proteins remained constant throughout the experiments (Table 1). The consumption of L-serine (Fig. 2c) in these fermentations decreased from 0.84±0.19 to 0.53±0.07 mmol g_{DW}^{-1}, which constitutes a decrease of 34±6 to 19±2% from the amount of L-serine in the medium. This suggests that glycine was produced mostly from L-threonine, rather than from L-serine, since L-threonine utilization (Fig. 2c) increased from 1.09±0.04 to 2.08±0.15 mmol g_{DW}^{-1}, which constitutes a reduction of 57±2 to 30±1% from the amount of L-threonine in the medium. However, the consumption of L-threonine was higher than the biosynthesis of glycine. LAB can degrade threonine into 2-oxobutanoate, which is a precursor in the biosynthesis of BCAAs. However, there was no downtrend in the utilization of external L-isoleucine, L-leucine and L-valine pools at different L-threonine concentrations (Fig. 2d).

The utilization of several amino acids, e.g. L-arginine (1.11-fold), L-asparagine (1.34-fold), L-cysteine (1.46-fold) and L-glutamine (1.31-fold), and the production of L-glutamate (1.19-fold) and L-ornithine (1.17-fold) were increased at higher L-threonine concentrations in feed (Fig. S2).

Table 1. Amino acid concentrations in cellular proteins during D-stat experiments

Mean results of three fermentations. Samples were taken throughout the fermentations (13 samples from the first, 3 from the second and 3 from the third fermentation) and each sample was analysed in three replicates. L-Tryptophan and L-cysteine could not be detected with this method, because these are degraded during hydrolysis. Also, L-asparagine is converted to L-aspartate and L-glutamine to L-glutamate during hydrolysis.

Amino acid	Concentration		% Total cellular protein
	mmol g_{DW}^{-1}	SE (%)	
Ala	0.495±0.024	4.8	8.3
Arg	0.142±0.007	4.8	5.2
Asn+Asp	0.430±0.021	5.0	11.5
Gln+Glu	0.511±0.021	4.2	15.4
Gly	0.335±0.017	5.0	4.5
His	0.066±0.006	9.7	2.1
Ile	0.209±0.009	4.3	5.6
Leu	0.313±0.013	4.3	8.3
Lys	0.337±0.014	4.0	10.1
Met	0.067±0.011	16.7	2.2
Phe	0.142±0.006	4.0	4.9
Pro	0.135±0.006	4.3	3.1
Ser	0.204±0.011	5.6	4.2
Thr	0.227±0.009	4.1	5.4
Tyr	0.093±0.003	3.0	3.6

Changes in protein expression

Proteome analysis was done for two D-stat experiments; expression was measured at three L-threonine concentrations in the feed (1.24, 2.86 and 3.99 mM) and compared with the first point. For instance, the expression of SHMT (GlyA) was increased at higher L-threonine concentrations, whereas the expressions of alcohol dehydrogenase (AdhE) and threonine dehydratase (IlvA) were decreased (Fig. 3a). L-Threonine as a substrate was definitely abundant in the medium, as the expression of threonine synthase (ThrC) was virtually unchanged during the experiment (Fig. 3a).

As for other notable changes at the proteome level, the expression of a betaine ABC transporter permease (BusAB), which is involved in osmotic stress, was increased (Fig. 3a). The expression of CysM, which is involved in cysteine biosynthesis from serine, was at first increased but then decreased by the end of the experiment (Fig. S2). Additionally, the expression of an enzyme involved in riboflavin biosynthesis (RibAB) was immensely increased. However, the fold change varied greatly between parallel experiments: it was 5.43 or 6.18 for one fermentation and 1.22 or 2.01 for the other.

Expression of some hypothetical proteins with the highest fold changes can be seen in Fig. 3b. Nevertheless, according to BLAST search, the nucleotide sequences of the genes encoding these proteins show no similarity to *ltaE* (a low-specificity TA) or *tdh* and *kbl* (involved in the conversion of L-threonine to 2-amino-3-ketobutyrate and subsequent production of glycine) in *E. coli* K-12 MG1655.

Experiment with ^{13}C,^{15}N-labelled L-threonine

In order to exactly determine L-threonine degradation products, a D-stat experiment was carried out at 0.2 h^{-1} with a feed containing 2.5 mM L-threonine. After stabilization was complete, the medium was switched to a feed that contained 2.5 mM ^{13}C,^{15}N-labelled L-threonine. Supplementary Movie S1 shows that after switching to a labelled medium *Lc. lactis* gradually incorporates labelled L-threonine into cells, converts it into glycine and secretes the latter amino acid to the environment. Note that L-threonine is the main source of extracellular glycine (Supplementary Movie S1).

Moreover, NMR analysis showed that other products biosynthesized from labelled L-threonine were acetaldehyde, acetaldehyde hydrate and ethanol (Fig. S3). The ratio of these three products (in sum) was virtually 1:1 with glycine (Fig. 4). Additionally, small labelled peaks were seen in NMR spectra, but the concentration of this unknown product was too low to adequately identify it. The peak was compared with an aminoacetone standard, but it did not match (Fig. S4). When samples were lyophilized, the unknown peak was no longer present, hinting that the compound might have been volatile or unstable.

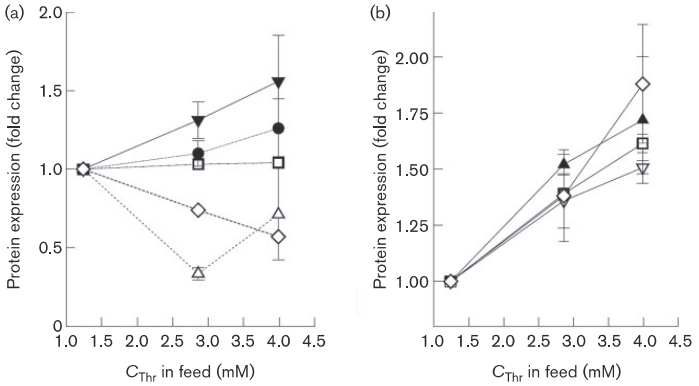

Fig. 3. Changes in protein expression during D-stat experiments at various L-threonine concentrations (C_{Thr}) in feed (mean ± SE of two fermentations). Samples were taken at C_{Thr} 1.24, 2.86 and 3.99 mM, and compared with C_{Thr} 1.24. (a) ◇, Alcohol dehydrogenase (AdhE); ●, SHMT (GlyA); △, threonine dehydratase (IlvA); □, threonine synthase (ThrC); ▼, betaine ABC transporter permease (BusAB). (b) Putative uncharacterized proteins for which mean expression changed at least 1.5-fold at different values of C_{Thr} and for which SE between the two analysed fermentations was less than 20 %. ◇, YrgH; □, YsfA; ▲, YsjD; ▽, YuaA.

DISCUSSION

Based on the results presented herein it can be concluded that if L-threonine is in excess in the growth environment it is primarily degraded to glycine and acetaldehyde, followed by the partial conversion of acetaldehyde to ethanol (Fig. 4). Lactate does not directly or indirectly originate from L-threonine; this was proved with the labelling experiment (Fig. S3) indicating that over-consumed serine was degraded via serine hydratase to pyruvate followed by lactate or acetate synthesis. The comparison of utilized L-serine (Fig. 2c) and L-serine in total cellular protein (Table 1) indicates that 60–74 % of consumed L-serine was directed to the biosynthesis of various substances. Decrease of the utilization of L-serine (Fig. 2c) might be related to a common transporter for serine and threonine. Noens & Lolkema (2014) showed that SerP1 transports L-serine, L-threonine and L-cysteine with high affinity. The decrease in L-serine consumption probably evoked the increase in L-asparagine utilization (Fig. S2) and degradation to L-aspartate followed by degradation to pyruvate. L-Asparagine degradation to L-aspartate occurs via L-asparaginase (AsnB), which was slightly upregulated (Fig. S2). In addition, the lower consumption of L-serine is apparently responsible for the elevated utilization of L-cysteine (Fig. S2), as the biosynthesis of the latter amino acid from L-serine is hampered (see expression of CysM in Fig. S2) and cells rely more on external L-cysteine pools.

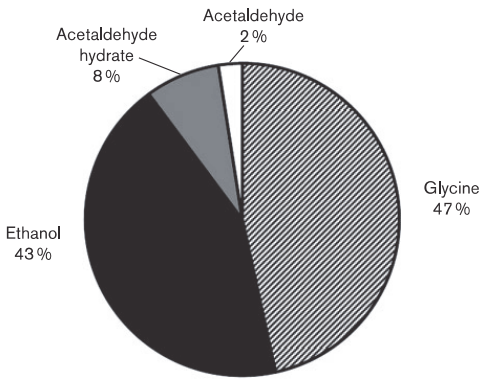

Fig. 4. Relative proportions of four products biosynthesized from $^{13}C,^{15}N$-labelled L-threonine (from NMR results). The sample was taken from the labelling experiment 10 h after the regular feed was switched to a medium containing $^{13}C,^{15}N$-labelled L-threonine. The concentration of L-threonine in the feed was 2.5 mM and 37 % of it was consumed by *Lc. lactis* IL1403.

SHMT might have TA activity, as shown by Chaves *et al.* (2002), although it is not a common feature in LAB (Ogawa *et al.*, 2000). Similarly, this activity of SHMT may be present in *Lc. lactis*, as the expression of SHMT (GlyA) was increased at higher L-threonine availability (Fig. 3a). Also, as several hypothetical proteins were upregulated at higher L-threonine concentrations (Fig. 3b), these may also be involved in the degradation pathways of L-threonine. In addition, higher L-threonine availability might increase intracellular pools of acetaldehyde, which in turn would induce the build-up of osmotic pressure [similarly to lactate in Loubiere *et al.* (1997) and Pieterse *et al.* (2005)].

This would explain the reduction in biomass yield (Fig. 2a) and the increase in BusAB expression (Fig. 3a). Also, in order to alleviate stress conditions, *Lc. lactis* increases the consumption of additional carbon and nitrogen sources (amino acids), as seen in Fig. S2. L-Arginine, for example, can be utilized to produce additional ATP for growth (Fernández & Zúñiga, 2006).

The production of glycine in unlabelled D-stat experiments increased at higher L-threonine availability, whereas the biosynthesis of ethanol was constant and acetaldehyde was present in minute amounts. This suggested that there might be an alternative glycine biosynthesis pathway present in *Lc. lactis* (the production of glycine from L-threonine rather than from 2-amino-3-oxobutanoate). However, a D-stat experiment with labelled L-threonine proved that the ratio of glycine and ethanol/acetaldehyde/acetaldehyde hydrate was almost 1 : 1, excluding this hypothesis. The conversion of acetaldehyde to ethanol by an alcohol dehydrogenase (AdhE) might be limited, which would explain why the production of ethanol was constant in unlabelled experiments. This is also supported by the fact that the expression of AdhE was reduced at higher L-threonine availability (Fig. 3a). When higher amounts of acetaldehyde are produced, the conversion rate of this compound to ethanol might stay constant and the residual acetaldehyde probably evaporates.

The utilization of L-threonine was on average twofold higher than the biosynthesis of glycine (Fig. 2c). Even if intracellular L-threonine and glycine concentrations (Table 1) are taken into account, 14 % of consumed L-threonine, on average, is degraded via unknown pathways. Thus, low amounts of other compounds were certainly produced from L-threonine as well. Regrettably, these metabolites could not be accurately identified, their concentrations being too low (Fig. S4). If L-threonine was converted to BCAAs, the bioavailability of these compounds should be increased, and thus the utilization of L-isoleucine, L-leucine and L-valine from the medium should be decreased. On the contrary, results show that the utilization of BCAAs is slightly increased at higher L-threonine availability (Fig. 2d) and the expression of the enzyme involved in the first reaction of BCAA biosynthesis (IlvA) is reduced (Fig. 3a). Hence, it is certain that BCAAs are not the compounds of interest.

Lastly, low amounts of produced glycine might have been directed to the biosynthesis of purines, which are precursors in the production of riboflavin (Burgess *et al.*, 2004). This claim is based on proteome results, which show that enzymes involved in riboflavin biosynthesis (RibAB) were upregulated at higher L-threonine availability. The positive effect of threonine supplementation and TA overexpression on riboflavin production has also been noticed in experiments with the filamentous hemiascomycete *Ashbya gossypii* (Monschau *et al.*, 1998). The facilitated production of riboflavin is also indicated by an increase in L-glutamine utilization (Fig. S2), since the latter amino acid is involved in the biosynthesis of nucleotides (Kilstrup *et al.*, 2005).

In conclusion, it was experimentally proven herein that a TA activity exists in *Lc. lactis* IL1403 and might be associated with GlyA. Incidentally, this is the principal pathway for L-threonine degradation in this particular strain. Moreover, L-threonine is preferred over L-serine as a substrate for glycine biosynthesis if the concentration of L-threonine is higher than that of L-serine, suggesting that they share a common transporter. Also, the conversion of acetaldehyde to ethanol is limited at higher L-threonine availability.

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Reduction of nutrient concentrations in a chemically defined medium increases amino acid usage efficiency in *Lactococcus lactis* IL1403

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Abstract

Lactococcus lactis exhibits potential for the production of value-added biochemicals. Optimization of media enables elucidation of interrelationships between metabolic pathways required for efficient growth of cells. This study examined the use of amino acids for biosynthetic and energetic purposes and how the flux of amino acids into biomass increases when availability of amino acids in the medium is limited.

The metabolism of *L. lactis* IL1403 was compared herein using three chemically defined media (BS7, K40, V19) and glucose-limited A-stat cultivation. In a medium with reduced amino acid content (K40) the flux of amino acids to proteins was increased in comparison to the initial medium (BS7) and to the medium with reduced metal ion content (V19). Important shift in arginine degradation was observed in K40 where arginine was mainly consumed for biosynthetic purposes instead of additional ATP generation through arginine deiminase pathway. Amino acids were grouped based on the target in metabolism: eight amino acids were consumed in higher amounts than needed for cellular protein and peptidoglycan synthesis, while four amino acids were synthesized to fulfil cellular requirements. The grouping of amino acids facilitates optimization of amino acid concentrations during the development of defined media for other lactococci.

Keywords

Lactic acid bacteria, continuous cultivation, chemically defined medium, medium optimization, amino acid metabolism.

Introduction

Lactic acid bacteria (LAB) are widely used in the food industry for the production of dairy, plant and meat fermented foods, due to their ability to contribute to the taste and texture and

to prevent food spoilage (Price *et al.*, 2012). LAB are particularly useful due to their ability to catabolize a wide variety of sugars (de Vos 1996) and for enabling *in situ* production of beneficial compounds in food (LeBlanc *et al.*, 2011, Li & Cao 2010, Ortiz *et al.*, 2013). Metabolically engineered LAB are efficient cell factories of functional biochemicals, e.g. the species *Lactococcus lactis* has emerged as a suitable host for the production of recombinant proteins (Morello *et al.*, 2008), ethanol (Solem *et al.*, 2013), alanine (Hols *et al.*, 1999), riboflavin (Sybesma *et al.*, 2004), 2,3-butanediol (Gaspar *et al.*, 2011) and other compounds.

When studying the metabolic pathways of compounds of interest or manufacturing microbial metabolites, the selection of a fermentation medium is critical. Rich but undefined media are typically inexpensive and provide high biomass yields, but the lots for inexpensive carbon and nitrogen sources may vary and thus influence fermentation performance. Utilization of a chemically defined medium (CDM) reduces inconstancy of the process, provides reproducible results and more explainable data, but can decrease biomass yield and increase the price of the fermentation. (Zhang & Greasham 1999; Zhang *et al.*, 2009). Additionally, CDMs simplify downstream processing and product purification of biosynthesized metabolites (Petry *et al.*, 2000; Torino *et al.*, 2005; Khan *et al.*, 2013) and enable incorporation of amino acid analogues or non-proteinogenic amino acids into proteins (El Khattabi *et al.*, 2008; Berntsson *et al.*, 2009).

CDMs for several LAB are available in literature and can be further optimized to meet the requirements of specific strains (Berry *et al.*, 1999, Chervaux *et al.*, 2000; Letort & Juillard 2001; Hébert *et al.*, 2004; Wegkamp *et al.*, 2010; Kim *et al.*, 2012). The species *L. lactis* has previously been determined to be auxotrophic for eight amino acids (Aller *et al.*, 2014, Zhang *et al.*, 2009). However, maximal specific growth rate in CDMs that contain only essential amino acids is low (Jensen & Hammer 1993; Cocaign-Bousquet *et al.*, 1995) and minimal media are thus unsuitable for studying the metabolism of LAB at higher μ values. Therefore, a different strategy should be used for developing defined media for LAB, i.e. selecting concentrations for amino acids in the medium based on whether these compounds are solely used for biomass protein and peptidoglycan synthesis or other targets as well.

In the present study the physiology of *L. lactis* IL1403 is compared in three CDMs (BS7, K40, V19) from a previous work (Aller *et al.*, 2014) at different specific growth rates using accelerostat (A-stat) continuous cultivations (Paalme *et al.*, 1995). In K40 and V19 media the concentrations of amino acids or mineral salts, respectively, have been reduced 2 to 8 times (compared with BS7) in order to create a more balanced medium, i.e. a medium with lower residual nutrient concentrations after the growth of cells. The main objective of this work is to evaluate the metabolic changes, especially in amino acid metabolism, that were induced by limited nutrient availability. We provide information that is useful for the development of synthetic media for other LAB that have similar amino acid auxotrophies with the IL1403 strain, i.e. which amino acids are mainly used for the formation of biomass proteins and which amino acids for other purposes. In addition, the obtained data may be implemented for increasing amino acid fluxes for the biosynthesis of recombinant proteins or amino acid degradation products by changing the composition of the medium.

Materials and methods

Microorganism

Lactococcus lactis subsp. *lactis* IL1403 was provided by INRA (Jouy-en-Josas, France). Inoculum was prepared from lyophilized stock culture stored at -80°C which was pre-grown twice in test tubes at 34°C on BS7 medium (Table 1).

Media

Three chemically defined media were used for cultivations: BS7, K40 and V19 (Table 1). These media were developed in a previous research (Aller *et al.*, 2014), where BS7 was the initial medium, while K40 and V19 were media with minimized amino acid or microelement content, respectively, that provided the best trade-off between optical density, specific growth rate and nutrient reduction rate.

Compounds were obtained from Serva Electrophoresis GmbH (Heidelberg, Germany) and Sigma-Aldrich (St. Louis, Missouri, USA) and were mixed together in the following order (to avoid precipitation): sugar, buffers, amino acids, mineral salts, vitamins, additional components. Media were sterilized by filtration, using stainless steel filter holders and nitrocellulose membrane filters (0.22 μm) (Merck Millipore, Darmstadt, Germany).

Cultivation conditions

The accelerostat (A-stat) continuous cultivations in glucose limitation were carried out in two parallels for each medium using 1.25 L Biobundle bioreactors with ADI1030 biocontrollers and „BioXpert 3“ control program, all provided by Applikon Biotechnology B.V. (Delft, The Netherlands). A detailed description of the fermentation equipment has been given in Adamberg *et al.*, 2009 and Lahtvee *et al.*, 2009. The following conditions were used: T=34°C, agitation speed 300 rpm and V=300 mL. pH was kept at 6.4 by addition of 2M NaOH and the gas enviroment was kept anaerobic by flushing the bioreactors with N_2. A constant dilution rate D=0.1 h^{-1} was applied after the culture in batch cultivation reached stationary phase. The culture was stabilized by pumping 5 cultivation volumes through the bioreactor and acceleration of dilution rate (a=0.006 h^{-2}) was initiated.

Samples for optical density and analytical measurements were collected after every 2 or 3 hours. **Optical densities** were measured with WPA CO 8000 Biowave Cell Density Meter (Biochrom GmbH; Berlin, Germany) at 600 nm. **The dry weight of biomass** was determined gravimetrically in three timepoints of each fermentation by washing freshly collected biomass in three parallels with destilled water (Rotanta 460R centrifuge, Hettich, Tuttlingen, Germany; 11500 rpm, 5 min, 4°C, three times). The remaining pellets were transferred onto aluminum foils, heated at 105°C for 24 h and weighed thereafter. The k value was 0.30±0.03 for BS7, 0.31±0.02 for K40 and 0.30±0.02 for V19.

Analytical methods

Cells in the samples were removed by centrifugation (14800 rpm, 5 min, 4°C; MicroCL 21R centrifuge; Thermo Fisher Scientific Inc.; Waltham, MA, USA) and the supernatant was used for the following analyses: (i) **Glucose and main fermentation end-products** (lactate, formate, acetate) were measured with liquid chromatography (Alliance 2795 system), refractive index detector (model 2414) and Empower software, all provided by Waters Corp. (Milford, MA, USA). The column used was BioRad HPX-87H (Hercules, CA, USA) at 35°C with isocratic elution of 5 mM H_2SO_4 at a flow rate 0.6 mL·min^{-1}. (ii) **Free amino acids** were detected with an amino acid analyser Acquity UPLC (Waters Corp.; Milford, MA, USA) according to the manufacturer's instructions. The samples were derivatized beforehand for 10 min at 55°C, using AccQ-Fluor reagent (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate; Waters Corp.). Data was processed with Empower software (Waters Corp.). (iii) **The concentration of Mg^{2+} ions** was detected as described in Supplementary material.

ATP spilling

ATP spilling was calculated with an in-house stoichiometric metabolic model as described in Supplementary material.

Results and discussion

Energetic efficiency was similar in all media

Glucose-limited A-stat fermentations were carried out on three CDMs in two parallels: (i) BS7 (the initial CDM); (ii) K40, where the concentrations of amino acids (except for asparagine, aspartate, glutamine, glutamate) were lowered 2-8 times compared with BS7 and (iii) V19, where the concentrations of mineral salts were lowered 2-4 times compared with BS7. Maximal specific growth rates (μ_{max}) were 0.57±0.04, 0.42±0.07 and 0.49±0.07 h^{-1} in BS7, K40 and V19, respectively (Table 1), and specific biomass productivity can be seen on Fig. 1a. Specific lactate productivity and the channeling of glucose to lactate can be seen on Fig. 1b and Fig. 1c, respectively. In V19 medium the fermentation was more homolactic than in BS7 or K40, as the production of byproducts (namely formate) was decreased (Fig. 1d). Specific acetate productivity can be seen in Fig. S1. The production of ethanol was not observed.

Flux calculations demonstrated that the usage of energy-wasting pathways decreased with the rise of specific growth rate, as can be seen by the drop of ATP spilling on Fig. 1e. ATP spilling is defined in this research as non-growth related ATP production, i.e. the difference between total ATP produced and total ATP spent for the synthesis of biomonomers. The production of unidentified compounds from glucose, i.e. products other than lactate, formate, acetate and ethanol decreased with the rise of μ (Fig. 1f).

Increased efficiency of amino acid usage in K40 medium

The specific consumption rate of all amino acids was either constant or increased with the rise of specific growth rate value. The consumption of arginine, cysteine, serine and threonine and the production of ornithine were lower in K40 than in other media (Fig. 2a, 2b, 2e, 2g, 2h). Amino acid utilization and production profiles were generally similar in BS7 and V19, except for glutamine and glutamate, which were consumed or produced less in case of V19 (Fig. 2c and 2d). Proline was consumed or produced in minute amounts in BS7/V19 or K40, respectively (Fig. 2f). The metabolism of other amino acids is presented in Fig. S2.

Fig. 3a and Fig. 3b show that several amino acids, namely arginine, asparagine/aspartate, glutamine, serine and threonine, are largely overconsumed, i.e. utilized for the production of unidentified byproducts in addition to biomass protein and peptidoglycan biosynthesis, especially at $\mu = 0.1\ h^{-1}$. Amino acid flux to proteins increased with specific growth rate in all three media. Isoleucine, leucine and methionine were slightly overconsumed, whereas the consumption of alanine, histidine, lysine, phenylalanine and valine was close to the requirements for protein formation, especially in the case of K40. More than 50% of glycine and more than 20% of tyrosine in cellular proteins originates from biosynthesis (Fig. 3c and 3d), whereas the remaining portions were obtained by consuming these amino acids from the medium. As K40 contained more serine than threonine (Table 1), serine was probably the main source for glycine biosynthesis due to competitive inhibition for the SerP1 transporter (Noens & Lolkema 2014).

As the flux of consumed amino acids to biomass proteins or byproducts varied between media, changing the composition of a CDM is one possible way of increasing or decreasing the biosynthesis of valuable or undesired substances originating from amino acid catabolism, e.g. aroma compounds, chemicals with antifungal activity, γ-aminobutyric acid, biogenic amines etc.

The flux through arginine deiminase pathway is diminshed in K40 medium

In the medium with lowered concentrations of amino acids (K40) cells avoid generation of additional ATP by reducing fluxes through the arginine-deiminase (ADI) pathway (Fig. 2a and 2e), which produces two moles of ammonia and one mole of ATP, ornithine and carbamoylphosphate per one mole of arginine. In BS7 and V19 media, 66 and 54% or 77 and 59% of arginine is converted to ornithine at $\mu = 0.1$ or 0.4 h^{-1}. In K40, however, 41% of arginine is converted to ornithine at $\mu = 0.1$ h^{-1} and only 2% at $\mu = 0.4$ h^{-1} (Fig. 3a and 3b). In BS7 and V19, 15-18% of consumed arginine is used for protein production, whereas in K40 the number is 59% at $\mu = 0.4$ h^{-1} (Fig. 3b). If arginine degradation through ADI pathway is decreased at higher μ values, other pathways should be present for obtaining carbamoylphosphate for pyrimidine biosynthesis, e.g. agmatine deiminase (AGDI) pathway. However, no putrescine production was observed (data not shown), although the genes of the AGDI cluster exist in *L. lactis* IL1403 (Ladero *et al.*, 2011). Hence, cells probably biosynthesized carbamoylphosphate from CO_2, which was obtained from glycolysis or degradation reactions of other amino acids.

Formation of glutamate from glutamine is decreased in V19 medium

The utilization of glutamine and concomitant glutamate production were decreased in a medium with lower Na^+ concentration (V19) (Fig. 2c and 2d). In BS7, K40 and V19, respectively, 46, 36 and 49% of glutamine was converted to glutamate at $\mu = 0.1$ h^{-1} (Fig. 3a) and 24, 20 or 13% at $\mu = 0.4$ h^{-1} (Fig. 3b). Presumably, this was evoked for three reasons: (i) the glutamine/glutamic acid transporter complex GlnPQ functions at the expense of ATP and thus glutamine overconsumption is avoided to maintain energy; (ii) uptake of amino acids by GlnPQ is more effective in the presence of higher osmolyte concentrations (Schuurman-Wolters & Poolman 2005); (iii) the need for acid stress allevation by ammonia production through the conversion of glutamine to glutamate is diminished, when less formate is produced (see V19 medium in Fig. 1d). According to Loubiere *et al.*, 1997, formate is more inhibitory to the cells than lactate, acetate and ethanol. In addition, proline in K40 medium was probably produced from glutamate (Fig. 2f), thereby regenerating $NAD(P)^+$.

Grouping of amino acids based on cellular requirements

Based on previous chapters the amino acids can be divided into five groups: (i) amino acids, that are largely overconsumed (arginine, asparagine/aspartate, glutamine, serine, threonine); (ii) amino acids, that are slightly overconsumed (isoleucine, leucine, methionine); (iii) amino acids, that are consumed in amounts close to the requirements for protein formation (alanine, histidine, lysine, phenylalanine, valine); (iv) amino acids, that are partially biosynthesized from other amino acids or compounds (glycine, tyrosine); (v) amino acids, that are completely synthesized from other amino acids (glutamate, proline). Therefore, when developing a new synthetic medium for specific strains of lactococci, amino acid concentrations for the group (iii) should correspond to the amounts needed for biomass proteins and peptidoglycan, whereas amounts of amino acids in the groups (i) and (ii) should correspond to the

requirements of other biosynthetic pathways (nucleotide synthesis), additional energy costs, regeneration of redox compounds or ammonia for balancing acid production. Amino acids from the groups (iv) and (v) may be omitted from the medium. Note that the requirements for some amino acids may increase at higher μ and amino acid limitation may lead to an earlier growth halt as seen by μ_{max} comparison of K40 and BS7 (Table 1).

Decreased residual nutrient concentrations and improved measurement accuracy

In comparison with BS7 medium, K40 contains lower amounts of amino acids. Thus, their percentual consumption (percentage consumed from the initial amount in the medium) has increased, resulting in lower residual concentrations of amino acids and a more balanced medium: more than 50% of 14 amino acids is consumed at $\mu = 0.1$ h^{-1} and over 80% of eight amino acids at $\mu = 0.4$ h^{-1} (Fig. 4). In comparison, in the experiments performed with *Lb. plantarum* by Teusink *et al.*, 2006 less than 17% of 16 amino acids were consumed from a defined medium (Table S1). Also, the accuracy for measuring metal ion utilization is increased in V19 compared with BS7 (Fig. S3).

Furthermore, K40 is 31% and V19 is 8% cheaper than BS7 (Table 1). The cost for the CDM of Dressaire *et al.*, 2008 is 7.48 $EUR \cdot L^{-1}$ and it is 3 times more expensive than K40. The cost for the minimal medium MS15 from Cocaign-Bousquet *et al.*, 1995 is 3.16 $EUR \cdot L^{-1}$, which is comparable to the price of BS7 (Table 1). Low-molecular media derived from yeast extract (YE) by membrane filtration (e.g. Zacharof & Lovitt 2013) are cheaper, but data interpretation is more aggravated in a filtered YE medium, as it contains hundreds of different peptides, nucleosides and other compounds. In addition, K40 offers higher maximal specific growth rate (0.42 *versus* 0.37 h^{-1}) than the minimal medium M15 (Cocaign-Bousquet *et al.*, 1995).

Conclusion

CDMs can be used for the production of microbial metabolites and are indispensable in metabolic research. In order to improve fermentation performance and understand the growth and metabolism of cells, the growth medium has to be tuned to match the cell requirements and the objective of the process. The authors propose K40 as a new efficient CDM for the metabolic research of lactococci in glucose-limited continuous cultivations. A strategy to design media for studying the metabolism of lactococci was presented by grouping amino acids according to cellular requirements for these compounds. Understanding of interrelationships between consumption, synthesis or degradation of amino acids and central pathways to balance ATP production or regeneration of NAD^+ is useful for improving the bioproduction of beneficial compounds.

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Tables

Table 1. Composition of chemically defined media used in this study (grams per liter). The cost of the media is calculated by using the prices of medium compounds in Sigma-Aldrich at 8th of July 2015.

	BS7	**K40**	**V19**
DESCRIPTION	Initial CDM	Concentrations of amino acid have been lowered 2-8 times (excl. Asn, Asp, Gln, Glu), compared with BS7	Concentrations of mineral salts have been lowered 2-4 times, compared with BS7
Maximal specfic growth rate (μ_{max}; h^{-1}), n=2	0.57±0.04	0.42±0.07	0.49±0.07
Amino acid/Glucose ratio (c-mol·c-mol^{-1})	0.44	0.20	0.44
Price (EUR·L^{-1})	3.21	2.22	2.97
COMPOSITION (g·L^{-1})			
SUGAR			
Glucose	4.5	4.5	4.5
BUFFERS			
K_2HPO_4	3.0	3.0	3.0
KH_2PO_4	2.5	2.5	2.5
AMINO ACIDS			
L-Alanine (Ala)	0.0782	0.0391	0.0782
L-Arginine (Arg)	0.1852	0.0926	0.1852
L-Asparagine (Asn)	0.0735	0.0735	0.0735
L-Aspartate (Asp)	0.0723	0.0723	0.0723
L-Cysteine (Cys)	0.0636	0.0079	0.0636
L-Glutamate (Glu)	0.0703	0.0703	0.0703
L-Glutamine (Gln)	0.1322	0.1322	0.1322
Glycine (Gly)	0.0578	0.0072	0.0578
L-Histidine (His)	0.0597	0.0075	0.0597
L-Isoleucine (Ile)	0.1018	0.0255	0.1018
L-Leucine (Leu)	0.2072	0.0518	0.2072
L-Lysine-HCl (Lys)	0.1576	0.0394	0.1576
L-Methionine (Met)	0.0407	0.0203	0.0407
L-Phenylalanine (Phe)	0.0860	0.0430	0.0860
L-Proline (Pro)	0.0917	0.0115	0.0917
L-Serine (Ser)	0.1634	0.0817	0.1634
L-Threonine (Thr)	0.0758	0.0379	0.0758
L-Tryptophan (Trp)	0.0164	0.0082	0.0164
L-Tyrosine (Tyr)	0.0295	0.0147	0.0295
L-Valine (Val)	0.1072	0.0268	0.1072
MINERAL SALTS			
$CaCl_2$	0.0500	0.0500	0.0125
$CoSO_4 \cdot 7H_2O$	0.0030	0.0030	0.0008
$CuSO_4 \cdot 5H_2O$	0.0030	0.0030	0.0015

$FeSO_4 \cdot 7H_2O$	0.0014	0.0014	0.0007
$MgSO_4 \cdot 7H_2O$	0.2000	0.2000	0.1000
$MnSO_4 \cdot H_2O$	0.0160	0.0160	0.0080
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.0030	0.0030	0.0008
NaCl	2.9000	2.9000	0.7250
$ZnSO_4 \cdot 7H_2O$	0.0050	0.0050	0.0013
VITAMINS			
Thiamine-HCl (B_1)	0.00051	0.00051	0.00051
Riboflavin (B_2)	0.00033	0.00033	0.00033
Nicotinamide (B_3)	0.00033	0.00033	0.00033
Ca-pantothenate (B_5)	0.00065	0.00065	0.00065
Pyridoxine (B_6)	0.00064	0.00064	0.00064
Biotin (B_7)	0.00031	0.00031	0.00031
Folic acid (B_9)	0.00121	0.00121	0.00121
ADDITIONAL COMPOUNDS			
Adenine	0.0250	0.0250	0.0250
Hypoxanthine	0.0250	0.0250	0.0250
Lipoic acid	0.00018	0.00018	0.00018

Figures

Fig. 1. Biomass yield and pyruvate metabolism. Average results of 2 fermentations per each medium: BS7 – black circles or black line; K40 – white triangles or dotted line; V19 – grey squares or grey line. **a.** Specific biomass productivity (gram of dry weight per gram of consumed glucose per hour). **b.** Specific lactate productivity (millimoles per gram cell dry weight per hour). **c.** Conversion of glucose to lactate (percentage of the total amount of consumed glucose). **d.** Specific formate productivity (millimoles per gram cell dry weight per hour). **e.** ATP spilling (millimoles per gram cell dry weight). **f.** Production of unidentified compounds (metabolites other than lactate, formate, acetate, ethanol) from glucose (millimoles per gram cell dry weight).

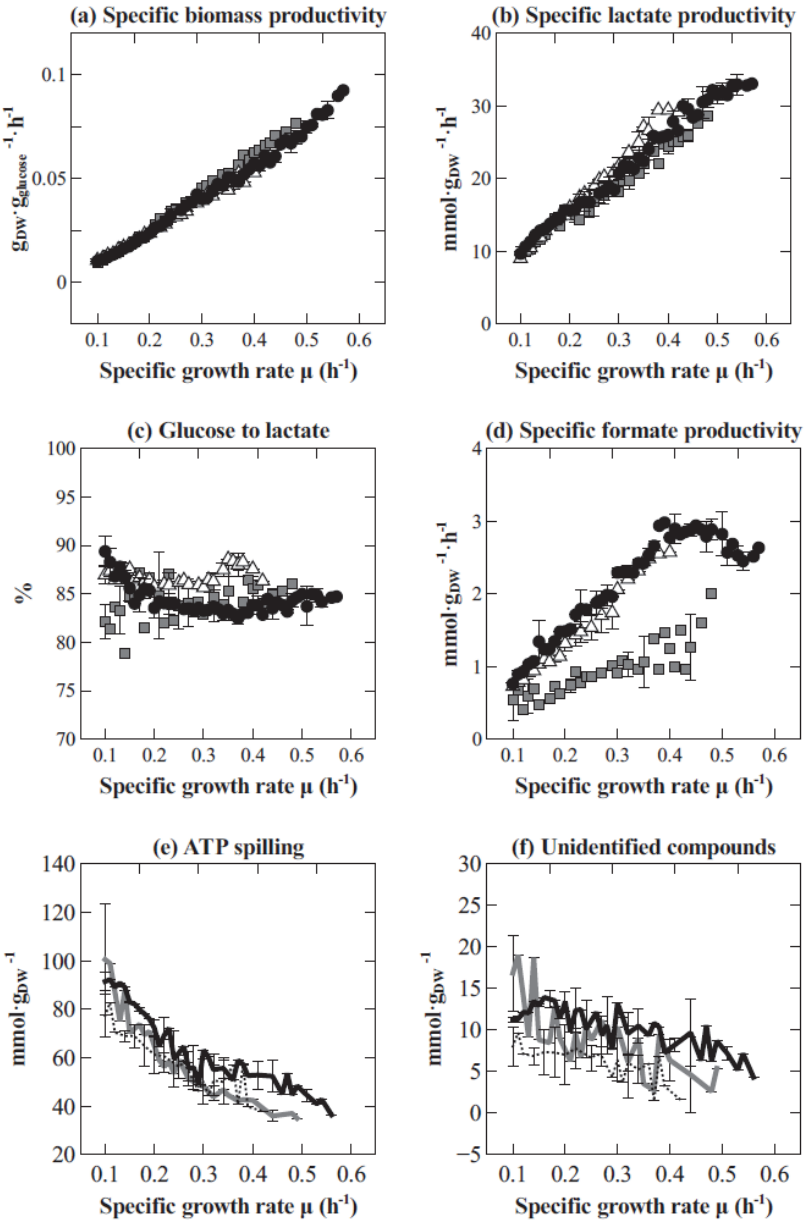


Fig. 2. Specific amino acid utilization rate. Average results of 2 fermentations per each medium (millimoles per gram cell dry weight per hour): BS7 – black circles; K40 – white triangles; V19 – grey squares. Negative values represent the production of amino acids. **a.** Arginine. **b.** Cysteine. **c.** Glutamine. **d.** Glutamate. **e.** Ornithine. **f.** Proline. **g.** Serine. **h.** Threonine.

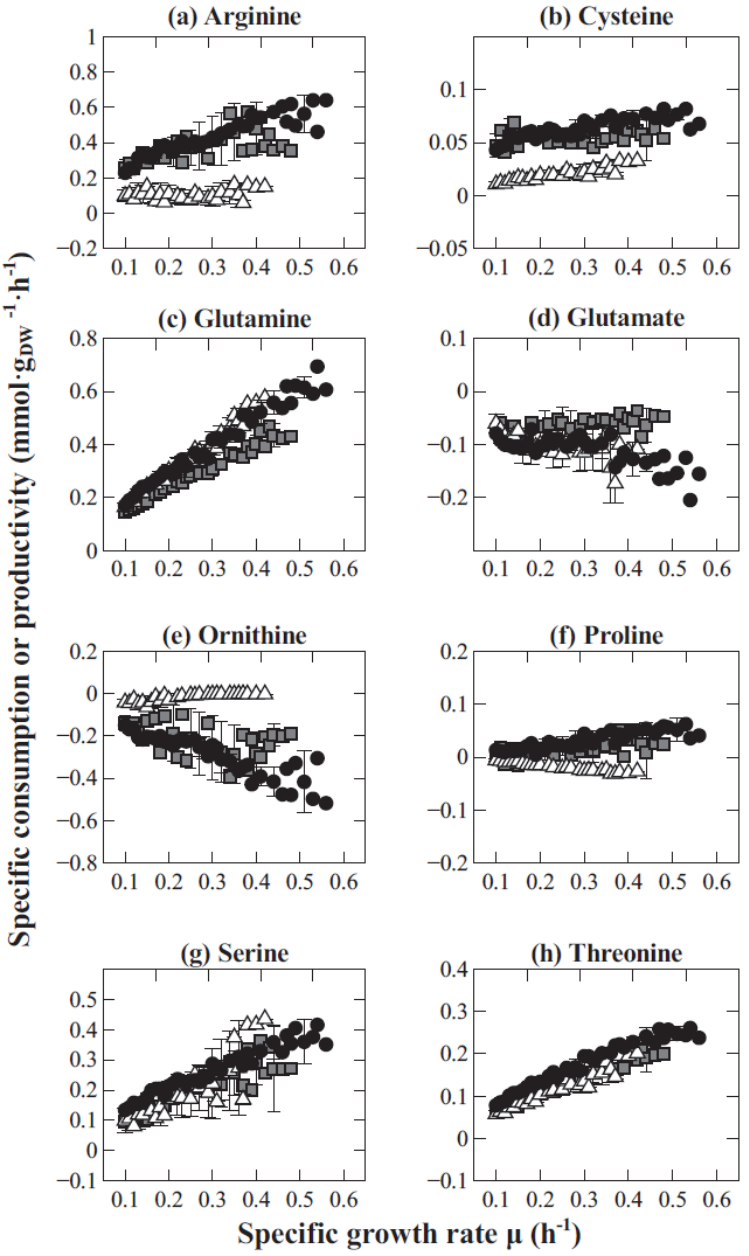


Fig. 3. Amino acid degradation products. Calculations are based on comparing amino acid utilization data of BS7, K40 and V19 with amino acid concentrations in cellular proteins and peptidoglycan*. **a. b.** The percentage of utilized amino acid used for the formation of cellular proteins (black bars); other compounds – arginine for ornithine and glutamine for glutamate (criss-crossed bars); and unidentified compounds (diagonally striped bars) at μ = 0.1 and 0.4 h^{-1}. **c. d.** The percentage of glycine or tyrosine in cellular proteins originating from glycine or tyrosine utilization (grey bars) and biosynthesis from other compounds (striped bars).

* The concentrations of amino acids in cellular proteins were taken from S1 in [51]. The analysis of cellular cysteine and tryptophan is unreliable with the method used therein as these amino acids are degraded during hydrolysis.

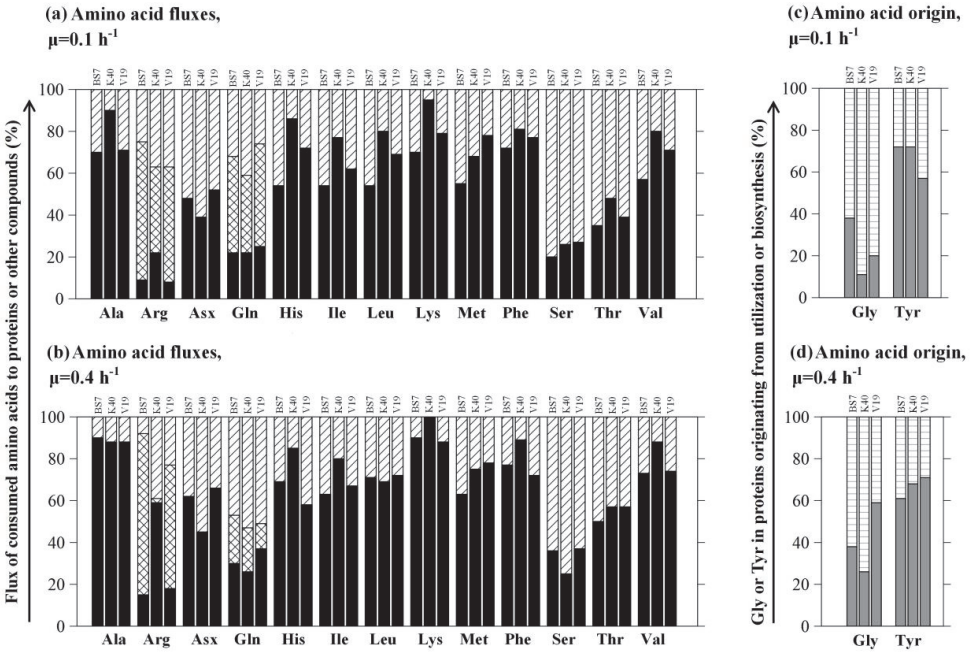
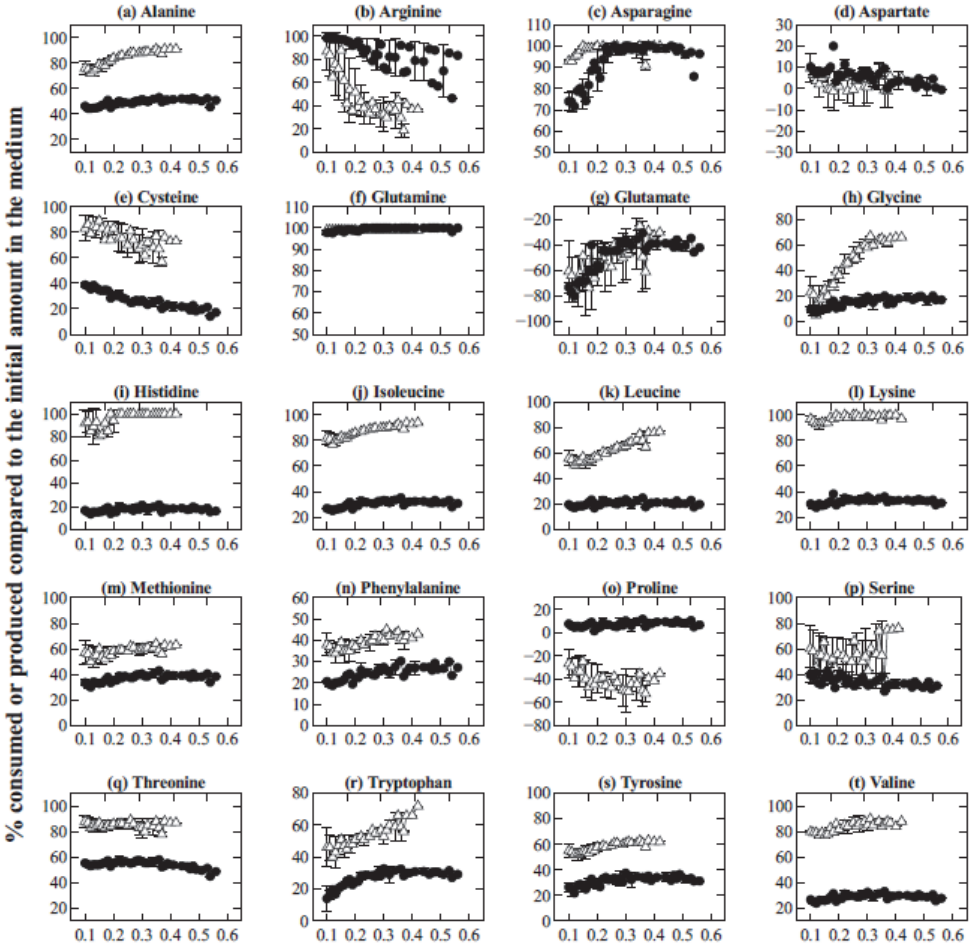


Fig. 4. Percentual amino acid consumption. Percentage of amino acid consumed or produced, compared to the initial amount in the medium. Average results of 2 fermentations per each medium: BS7 – black circles; K40 – white triangles. Negative values represent production.


(a) Alanine
(b) Arginine
(c) Asparagine
(d) Aspartate
(e) Cysteine
(f) Glutamine
(g) Glutamate
(h) Glycine
(i) Histidine
(j) Isoleucine
(k) Leucine
(l) Lysine
(m) Methionine
(n) Phenylalanine
(o) Proline
(p) Serine
(q) Threonine
(r) Tryptophan
(s) Tyrosine
(t) Valine
% consumed or produced compared to the initial amount in the medium
Specific growth rate μ (h-1)

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IV Lahtvee PJ, Adamberg K, Arike L, Nahku R, **Aller K**, Vilu R (2011) Multi-omics approach to study the growth efficiency and amino acid metabolism in *Lactococcus lactis* at various specific growth rates. Microb Cell Fact 10(12).

MICROBIAL CELL FACTORIES

RESEARCH Open Access

Multi-omics approach to study the growth efficiency and amino acid metabolism in *Lactococcus lactis* at various specific growth rates

Petri-Jaan Lahtvee[1,2], Kaarel Adamberg[2,3], Liisa Arike[2,3], Ranno Nahku[1,2], Kadri Aller[1,2], Raivo Vilu[1,2*]

Abstract

Background: *Lactococcus lactis* is recognised as a safe (GRAS) microorganism and has hence gained interest in numerous biotechnological approaches. As it is fastidious for several amino acids, optimization of processes which involve this organism requires a thorough understanding of its metabolic regulations during multisubstrate growth.

Results: Using glucose limited continuous cultivations, specific growth rate dependent metabolism of *L. lactis* including utilization of amino acids was studied based on extracellular metabolome, global transcriptome and proteome analysis. A new growth medium was designed with reduced amino acid concentrations to increase precision of measurements of consumption of amino acids. Consumption patterns were calculated for all 20 amino acids and measured carbon balance showed good fit of the data at all growth rates studied. It was observed that metabolism of *L. lactis* became more efficient with rising specific growth rate in the range 0.10 - 0.60 h^{-1}, indicated by 30% increase in biomass yield based on glucose consumption, 50% increase in efficiency of nitrogen use for biomass synthesis, and 40% reduction in energy spilling. The latter was realized by decrease in the overall product formation and higher efficiency of incorporation of amino acids into biomass. *L. lactis* global transcriptome and proteome profiles showed good correlation supporting the general idea of transcription level control of bacterial metabolism, but the data indicated that substrate transport systems together with lower part of glycolysis in *L. lactis* were presumably under allosteric control.

Conclusions: The current study demonstrates advantages of the usage of strictly controlled continuous cultivation methods combined with multi-omics approach for quantitative understanding of amino acid and energy metabolism of *L. lactis* which is a valuable new knowledge for development of balanced growth media, gene manipulations for desired product formation etc. Moreover, collected dataset is an excellent input for developing metabolic models.

Background

Lactococcus (*L.*) *lactis* is the most intensively studied lactic acid bacterium and it has a great industrial importance. In addition to its wide usage in the dairy industry, *L. lactis* subsp. *lactis* IL1403 was the first lactic acid bacterium whose genome was sequenced [1], and it is extensively used for production of different metabolic products and recombinant proteins [reviews in [2-4]]. As this bacterium is generally recognised as safe (GRAS), there has been increasing interest in its use as a live vector for mucosal delivery of therapeutic proteins, including nasal and gastrointestinal vaccines [5,6]. However, there exists a remarkable lack of knowledge about the peculiarities of *L. lactis* metabolic regulation, especially regarding amino acid metabolism. There are several defined media designed for *L. lactis* [7-9], however, these are unbalanced and concentrations of individual amino acids are quite high, making their consumption measurements inaccurate as utilization by the cells is small compared to the total content. Lack of reliable information on consumption patterns and regulation of amino acid metabolism hinders design of cheaper balanced complex media and optimization of bioprocesses.

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Systems biology approaches where 'omics' methods are combined with advanced cultivation methods, computational and mathematical models form a solid platform for elucidating quantitative peculiarities of metabolism and its regulation in microorganisms. Transcriptome and proteome expression in *L. lactis* have been measured and compared several times in various phases of batch cultivations [10,11]. A multi-omics study where *L. lactis* was cultivated at steady state conditions was carried out by Dressaire et al. [12,13]. They characterized *L. lactis* at the transcriptome level in isoleucine limited chemostat cultures, calculated translation efficiencies based on proteome and transcriptome levels, and showed that energy costs associated with protein turnover in cells are bigger at low growth rates in comparison with higher ones.

To provide more comprehensive knowledge about amino acid metabolism in *L. lactis* we developed a new medium, which allowed studying quantitative patterns of amino acid consumption. To further link amino acid metabolism with the overall physiological state of cells, growth rate dependent trancriptomes, proteomes and extracellular metabolomes were measured and studied together with carbon, nitrogen and ATP, redox balance analyses. *L. lactis* was cultivated in accelerostat (A-stat) continuous cultures as this method allows acquisition of vast amount of data on quasi steady state growing cells and precise determination of growth characteristics, especially investigation of dependences of growth characteristics on residual concentrations of growth limiting substrate (e.g. glucose) which determines the specific growth rate of cells (μ).

Results

L. lactis growth characteristics

L. lactis was cultivated in A-stat culture where after stabilisation in chemostat at dilution rate 0.10 h^{-1}, specific growth rate (μ) was smoothly increased until the maximal μ (μ_{max}) was reached at 0.59 ± 0.02 h^{-1} (average value of five independent experiments ± standard deviation; Figure 1). To obtain higher precision in the determination of amino acid consumption patterns, concentrations of most amino acids in the growth medium were reduced *ca* 3 times compared to the chemically defined medium (CDM) [14], exceptions being arginine and glutamine, whose concentrations were increased in the medium to avoid amino group shortage during the growth (see Methods). The residual glucose concentration remained below detection limit (<0.1 mM) between μ 0.10 h^{-1} and 0.59 ± 0.02 h^{-1} in all five independent experiments. It is important to note that constant protein content (45 ± 2% of cell dry weight) and constant amino acid composition of the protein fraction was observed in the full range of μ from 0.10 to 0.55 h^{-1}

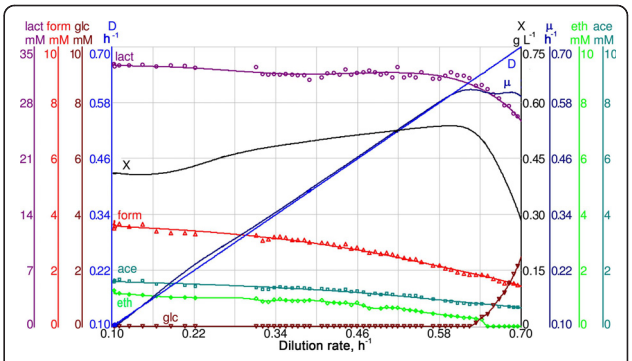

Figure 1 Typical A-stat cultivation, where dilution rate dependent metabolism of *L. lactis* is illustrated. D - dilution rate (h^{-1}); X - biomass concentration (g (dry cellular weight) L^{-1}); μ - specific growth rate (h^{-1}); lact, form, glc, eth, ace - lactate, formate, glucose, ethanol, acetate concentration in bioreactor, respectively (mM). D, μ and X are monitored constantly; metabolite concentrations are measured with a frequency of approximately 0.01 h^{-1}.

(Additional file 1, Table S1). RNA content increased from 6.5 ± 1.0% to 9.5 ± 1.5% in cell dry weight in between the latter μ values. The biomass yield per consumed carbon (Y_{XC}) increased from 0.13 ± 0.00 to 0.17 ± 0.01 C-$mol_{biomass}$ C-mol_{carbon}^{-1} when μ was raised from 0.20 ± 0.02 h^{-1} to 0.52 ± 0.04 h^{-1} (Additional file 2, Table S1). It was realized by decrease of by-product formation per biomass from 89.6 to 62.3 mmol gdw^{-1} (sum of Y_{lact}, Y_{ace} and Y_{eth}, Additional file 2, Table S1). Corresponding yield of these by-products (lactate, acetate, ethanol) per consumed glucose decreased from 2.05 to 1.88 $mol_{products}$ mol_{glc}^{-1}, with lactate yield per consumed glucose Y_{lg} = 1.83 ± 0.03 mol_{lact} mol_{glc}^{-1} remaining constant. As by-product formation exceeded maximal possible yield (2 mol mol^{-1}) per consumed glucose at growth rates below 0.4 h^{-1} (Additional file 1, Table S2) it indicated that part of the amino acids should have been catabolised to pyruvate and eventually to by-products. The overall consumption of amino acids decreased from 12.5 ± 0.5 mmol gdw^{-1} to 9.3 ± 0.3 mmol gdw^{-1} with increasing μ (Additional file 2, Figure S1), exceeding two to three times that required for synthesis of proteins in biomass (4.2 ± 0.1 mmol gdw^{-1}, Additional file 1, Table S1), and constituting always 21 ± 1% (52 to 39 C-mmol gdw^{-1}) of all the total carbon utilised by cells throughout the μ range studied.

For proof of principle, a chemostat experiment was carried out at a dilution rate of 0.45 h^{-1} and the data obtained were compared with the data obtained at the same μ value in A-stat experiments. The measured substrate and product yields in chemostat culture had values in the range of presented standard deviations for A-stat data (Additional file 2, Table S2) which shows

that quasi steady state data from A-stat is comparable to chemostat.

Amino acid consumption profiles

Based on amino acid concentrations in the cultivation broth, consumption patterns ($mmol_{AA}$ gdw^{-1}) for all the 20 amino acids were calculated (Figure 2 and Additional file 2, Figure S2). The most abundantly consumed amino acid throughout the μ range studied was glutamine (Additional file 2, Figure S2). Asparagine, arginine, serine, threonine, alanine, leucine, isoleucine and cysteine were the next most intensively consumed amino acids which consumption exceeded notably the amounts necessary for biomass formation. Lysine, phenylalanine and valine were consumed in slightly higher amounts than needed for biomass production. Consumption of aspartate, histidine, and proline were in the range of measurement errors, hence, their consumption can be considered minimal or nonexistent. It has been shown that the latter amino acids are non-essential for the growth of *L. lactis* [8].

In more detail, specific growth rate dependent consumptions of asparagine, threonine and cysteine per biomass were constant in the μ range of 0.10 - 0.20 h^{-1}, but decreased 30 to 40% from μ = 0.20 h^{-1} until μ_{max} value (Figure 2 and Additional file 2, Figure S2). Consumption of arginine decreased rapidly in the μ range of 0.10 - 0.35 h^{-1} from 2.15 ± 0.04 mmol gdw^{-1} and levelled at 0.44 ± 0.07 mmol gdw^{-1} at higher growth rates (Figure 2) - at an amount greater than necessary for biomass production (0.20 ± 0.02 mmol gdw^{-1}). Decreasing trends in the μ range 0.10 - 0.35 h^{-1} were observed for the production of ornithine and for the production of the only amino acid produced - glutamate. Glycine was the only amino acid which consumption increased during increasing μ (Figure 2), however, its consumption was always lower than its need for biomass formation. Consumption of other amino acids (Gln, Ile, His, Leu, Lys, Met, Phe, Tyr, Trp, Val) did not change significantly throughout the studied μ range, indicating also a more efficient use of amino acids at higher μ values as growth yields based on carbon and nitrogen consumption increased.

Carbon, nitrogen and ATP balances

Carbon recovery which was calculated based on glucose and amino acid consumptions, product and biomass formation was 100 ± 2% over the entire μ range (Additional file 2, Figure S3). However, nitrogen recovery, calculated based on amino acid utilization and ornithine, glutamate and biomass formation, was 55 ± 3% (Additional file 2, Figure S3). Amino acids were the main nitrogen source in the medium, comprising more than 99% of the consumed nitrogen by the cultivated bacterium. Based on amino acid utilization, the total consumption of nitrogen decreased from 22 to 14 mmol gdw^{-1} between the μ range 0.10 - 0.59 ± 0.02 h^{-1}. On the basis of monomer composition, N-molar content in the biomass was found to be constant at 7.2 mmol gdw^{-1} during the studied μ range. Concomitantly, nitrogen incorporation into the biomass increased from 33 to 50% from total consumed nitrogen in amino acids with increasing μ. The rest of nitrogen (50-67%) could have been metabolised through arginine deiminase (ADI) pathway, by excreting other amino acids (glutamate, aspartate) or through deamination reactions (ammonium). Activity of the ADI pathway decreased in the μ range 0.10 - 0.35 h^{-1} and nitrogen excretion to ornithine and synthesis of exogenous NH_3 declined from 4.7 to 0.5 mmol gdw^{-1} (21 to 4% from total nitrogen consumed) in the above μ range. In addition, 0.4 to 0.06 mmol gdw^{-1} of nitrogen was excreted as glutamate and 0.1 mmol gdw^{-1} through transamination reactions with the formation of the following compounds detected and quantified by mass-spectrometry: 4-hydroxyphenylpyruvic acid, hydroxyphenyllactic acid, 2-hydroxy-3-methylbutyric acid, 2-hydroxyisocaproic acid and L-3-phenyllactic acid from tyrosine,

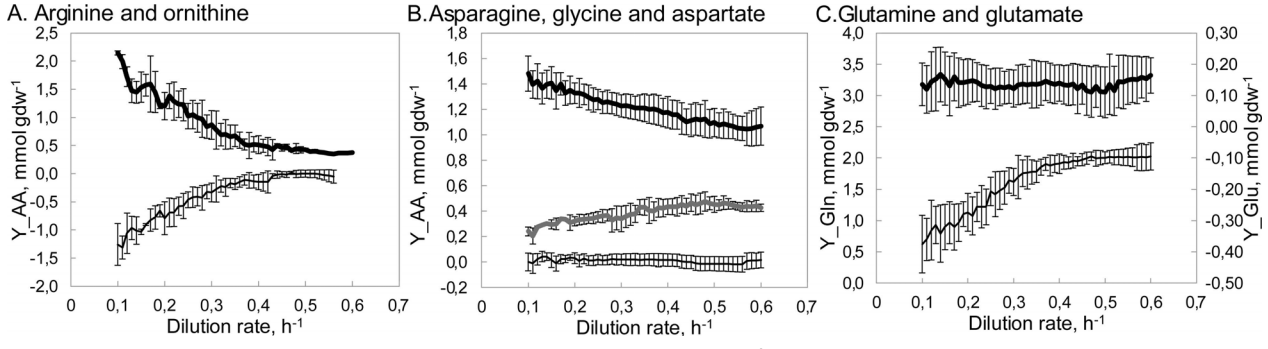

Figure 2 *L. lactis* dilution rate dependent amino acid consumptions (mmol gdw^{-1}) for (A) arginine (thick line) and ornithine (thin line); (B) asparagine (thick line), glycine (dashed line) and aspartate (thin line); (C) glutamine (thick line) and glutamate (thin line). Negative numbers on chart represent production. Refer to Additional file 2, Figure S2 for consumption yields of all amino acids.

phenylalanine or branched chain amino acids (data not shown). The left-over of consumed nitrogen was 9.5 - 6.6 mmol gdw^{-1} (contributing 44 - 48% from total nitrogen) in the μ range of 0.1 - 0.6 h^{-1}. This nitrogen must have been excreted as NH_3 if the excess of consumed amino acids not incorporated into protein fraction of biomass would have been converted to pyruvate. The latter assumption is supported by the fact that the carbon was fully recovered during the growth. Reduction of carbon and nitrogen wasting led to the increase of the biomass yields based on carbon (including glucose and amino acids) and nitrogen consumption 1.3 and 1.5 times, respectively (from 0.12 to 0.15 C-mol $C\text{-mol}^{-1}$ and from 0.33 to 0.50 N-mol $N\text{-mol}^{-1}$), in parallel with the increase of μ from 0.10 to 0.59 ± 0.02 h^{-1}.

Based on biomass monomer composition and the stoichiometry of ATP, NAD(P)H and central metabolites for monomer production, μ dependent ATP and NAD(P)H balance calculations were carried out (Additional file 1, Tables S3-S5). Calculations indicated that more ATP was produced than necessary for biomass formation. Presumably the ATP synthesized in excess was wasted in futile cycles. Calculated energy spilling was constant at 60 mmol ATP gdw^{-1} in the range of the μ 0.10 - 0.15 h^{-1} and decreased afterwards to 36 mmol gdw^{-1} at μ_{max}, indicating that the metabolism was the most efficient near μ_{max} conditions (Additional file 1, Table S5). Similarly calculated NAD(P)H misbalance (spilling) decreased from 3.5 mmol gdw^{-1} at low growth rates to 0 mmol gdw^{-1} at specific growth rate >0.45 h^{-1} (Additional file 1, Table S5). However, latter improvement of balance is inside the range of errors of lactate measurements (as lactate dehydrogenase is the main NAD regeneration reaction in lactic acid bacteria). Therefore a conclusion that redox balance was maintained throughout the studied growth conditions should be drawn.

Transcriptome and proteome response

Transcriptomes and proteomes at four different quasi steady state μ values (0.17, 0.24, 0.44, 0.52 h^{-1}) were compared to steady state μ = 0.10 h^{-1} (additional info in Methods). Changes in gene and protein expression levels for the most relevant reactions between μ 0.52 and 0.10 h^{-1} are illustrated on Figure 3 and 4; a full list of measured gene and protein expression changes at various μ values can be found in Additional file 3. In this section we discuss changes of mRNA and protein expressions significant with *P* value ≤ 0.05 for μ 0.52 ± 0.03 h^{-1} *vs.* 0.10 h^{-1}.

Mannose uptake genes *ptnAB*, which are responsible for glucose transport in *L. lactis*, and *ptsI* were up-regulated 2.1 to 4.3-fold at the transcriptome level at higher growth rates (above 0.44 h^{-1}). However, corresponding enzymes did not show any remarkable change in the same growth rate range as measured in the proteome. Transporter genes for additional sugars (not present in our medium) like galactose (by *galE*) and cellobiose (by *ptcABC* and *yidB*) were 1.8 to 2.9-fold down-regulated at higher specific growth rates at the transcriptome level, whereas a 2.2- to 2.8-fold repression of PtcAB was measured for proteome. This down-regulation is known to be the consequence of carbon catabolite repression which is extensively studied also in other bacteria like *E. coli* and *B. subtilis* [15,16].

Expression in the upper part of glycolysis did not change significantly during increase of μ. However, the lower part of glycolysis (from *fbaA* to *eno*) was 1.8- to 4-times up-regulated at the transcriptome level, but only Pmg showed significant 1.6-fold up-regulation at the proteome level at the growth rates higher than 0.44 h^{-1} (Figure 3). The pentose phosphate pathway showed a 1.3- to 2.0-fold down-regulation in genes *deoBC, rpiA, zwf, tkt, ywcC* (Additional file 3), which might be explained by a lower NADPH requirements at higher μ conditions. Despite the down-regulation of pentose phosphate pathway, genes encoding proteins involved in purine and pyrimidine metabolism were up-regulated. Moderate, 1.5- to 3.0-fold up-regulation both at the transcriptome and proteome level of the operon PurA-BEFLMQ was observed. With the increase of purine and pyrimidine metabolism, the need for amino group transfer from glutamine should have been also increased with rising specific growth rate. In agreement with this, expression of the genes in the first steps of purine and pyrimidine synthesis, *purF* increased and *carAB* remained constant respectively, with the increase of μ. High glutamine availability was maintained presumably by increased expression of glutamine transporter (*glnQP*) and glutamine synthetase (*glnA*).

Considering pyruvate metabolism, decreased acetate production was in accordance with the significant down-regulation of genes *eutD* and *ackA2* and their corresponding enzymes (see Figure 3). However, decreased production of formate and lactate seemed not to be regulated similarly with acetate - Pfl and Ldh showed no major changes neither in gene nor protein expression levels confirming that Ldh is regulated rather by the $NADH/NAD^+$ ratio than by transcription and/or translation, as proposed in literature [17]. Although ethanol production decreased, AdhE expression increased 7.3- and 1.8-fold in transcriptome and proteome analysis, respectively. This might be related to the incorporation of ethanol formation pathway intermediate, acetaldehyde, to acetyl-CoA synthesis from deoxyribose. Pyruvate dehydrogenase subunits (PdhABCD) were 2- to 3-fold down-regulated at both levels (Figure 3).

It is well known, that *L. lactis* can direct part of the consumed (or *de novo* synthesised) serine into pyruvate by *sdaA* and *ilvA* - this flux could form up to 10% of overall

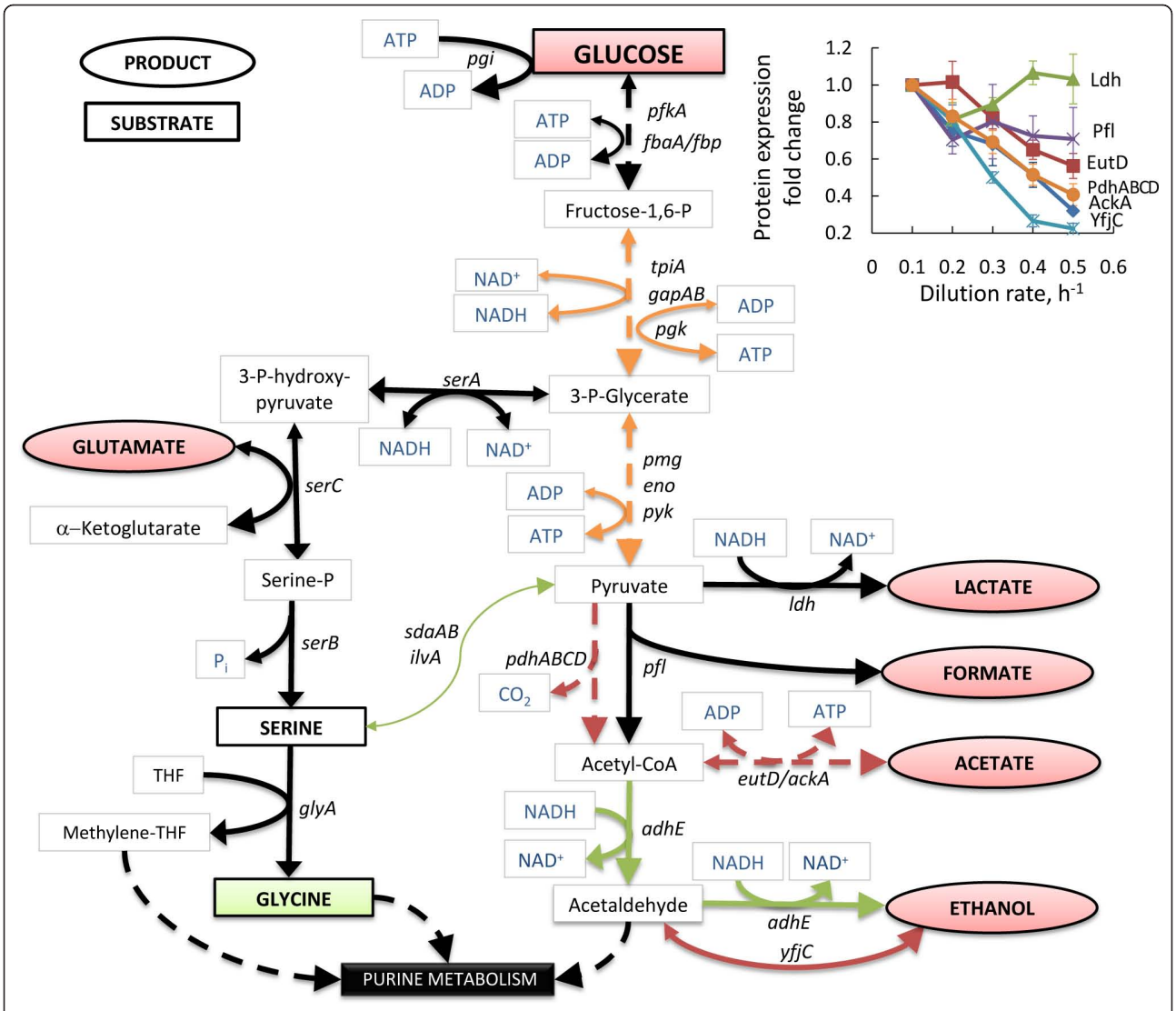

Figure 3 Overview of central carbon metabolism in *L. lactis* at various specific growth rates (μ). Black and capitalised metabolites were measured extracellular. Measured metabolites in boxes/ellipses were consumed/produced, respectively. Red/green/white background represents decrease/increase/no change, respectively, in metabolite consumption or production with increasing μ. Red arrows indicate decrease, green arrows increase and black arrows no significant change in transcriptome and proteome expressions when μ 0.5 h^{-1} is compared with μ 0.1 h^{-1}. Orange arrows represent increase only at transcriptome level with increasing μ. Arrowheads indicate the assumed reaction directions. More specific protein expression fold changes are illustrated on chart.

pyruvate flux [18]. In the current study, these noted genes were 1.4- to 2.2-fold up-regulated comparing μ = 0.50 to μ = 0.10 h^{-1}. In concordance with the sharp decrease of arginine consumption from μ 0.10 h^{-1} up to μ 0.35 h^{-1}, the 2.3- to 4.5-fold decrease in protein expression of ArcAB, which converts arginine to ornithine, was observed during the increase of μ (Figure 4).

Discussion

Carbon balance and growth efficiency

Growth conditions have a strong influence on specific growth rate (μ), macromolecular composition of biomass (*i.e.* ribosomal content) and cell size of microorganisms [18,19]. In this study, a gradual change to more efficient carbon metabolism with the increase of μ was observed for *L. lactis* (Figure 1). The first shift in *L. lactis* metabolism took place at μ 0.20 ± 0.02 h^{-1}, when biomass yield (Y_{XC}) per consumed carbon started to increase. Thirty percent increase with the increase of μ from 0.10 to 0.60 h^{-1} was achieved by reduction of fermentation by-products synthesis (acetate, formate, ethanol). Concomitantly to the increase of biomass yield, calculated ATP balance showed decreased energy spilling. It has been postulated that higher energy spilling at lower μ conditions could be

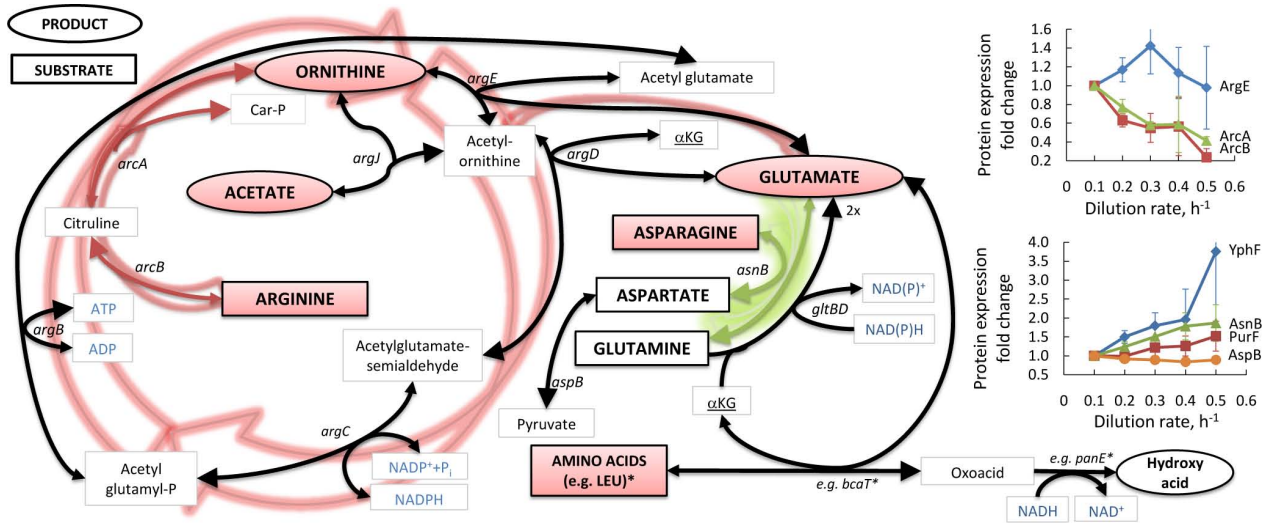

Figure 4 Overview of arginine and glutamine metabolism in *L. lactis* at various specific growth rates (μ). Black and capitalised metabolites were measured extracellular. Measured metabolites in boxes/ellipses were consumed/produced, respectively. Red/white background represents decrease/no change, respectively, in metabolite consumption or production with increasing μ. Red arrows indicate decrease, green arrows increase and black arrows no significant change in transcriptome and proteome expressions when μ 0.5 h^{-1} is compared with μ 0.1 h^{-1}. Arrowheads indicate the assumed reaction directions. Underlined metabolites exist several times on chart. More specific protein expression fold changes are illustrated on chart. Proteins PurF and YphF, represented only on charts, are involved in purine metabolism and converting glutamine to glutamate. THF - tetrahydrofolate; αKG - α-ketoglutarate; Car-P - carbamoyl-phosphate, * - represents example pathway components from literature [38,39].

caused by greater costs of turnover of macromolecules and sensory molecules, establishment of ion gradients across the cell membrane *etc* [20]. Dressaire et al. [12] calculated the degradation rates for proteins and found that protein median half-lives were *ca* 10-fold shorter at μ = 0.10 h^{-1} than at μ_{max}. As ATP is consumed during protein degradation [21] this non-growth related expenditure might form a higher proportion of the total energy synthesized at lower μ conditions than at higher growth rates.

Nitrogen metabolism

With the increase of specific growth rate from 0.10 to 0.60 h^{-1} biomass yield Y_{XN} increased 1.5 times showing that cells used nitrogen more effectively for biomass production. The most important amino acid that plays role in the observed reduction of nitrogen wasting was arginine (arginine consumption decreased from 1.5 to 0.5 mmol gdw^{-1} with increase of μ from 0.1 to 0.35 h^{-1}). Throughout the μ range studied, arginine consumption was 0.3 to 1.3 mmol gdw^{-1} higher than spent for biomass synthesis and majority of the consumed arginine was transformed to ornithine (0.05 to 1.2 mmol gdw^{-1}), especially at lower specific growth rates, which indicates energy limitation of cells. However, not all arginine left over from calculated requirements for biosynthesis (0.1 to 0.25 mmol gdw^{-1}) was converted to ornithine. Based on annotated network of *L. lactis* there is no route for consumption of ornithine other than that leading to the synthesis of glutamate (mediated by ArgCDJFG, which were reduced with increase of specific growth rates especially after 0.4 h^{-1}). Although the mechanisms of arginine overconsumption in addition to ornithine production are not known, correlation between ornithine production and glutamate synthesis was 0.99, which shows that these syntheses were most probably coupled. Production of glutamate has also been observed before, when both glutamine and glutamate were present in the cultivation medium [8,22].

Nitrogen wasting through glutamine metabolism was not decreased during the increase of specific growth rate. Glutamine, the most consumed amino acid (glutamine consumption covers 30 to 50% of total nitrogen consumed, at μ 0.10 and 0.60 h^{-1}, respectively), is used for synthesis of biomass proteins and it is the donor of amino groups in purine, pyrimidine and in aminosugar production pathways (glutamine and glutamate requirements for transamination reactions in aminosugar and nucleotide synthesis was in average 1.35 mmol gdw^{-1}). It should be noted that glutamine synthetase (*glnA*) was highly expressed (having array spot intensity values up to four times higher than these of average values of all genes) and increased with increase of μ in parallel to high consumption of the amino acid. Although we cannot argue over the direction of reactions on the basis of our experimental data, it could be assumed that maintenance of high intracellular concentrations of glutamine

in the cells in the result of intense synthesis and consumption flows might be necessary to keep the transfer of amino group effective.

The third biggest part of nitrogen wasting could be associated with the consumption of asparagine, which was reduced from 1.4 to 1.1 mmol gdw^{-1} with increase of μ from 0.10 to 0.60 h^{-1}. Asparagine and aspartate (which was not consumed and therefore should have been produced from asparagine) are required for syntheses of nucleotides (in average 0.35 mmol gdw^{-1}) and proteins (in average 0.4 mmol gdw^{-1}). It was shown that 0.35 to 0.65 mmol gdw^{-1} of asparagine was not used for biosynthesis. Asparagine can be metabolised through asparaginase (*ansB*) - however its expression was in the range of threshold values in the mRNA array and corresponding protein was not detected. Instead of that the high expression (array spot intensity values up to seven times higher than these of average values of all genes) of asparagine synthetase (*asnB*), which expression even increased with increase of specific growth rate was observed. Similarly to glutamine it could be assumed that overconsumption of asparagine and high expression of the relevant synthesis genes might be necessary to keep the transfer of amino group effective. Energetically transport of asparagine in *L. lactis* is much more efficient than aspartate [23], moreover, asparagine is probably preferentially directed towards the production of aspartate [24,25]. Surplus of aspartate in its turn can be directed into pyruvate by AspB (Figure 4).

The role of other amino acids (other than glutamine, arginine and aspartate) in nitrogen wasting can be considered minimal as over-consumptions (amounts greater than necessary for biomass production) of these amino acids were below 0.2 mmol gdw^{-1} (cysteine, serine, threonine) or 0.1 mmol gdw^{-1} (all other not mentioned above).

Omics comparison

Good correlation with a Pearson coefficient up to 0.69 was observed between 600 measured protein and gene expression data (Figure 5). An interesting phenomenon was seen at μ values 0.52 ± 0.03 h^{-1} and 0.42 ± 0.02 h^{-1} compared to 0.10 h^{-1}: a large amount of genes up-regulated at the transcriptome level showed only small or no change at the proteome level (Figure 5). The vast majority of members in this group were related to ribosomal subunits (74% from all detected ribosomal proteins), as well as lower glycolysis (FbaA, GapB, Pgk, Eno) and amino acid or peptide transport (BusAB, GlnPQ, GltPS, OptCD, PepCPX, PtnABD, PtsHI). Up-regulation at the transcriptome level and no significant change at proteome level during anaerobic growth of *L. lactis* in lower part of glycolysis have also been noticed before [11,12]. Despite the relatively good

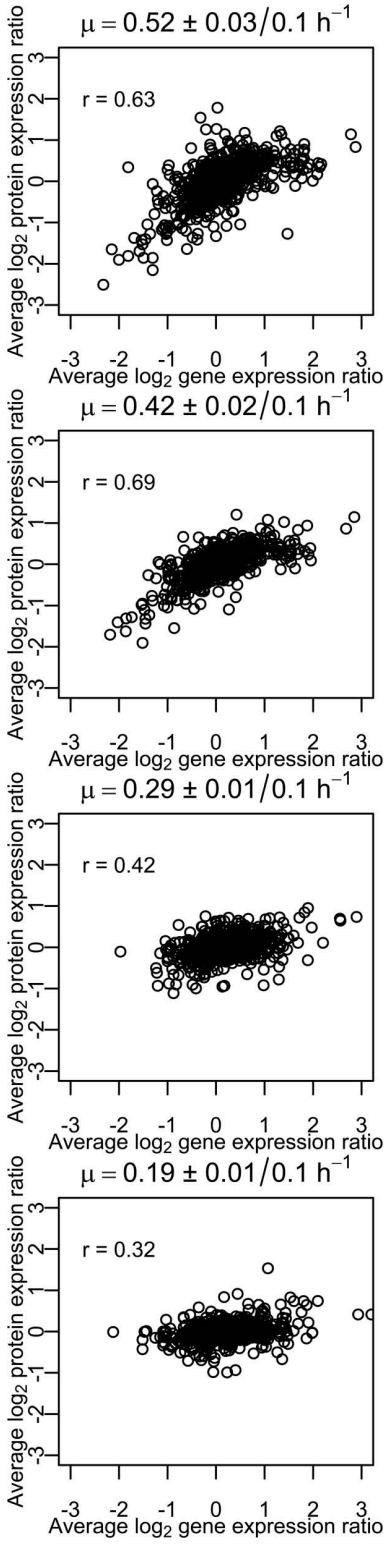

Figure 5 *L. lactis* transcriptome and proteome correlation at various specific growth rates. "R" value on chart represents Pearson coefficient. Six hundred proteins, with a standard deviation less than 30% and their corresponding genes are indicated on a graph.

correlation between the transcriptomic and proteomic data, several important regulations were observed only at trancriptome level. The data obtained indicated importance of taking into account the possibility of allosteric regulation, and carrying out measurements of fluxome in addition to transcriptome and proteome to fully characterize regulation of metabolic pathways.

By scanning the entire range of specific growth rates using A-stat experiments, it is possible to continuously monitor the steady state metabolism using on-line sensors or frequently collected samples for at-line analyses. Reproducibility of growth characteristics in A-stat were compared with chemostat at μ 0.45 h^{-1}. All measured substrate consumption and product formation yields (including amino acids) remained within mentioned standard deviation ranges indicating the accordance of quasi steady state and steady state data (Additional file 2, Table S2). Recently, similar comparisons at the global transcriptome level were conducted with *E. coli* achieving very good correlation with a Pearson coefficient up to 0.96 [26]. In both studies, it was shown that the A-stat cultivation technique allows precise monitoring the sequence of metabolic switch points.

Conclusions

Distinct ratios of glucose and amino acids in the growth media are very important for biomass yield optimization as carbon and nitrogen metabolism are tightly coupled in *L. lactis*. High biomass yields are crucial for producing vaccines using microorganisms and nutrient limitations can strongly affect achieving the desired results. As was shown in this study, some amino acids were consumed in large amounts (glutamine, asparagine, arginine) and more efficient growth might not be achieved by insufficient supply of these compounds. There have been several attempts to optimize the media for lactococci using a single omission technique [7,8], however, a systematic approach taking into account that amino acid requirements depend on environmental conditions (*e.g.* at various μ values) has not yet been fully realized as it is difficult using only batch cultivation. The current work combining systematic continuous cultivation approach with omics methods is therefore of high value for better media design, as well as for understanding principles of metabolism of the bacteria.

Using steady state cultivation methods and a systems biology approach for characterisation of *L. lactis* metabolism, it was possible to demonstrate a shift to more efficient metabolism at higher growth rates by increasing the biomass yield, change towards homolactic fermentation, and decreasing the flux through alternative energy generation pathways with lower efficiency than glycolysis e.g. acetate formation and the ADI pathway.

This study demonstrates the necessity of using strictly controlled continuous cultivation methods in combination with a multi-omics approach and element balance calculations to gain quantitative understanding of the regulation of complex global metabolic networks, important for strain dependent media optimisation or the design of efficient producer cells. However, questions about rationale of 2-3 times over-consumption of amino acids by cells and principles of properly balanced media remain to be answered in full in the future studies.

Methods

Microorganism and medium

The strain used throughout these experiments *Lactococcus lactis* subsp. *lactis* IL1403 was kindly provided by Dr. Ogier from INRA (Jouy-en-Josas, France). Inoculum was prepared using a lyophilized stock culture stored at -80°C which was pre-grown twice on the cultivation medium. Chemically defined medium with a reduced amino acid concentrations were developed especially for better detection of amino acids. Media contained 70% GIBCO™ F-12 Nutrient Mixture (Invitrogen Corporation, Carlsbad, CA) and 30% modified CDM (composition in [27]). This combination gave the best trade-off for growth yield and maximal growth rate. Composition of the final medium was as follows (mg L^{-1}): limiting substrate D-Glucose - 3500; L-Alanine - 78; L-Arginine - 185; L-Asparagine - 74; L-Aspartic acid - 72; L-Cysteine - 64; L-Glutamic acid - 70; L-Glutamine - 132; Glycine - 58; L-Histidine - 60; L-Isoleucine - 102; L-Leucine - 207; L-Lysine - 158; L-Methionine - 41; L-Phenylalanine - 86; L-Proline - 92; L-Serine - 163; L-Threonine - 76; L-Trypthophan - 16; L-Tyrosine - 29; L-Valine - 107; Biotin - 0.305; Choline chloride - 9.8; D-Pantothenate - 0.65; Folic Acid - 1.21; Niacinamide - 0.325; Pyridoxine hydrochloride - 0.642; Riboflavin - 0.326; Thiamine hydrochloride - 0.51; Vitamin B12 - 0.98; i-Inositol - 12.6; $CaCl_2$ - 28; $CuSO_4 \times 5H_2O$ - 0.272; $FeSO_4 \times 7H_2O$ - 0.71; $MgCl_2$ - 58; KCl - 157; NaCl - 5580; Na_2PO_4 - 99; $ZnSO_4 \times 7H_2O$ - 1; Hypoxanthine-Na - 3; Linoleic Acid - 0.1; Lipoic Acid - 0.1; Phenol Red - 0.8; Putrescine × 2HCl - 0.1; Na-Pyruvate - 77; Thymidine - 0.5.

A-stat cultivations

A-stat cultivations were carried out in a 1 L Biobundle bioreactor (Applikon, Schiedam, the Netherlands) controlled by an ADI1030 biocontroller (Applikon) and a cultivation control program "BioXpert NT" (Applikon) (detailed description in [28], with an addition of an *in situ* OD sensor (model TruCell2; Finesse, San Jose, CA)). Cultivations were carried out under anaerobic conditions (N_2-environment) with an agitation speed of 300 rpm at 34°C and pH 6.4. Five parallel A-stat experiments were carried out where after a batch phase,

constant dilution rate (D = 0.1 h^{-1}) was initiated. Culture was stabilised until constant optical density and titration rate, pumping through at least 5 volumes of medium. After achieving steady state conditions, acceleration of dilution rate (a = 0.01 h^{-2}) was started. Additionally, a steady state chemostat experiment was carried out at a dilution rate of 0.45 h^{-1} and results were compared with data collected from the A-stat experiment at the same dilution rate. Average yield and metabolic switch point values with their standard deviations were calculated based on five independent experiments, additionally taking into account chemostat experiment values at a dilution rate of 0.45 h^{-1}.

Analytical methods and growth characteristics

Biomass was constantly monitored by measuring the optical density at 600 nm; biomass dry weight was determined gravimetrically. Biomass correlation constant K was 0.372 ± 0.005 and was not specific growth rate dependent. Levels of glucose, lactate, formate, acetate and ethanol in the culture medium were measured with liquid chromatography (Alliance 2795 system, Waters Corp., Milford, MA), using a BioRad HPX-87H column (Hercules, CA) with isocratic elution of 5 mM H_2SO_4 at a flow rate of 0.6 mL min^{-1} and at 35°C. A refractive index detector (model 2414; Waters Corp.) was used for detection and quantification of substances. The detection limit for the analytical method was 0.1 mM. Samples from culture medium were centrifuged (14,000 × *g*, 4 min); supernatants were collected and analyzed immediately or stored at -20°C until analysis. Free amino acid concentrations were determined from the same sample (analysing frequency *ca* 0.02 h^{-1}) with an amino acid analyzer (Acquity UPLC; Waters Corp.) according to the manufacturer's instructions. Empower software (Waters Corp.) was used for the data processing. For measuring amino acid concentrations in protein content, biomass was hydrolysed with 6 M HCl for 20 h at 120°C. From hydrolyte, amino acids were determined as free amino acids described above. Protein content from biomass dry cell weight was calculated based on amino acid analysis and, additionally, measured using the Lowry method [29], where bovine serum albumin was used as a standard. For measurement of DNA content in biomass genomic DNA was extracted and measured using instructions of RTP® Bacteria DNA Mini Kit (Invitec, Germany). Detailed protocol for fatty acid quantification is described in [30]. Growth characteristics μ, Y_{XS}, $Y_{Substrate}$, $Y_{Product}$ were calculated as described previously [27,28]. For consumption calculations, measured medium concentrations were used.

Carbon, nitrogen and ATP balance calculations

For carbon balance calculations C-molar concentrations of measured substrates, products and biomass were used (biomass C-molar concentration with a value 0.03625 C-mol gdw^{-1} was calculated based on monomer composition). For nitrogen balance calculations N-molar amino acid consumptions, production of ornithine and glutamate, ADI pathway activity and biomass composition (0.00725 N-mol gdw^{-1}) were taken into account.

For calculations of ATP and NAD(P)H balance measured biomass, amino acid, RNA, DNA and fatty acid contents were used. Other necessary data were adapted from literature [31]. Stoichiometry of ATP, NAD(P)H and central metabolites for monomer production were taken from the Kyoto Encyclopaedia of Genes and Genomes database http://www.kegg.jp/, with an assumption that amino acids were not synthesized. Specific calculations are presented in Additional file 1.

Gene expression profiling

Agilent's DNA microarrays (Santa Clara, CA) were designed in eArray web portal in 8 × 15K format, containing 7 unique probes per target https://earray.chem.agilent.com/earray/. Target sequences for 2234 genes were downloaded from Kyoto Encyclopaedia of Genes and Genomes ftp://ftp.genome.jp/pub/kegg/genes/organisms/lla/l.lactis.nuc.

For microarray analysis, steady state chemostat culture of *L. lactis* IL1403 was used as reference (D = 0.10 h^{-1}). Subsequent quasi steady state points from A-stat experiment at specific growth rates 0.52 ± 0.03; 0.42 ± 0.02; 0.29 ± 0.01 h^1 in biological duplicates and 0.17 h^{-1} were compared to the reference sample. Transcript change was considered significant if the *P* value between parallel experiments was less than 0.05.

Total RNA was extracted and quantified, cDNA synthesised and labelled as described previously [27], with minor modification: 11 μg of total RNA was used for cDNA synthesis. Hybridization, slide washing and scanning was performed using standard Agilent's reagents and hardware http://www.chem.agilent.com. Gene expression data was analyzed as described before [27], except global lowess normalization was used. Spots with intensities lower than 100 units in both channels and outliers among technical replicates (according [32]) were filtered. After filtering, seven technical replicates showed average standard deviation <10%. Gene (and protein) expression measurement results are shown in Additional file 3. DNA microarray data is also available at NCBI Gene Expression Omnibus (Reference series: GSE26536).

Protein expression profiling

For protein expression analysis, the steady state chemostat culture of *L. lactis* IL1403 was used as reference (μ = 0.10 h^{-1}). Quasi steady state points at μ = 0.20 ± 0.01, 0.30 ± 0.02, 0.42 ± 0.01 and 0.50 ± 0.01 h^{-1} were

compared with the reference sample. Three biological replicates were analysed.

Samples intended for proteome analysis were collected, washed with PBS (0.137 M NaCl, 2.7 mM KCl, 10.0 mM Na_2HPO_4, 1.4 mM KH_2PO_4), flash frozen in liquid nitrogen and stored at -80°C prior to protein extraction.

Proteins were extracted in ice-cold SDS-buffer (100 mM Tris-HCl (pH 6.5), 1% SDS (w/v)). Cells were disrupted as a result of agitating the suspension with glass-beads at 4°C for 30 minutes. After centrifugation for 30 min at 4°C, the supernatant was collected and the protein concentration was determined by 2D Quant kit (Amersham Biosciences, Buckinghamshire, UK) and protein samples were stored at -80°C until further analysis.

Aliquots of 100 μg cloroform/MeOH chloroform precipitated proteins from each sample were processed for labeling with iTRAQ 4plex reagents (Applied Biosystems, Foster City, CA) according to manufacturer's instructions. Briefly, precipitated proteins were dissolved in 0.5 M triethylammonium bicarbonate (TEAB) and 0.1% SDS, disulfide bonds were reduced in 5 mM Tris-(2-carboxyethyl) phosphine (TCEP) for 1 h at 60°C, followed by blocking cycteine residues in 10 mM methyl methanethiosulfonate (MMTS) for 30 min at room temperature, before digestion with trypsin (1:40, enzyme to protein ratio) overnight at 37°C. For labeling, each iTRAQ reagent was dissolved in 70 μl of ethanol and added to the respective peptide mixture. After 1 h incubation at room temperature the reactions were stopped by adding 100 μl milliQ water and incubating for 30 min. All four samples were mixed together and ethanol was removed by drying in a vacuum concentrator (Model 5301, Eppendorf, Cambridgeshire, UK).

The combined peptide mixtures were separated into 10 fractions with a cation exchange cartridge system (Applied Biosystems, Foster City, CA) by different KH_2PO_4 concentrations (10-1000 mM) and cleaned by StageTips [33]. All fractions were analyzed twice by LC-MS/MS using an Agilent 1200 series nanoflow system (Agilent Technologies, Santa Clara, CA) connected to a Thermo Scientific LTQ Orbitrap mass-spectrometer (Thermo Electron, San Jose, CA) equipped with a nanoelectrospray ion source (Proxeon, Odense, Denmark). Purified peptides were dissolved in 0.5% formic acid and loaded on self-packed fused silica emitter (150 mm × 0.075 mm; Proxeon) packed with Repropur-Sil C18-AQ 3 μm particles (Dr. Maisch, Germany) using a flow rate of 0.7 μl min^{-1}. Peptides were separated with a 180 min gradient from 3 - 40% B (A: 0.1% formic acid, B: 0.1% formic acid/80% acetonitrile) using a flow-rate of 200 nl min^{-1} and sprayed directly into LTQ Orbitrap mass-spectrometer operated at 180°C capillary temperature and 2.4 kV spray voltage.

Mass spectrometry method combined HCD and CID spectrums as described in Köcher et al. [34]. Briefly, full mass spectra were acquired in profile mode, with mass range from *m/z* 300 to 1800 at resolving power of 60000 (FWHM). Up to four data-dependent MS/MS scans with CID and four scans with HCD tandem mass spectrometry experiment triggered from the same precursor ion were acquired in centroid mode for each FTMS full-scan spectrum. CID was carried out with a target signal value of 10 000 in the linear ion trap, collision energy of 35%, Q value of 0.25 and an activation time of 30 ms. HCD-generated ions were detected in the Orbitrap using the target signal value of 10 000, collision energy of 35% and an activation time of 40 ms. Each fragmented ion was dynamically excluded for 60s.

Raw files were extracted to .mgf files by MM File Conversion Tools http://searcher.rrc.uic.edu/cgi-bin/mm-cgi/downloads.py. Each .mgf file was converted to a QuantMerge file [34]. All files from the same sample were merged together. Data generated was searched against *L. lactis* IL1403 NCBI database (22092009) by MassMatrix search tool [35]. A reversed decoy database was used for false positives detection. In all cases, a peptide mass tolerance of 5 ppm was used and fragment ion masses were searched with a 0.6 Da mass window. Two missed cleavage sites for trypsin were allowed. Betamethylthiolation of a cysteine was set as a fixed modification and oxidation of methionine as a variable modification. Quantification was set as iTRAQ and quantification statistics as arithmetic mean. Only proteins with confidence intervals of more than 95% were allowed for further data analysis (Additional file 3). Proteomic analysis raw data is available at the PRIDE database [36]http://www.ebi.ac.uk/pride under accession numbers 13105-13162 (username: review17185, password: wyd*b6_6). The data was converted using PRIDE Converter http://code.google.com/p/pride-converter[37]. Protein expression change was considered significant if the *P* value between parallel experiments was less than 0.05.

Additional material

Additional file 1: Specific growth rate dependent ATP and NAD(P)H balance calculations for A-stat experiments with *Lactococcus lactis* subsp. *lactis* IL1403.

Additional file 2: Supplementary figures and tables.

Additional file 3: Specific growth rate dependent mRNA and protein expression changes from A-stat experiments with *Lactococcus lactis* subsp. *lactis* IL1403. The expression fold change is given accordingly: sample at respective specific growth rate (quasi steady state) is divided by steady state chemostat sample (0.10 h-1). Average log2 gene and protein expression changes were calculated from "n" number of parallel A-stat experiments. In gene expression analysis spots with intensities lower than 100 units in both channels and outliers among technical replicates (according Rorabacher, 1991) were filtered. In

protein expression analysis, proteins identified with a confidence interval more the 95% and appearances in all mentioned parallels are presented.

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Authors' contributions
PJL, KAd, RV designed experiments and conceived the project. PJL, KAl carried out experiments. PJL, RN, LA, KAl contributed in analytics and data analysis. KAd was responsible for mathematical calculations. PJL drafted the manuscript. KAd helped drafting the manuscript. RV, RN, LA edited the manuscript. All authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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ELECTRONIC APPENDIX

Table S6. Chemically defined media from the literature which contain numerous nutrients in excess and are thus suitable for the cultivation of multiple species of lactic acid bacteria.

Medium	CDM	DM1	DML	MCD [1]	ZBM1	ZBM2
Strain	Wine LAB [2]	Lactobacilli [3]	Meat lactobacilli [4]	Lactococci, enterococci [5]	Lactococci, streptococci, enterococci [6]	Lactococci, streptococci, enterococci [6]
Reference	[212]	[187]	[102]	[190], modified by [16]	[79]	[79]
COMPOSITION (g·L^{-1})						
SUGARS						
Glucose		10	10	10	15	15
Ribose	10					
BUFFERS						
K_2HPO_4	1.0	3.1		7.5 (2.5) [7]	6.4	7.3
KH_2PO_4		1.5	1.0	9.0 (3.0) [7]	3.1	3.6
MOPS					15.0	13.1
Tricine					1.5	1.3
AMINO ACIDS						
L-Alanine (Ala) [8]	0.2000	0.2000	0.1600	0.2400	0.4000	0.4800
L-Arginine (Arg)	0.7500	0.2000	0.0413 [9]	0.1200	0.7200	0.7200
L-Asparagine (Asn)	0.1500	0.1000	0.4000	0.3400	0.5000	0.6000
L-Aspartate (Asp)	0.3500	0.3000			0.0500	0.0600
L-Cysteine (Cys)	0.2000	0.1000	0.1380 [10]	0.1700	0.1537 [11]	0.1845 [12]
L-Glutamine (Gln)	0.2000		0.3000	0.5100	0.6000	0.7200
L-Glutamate (Glu)	0.5000	0.3000			0.6000	0.7200
Glycine (Gly)	0.5000	0.2000	0.1000	0.1700	0.3000	0.3600
L-Histidine (His)	0.5000	0.1619 [13]	0.0740 [14]	0.1100	0.1700	0.1700
L-Isoleucine (Ile)	0.2000	0.1000	0.0500	0.2000	0.2400	0.2400
L-Leucine (Leu)	0.2000	0.2000	0.0600	0.4700	1.0000	1.0000
Lysine-HCl (Lys)	0.2084 [15]	0.2000	0.1000	0.3500	0.5000	0.6000
L-Methionine (Met)	0.1500	0.1000	0.0250	0.1200	0.0600	0.0600
L-Phenylalanine (Phe)	0.2000	0.1000	0.0400	0.2800	0.4000	0.4800
L-Proline (Pro)	0.5000	0.2000	0.0400	0.6800	0.7000	0.8400
L-Serine (Ser)	0.4000	0.1000	0.0800	0.3400	0.5000	0.6000
L-Threonine (Thr)	0.3500	0.1000	0.0500	0.2300	0.5000	0.6000
L-Tryptophan (Trp)	0.2000	0.1000	0.1000	0.0500	0.2000	0.2400
L-Tyrosine (Tyr)	0.2000	0.1000	0.0400	0.2900	0.3000	0.3600
L-Valine (Val)	0.2000	0.1000	0.0600	0.3300	0.7000	0.7000
MINERAL SALTS						
$CaCl_2·2H_2O$	0.5828 [16]			0.0500	0.0400	0.0400
$CoCl_2·6H_2O$				0.0025	0.0002	0.0002
$CoSO_4$		0.5000				
$CuSO_4·5H_2O$	0.0000				0.0002	0.0002
$FeSO_4·7H_2O$	0.0200			0.0137 [17]	0.0040	0.0040
$MgCl_2·6H_2O$				0.2000		
$MgSO_4·7H_2O$	0.1000	0.5000	0.1500		1.0000	1.0000
$MnSO_4·4H_2O$	0.1000	0.0264 [18]	0.0201 [19]		0.0004	0.0004
$(NH_4)_6Mo_7O_{24}·4H_2O$					0.0002	0.0002
NaCl		0.0200			3.0000	3.0000
$ZnSO_4·7H_2O$	0.0001			0.0050	0.0050	0.0050
VITAMINS						
Thiamine-HCl (B_1) [20]	0.00100		0.00100	0.00127 [21]	0.00056	0.00056
Riboflavin (B_2)	0.00200	0.01000	0.00050	0.00100	0.00090	0.00090
Niacin (Nicotinic acid; B_3)	0.00200	0.01000	0.00100	0.00100	0.00090	0.00090
Ca-pantothenate (B_5)	0.00200	0.01000	0.00109 [22]	0.00100	0.00120	0.00120
Pyridoxal-HCl (B_6)		0.01000	0.00050		0.00480	0.00480
Pyridoxamine (B_6)				0.00500		
Pyridoxine (B_6)	0.00165 [23]			0.00200		
Biotin (B_7)	0.00200	0.00100	0.00001	0.01000	0.00600	0.00600
***Myo*-inositol (formerly B_8)**		0.01000			0.00200	0.00200
Folic acid (B_9)	0.00200	0.00020	0.00010	0.00100	0.00056	0.00056
Cyanocobalamine (B_{12})	0.00010	0.00002		0.00100		
***para*-Aminobenzoic acid (*p* ABA, formerly B_x)**	0.00010	0.00020		0.01000	0.00006	0.00006
Ascorbic acid (C)		0.50000				
NUCLEOBASES, NUCLEOSIDES						
Adenine (A)	0.0073 [24]		0.0250	0.0050	0.0110	0.0110
Cytosine (C)	0.0100					
Guanine (G)	0.0081 [25]	0.1000		0.0010	0.0056	0.0056
Thymine (T)	0.0100	0.1000				
Uracil (U)	0.0100		0.0250	0.0050	0.0230	0.0230
Xanthine	0.0050		0.0250	0.0050	0.0038	0.0038
Cytidine		0.1000				
Deoxyadenosine (dA)		0.1000				
Deoxythymidine (dT)				0.0050		
Deoxyuridine (dU)		0.1000				
Inosine (I)				0.0050		
Orotic acid				0.0050		
FATTY ACID PRECURSORS						
K-acetate		10.00000			0.90000	0.90000
Na-acetate				1.00000		
Lipoic acid (DL-6,8-Thioctic acid)				0.00250	0.00100	0.00100
Tween 80		1.00000	0.55000		0.50000	0.50000
OTHER COMPOUNDS						
$(NH_4)_2SO_4$					1.0	1.0
Ammonium citrate [26]		2.0		0.6		
Ca-lactate		1.0				
Choline chloride	0.00200					
D,L-aminobutyrate		0.1				
EDTA					0.00750	0.00750
Glutathione					0.01500	0.01500
H_3BO_3					0.00075	0.00075
K_2SO_4					0.02300	0.02300
KI					0.00011	0.00011
Nitrilotriacetic acid					0.00750	0.00750
Succinic acid			11.9			

[1] The exact composition has been taken from [91].

[2] Suitable for several species of *O. oeni* , *Lb. brevis* , *Lb. buchneri* , *Lb. hilgardii* , *Lb. plantarum* , *Lb. pasteurianus* , *Lb. delbrueckii* , *P. pentosaceus* .

[3] Suitable for *Lb. johnsonii* ATCC33200, ATCC11506, La1 (NCC533), DSM20553; *Lb. gasseri* DSM20243; *Lb. gallinarum* DSM33199; *Lb. casei* ATCC393; *Lb. paracasei* NCDO 151; *Lb. delbrueckii* subsp. *delbrueckii* DSM20074; *Lb. plantarum* NCDO1193; *Lb. lactis* ATCC7830.

[4] Suitable for *Lb. plantarum* NCFB1752, NC8; *Lb. sake* Lb16, NCFB2714; *Lb. pentosus* NCFB363; *Lb. curvatus* NCFB2739.

[5] This CDM has been used for studying the following strains: *L. cremoris* MG1363, Wg2 [15-16, 190], *L. lactis* IL1403, ML3, NCDO2118 [16, 91, 192], *Lb. plantarum* WCFS1 [9, 106, 113].

[6] The following strains were tested: *L. lactis* IL1403, LM0230, ML3; *L. lactis* biovar *diacetylactis* 18-16, DCR3, UCD172; *L. cremoris* FG2, MG1363, SK11; *S. thermophilus* MTC330, MTC360; *E. faecalis* KA177, OG1RF.

[7] Concentrations in brackets were used in pH-regulated bioreactors

[8] [102] and [187] used DL-alanine

[9] 0.05 g·L^{-1} of Arginine-HCl

[10] 0.2 g·L^{-1} of Cysteine-HCl·H_2O

[11] 0.2 g·L^{-1} of Cysteine-HCl

[12] 0.24 g·L^{-1} of Cysteine-HCl

[13] 0.2 g·L^{-1} of Histidine-HCl

[14] 0.1 g·L^{-1} of Histidine-HCl·H_2O

[15] 0.25 g·L^{-1} of Lysine-2HCl

[16] 0.44 g·L^{-1} of $CaCl_2$

[17] 0.011 g·L^{-1} of $FeSO_4·4H_2O$

[18] 0.02 g·L^{-1} of $MnSO_4·H_2O$

[19] 0.0152 g·L^{-1} of $MnSO_4·H_2O$

[20] Synonyms: Thiamine dichloride

[21] 0.001 g·L^{-1} of Thiamine

[22] 0.001 g·L^{-1} of Pantothenic acid

[23] 0.002 g·L^{-1} of Pyridoxine-HCl

[24] 0.01 g·L^{-1} of Adenine sulfate

[25] 0.01 g·L^{-1} of Guanine-HCl

[26] Synonyms: Diammonium hydrogen citrate

Table S7. Chemically defined media from the literature with definite applications or for specific species of lactic acid bacteria.

Medium	CDM	CDM	CDM	CDM	CDM1	CDM2	CDM3	CDM-1G	LPSM	MPL	MS10	SA	SCDM
Strain	*Lb. bulgaricus* NCFB2772	*Lb. rhamnosus* ATCC10863	*Lb. bulgaricus* CNRZ1187, CNRZ416	*Lb. johnsonii* NCC533	*Lb. rhamnosus* E-97800, *Lb. brevis* ATCC8287	*Lb. reuteri* SD2112	*L. lactis* MG1363	*L. lactis* IO-1	*Lb. plantarum* N4, N8	*Lb. bulgaricus* CNRZ397 [3]	*L. lactis* IL1403, NCDO2118	*L. lactis* , *L.cremoris* [2]	*Lb. helveticus* CRL 974, CRL1062
Reference	[96]	[191]	[82]	[213]	[214]	[214]	[214]	[101]	[103]	[94]	[91]	[92]	[97]
Specific applications	Quantitative and qualitative production of EPS	High levels of growth and lactic acid production	EPS production		Protein radiolabelling and proteomic analyses of lactobacilli					Phage and EPS production, protein labelling			
GROWTH OF CELLS													
OD (λ=600 nm, if not stated otherwise)	1.4			2-3				2.05±0.05		2		1.6-3.0 (450 nm) [1]	1.40-1.50 (560 nm) [4]
μ (h^{-1})	0.18	0.49		1.6				0.71±0.09	0.44-0.56 [5]	0.59 [6]	0.5-0.6 [7]	0.22-0.73 [8]	0.28-0.34 [9]
COMPOSITION (g·L^{-1})													
SUGARS													
Fructose					5 [10]								
Glucose	20	80	(30) [11]	20	25	25	25	10	10		10	9	10
Lactose			(30) [11]							20			
BUFFERS													
K_2HPO_4		2.3 [12]	2.0	1.5	0.6	0.6	0.6	2.8			7.5 (2.5) [13]		3.0
KH_2PO_4	1.0	3.0	2.0	3.1	0.6	0.6	0.6	1.2	2.0		9.0 (3.0) [13]	0.2	3.0
MOPS												8.4	
Tricine												0.7	
AMINO ACIDS													
L-Alanine (Ala) [14]	0.0980	0.5000	0.2000	0.2000	0.5000	0.5000	0.5000	0.1000	0.2000	0.1000	0.2400	0.3029	0.1000
L-Arginine (Arg)	0.0871	0.5000	0.2000	0.2000				0.1000	0.3000	0.3200	0.1200	0.1916	0.1000
L-Asparagine (Asn)	0.0925	1.5000		0.2000				0.0880 [15]	0.2000	0.3000	0.3400	0.1057	0.2000
L-Aspartate (Asp)	0.1065	1.0000	0.3000	0.2000	1.1000	1.1000	1.1000	0.1000	0.2000	0.5000			0.2000
L-Cysteine (Cys)	0.3029	1.5000	0.4000	0.2000				0.1000	0.1537 [16]	0.2152 [17]	0.1700	0.0969	0.2000
L-Glutamine (Gln)	0.1023	1.0000			1.0000	1.0000	1.0000	0.1000		0.3000	0.5100	0.1023	0.2000
L-Glutamate (Glu)	0.1030	1.5000	0.3000	0.6000	1.2000	1.2000	0.8800	0.1000	0.1500	0.3800		0.3090	0.2000
Glycine (Gly)	0.0976	0.5000	0.2000	0.4000	0.4000	0.4000	0.5600	0.1000	0.3000	0.1600	0.1700	0.2027	0.1000
L-Histidine (His)	0.0931	0.5000	0.2000	0.4000	0.4400	0.4400	0.5300	0.1000	0.1619 [18]	0.2000	0.1100	0.0465	0.1000
L-Isoleucine (Ile)	0.1049	0.5000	0.2000	0.1000	0.5000	0.5000	0.6000	0.1000	0.2000	0.3600	0.2000	0.1049	0.1000
L-Leucine (Leu)	0.1049	0.5000	0.2000	0.2000	0.5000	0.5000	0.6000	0.1000	0.3000	0.6000	0.4700	0.1049	0.1000
Lysine-HCl (Lys)	0.1096	1.4993 [19]	0.2499 [20]	0.2499 [20]	1.3119 [21]	1.3119 [21]	1.3119 [21]	0.1249 [22]	0.3000	0.4400	0.3500	0.2557	0.1249 [22]
L-Methionine (Met)	0.1044	0.5000	0.2000	0.1000	0.2000	0.2000	0.2000	0.1000	0.2000	0.1200	0.1200	0.1044	0.1000
L-Phenylalanine (Phe)	0.0991	0.5000	0.2000	0.2000	0.5000	0.5000	0.5000	0.1000	0.2000	0.3400	0.2800	0.1982	0.1000
L-Proline (Pro)	0.1036	0.5000	0.2000	0.2000	0.4000	0.4000	0.4800	0.1000	0.3000	0.9200	0.6800	0.2993	0.1000
L-Serine (Ser)	0.1051	1.5000	0.2000	0.2000	1.5500	1.5500	0.6000	0.1000	0.3000	0.3600	0.3400	0.3048	0.1000
L-Threonine (Thr)	0.0953	0.5000	0.2000	0.1000	0.5000	0.5000	0.6400	0.1000	0.2000	0.3000	0.2300	0.2025	0.1000
L-Tryptophan (Trp)	0.1021	0.5000	0.2000	0.2000				0.1000	0.2000	0.1000	0.0500	0.1021	0.1000
L-Tyrosine (Tyr)	0.1087	0.5000	0.2000	0.1000				0.1000	0.3000	0.1200	0.2900	0.0544	0.1000
L-Valine (Val)	0.1054	0.5000	0.2000	0.2000	1.0000	1.0000	1.0000	0.1000	0.3000	0.4800	0.3300	0.1054	0.1000
MINERAL SALTS													
$CaCl_2$		0.0755 [23]	0.2000							0.2700		5.5·10^{-5}	
$CoCl_2·6H_2O$	0.0002									7.0·10^{-6}		7.7·10^{-4} [24]	
$CoSO_4·7H_2O$		0.0100											
$CuCl_2·2H_2O$	1.7·10^{-6}												
$CuSO_4$		0.0064 [25]								1.6·10^{-6}		1.6·10^{-6}	
$FeCl_2·4H_2O$	0.0015												
$FeCl_3·6H_2O$								0.0010					
$FeSO_4·7H_2O$		0.0200	0.0200	0.0200	0.0100	0.0100	0.0100		0.0100	6.6·10^{-5}		0.0028 [26]	
$MgCl_2·6H_2O$											0.2000	0.1057 [27]	
$MgSO_4·7H_2O$	0.1972	0.5000	0.5000	0.5000	0.2562 [28]	0.2562 [28]	0.2562 [28]	0.3500	0.1500	0.2000			0.2000
$MnCl_2$	0.0378 [29]											1.0·10^{-5}	
$MnSO_4·4H_2O$		0.0500	0.0660 [30]	0.0264 [31]	0.0100	0.0100	0.0100	0.0463 [32]	0.0200	0.0264 [31]			
$(NH_4)_6Mo_7O_{24}·4H_2O$		0.0100								3.7·10^{-6}		3.7·10^{-6}	
$Na_2MoO_2·2H_2O$	4.8·10^{-5}												
NaCl				0.0200	0.0100	0.0100	0.0100	0.0100				2.9220	
$NiCl_2$	1.3·10^{-5}												
$NiSO_4·6H_2O$		0.0100											
$ZnCl_2$	6.8·10^{-5}												
$ZnSO_4·7H_2O$		0.0100								2.9·10^{-6}		2.9·10^{-4} [33]	
VITAMINS													
Thiamine (B_1)	0.00016	0.00500			0.00500	0.00500	0.00500	0.00079 [34]	0.00079 [34]			0.00080 [35]	
Riboflavin (B_2)	0.00010	0.00500	0.00200	0.00050	0.00500	0.00300	0.00500	0.00100	0.00100	0.00020	0.00100 [36]	0.00098	0.00100
Niacin (Nicotinic acid; B_3)	0.00020	0.00500	0.00200	0.00050	0.00400	0.00300	0.00500	0.00100	0.00100	0.00017	0.00100		0.00100
Niacinamide (B_3)												0.00098	
Ca-pantothenate (B_5)	9.5·10^{-5}	0.00543 [37]	0.00200	0.00050	0.00500	0.00300	0.00500	0.00100	0.00100	0.00010	0.00100	0.00048 [38]	0.00100
Pyridoxal (B_6)			0.00200						0.00060 [39]	0.00005		0.00167 [40]	0.00200
Pyridoxamine (B_6)	0.00035										0.00500		
Pyridoxine (B_6)	5.1·10^{-5}	0.00500		0.00050	0.00400	0.00400	0.00400	0.00100					
Biotin (B_7)	2.0·10^{-5}	0.00500	0.00100	0.00005	0.00500	0.00100	0.00500	0.00001	0.01000		0.01000	9.8·10^{-5}	
***Myo* -inositol (formerly B_8)**		0.00500		0.00050	0.00500	0.00500	0.00500						
Folic acid (B_9)	4.4·10^{-5}	0.00500	0.00050	0.00010	0.00500	0.00050	0.00500	0.00001	0.00100	0.00003		0.00102	
Cyanocobalamine (B_{12})	9.5·10^{-5}	0.00500	0.00100	0.00001	0.00500	0.00500	0.00500		0.00100	0.00001			
***para* -Aminobenzoic acid (pABA, formerly B_x)**	9.6·10^{-5}	0.00500	0.00050	0.00010	0.00500	0.00060	0.00500		0.01000				
Ascorbic acid (C)				0.00050				0.50000					
NUCLEOBASES, NUCLEOSIDES													
Adenine (A)	0.0095		0.0200		0.0100	0.0100	0.0100		0.0500	0.0500			
Guanine (G)	0.0106				0.0100	0.0100	0.0100		0.0403 [41]	0.0500			
Uracil (U)	0.0101				0.0100	0.0100	0.0100			0.0500			0.0100
Xanthine	0.0106		0.0400		0.0100	0.0100	0.0100			0.0500			
Deoxyguanosine (dG)									0.0500				
Cytidylic acid									0.0500				
Inosine (I)				10.0000									
Orotic acid			0.5000										
FATTY ACID PRECURSORS													
K-acetate				10.00					10.00	7.35			
Na-acetate	6.75	2.00	4.00		20.00	20.00	20.00					1.23	6.00
Lipoic acid (DL-6,8-Thioctic acid)	5.0·10^{-5}												
Tween 80	1.06 [42]	1.06 [42]		1.00	1.06 [42]	1.06 [42]			1.0·10^{-3}	1.00			1.00
OTHER COMPOUNDS													
$(NH_4)_2SO_4$								0.40			0.18		
Ammonium citrate [43]		1.00		2.00									
Ca-lactate				1.00									
Citric acid	0.83												
D,L-aminobutyrate				0.10									
Disodium glycerolphosphate $5H_2O$								19.00					
H_3BO_3	6.2·10^{-6}									2.5·10^{-5}		2.5·10^{-5}	
K_2SO_4												0.05	
KCl								0.50					
Na_2HPO_4	2.00									0.30			
Na-citrate-$2H_2O$								0.20					
$NaH_2PO_4·H_2O$										0.80			
NH4Cl	1.00				3.00	3.00	3.00					0.51	
Sodium thioglycollate									0.50	0.50			
Triammonium citrate			1.00										

[1] In addition, if the vitamin comnosition was altered. MPL sunoorted growth of several strains of *Lb. buchneri* . *Lb. fermentum* . *Lb. plantarum* . *Lb. zeae* . *Leuc. mesenteroides* . *S. thermophilus* .

[2] *L. lactis* MG1363, MG1614, NCDO712, C2, IL1403; *L. cremoris* 3107, 901-1, 936-1, Wg2.

[3] *L. lactis* C2 2.1±0.4. IL1403 2.5±0.1. MG1363 3.0±0.3. MG1614 2.9±0.3 and NCDO712 2.5±0.3 h^{-1}. *L. cremoris* 3107 1.6±0.3. 901-1 2.0±0.4. 936-1 2.0±0.1 and Wg2 1.7±0.8 h^{-1}.

[4] 1.40±0.08 for strain CRL974 and 1.50±0.07 for CRL1062.

[5] 0.44 h^{-1} for strain N8 and 0.56 h^{-1} for N4.

[6] The calculation is based on the doubling time of 70 min.

[7] 0.5 h^{-1} for strain IL1403 and 0.6 h^{-1} for NCDO2118.

[8] *L. lactis* C2 0.63±0.13. IL1403 0.64±0.04. MG1363 0.73±0.03. MG1614 0.63±0.04 and NCDO712 0.55±0.04 h^{-1}. *L. cremoris* 3107 0.45±0.02. 901-1 0.41±0.03. 936-1 0.44±0.03 and Wg2 0.22±0.05 h^{-1}.

[9] 0.28±0.02 h^{-1} for strain CRL974 and 0.34±0.02 h^{-1} for CRL1062.

[10] Fructose is added onlv for the cultivation of *Lb. brevis* .

[11] The carbon source can be either glucose or lactose.

[12] 3 g·L^{-1} of $K_2HPO_4·3H_2O$

[13] Concentrations in brackets were used in pH-regulated bioreactors

[14] [103] and [213] used DL-alanine

[15] 0.1 g·L^{-1} of Asparagine-H_2O

[16] 0.2 g·L^{-1} of Cysteine-HCl

[17] 0.28 g·L^{-1} of Cysteine-HCl

[18] 0.2 g·L^{-1} of Histidine-HCl

[19] 1.2 g·L^{-1} of Lysine

[20] 0.2 g·L^{-1} of Lysine

[21] 1.05 g·L^{-1} of Lysine

[22] 0.1 g·L^{-1} of Lysine

[23] 0.1 g·L^{-1} of $CaCl_2·2H_2O$

[24] 0.03 µM of $CoCl_2$

[25] 0.01 g·L^{-1} of $CuSO_4·5H_2O$

[26] 0.01 mM of $FeSO_4$

[27] 0.52 mM of $MgCl_2$

[28] 0.2 g·L^{-1} of $MgSO_4·4H_2O$

[29] 0.3 mM of $MnCl_2·4H_2O$

[30] 0.05 g·L^{-1} of $MnSO_4·H_2O$

[31] 0.02 g·L^{-1} of $MnSO_4·H_2O$

[32] 0.05 g·L^{-1} of $MnSO_4·5H_2O$

[33] 0.01 µM of $ZnSO_4$

[34] 0.001 g·L^{-1} of Thiamine-HCl

[35] 0.003 mM of Thiamine-HCl

[36] Riboflavin is added only for the cultivation of IL1403 strain.

[37] 0.005 g·L^{-1} of Pantothenic acid

[38] 0.002 mM of Pantothenic acid

[39] 0.001 g·L^{-1} of Pvridoxal-ethvl-acetal-HCl

[40] 0.01 mM of Pyridoxal-HCl

[41] 0.05 g·L^{-1} of Guanine-HCl

[42] 1 mL·L^{-1} (0.1% v/v) of Tween 80, if the density is 1.06 g·mL^{-1}

[43] Synonyms: Diammonium citrate

Table S8. Minimal defined media from the literature for lactic acid bacteria. These media have been minimized in the number of nutrients and their concentrations.

Medium	BL	CDM	CDM	LMCDM	MDM	MS13	MS14	MS15	PMM5	PMM7	SDM
Strain	*L. lactis* NCDO712	*Leuc. mesenteroides* ATCC8293	*S. thermophilus* [1]	*Leuc. dextranicum* , *Leuc. mesenteroides* [2]	*Lb. lactis* CRL581, CRL654	*L. lactis* NCDO2118	*L. lactis* NCDO2118	*L. lactis* IL1403	*Lb. plantarum* WCFS1	*Lb. plantarum* WCFS1	*E. faecalis* B951
Reference	[92]	[99]	[100]	[95]	[98]	[91]	[86]	[91]	[113]	[113]	[81]
GROWTH OF CELLS											
OD (λ=600 nm, if not stated otherwise)	2.3 (450 nm)	2		1.67±0.18 (620 nm) [3]		2.7 (580 nm)		2.7 (580 nm)	0.10±0.02	1.10±0.05	
μ_{max} (h^{-1})	0.28	0.22±0.01	0.38-0.64	0.85±0.10 [3]	0.30-0.32 [4]	0.23	0.2	0.37		0.15	
COMPOSITION ($g \cdot L^{-1}$)											
SUGARS											
Fructose		20									
Glucose	9				10	10	20	10	11	11	15
Lactose			5	10							
BUFFERS											
K_2HPO_4			2.5	3.0	3.0	7.5 (2.5) [5]	7.5 (2.5) [5]	7.5 (2.5) [5]			7.0
KH_2PO_4	0.2	6.0	3.0	2.5	3.0	9.0 (3.0) [5]	9.0 (3.0) [5]	9.0 (3.0) [5]			2.0
Phosphate									6.0	6.0	
MOPS	39.8										
Tricine	0.7										
AMINO ACIDS											
L-Alanine (Ala)					0.1000						
L-Arginine (Arg)		0.1034 [6]		0.1250	0.1000			0.1200	0.1250	0.1250	1.0000
L-Asparagine (Asn)	0.105696										
L-Aspartate (Asp)					0.2000						
L-Cysteine (Cys)		0.1922 [7]	0.1500	0.2500	0.2000					0.1300	1.0000
L-Glutamine (Gln)	0.102298	0.5000	0.2000	0.3900							
L-Glutamate (Glu)	0.3090				0.2000	0.1000	0.6000	0.1000	0.5000	0.5000	1.0000
Glycine (Gly)								0.1100			1.0000
L-Histidine (His)	0.0465	0.1215 [8]		0.1500	0.1000						1.0000
L-Isoleucine (Ile)	0.1049	0.2130	0.0500	0.2130	0.1000	0.2000	0.3300	0.2000	0.2100	0.2100	1.0000
L-Leucine (Leu)	0.1968	0.4750	0.1000	0.4750	0.1000	0.4700	0.6600	0.4700	0.4750	0.4750	1.0000
L-Lysine (Lys)					0.1000						1.0000
L-Methionine (Met)	0.0746	0.2500	0.0600	0.1250	0.1000	0.1200	0.1200	0.1200	0.1250	0.1250	1.0000
L-Phenylalanine (Phe)		0.2750		0.2750	0.1000				0.2750	0.2750	1.0000
L-Proline (Pro)		0.6750		0.6750							
L-Serine (Ser)					0.1000	0.3400	2.0800	0.3400			1.0000
L-Threonine (Thr)		0.2250		0.2250				0.2300	0.2250	0.2250	1.0000
L-Tryptophan (Trp)		0.0500		0.0500	0.1000				0.0500	0.0500	1.0000
L-Tyrosine (Tyr)					0.1000				0.2500	0.2500	
L-Valine (Val)	0.3046	0.5000	0.2000	0.3250	0.1000	0.3300	0.3300	0.3300	0.3250	0.3250	1.0000
MINERAL SALTS											
$CaCl_2$	$5.5 \cdot 10^{-5}$		0.0075 [9]								
$CoCl_2$	$3.9 \cdot 10^{-6}$										
$CuSO_4$	$1.6 \cdot 10^{-6}$										
$FeSO_4 \cdot 7H_2O$	0.0028 [10]										0.0080
$MgCl_2 \cdot 6H_2O$	0.1057 [11]	0.2000	0.1600	0.2000		0.2000	0.2000	0.2000	0.4271 [12]	0.4271 [12]	
$MgSO_4 \cdot 7H_2O$					0.2000						0.2048 [13]
$MnCl_2$	$1.0 \cdot 10^{-5}$								0.0160	0.0160	
$MnSO_4 \cdot H_2O$		0.1000		0.0100							
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	$3.7 \cdot 10^{-6}$										
NaCl	2.9220										
$ZnSO_4$	$1.6 \cdot 10^{-6}$										
VITAMINS											
Thiamine-HCl (B_1) [14]	0.00101181	0.00100	$5.0 \cdot 10^{-6}$	0.00100							
Riboflavin (B_2)	0.00098		0.00005	0.00100	0.00100	0.00100	0.00100	0.00100	0.00100	0.00100	0.00200
Niacin (Nicotinic acid; B_3)		0.01000	0.00010	0.00100	0.00100	0.00100	0.00100	0.00100	0.00100	0.00100	0.00200
Niacinamide (B_3)	0.00098										
Ca-pantothenate (B_5)	0.00048 [15]	0.001	0.0001	0.00100	0.00109 [16]	0.00100	0.00100	0.00100	0.00109 [16]	0.00109 [16]	0.02000
Pyridoxal-HCl (B_6)	0.0020362			0.00200	0.00244 [17]						0.02436 [18]
Pyridoxamine (B_6)			0.00066 [19]			0.00500	0.00500	0.00500		0.00500	
Biotin (B_7)	$9.8 \cdot 10^{-5}$					0.01000	0.01000	0.01000			
Folic acid (B_9)	0.00115	0.01000		0.00100							0.00200
Cyanocobalamine (B_{12})					0.00100						
Ascorbic acid (C)		0.50000	0.50000						0.50000	0.50000	
NUCLEOBASES, NUCLEOSIDES											
Adenine (A)		0.0100		0.0100	0.0100						0.0147 [20]
Guanine (G)					0.0100						
Uracil (U)		0.0100		0.0100	0.0100						0.0200
Deoxythymidine (dT)				0.0050							
Inosine (I)		0.0050		0.0050							
FATTY ACID PRECURSORS											
Na-acetate	1.23	5.00	1.00	1.00	5.00				1.00	1.00	
Tween 80		1.06 [21]		1.06 [21]							
OTHER COMPOUNDS											
Ammonium citrate			0.60							0.60	
H_3BO_3	$2.5 \cdot 10^{-5}$										
K_2SO_4	0.0488										
NH_4Cl	0.5082										
Triammonium citrate		2.00		0.60							
Urea			0.24								

[1] Strains ST1, ST2, ST7, ST8, ST11, ST14, ST16, ST18, ST21, ST22, ST23, ST24, ST25, ST26, Z302

[2] *Leuc. dextranicum* CNRZ1694, CNRZ1742, CNRZ1744 and *Leuc. mesenteroides* CNRZ1463, CNRZ1697, CNRZ1750.

[3] Average of all strains tested.

[4] 0.32 h^{-1} for strain CRL 581 and 0.30 h^{-1} for CRL 654.

[5] Concentrations in brackets were used in pH-regulated bioreactors

[6] 0.125 $g \cdot L^{-1}$ of Arginine-HCl

[7] 0.25 $g \cdot L^{-1}$ of Cysteine-HCl

[8] 0.15 $g \cdot L^{-1}$ of Histidine-HCl

[9] 0.01 $g \cdot L^{-1}$ of $CaCl_2 \cdot 2H_2O$

[10] 0.01 mM of $FeSO_4$

[11] 0.52 mM of $MgCl_2$

[12] 0.2 $g \cdot L^{-1}$ of $MgCl_2$

[13] 0.1 $g \cdot L^{-1}$ of $MgSO_4$

[14] Synonyms: Thiamine dichloride

[15] 0.002 mM of Pantothenic acid

[16] 0.001 $g \cdot L^{-1}$ of Pantothenic acid

[17] 0.002 $g \cdot L^{-1}$ of Pyridoxal

[18] 0.02 $g \cdot L^{-1}$ of Pyridoxal

[19] 0.0008 $g \cdot L^{-1}$ of Pyridoxamine-HCl

[20] 0.02 $g \cdot L^{-1}$ of Adenine sulfate

[21] 1 $mL \cdot L^{-1}$ of Tween 80, if the density is 1.06 $g \cdot mL^{-1}$

Table S9. Chemically defined media with **minimal amino acid concentrations** that were developed in this thesis for *L. lactis* IL1403. These media were used for batch cultivations on **microtiter plates** with the **cultivation volume 0.24 mL.**
The difference of amino acid concentrations with the initial medium BS7 has been shown for each medium (reduced 2, 4, 8 or 16 times).
The concentrations of buffers, mineral salts and vitamins were the same as for the medium BS7 (see Table 5 in Materials and Methods section of the thesis), but these media did not contain adenine, hypoxanthine and lipoic acid.

Medium	JA	JB	JC	JD	JE	JF	JG	JH	JI	JJ	JM	JN	JO	JP	JR	JS	JT	JU	JV	JW
OD_{max} (600 nm), n=3	0.234±0.014	0.167±0.003	0.197±0.014	0.205±0.002	0.201±0.004	0.212±0.001	0.233±0.020	0.180±0.005	0.194±0.005	0.150±0.005	0.192±0.004	0.206±0.022	0.220±0.010	0.206±0.002	0.205±0.013	0.192±0.008	0.205±0.007	0.174±0.006	0.195±0.002	0.192±0.006
μ_{max} (h^{-1}), n=3	0.33±0.05	0.37±0.01	0.39±0.02	0.40±0.04	0.41±0.01	0.40±0	0.44±0.01	0.34±0.02	0.39±0.01	0.38±0.01	0.40±0.01	0.40±0	0.42±0	0.42±0.01	0.41±0.01	0.41±0	0.42±0.01	0.41±0.01	0.41±0.02	0.37±0.01
COMPOSITION ($g \cdot L^{-1}$)	**JA**	**JB**	**JC**	**JD**	**JE**	**JF**	**JG**	**JH**	**JI**	**JJ**	**JM**	**JN**	**JO**	**JP**	**JR**	**JS**	**JT**	**JU**	**JV**	**JW**
SUGAR																				
Glucose	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5
BUFFERS																				
K_2HPO_4	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9
KH_2PO_4	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75
MOPS	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5
AMINO ACIDS																				
L-Alanine (Ala)	0.0196 4x↓	0.0196 4x↓	0.0196 4x↓	0.0196 4x↓	0.0196 4x↓	0.0196 4x↓	0.0196 4x↓	0.0196 4x↓	0.0196 4x↓	0.0196 4x↓	0.0196 4x↓	0.0196 4x↓	0.0196 4x↓	0.0196 4x↓	0.0196 4x↓	0.0196 4x↓	0.0196 4x↓	0.0196 4x↓	0.0196 4x↓	0.0196 4x↓
L-Arginine (Arg)	0.0463 4x↓	0.0463 4x↓	0.0463 4x↓	0.0463 4x↓	0.0463 4x↓	0.0463 4x↓	0.0463 4x↓	0.0463 4x↓	0.0463 4x↓	0.0463 4x↓	0.0463 4x↓	0.0463 4x↓	0.0463 4x↓	0.0463 4x↓	0.0463 4x↓	0.0232 8x↓	0.0463 4x↓	0.0463 4x↓	0.0232 8x↓	0.0463 4x↓
L-Asparagine (Asn)	0.0184 4x↓	0.0184 4x↓	0.0184 4x↓	0.0184 4x↓	0.0184 4x↓	0.0184 4x↓	0.0184 4x↓	0.0184 4x↓	0.0184 4x↓	0.0092 8x↓	0.0184 4x↓	0.0184 4x↓	0.0184 4x↓	0.0184 4x↓	0.0184 4x↓	0.0184 4x↓	0.0184 4x↓	0.0184 4x↓	0.0184 4x↓	0.0184 4x↓
L-Aspartate (Asp)	0.0181 4x↓	0.0181 4x↓	0.0181 4x↓	0.0181 4x↓	0.0181 4x↓	0.0181 4x↓	0.0181 4x↓	0.0181 4x↓	0.0181 4x↓		0.0181 4x↓	0.0181 4x↓	0.0181 4x↓	0.0181 4x↓	0.0181 4x↓	0.0181 4x↓	0.0181 4x↓	0.0181 4x↓	0.0181 4x↓	0.0181 4x↓
L-Cysteine (Cys)	0.0159 4x↓	0.0159 4x↓	0.0159 4x↓	0.0159 4x↓	0.0159 4x↓	0.0159 4x↓	0.0159 4x↓	0.0159 4x↓	0.0159 4x↓	0.0159 4x↓	0.0159 4x↓	0.0159 4x↓	0.0159 4x↓	0.0159 4x↓	0.0159 4x↓	0.0159 4x↓	0.0159 4x↓	0.0159 4x↓	0.0159 4x↓	0.0159 4x↓
L-Glutamine (Gln)	0.0331 4x↓	0.0331 4x↓	0.0331 4x↓	0.0331 4x↓	0.0331 4x↓	0.0331 4x↓	0.0331 4x↓	0.0331 4x↓	0.0331 4x↓	0.0165 8x↓	0.0331 4x↓	0.0331 4x↓	0.0331 4x↓	0.0331 4x↓	0.0331 4x↓	0.0331 4x↓	0.0331 4x↓	0.0331 4x↓	0.0331 4x↓	0.0331 4x↓
L-Glutamate (Glu)	0.0176 4x↓	0.0176 4x↓	0.0176 4x↓	0.0176 4x↓	0.0176 4x↓	0.0176 4x↓	0.0176 4x↓	0.0176 4x↓			0.0176 4x↓	0.0176 4x↓	0.0176 4x↓	0.0176 4x↓	0.0176 4x↓	0.0176 4x↓	0.0176 4x↓	0.0176 4x↓	0.0176 4x↓	0.0176 4x↓
Glycine (Gly)	0.0144 4x↓	0.0144 4x↓	0.0144 4x↓	0.0144 4x↓	0.0144 4x↓	0.0144 4x↓	0.0144 4x↓	0.0144 4x↓	0.0144 4x↓	0.0144 4x↓	0.0144 4x↓	0.0144 4x↓	0.0144 4x↓	0.0144 4x↓	0.0144 4x↓	0.0144 4x↓	0.0144 4x↓	0.0144 4x↓	0.0144 4x↓	0.0144 4x↓
L-Histidine (His)	0.0149 4x↓	0.0149 4x↓	0.0149 4x↓	0.0149 4x↓	0.0149 4x↓	0.0149 4x↓	0.0149 4x↓	0.0149 4x↓	0.0149 4x↓	0.0149 4x↓	0.0149 4x↓	0.0149 4x↓	0.0149 4x↓	0.0149 4x↓	0.0149 4x↓	0.0149 4x↓	0.0149 4x↓	0.0149 4x↓	0.0149 4x↓	0.0149 4x↓
L-Isoleucine (Ile)	0.0255 4x↓	0.0255 4x↓	0.0127 8x↓	0.0064 16x↓	0.0064 16x↓	0.0127 8x↓	0.0255 4x↓	0.0255 4x↓	0.0255 4x↓	0.0255 4x↓	0.0255 4x↓	0.0255 4x↓	0.0255 4x↓	0.0255 4x↓	0.0255 4x↓	0.0255 4x↓	0.0255 4x↓	0.0255 4x↓	0.0255 4x↓	0.0255 4x↓
L-Leucine (Leu)	0.0259 8x↓	0.0129 16x↓	0.0518 4x↓	0.0518 4x↓	0.0129 16x↓	0.0259 8x↓	0.0518 4x↓	0.0518 4x↓	0.0518 4x↓	0.0518 4x↓	0.0518 4x↓	0.0518 4x↓	0.0518 4x↓	0.0518 4x↓	0.0518 4x↓	0.0518 4x↓	0.0518 4x↓	0.0518 4x↓	0.0518 4x↓	0.0518 4x↓
L-Lysine-HCl (Lys)	0.0394 4x↓	0.0394 4x↓	0.0394 4x↓	0.0394 4x↓	0.0394 4x↓	0.0394 4x↓	0.0394 4x↓	0.0394 4x↓	0.0394 4x↓	0.0394 4x↓	0.0394 4x↓	0.0394 4x↓	0.0394 4x↓	0.0394 4x↓	0.0394 4x↓	0.0394 4x↓	0.0197 8x↓	0.0098 16x↓	0.0197 8x↓	0.0394 4x↓
L-Methionine (Met)	0.0102 4x↓	0.0102 4x↓	0.0102 4x↓	0.0102 4x↓	0.0102 4x↓	0.0102 4x↓	0.0102 4x↓	0.0102 4x↓	0.0102 4x↓	0.0102 4x↓	0.0102 4x↓	0.0102 4x↓	0.0102 4x↓	0.0102 4x↓	0.0102 4x↓	0.0102 4x↓	0.0102 4x↓	0.0102 4x↓	0.0102 4x↓	0.0102 4x↓
L-Phenylalanine (Phe)	0.0215 4x↓	0.0215 4x↓	0.0215 4x↓	0.0215 4x↓	0.0215 4x↓	0.0215 4x↓	0.0215 4x↓	0.0215 4x↓	0.0215 4x↓	0.0215 4x↓	0.0215 4x↓	0.0215 4x↓	0.0108 8x↓	0.0054 16x↓		0.0215 4x↓	0.0215 4x↓	0.0215 4x↓	0.0215 4x↓	0.0215 4x↓
L-Proline (Pro)	0.0229 4x↓	0.0229 4x↓	0.0229 4x↓	0.0229 4x↓	0.0229 4x↓	0.0229 4x↓	0.0229 4x↓	0.0229 4x↓	0.0229 4x↓	0.0229 4x↓	0.0229 4x↓	0.0229 4x↓	0.0229 4x↓	0.0229 4x↓	0.0229 4x↓	0.0229 4x↓	0.0229 4x↓	0.0229 4x↓	0.0229 4x↓	0.0229 4x↓
L-Serine (Ser)	0.0408 4x↓	0.0408 4x↓	0.0408 4x↓	0.0408 4x↓	0.0408 4x↓	0.0408 4x↓	0.0408 4x↓	0.0408 4x↓	0.0408 4x↓	0.0408 4x↓	0.0408 4x↓	0.0408 4x↓	0.0408 4x↓	0.0408 4x↓	0.0408 4x↓	0.0408 4x↓	0.0408 4x↓	0.0408 4x↓	0.0408 4x↓	0.0408 4x↓
L-Threonine (Thr)	0.0190 4x↓	0.0190 4x↓	0.0190 4x↓	0.0190 4x↓	0.0190 4x↓	0.0190 4x↓	0.0190 4x↓	0.0190 4x↓	0.0190 4x↓	0.0190 4x↓	0.0190 4x↓	0.0190 4x↓	0.0190 4x↓	0.0190 4x↓	0.0190 4x↓	0.0190 4x↓	0.0190 4x↓	0.0190 4x↓	0.0095 8x↓	
L-Tryptophan (Trp)	0.0041 4x↓	0.0041 4x↓	0.0041 4x↓	0.0041 4x↓	0.0041 4x↓	0.0041 4x↓	0.0041 4x↓	0.0041 4x↓	0.0041 4x↓	0.0041 4x↓	0.0021 8x↓	0.0010 16x↓	0.0021 8x↓	0.0010 16x↓	0.0010 16x↓	0.0041 4x↓	0.0041 4x↓	0.0041 4x↓	0.0041 4x↓	0.0041 4x↓
L-Tyrosine (Tyr)	0.0074 4x↓	0.0074 4x↓	0.0074 4x↓	0.0074 4x↓	0.0074 4x↓	0.0074 4x↓	0.0074 4x↓	0.0074 4x↓	0.0074 4x↓	0.0074 4x↓	0.0037 8x↓	0.0018 16x↓	0.0037 8x↓	0.0018 16x↓	0.0018 16x↓	0.0074 4x↓	0.0074 4x↓	0.0074 4x↓	0.0074 4x↓	0.0074 4x↓
L-Valine (Val)	0.0268 4x↓	0.0268 4x↓	0.0268 4x↓	0.0268 4x↓	0.0268 4x↓	0.0268 4x↓	0.0134 8x↓	0.0067 16x↓	0.0268 4x↓	0.0268 4x↓	0.0268 4x↓	0.0268 4x↓	0.0268 4x↓	0.0268 4x↓	0.0268 4x↓	0.0268 4x↓	0.0268 4x↓	0.0268 4x↓	0.0268 4x↓	0.0268 4x↓
MINERAL SALTS																				
$CaCl_2$	0.0500	0.0500	0.0500	0.0500	0.0500	0.0500	0.0500	0.0500	0.0500	0.0500	0.0500	0.0500	0.0500	0.0500	0.0500	0.0500	0.0500	0.0500	0.0500	0.0500
$CoSO_4 \cdot 7H_2O$	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030
$CuSO_4 \cdot 5H_2O$	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030
$FeSO_4 \cdot 7H_2O$	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014
$MgSO_4 \cdot 7H_2O$	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000
$MnSO_4 \cdot H_2O$	0.0160	0.0160	0.0160	0.0160	0.0160	0.0160	0.0160	0.0160	0.0160	0.0160	0.0160	0.0160	0.0160	0.0160	0.0160	0.0160	0.0160	0.0160	0.0160	0.0160
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030
NaCl	2.9000	2.9000	2.9000	2.9000	2.9000	2.9000	2.9000	2.9000	2.9000	2.9000	2.9000	2.9000	2.9000	2.9000	2.9000	2.9000	2.9000	2.9000	2.9000	2.9000
$ZnSO_4 \cdot 7H_2O$	0.0050	0.0050	0.0050	0.0050	0.0050	0.0050	0.0050	0.0050	0.0050	0.0050	0.0050	0.0050	0.0050	0.0050	0.0050	0.0050	0.0050	0.0050	0.0050	0.0050
VITAMINS																				
Thiamine-HCl (B_1)	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051
Riboflavin (B_2)	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033
Nicotinamide (B_3)	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033
Ca-pantothenate (B_5)	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065
Pyridoxine (B_6)	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064
Biotin (B_7)	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031
Folic acid (B_9)	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121

Table S10. Chemically defined media with **minimal amino acid concentrations** that were developed in this thesis for *L. lactis* IL1403. These media were used for batch cultivations in **test tubes**.
The difference of amino acid concentrations with the initial medium BS7 has been shown for each medium (reduced 2, 4 or 8 times).
The concentrations of buffers, mineral salts and vitamins were the same as for the medium BS7 (see Table 5 in Materials and Methods section of the thesis), but these media did not contain adenine and hypoxanthine.
The composition and growth results for K40 medium can be seen in Table 5 (Materials and Methods section of the thesis).

Medium	K1	K2	K3	K4	K5	K6	K7	K8	K9	K10	K11	K12	K13	K14	K15	K16	K17	K18
OD$_{max}$ (600 nm), n=3	1.06±0.03	1.11±0.04	0.96±0.01	0.97±0.02	1.07±0.02	1.04±0.03	1.16±0.01	1.10±0.02	0.99±0.02	1.15±0.02	1.17±0.02	1.25±0.01	1.12±0.03	1.11±0.03	1.11±0.02	1.12±0.03	1.15±0.01	1.20±0.03
μ_{max} (h^{-1}), n=3	0.39±0.04	0.35±0.12	0.36±0.04	0.34±0.04	0.34±0.10	0.35±0.03	0.41±0.08	0.39±0.04	0.31±0.08	0.51±0.01	0.45±0.06	0.42±0.04	0.55±0.05	0.58±0.06	0.45±0.01	0.51±0.07	0.46±0.04	0.44±0.08
Lag-phase (h)	8.0	8.0	8.0	7.0	8.0	7.0	8.0	8.0	8.0	7.0	7.0	6.0	8.5	8.0	8.5	8.0	8.0	8.5
Exponential phase (h)																		
COMPOSITION (g·L^{-1})	K1	K2	K3	K4	K5	K6	K7	K8	K9	K10	K11	K12	K13	K14	K15	K16	K17	K18
SUGAR																		
Glucose	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5
BUFFERS																		
K_2HPO_4	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9
KH_2PO_4	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75
MOPS	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5
AMINO ACIDS																		
L-Alanine (Ala)	0.0391 2x↓	0.0391 2x↓	0.0391 2x↓	0.0391 2x↓	0.0391 2x↓	0.0391 2x↓	0.0391 2x↓	0.0391 2x↓	0.0391 2x↓	0.0391 2x↓	0.0391 2x↓	0.0391 2x↓	0.0391 2x↓	0.0391 2x↓	0.0196 4x↓	0.0196 4x↓	0.0196 4x↓	0.0196 4x↓
L-Arginine (Arg)	0.0926 2x↓	0.0926 2x↓	0.0926 2x↓	0.0926 2x↓	0.0926 2x↓	0.0926 2x↓	0.0926 2x↓	0.0926 2x↓	0.0926 2x↓	0.0926 2x↓	0.0926 2x↓	0.0926 2x↓	0.0463 4x↓	0.0232 8x↓	0.0926 2x↓	0.0463 4x↓	0.0232 8x↓	0.0463 4x↓
L-Asparagine (Asn)	0.0735	0.0735	0.0735	0.0735	0.0735	0.0735	0.0735	0.0735	0.0735	0.0735	0.0735	0.0735	0.0735	0.0735	0.0735	0.0735	0.0735	0.0735
L-Aspartate (Asp)																		
L-Cysteine (Cys)	0.0159 4x↓	0.0318 2x↓	0.0079 8x↓	0.0079 8x↓	0.0159 4x↓	0.0159 4x↓	0.0318 2x↓	0.0318 2x↓	0.0079 8x↓	0.0079 8x↓	0.0159 4x↓	0.0079 8x↓	0.0079 8x↓	0.0079 8x↓	0.0079 8x↓	0.0079 8x↓	0.0079 8x↓	0.0159 4x↓
L-Glutamine (Gln)	0.1322	0.1322	0.1322	0.1322	0.1322	0.1322	0.1322	0.1322	0.1322	0.1322	0.1322	0.1322	0.1322	0.1322	0.1322	0.1322	0.1322	0.1322
L-Glutamate (Glu)																		
Glycine (Gly)	0.0072 8x↓	0.0072 8x↓	0.0072 8x↓	0.0072 8x↓	0.0072 8x↓	0.0072 8x↓	0.0072 8x↓	0.0072 8x↓	0.0072 8x↓	0.0072 8x↓	0.0072 8x↓	0.0072 8x↓	0.0072 8x↓	0.0072 8x↓	0.0072 8x↓	0.0072 8x↓	0.0072 8x↓	0.0072 8x↓
L-Histidine (His)	0.0075 8x↓	0.0075 8x↓	0.0075 8x↓	0.0075 8x↓	0.0075 8x↓	0.0075 8x↓	0.0075 8x↓	0.0075 8x↓	0.0075 8x↓	0.0075 8x↓	0.0075 8x↓	0.0075 8x↓	0.0075 8x↓	0.0075 8x↓	0.0075 8x↓	0.0075 8x↓	0.0075 8x↓	0.0075 8x↓
L-Isoleucine (Ile)	0.0255 4x↓	0.0255 4x↓	0.0255 4x↓	0.0255 4x↓	0.0255 4x↓	0.0255 4x↓	0.0255 4x↓	0.0255 4x↓	0.0255 4x↓	0.0255 4x↓	0.0255 4x↓	0.0255 4x↓	0.0255 4x↓	0.0255 4x↓	0.0255 4x↓	0.0255 4x↓	0.0255 4x↓	0.0255 4x↓
L-Leucine (Leu)	0.0518 4x↓	0.0518 4x↓	0.0518 4x↓	0.0518 4x↓	0.0518 4x↓	0.0518 4x↓	0.0518 4x↓	0.0518 4x↓	0.0518 4x↓	0.0518 4x↓	0.0518 4x↓	0.0518 4x↓	0.0518 4x↓	0.0518 4x↓	0.0518 4x↓	0.0518 4x↓	0.0518 4x↓	0.0518 4x↓
L-Lysine-HCl (Lys)	0.0394 4x↓	0.0394 4x↓	0.0394 4x↓	0.0394 4x↓	0.0394 4x↓	0.0394 4x↓	0.0394 4x↓	0.0394 4x↓	0.0394 4x↓	0.0394 4x↓	0.0394 4x↓	0.0394 4x↓	0.0394 4x↓	0.0394 4x↓	0.0394 4x↓	0.0394 4x↓	0.0394 4x↓	0.0394 4x↓
L-Methionine (Met)	0.0102 4x↓	0.0102 4x↓	0.0203 2x↓	0.0407	0.0203 2x↓	0.0407	0.0203 2x↓	0.0407	0.0102 4x↓	0.0102 4x↓	0.0102 4x↓	0.0203 2x↓	0.0102 4x↓	0.0102 4x↓	0.0102 4x↓	0.0102 4x↓	0.0102 4x↓	0.0203 2x↓
L-Phenylalanine (Phe)	0.0430 2x↓	0.0430 2x↓	0.0430 2x↓	0.0430 2x↓	0.0430 2x↓	0.0430 2x↓	0.0430 2x↓	0.0430 2x↓	0.0430 2x↓	0.0430 2x↓	0.0430 2x↓	0.0430 2x↓	0.0430 2x↓	0.0430 2x↓	0.0430 2x↓	0.0430 2x↓	0.0430 2x↓	0.0430 2x↓
L-Proline (Pro)	0.0115 8x↓	0.0115 8x↓	0.0115 8x↓	0.0115 8x↓	0.0115 8x↓	0.0115 8x↓	0.0115 8x↓	0.0115 8x↓	0.0229 4x↓	0.0458 2x↓	0.0229 4x↓	0.0229 4x↓	0.0115 8x↓	0.0115 8x↓	0.0115 8x↓	0.0115 8x↓	0.0115 8x↓	0.0229 4x↓
L-Serine (Ser)	0.0817 2x↓	0.0817 2x↓	0.0817 2x↓	0.0817 2x↓	0.0817 2x↓	0.0817 2x↓	0.0817 2x↓	0.0817 2x↓	0.0817 2x↓	0.0817 2x↓	0.0817 2x↓	0.0817 2x↓	0.0817 2x↓	0.0817 2x↓	0.0817 2x↓	0.0817 2x↓	0.0817 2x↓	0.0817 2x↓
L-Threonine (Thr)	0.0379 2x↓	0.0379 2x↓	0.0379 2x↓	0.0379 2x↓	0.0379 2x↓	0.0379 2x↓	0.0379 2x↓	0.0379 2x↓	0.0379 2x↓	0.0379 2x↓	0.0379 2x↓	0.0379 2x↓	0.0379 2x↓	0.0379 2x↓	0.0379 2x↓	0.0379 2x↓	0.0379 2x↓	0.0379 2x↓
L-Tryptophan (Trp)	0.0082 2x↓	0.0082 2x↓	0.0082 2x↓	0.0082 2x↓	0.0082 2x↓	0.0082 2x↓	0.0082 2x↓	0.0082 2x↓	0.0082 2x↓	0.0082 2x↓	0.0082 2x↓	0.0082 2x↓	0.0082 2x↓	0.0082 2x↓	0.0082 2x↓	0.0082 2x↓	0.0082 2x↓	0.0082 2x↓
L-Tyrosine (Tyr)	0.0147 2x↓	0.0147 2x↓	0.0147 2x↓	0.0147 2x↓	0.0147 2x↓	0.0147 2x↓	0.0147 2x↓	0.0147 2x↓	0.0147 2x↓	0.0147 2x↓	0.0147 2x↓	0.0147 2x↓	0.0147 2x↓	0.0147 2x↓	0.0147 2x↓	0.0147 2x↓	0.0147 2x↓	0.0147 2x↓
L-Valine (Val)	0.0268 4x↓	0.0268 4x↓	0.0268 4x↓	0.0268 4x↓	0.0268 4x↓	0.0268 4x↓	0.0268 4x↓	0.0268 4x↓	0.0268 4x↓	0.0268 4x↓	0.0268 4x↓	0.0268 4x↓	0.0268 4x↓	0.0268 4x↓	0.0268 4x↓	0.0268 4x↓	0.0268 4x↓	0.0268 4x↓
MINERAL SALTS																		
$CaCl_2$	0.0500	0.0500	0.0500	0.0500	0.0500	0.0500	0.0500	0.0500	0.0500	0.0500	0.0500	0.0500	0.0500	0.0500	0.0500	0.0500	0.0500	0.0500
$CoSO_4·7H_2O$	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030
$CuSO_4·5H_2O$	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030
$FeSO_4·7H_2O$	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014
$MgSO_4·7H_2O$	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000
$MnSO_4·H_2O$	0.0160	0.0160	0.0160	0.0160	0.0160	0.0160	0.0160	0.0160	0.0160	0.0160	0.0160	0.0160	0.0160	0.0160	0.0160	0.0160	0.0160	0.0160
$(NH_4)_6Mo_7O_{24}·4H_2O$	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030
NaCl	2.9000	2.9000	2.9000	2.9000	2.9000	2.9000	2.9000	2.9000	2.9000	2.9000	2.9000	2.9000	2.9000	2.9000	2.9000	2.9000	2.9000	2.9000
$ZnSO_4·7H_2O$	0.0050	0.0050	0.0050	0.0050	0.0050	0.0050	0.0050	0.0050	0.0050	0.0050	0.0050	0.0050	0.0050	0.0050	0.0050	0.0050	0.0050	0.0050
VITAMINS																		
Thiamine-HCl (B_1)	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051
Riboflavin (B_2)	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033
Nicotinamide (B_3)	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033
Ca-pantothenate (B_5)	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065
Pyridoxine (B_6)	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064
Biotin (B_7)	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031
Folic acid (B_9)	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121
OTHER COMPOUNDS																		
Lipoic acid	0.00018	0.00018	0.00018	0.00018	0.00018	0.00018	0.00018	0.00018	0.00018	0.00018	0.00018	0.00018	0.00018	0.00018	0.00018	0.00018	0.00018	0.00018

Medium	K19	K20	K21	K22	K23	K24	K25	K26	K27	K28	K29	K30	K31	K32	K33	K34	K35	K36
OD$_{max}$ (600 nm), n=3	1.15±0.02	1.09±0.03	1.14±0.03	1.14±0.02	1.17±0.02	1.12±0.02	1.07±0.01	1.08±0.03	1.12±0.03	1.13±0.01	1.24±0.01	1.08±0.02	1.14±0.04	1.12±0	1.13±0	1.09±0.04	1.11±0.03	1.12±0.02
μ_{max} (h^{-1}), n=3	0.56±0.02	0.48±0.08	0.42±0.09	0.40±0.07	0.33±0.05	0.43±0.03	0.44±0.08	0.48±0.09	0.53±0.07	0.44±0.07	0.38±0.08	0.44±0.05	0.50±0.01	0.44±0.04	0.41±0.01	0.53±0.07	0.37±0.01	0.39±0.08
Lag-phase (h)	9.0	8.0	8.0	8.5	10.0	9.0	6.0	6.0	6.0	6.0	6.0	6.0	6.0	6.5	6.5	6.5	6.5	6.0
Exponential phase (h)																		
COMPOSITION (g·L^{-1})	K19	K20	K21	K22	K23	K24	K25	K26	K27	K28	K29	K30	K31	K32	K33	K34	K35	K36
SUGAR																		
Glucose	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5
BUFFERS																		
K_2HPO_4	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9
KH_2PO_4	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75
MOPS	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5
AMINO ACIDS																		
L-Alanine (Ala)	0.0391 2x↓	0.0391 2x↓	0.0391 2x↓	0.0391 2x↓	0.0391 2x↓	0.0391 2x↓	0.0391 2x↓	0.0391 2x↓	0.0391 2x↓	0.0391 2x↓	0.0391 2x↓	0.0391 2x↓	0.0391 2x↓	0.0391 2x↓	0.0391 2x↓	0.0391 2x↓	0.0391 2x↓	0.0391 2x↓
L-Arginine (Arg)	0.0463 4x↓	0.0463 4x↓	0.0463 4x↓	0.0926 2x↓	0.0926 2x↓	0.0926 2x↓	0.0926 2x↓	0.0926 2x↓	0.0926 2x↓	0.0926 2x↓	0.0926 2x↓	0.0926 2x↓	0.0926 2x↓	0.0926 2x↓	0.0926 2x↓	0.0926 2x↓	0.0926 2x↓	0.0926 2x↓
L-Asparagine (Asn)	0.0735	0.0735	0.0735	0.0735	0.0735	0.0735	0.0735	0.0735	0.0735	0.0735	0.0735	0.0735	0.0735	0.0735	0.0735	0.0735	0.0735	0.0735
L-Aspartate (Asp)																		
L-Cysteine (Cys)	0.0159 4x↓	0.0079 8x↓	0.0159 4x↓	0.0079 8x↓	0.0159 4x↓	0.0079 8x↓	0.0079 8x↓	0.0079 8x↓	0.0079 8x↓	0.0079 8x↓	0.0159 4x↓	0.0079 8x↓	0.0079 8x↓	0.0079 8x↓	0.0079 8x↓	0.0079 8x↓	0.0159 4x↓	0.0159 4x↓
L-Glutamine (Gln)	0.1322	0.1322	0.1322	0.0661 2x↓	0.0661 2x↓	0.0661 2x↓	0.1322	0.1322	0.1322	0.1322	0.0661 2x↓	0.1322	0.1322	0.1322	0.1322	0.1322	0.0661 2x↓	0.1322
L-Glutamate (Glu)																		
Glycine (Gly)	0.0072 8x↓	0.0072 8x↓	0.0072 8x↓	0.0072 8x↓	0.0072 8x↓	0.0072 8x↓	0.0072 8x↓	0.0072 8x↓	0.0072 8x↓	0.0072 8x↓	0.0072 8x↓	0.0072 8x↓	0.0072 8x↓	0.0072 8x↓	0.0072 8x↓	0.0072 8x↓	0.0072 8x↓	0.0072 8x↓
L-Histidine (His)	0.0075 8x↓	0.0075 8x↓	0.0075 8x↓	0.0075 8x↓	0.0075 8x↓	0.0075 8x↓	0.0075 8x↓	0.0075 8x↓	0.0075 8x↓	0.0075 8x↓	0.0075 8x↓	0.0075 8x↓	0.0075 8x↓	0.0075 8x↓	0.0075 8x↓	0.0075 8x↓	0.0075 8x↓	0.0075 8x↓
L-Isoleucine (Ile)	0.0255 4x↓	0.0255 4x↓	0.0255 4x↓	0.0255 4x↓	0.0255 4x↓	0.0255 4x↓	0.0255 4x↓	0.0509 2x↓	0.0255 4x↓	0.0255 4x↓	0.0255 4x↓	0.0509 2x↓	0.0509 2x↓	0.0509 2x↓	0.0509 2x↓	0.0509 2x↓	0.0509 2x↓	0.0509 2x↓
L-Leucine (Leu)	0.0518 4x↓	0.0518 4x↓	0.0518 4x↓	0.0518 4x↓	0.0518 4x↓	0.0518 4x↓	0.0518 4x↓	0.1036 2x↓	0.1036 2x↓	0.0518 4x↓	0.0518 4x↓	0.0518 4x↓	0.0518 4x↓	0.0518 4x↓	0.0518 4x↓	0.0518 4x↓	0.0518 4x↓	0.0518 4x↓
L-Lysine-HCl (Lys)	0.0394 4x↓	0.0394 4x↓	0.0394 4x↓	0.0394 4x↓	0.0394 4x↓	0.0394 4x↓	0.0394 4x↓	0.0394 4x↓	0.0394 4x↓	0.0394 4x↓	0.0394 4x↓	0.0394 4x↓	0.0394 4x↓	0.0394 4x↓	0.0394 4x↓	0.0394 4x↓	0.0394 4x↓	0.0394 4x↓
L-Methionine (Met)	0.0203 2x↓	0.0203 2x↓	0.0203 2x↓	0.0102 4x↓	0.0203 2x↓	0.0203 2x↓	0.0102 4x↓	0.0102 4x↓	0.0102 4x↓	0.0203 2x↓	0.0203 2x↓	0.0102 4x↓	0.0102 4x↓	0.0102 4x↓	0.0102 4x↓	0.0203 2x↓	0.0203 2x↓	0.0203 2x↓
L-Phenylalanine (Phe)	0.0430 2x↓	0.0430 2x↓	0.0430 2x↓	0.0430 2x↓	0.0430 2x↓	0.0430 2x↓	0.0430 2x↓	0.0430 2x↓	0.0430 2x↓	0.0430 2x↓	0.0430 2x↓	0.0215 4x↓	0.0430 2x↓	0.0215 4x↓	0.0215 4x↓	0.0215 4x↓	0.0215 4x↓	0.0215 4x↓
L-Proline (Pro)	0.0229 4x↓	0.0115 8x↓	0.0115 8x↓	0.0115 8x↓	0.0115 8x↓	0.0115 8x↓	0.0115 8x↓	0.0115 8x↓	0.0115 8x↓	0.0115 8x↓	0.0115 8x↓	0.0115 8x↓	0.0115 8x↓	0.0115 8x↓	0.0115 8x↓	0.0115 8x↓	0.0115 8x↓	0.0115 8x↓
L-Serine (Ser)	0.0817 2x↓	0.0817 2x↓	0.0817 2x↓	0.0817 2x↓	0.0817 2x↓	0.0817 2x↓	0.0817 2x↓	0.0817 2x↓	0.0817 2x↓	0.0817 2x↓	0.0817 2x↓	0.0817 2x↓	0.0817 2x↓	0.0817 2x↓	0.0817 2x↓	0.0817 2x↓	0.0817 2x↓	0.0817 2x↓
L-Threonine (Thr)	0.0379 2x↓	0.0379 2x↓	0.0379 2x↓	0.0379 2x↓	0.0379 2x↓	0.0379 2x↓	0.0379 2x↓	0.0379 2x↓	0.0379 2x↓	0.0379 2x↓	0.0379 2x↓	0.0379 2x↓	0.0379 2x↓	0.0379 2x↓	0.0379 2x↓	0.0379 2x↓	0.0379 2x↓	0.0379 2x↓
L-Tryptophan (Trp)	0.0082 2x↓	0.0082 2x↓	0.0082 2x↓	0.0082 2x↓	0.0082 2x↓	0.0082 2x↓	0.0082 2x↓	0.0082 2x↓	0.0082 2x↓	0.0082 2x↓	0.0082 2x↓	0.0082 2x↓	0.0082 2x↓	0.0082 2x↓	0.0082 2x↓	0.0082 2x↓	0.0082 2x↓	0.0082 2x↓
L-Tyrosine (Tyr)	0.0147 2x↓	0.0147 2x↓	0.0147 2x↓	0.0147 2x↓	0.0147 2x↓	0.0147 2x↓	0.0147 2x↓	0.0147 2x↓	0.0147 2x↓	0.0147 2x↓	0.0147 2x↓	0.0147 2x↓	0.0074 4x↓	0.0074 4x↓	0.0037 8x↓	0.0074 4x↓	0.0074 4x↓	0.0074 4x↓
L-Valine (Val)	0.0268 4x↓	0.0268 4x↓	0.0268 4x↓	0.0268 4x↓	0.0268 4x↓	0.0268 4x↓	0.0268 4x↓	0.0268 4x↓	0.0268 4x↓	0.0268 4x↓	0.0268 4x↓	0.0268 4x↓	0.0268 4x↓	0.0268 4x↓	0.0268 4x↓	0.0268 4x↓	0.0268 4x↓	0.0268 4x↓
MINERAL SALTS																		
$CaCl_2$	0.0500	0.0500	0.0500	0.0500	0.0500	0.0500	0.0500	0.0500	0.0500	0.0500	0.0500	0.0500	0.0500	0.0500	0.0500	0.0500	0.0500	0.0500
$CoSO_4·7H_2O$	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030
$CuSO_4·5H_2O$	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030
$FeSO_4·7H_2O$	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014
$MgSO_4·7H_2O$	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000
$MnSO_4·H_2O$	0.0160	0.0160	0.0160	0.0160	0.0160	0.0160	0.0160	0.0160	0.0160	0.0160	0.0160	0.0160	0.0160	0.0160	0.0160	0.0160	0.0160	0.0160
$(NH_4)_6Mo_7O_{24}·4H_2O$	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030
NaCl	2.9000	2.9000	2.9000	2.9000	2.9000	2.9000	2.9000	2.9000	2.9000	2.9000	2.9000	2.9000	2.9000	2.9000	2.9000	2.9000	2.9000	2.9000
$ZnSO_4·7H_2O$	0.0050	0.0050	0.0050	0.0050	0.0050	0.0050	0.0050	0.0050	0.0050	0.0050	0.0050	0.0050	0.0050	0.0050	0.0050	0.0050	0.0050	0.0050
VITAMINS																		
Thiamine-HCl (B_1)	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051
Riboflavin (B_2)	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033
Nicotinamide (B_3)	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033
Ca-pantothenate (B_5)	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065
Pyridoxine (B_6)	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064
Biotin (B_7)	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031
Folic acid (B_9)	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121
OTHER COMPOUNDS																		
Lipoic acid	0.00018	0.00018	0.00018	0.00018	0.00018	0.00018	0.00018	0.00018	0.00018	0.00018	0.00018	0.00018	0.00018	0.00018	0.00018	0.00018	0.00018	0.00018

Medium	K37	K38	K39	K41	K42	K43	K44	MA	MB	MC	MD	ME	MF	MG	MH	NA
OD$_{max}$ (600 nm), n=3	1.13±0.02	1.19±0.01	1.24±0.03	1.26±0.03	1.19±0.03	1.06±0.04	1.23±0.02	1.11±0.02	1.01±0.03	1.03±0.03	1.08±0.02	1.06±0.02	1.03±0.04	1.01±0.02	1.04±0.03	0.74±0.01
μ_{max} (h^{-1}), n=3	0.47±0.09	0.46±0.02	0.53±0.02	0.53±0.01	0.48±0.10	0.54±0.01	0.48±0.10	0.57±0.02	0.55±0.09	0.50±0.11	0.57±0.05	0.53±0.04	0.56±0.06	0.48±0.09	0.45±0.06	0.37±0.13
Lag-phase (h)	5.5	6.0	6.0	6.0	6.0	7.0	6.0	5.0	5.5	5.5	5.0	6.0	6.0	7.0	6.0	6.0
Exponential phase (h)								6.0	5.5	5.5	6.0	5.0	5.0	5.0	5.0	6.0
COMPOSITION (g·L^{-1})	K37	K38	K39	K41	K42	K43	K44	MA	MB	MC	MD	ME	MF	MG	MH	NA
SUGAR																
Glucose	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5
BUFFERS																
K_2HPO_4	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9
KH_2PO_4	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75
MOPS	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5
AMINO ACIDS																
L-Alanine (Ala)	0.0391 2x↓	0.0391 2x↓	0.0391 2x↓	0.0391 2x↓	0.0391 2x↓	0.0391 2x↓	0.0391 2x↓	0.0391 2x↓	0.0391 2x↓	0.0391 2x↓	0.0391 2x↓	0.0391 2x↓	0.0391 2x↓	0.0391 2x↓	0.0391 2x↓	0.0196 4x↓
L-Arginine (Arg)	0.0926 2x↓	0.0926 2x↓	0.0926 2x↓	0.0926 2x↓	0.0926 2x↓	0.0926 2x↓	0.0926 2x↓	0.0926 2x↓	0.0926 2x↓	0.0926 2x↓	0.0926 2x↓	0.0926 2x↓	0.0926 2x↓	0.0926 2x↓	0.0926 2x↓	0.0463 4x↓
L-Asparagine (Asn)	0.0735	0.0735	0.0735	0.0735	0.0735	0.0735	0.0735	0.0368 2x↓	0.0368 2x↓	0.0368 2x↓	0.0368 2x↓	0.0368 2x↓	0.0368 2x↓	0.0368 2x↓	0.0368 2x↓	0.0184 4x↓
L-Aspartate (Asp)	0.0723	0.0723	0.0723	0.0362 2x↓	0.0362 2x↓	0.0362 2x↓	0.0362 2x↓	0.0362 2x↓	0.0362 2x↓	0.0362 2x↓	0.0362 2x↓	0.0362 2x↓		0.0362 2x↓	0.0362 2x↓	0.0181 4x↓
L-Cysteine (Cys)	0.0159 4x↓	0.0079 8x↓	0.0079 8x↓	0.0159 4x↓	0.0079 8x↓	0.0079 8x↓	0.0079 8x↓	0.0318 2x↓	0.0318 2x↓	0.0318 2x↓	0.0318 2x↓	0.0318 2x↓	0.0318 2x↓	0.0318 2x↓	0.0318 2x↓	0.0159 4x↓
L-Glutamine (Gln)	0.1322	0.1322	0.1322	0.1322	0.1322	0.1322	0.1322	0.0661 2x↓	0.0661 2x↓	0.0661 2x↓	0.0661 2x↓	0.0661 2x↓	0.0661 2x↓	0.0661 2x↓	0.0661 2x↓	0.0331 4x↓
L-Glutamate (Glu)	0.0703	0.0703	0.0703	0.0351 2x↓	0.0351 2x↓	0.0351 2x↓	0.0351 2x↓		0.0351 2x↓	0.0351 2x↓	0.0351 2x↓	0.0351 2x↓		0.0351 2x↓	0.0351 2x↓	
Glycine (Gly)	0.0072 8x↓	0.0072 8x↓	0.0072 8x↓	0.0072 8x↓	0.0072 8x↓	0.0072 8x↓	0.0072 8x↓	0.0289 2x↓	0.0289 2x↓	0.0289 2x↓	0.0289 2x↓	0.0289 2x↓	0.0289 2x↓	0.0289 2x↓	0.0289 2x↓	0.0144 4x↓
L-Histidine (His)	0.0075 8x↓	0.0075 8x↓	0.0075 8x↓	0.0075 8x↓	0.0075 8x↓	0.0075 8x↓	0.0075 8x↓	0.0299 2x↓	0.0299 2x↓	0.0299 2x↓	0.0299 2x↓	0.0299 2x↓	0.0299 2x↓	0.0299 2x↓	0.0299 2x↓	0.0149 4x↓
L-Isoleucine (Ile)	0.0255 4x↓	0.0255 4x↓	0.0255 4x↓	0.0255 4x↓	0.0255 4x↓	0.0255 4x↓	0.0255 4x↓	0.0509 2x↓	0.0509 2x↓	0.0509 2x↓	0.0509 2x↓	0.0509 2x↓	0.0509 2x↓	0.0509 2x↓	0.0509 2x↓	0.0255 4x↓
L-Leucine (Leu)	0.0518 4x↓	0.0518 4x↓	0.1036 2x↓	0.0518 4x↓	0.0518 4x↓	0.1036 2x↓	0.0518 4x↓	0.1036 2x↓	0.1036 2x↓	0.1036 2x↓	0.0518 4x↓	0.1036 2x↓	0.1036 2x↓	0.1036 2x↓	0.1036 2x↓	0.0518 4x↓
L-Lysine-HCl (Lys)	0.0394 4x↓	0.0394 4x↓	0.0394 4x↓	0.0394 4x↓	0.0394 4x↓	0.0394 4x↓	0.0394 4x↓	0.0788 2x↓	0.0788 2x↓	0.0788 2x↓	0.0788 2x↓	0.0788 2x↓	0.0788 2x↓	0.0788 2x↓	0.0394 4x↓	0.0394 4x↓
L-Methionine (Met)	0.0407	0.0102 4x↓	0.0102 4x↓	0.0407	0.0102 4x↓	0.0102 4x↓	0.0203 2x↓	0.0203 2x↓	0.0203 2x↓	0.0203 2x↓	0.0203 2x↓	0.0203 2x↓	0.0203 2x↓	0.0203 2x↓	0.0203 2x↓	0.0102 4x↓
L-Phenylalanine (Phe)	0.0430 2x↓	0.0430 2x↓	0.0430 2x↓	0.0430 2x↓	0.0430 2x↓	0.0430 2x↓	0.0430 2x↓	0.0430 2x↓		0.0430 2x↓	0.0430 2x↓	0.0430 2x↓	0.0430 2x↓	0.0215 4x↓	0.0430 2x↓	0.0215 4x↓
L-Proline (Pro)	0.0115 8x↓	0.0458 2x↓	0.0115 8x↓	0.0115 8x↓	0.0458 2x↓	0.0115 8x↓	0.0115 8x↓	0.0458 2x↓	0.0458 2x↓	0.0458 2x↓	0.0458 2x↓	0.0458 2x↓	0.0458 2x↓	0.0458 2x↓	0.0458 2x↓	0.0229 4x↓
L-Serine (Ser)	0.0817 2x↓	0.0817 2x↓	0.0817 2x↓	0.0817 2x↓	0.0817 2x↓	0.0817 2x↓	0.0817 2x↓	0.0817 2x↓	0.0817 2x↓	0.0817 2x↓	0.0817 2x↓	0.0817 2x↓	0.0817 2x↓	0.0817 2x↓	0.0817 2x↓	0.0408 4x↓
L-Threonine (Thr)	0.0379 2x↓	0.0379 2x↓	0.0379 2x↓	0.0379 2x↓	0.0379 2x↓	0.0379 2x↓	0.0379 2x↓	0.0379 2x↓	0.0379 2x↓	0.0379 2x↓	0.0379 2x↓	0.0379 2x↓	0.0379 2x↓	0.0379 2x↓	0.0379 2x↓	0.0190 4x↓
L-Tryptophan (Trp)	0.0082 2x↓	0.0082 2x↓	0.0082 2x↓	0.0082 2x↓	0.0082 2x↓	0.0082 2x↓	0.0021 8x↓	0.0082 2x↓	0.0082 2x↓	0.0082 2x↓	0.0082 2x↓	0.0082 2x↓	0.0082 2x↓	0.0041 4x↓	0.0082 2x↓	0.0041 4x↓
L-Tyrosine (Tyr)	0.0147 2x↓	0.0147 2x↓	0.0147 2x↓	0.0147 2x↓	0.0147 2x↓	0.0147 2x↓	0.0147 2x↓	0.0147 2x↓	0.0147 2x↓		0.0147 2x↓	0.0147 2x↓	0.0147 2x↓	0.0074 4x↓	0.0147 2x↓	0.0074 4x↓
L-Valine (Val)	0.0268 4x↓	0.0268 4x↓	0.0268 4x↓	0.0268 4x↓	0.0268 4x↓	0.0268 4x↓	0.0268 4x↓	0.0536 2x↓	0.0536 2x↓	0.0536 2x↓	0.0536 2x↓	0.0268 4x↓	0.0536 2x↓	0.0536 2x↓	0.0536 2x↓	0.0268 4x↓
MINERAL SALTS																
$CaCl_2$	0.0500	0.0500	0.0500	0.0500	0.0500	0.0500	0.0500	0.0500	0.0500	0.0500	0.0500	0.0500	0.0500	0.0500	0.0500	0.0500
$CoSO_4·7H_2O$	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030
$CuSO_4·5H_2O$	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030
$FeSO_4·7H_2O$	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014
$MgSO_4·7H_2O$	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000
$MnSO_4·H_2O$	0.0160	0.0160	0.0160	0.0160	0.0160	0.0160	0.0160	0.0160	0.0160	0.0160	0.0160	0.0160	0.0160	0.0160	0.0160	0.0160
$(NH_4)_6Mo_7O_{24}·4H_2O$	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030
NaCl	2.9000	2.9000	2.9000	2.9000	2.9000	2.9000	2.9000	2.9000	2.9000	2.9000	2.9000	2.9000	2.9000	2.9000	2.9000	2.9000
$ZnSO_4·7H_2O$	0.0050	0.0050	0.0050	0.0050	0.0050	0.0050	0.0050	0.0050	0.0050	0.0050	0.0050	0.0050	0.0050	0.0050	0.0050	0.0050
VITAMINS																
Thiamine-HCl (B_1)	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051
Riboflavin (B_2)	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033
Nicotinamide (B_3)	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033
Ca-pantothenate (B_5)	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065
Pyridoxine (B_6)	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064
Biotin (B_7)	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031
Folic acid (B_9)	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121
OTHER COMPOUNDS																
Lipoic acid	0.00018	0.00018	0.00018	0.00018	0.00018	0.00018	0.00018	0.00018	0.00018	0.00018	0.00018	0.00018	0.00018	0.00018	0.00018	0.00018

Medium	NB	NC	ND	NE	NF	NG	NH	OC	OD	OE	OF	OG	OH	OI	OJ	OL	OM	PB	PC	PD	PE
OD$_{max}$ (600 nm), n=3	0.74±0.03	0.73±0.02	0.73±0.03	0.72±0.03	0.71±0.02	0.68±0.02	0.73±0.02	1.19±0.03	1.04±0.03	1.08±0.07	1.14±0.03	1.15±0.04	1.11±0.02	1.07±0.04	1.00±0.03	1.19±0.02	1.16±0.02	1.28±0.03	1.18±0.01	1.34±0.07	1.25±0.02
μ_{max} (h^{-1}), n=3	0.53±0.11	0.35±0.01	0.41±0.05	0.37±0.15	0.39±0.12	0.30±0.07	0.35±0.08	0.55±0.03	0.39±0.09	0.51±0.10	0.49±0.07	0.40±0.12	0.47±0.05	0.42±0.08	0.35±0.02	0.43±0	0.29±0.03				
Lag-phase (h)	6.0	6.0	6.0	6.0	6.0	6.0	6.0	5.5	5.5	5.5	5.5	5.5	5.5	6.0	9.0	7.0	9.5	8.5	8.0	8.0	8.0
Exponential phase (h)	6.0	6.0	6.0	6.0	6.0	6.0	6.0	5.5	5.5	5.5	5.5	6.5	6.5	5.0	5.0	5.0	5.5				
COMPOSITION (g·L^{-1})	NB	NC	ND	NE	NF	NG	NH	OC	OD	OE	OF	OG	OH	OI	OJ	OL	OM	PB	PC	PD	PE
SUGAR																					
Glucose	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5
BUFFERS																					
K_2HPO_4	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9
KH_2PO_4	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75
MOPS	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5
AMINO ACIDS																					
L-Alanine (Ala)	0.0196 4x↓	0.0196 4x↓	0.0196 4x↓	0.0196 4x↓	0.0196 4x↓	0.0196 4x↓	0.0196 4x↓	0.0391 2x↓	0.0391 2x↓	0.0391 2x↓	0.0391 2x↓	0.0391 2x↓	0.0391 2x↓	0.0391 2x↓	0.0196 4x↓	0.0391 2x↓	0.0391 2x↓	0.0391 2x↓	0.0391 2x↓	0.0391 2x↓	0.0391 2x↓
L-Arginine (Arg)	0.0463 4x↓	0.0463 4x↓	0.0463 4x↓	0.0463 4x↓	0.0463 4x↓	0.0463 4x↓	0.0463 4x↓	0.0463 4x↓	0.0463 4x↓	0.0463 4x↓	0.0463 4x↓	0.0463 4x↓	0.0463 4x↓	0.0463 4x↓	0.0232 8x↓	0.0926 2x↓	0.0926 2x↓	0.0926 2x↓	0.0926 2x↓	0.0926 2x↓	0.0926 2x↓
L-Asparagine (Asn)	0.0184 4x↓	0.0184 4x↓	0.0184 4x↓	0.0184 4x↓	0.0184 4x↓	0.0184 4x↓	0.0184 4x↓	0.0735	0.0368 2x↓	0.0368 2x↓	0.0368 2x↓	0.0368 2x↓	0.0368 2x↓	0.0368 2x↓	0.0735	0.0735	0.0368 2x↓	0.0735	0.0735	0.0735	0.0735
L-Aspartate (Asp)	0.0181 4x↓	0.0181 4x↓	0.0181 4x↓	0.0181 4x↓		0.0181 4x↓	0.0181 4x↓														
L-Cysteine (Cys)	0.0159 4x↓	0.0159 4x↓	0.0159 4x↓	0.0159 4x↓	0.0159 4x↓	0.0159 4x↓	0.0159 4x↓	0.0159 4x↓	0.0159 4x↓	0.0159 4x↓	0.0159 4x↓	0.0159 4x↓	0.0159 4x↓	0.0159 4x↓	0.0079 8x↓	0.0079 8x↓	0.0079 8x↓	0.0079 8x↓	0.0079 8x↓	0.0079 8x↓	0.0079 8x↓
L-Glutamine (Gln)	0.0331 4x↓	0.0331 4x↓	0.0331 4x↓	0.0331 4x↓	0.0331 4x↓	0.0331 4x↓	0.0331 4x↓	0.1322	0.0661 2x↓	0.0661 2x↓	0.0661 2x↓	0.0661 2x↓	0.0661 2x↓	0.0661 2x↓	0.1322	0.1322	0.0661 2x↓	0.1322	0.1322	0.1322	0.1322
L-Glutamate (Glu)	0.0176 4x↓	0.0176 4x↓	0.0176 4x↓	0.0176 4x↓		0.0176 4x↓	0.0176 4x↓														
Glycine (Gly)	0.0144 4x↓	0.0144 4x↓	0.0144 4x↓	0.0144 4x↓	0.0144 4x↓	0.0144 4x↓	0.0144 4x↓	0.0144 4x↓	0.0144 4x↓	0.0144 4x↓	0.0144 4x↓	0.0144 4x↓	0.0144 4x↓	0.0144 4x↓	0.0072 8x↓	0.0072 8x↓	0.0072 8x↓	0.0072 8x↓	0.0072 8x↓	0.0072 8x↓	0.0072 8x↓
L-Histidine (His)	0.0149 4x↓	0.0149 4x↓	0.0149 4x↓	0.0149 4x↓	0.0149 4x↓	0.0149 4x↓	0.0149 4x↓	0.0149 4x↓	0.0149 4x↓	0.0149 4x↓	0.0149 4x↓	0.0149 4x↓	0.0149 4x↓	0.0149 4x↓	0.0075 8x↓	0.0075 8x↓	0.0075 8x↓	0.0075 8x↓	0.0075 8x↓	0.0075 8x↓	0.0075 8x↓
L-Isoleucine (Ile)	0.0255 4x↓	0.0255 4x↓	0.0255 4x↓	0.0255 4x↓	0.0255 4x↓	0.0255 4x↓	0.0255 4x↓	0.0509 2x↓	0.0509 2x↓	0.0509 2x↓	0.0509 2x↓	0.0509 2x↓	0.0509 2x↓	0.0509 2x↓	0.0255 4x↓	0.0255 4x↓	0.0255 4x↓	0.0255 4x↓	0.0255 4x↓	0.0255 4x↓	0.0255 4x↓
L-Leucine (Leu)	0.0518 4x↓	0.0518 4x↓	0.0259 8x↓	0.0518 4x↓	0.0518 4x↓	0.0518 4x↓	0.0518 4x↓	0.1036 2x↓	0.1036 2x↓	0.1036 2x↓	0.0518 4x↓	0.0518 4x↓	0.0518 4x↓	0.0518 4x↓	0.0518 4x↓	0.0518 4x↓	0.0518 4x↓	0.0518 4x↓	0.0518 4x↓	0.0518 4x↓	0.0518 4x↓
L-Lysine-HCl (Lys)	0.0394 4x↓	0.0394 4x↓	0.0394 4x↓	0.0394 4x↓	0.0394 4x↓	0.0394 4x↓	0.0197 8x↓	0.0394 4x↓	0.0394 4x↓	0.0394 4x↓	0.0394 4x↓	0.0394 4x↓	0.0394 4x↓	0.0394 4x↓	0.0197 8x↓	0.0394 4x↓	0.0394 4x↓	0.0394 4x↓	0.0394 4x↓	0.0394 4x↓	0.0394 4x↓
L-Methionine (Met)	0.0102 4x↓	0.0102 4x↓	0.0102 4x↓	0.0102 4x↓	0.0102 4x↓	0.0102 4x↓	0.0102 4x↓	0.0102 4x↓	0.0102 4x↓	0.0102 4x↓	0.0102 4x↓	0.0102 4x↓	0.0102 4x↓	0.0102 4x↓	0.0051 8x↓	0.0102 4x↓	0.0102 4x↓	0.0102 4x↓	0.0102 4x↓	0.0102 4x↓	0.0102 4x↓
L-Phenylalanine (Phe)		0.0215 4x↓	0.0215 4x↓	0.0215 4x↓	0.0215 4x↓	0.0108 8x↓	0.0215 4x↓	0.0215 4x↓	0.0215 4x↓	0.0430 2x↓	0.0215 4x↓	0.0430 2x↓	0.0430 2x↓		0.0108 8x↓	0.0430 2x↓	0.0430 2x↓	0.0430 2x↓	0.0430 2x↓	0.0430 2x↓	0.0430 2x↓
L-Proline (Pro)	0.0229 4x↓	0.0229 4x↓	0.0229 4x↓	0.0229 4x↓	0.0229 4x↓	0.0229 4x↓	0.0229 4x↓	0.0229 4x↓	0.0229 4x↓	0.0229 4x↓	0.0229 4x↓	0.0229 4x↓	0.0229 4x↓	0.0229 4x↓	0.0115 8x↓	0.0115 8x↓	0.0115 8x↓	0.0115 8x↓	0.0115 8x↓	0.0115 8x↓	0.0115 8x↓
L-Serine (Ser)	0.0408 4x↓	0.0408 4x↓	0.0408 4x↓	0.0408 4x↓	0.0408 4x↓	0.0408 4x↓	0.0408 4x↓	0.0817 2x↓	0.0817 2x↓	0.0817 2x↓	0.0817 2x↓	0.0817 2x↓	0.0817 2x↓	0.0817 2x↓	0.0408 4x↓	0.0817 2x↓	0.0817 2x↓	0.0817 2x↓	0.0817 2x↓	0.0817 2x↓	0.0817 2x↓
L-Threonine (Thr)	0.0190 4x↓	0.0190 4x↓	0.0190 4x↓	0.0190 4x↓	0.0190 4x↓	0.0190 4x↓	0.0190 4x↓	0.0379 2x↓	0.0379 2x↓	0.0379 2x↓	0.0379 2x↓	0.0379 2x↓	0.0379 2x↓	0.0379 2x↓	0.0190 4x↓	0.0379 2x↓	0.0379 2x↓	0.0379 2x↓	0.0379 2x↓	0.0379 2x↓	0.0379 2x↓
L-Tryptophan (Trp)	0.0041 4x↓	0.0041 4x↓	0.0041 4x↓	0.0041 4x↓	0.0041 4x↓	0.0021 8x↓	0.0041 4x↓	0.0041 4x↓	0.0041 4x↓	0.0082 2x↓	0.0041 4x↓	0.0082 2x↓	0.0082 2x↓	0.0082 2x↓	0.0021 8x↓	0.0082 2x↓	0.0082 2x↓	0.0082 2x↓	0.0082 2x↓	0.0082 2x↓	0.0082 2x↓
L-Tyrosine (Tyr)	0.0074 4x↓		0.0074 4x↓	0.0074 4x↓	0.0074 4x↓	0.0037 8x↓	0.0074 4x↓	0.0074 4x↓	0.0074 4x↓	0.0147 2x↓	0.0074 4x↓	0.0147 2x↓		0.0147 2x↓	0.0037 8x↓	0.0147 2x↓	0.0147 2x↓	0.0147 2x↓	0.0147 2x↓	0.0147 2x↓	0.0147 2x↓
L-Valine (Val)	0.0268 4x↓	0.0268 4x↓	0.0268 4x↓	0.0134 8x↓	0.0268 4x↓	0.0268 4x↓	0.0268 4x↓	0.0268 4x↓	0.0268 4x↓	0.0268 4x↓	0.0268 4x↓	0.0268 4x↓	0.0268 4x↓	0.0268 4x↓	0.0134 8x↓	0.0268 4x↓	0.0268 4x↓	0.0268 4x↓	0.0268 4x↓	0.0268 4x↓	0.0268 4x↓
MINERAL SALTS																					
$CaCl_2$	0.0500	0.0500	0.0500	0.0500	0.0500	0.0500	0.0500	0.0500	0.0500	0.0500	0.0500	0.0500	0.0500	0.0500	0.0500	0.0500	0.0500	0.0500	0.0500	0.0500	0.0500
$CoSO_4·7H_2O$	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030
$CuSO_4·5H_2O$	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030
$FeSO_4·7H_2O$	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014	0.0014
$MgSO_4·7H_2O$	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000
$MnSO_4·H_2O$	0.0160	0.0160	0.0160	0.0160	0.0160	0.0160	0.0160	0.0160	0.0160	0.0160	0.0160	0.0160	0.0160	0.0160	0.0160	0.0160	0.0160	0.0160	0.0160	0.0160	0.0160
$(NH_4)_6Mo_7O_{24}·4H_2O$	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030
NaCl	2.9000	2.9000	2.9000	2.9000	2.9000	2.9000	2.9000	2.9000	2.9000	2.9000	2.9000	2.9000	2.9000	2.9000	2.9000	2.9000	2.9000	2.9000	2.9000	2.9000	2.9000
$ZnSO_4·7H_2O$	0.0050	0.0050	0.0050	0.0050	0.0050	0.0050	0.0050	0.0050	0.0050	0.0050	0.0050	0.0050	0.0050	0.0050	0.0050	0.0050	0.0050	0.0050	0.0050	0.0050	0.0050
VITAMINS																					
Thiamine-HCl (B_1)	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051
Riboflavin (B_2)	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00065	0.00033	0.00098	0.00033
Nicotinamide (B_3)	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00065	0.00033	0.00098
Ca-pantothenate (B_5)	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065
Pyridoxine (B_6)	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064
Biotin (B_7)	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031
Folic acid (B_9)	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121
OTHER COMPOUNDS																					
Lipoic acid	0.00018	0.00018	0.00018	0.00018	0.00018	0.00018	0.00018	0.00018	0.00018	0.00018	0.00018	0.00018	0.00018	0.00018	0.00018	0.00018	0.00018	0.00018	0.00018	0.00018	0.00018

Table S11. Chemically defined media with **minimal metal ion concentrations** that were developed in this thesis for *L. lactis* IL1403. These media were used for batch cultivations on **microtiter plates** with the **cultivation volume 0.3 mL**.[1]
The difference of mineral salts concentrations with the initial medium BS7 has been shown for each medium (reduced 2, 4, 8 or 10 times).
The concentrations of buffers, amino acids and vitamins were the same as for the medium BS7 (see Table 5 in Materials and Methods section of the thesis), but these media did not contain adenine and hypoxanthine.
The composition and growth results for V19 medium can be seen in Table 5 (Materials and Methods section of the thesis).

Medium	V2	V3	V12	V13	V14	V17	V18	V20	V24	V25	V26	V27	V28	V29	V30	V31	V32
OD_{max} (600 nm), n=3	0.554±0.008	0.584±0.005	0.581±0.002	0.560±0.002	0.528±0.006	0.455±0.007	0.458±0.006	0.487±0.008	0.489±0.003	0.473±0.018	0.510±0.010	0.468±0.008	0.514±0.005	0.523±0.009	0.545±0.008	0.517±0.005	0.504±0.009
μ_{max} (h^{-1}), n=3	0.54±0	0.49±0.03	0.60±0.01	0.58±0	0.55±0.01	0.40±0.02	0.40±0.06	0.63±0.06	0.68±0.03	0.56±0.10	0.61±0.07	0.65±0.09	0.50±0.06	0.62±0.07	0.55±0.03	0.62±0.06	0.60±0.09
COMPOSITION ($g \cdot L^{-1}$)	**V2**	**V3**	**V12**	**V13**	**V14**	**V17**	**V18**	**V20**	**V24**	**V25**	**V26**	**V27**	**V28**	**V29**	**V30**	**V31**	**V32**
SUGAR																	
Glucose	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5
BUFFERS																	
K_2HPO_4	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9
KH_2PO_4	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75	0.75
MOPS	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5	7.5
AMINO ACIDS																	
L-Alanine (Ala)	0.0782	0.0782	0.0782	0.0782	0.0782	0.0782	0.0782	0.0782	0.0782	0.0782	0.0782	0.0782	0.0782	0.0782	0.0782	0.0782	0.0782
L-Arginine (Arg)	0.1852	0.1852	0.1852	0.1852	0.1852	0.1852	0.1852	0.1852	0.1852	0.1852	0.1852	0.1852	0.1852	0.1852	0.1852	0.1852	0.1852
L-Asparagine (Asn)	0.0735	0.0735	0.0735	0.0735	0.0735	0.0735	0.0735	0.0735	0.0735	0.0735	0.0735	0.0735	0.0735	0.0735	0.0735	0.0735	0.0735
L-Aspartate (Asp)	0.0723	0.0723	0.0723	0.0723	0.0723	0.0723	0.0723	0.0723	0.0723	0.0723	0.0723	0.0723	0.0723	0.0723	0.0723	0.0723	0.0723
L-Cysteine (Cys)	0.0636	0.0636	0.0636	0.0636	0.0636	0.0636	0.0636	0.0636	0.0636	0.0636	0.0636	0.0636	0.0636	0.0636	0.0636	0.0636	0.0636
L-Glutamine (Gln)	0.1322	0.1322	0.1322	0.1322	0.1322	0.1322	0.1322	0.1322	0.1322	0.1322	0.1322	0.1322	0.1322	0.1322	0.1322	0.1322	0.1322
L-Glutamate (Glu)	0.0703	0.0703	0.0703	0.0703	0.0703	0.0703	0.0703	0.0703	0.0703	0.0703	0.0703	0.0703	0.0703	0.0703	0.0703	0.0703	0.0703
Glycine (Gly)	0.0578	0.0578	0.0578	0.0578	0.0578	0.0578	0.0578	0.0578	0.0578	0.0578	0.0578	0.0578	0.0578	0.0578	0.0578	0.0578	0.0578
L-Histidine (His)	0.0597	0.0597	0.0597	0.0597	0.0597	0.0597	0.0597	0.0597	0.0597	0.0597	0.0597	0.0597	0.0597	0.0597	0.0597	0.0597	0.0597
L-Isoleucine (Ile)	0.1018	0.1018	0.1018	0.1018	0.1018	0.1018	0.1018	0.1018	0.1018	0.1018	0.1018	0.1018	0.1018	0.1018	0.1018	0.1018	0.1018
L-Leucine (Leu)	0.2072	0.2072	0.2072	0.2072	0.2072	0.2072	0.2072	0.2072	0.2072	0.2072	0.2072	0.2072	0.2072	0.2072	0.2072	0.2072	0.2072
L-Lysine-HCl (Lys)	0.1576	0.1576	0.1576	0.1576	0.1576	0.1576	0.1576	0.1576	0.1576	0.1576	0.1576	0.1576	0.1576	0.1576	0.1576	0.1576	0.1576
L-Methionine (Met)	0.0407	0.0407	0.0407	0.0407	0.0407	0.0407	0.0407	0.0407	0.0407	0.0407	0.0407	0.0407	0.0407	0.0407	0.0407	0.0407	0.0407
L-Phenylalanine (Phe)	0.0860	0.0860	0.0860	0.0860	0.0860	0.0860	0.0860	0.0860	0.0860	0.0860	0.0860	0.0860	0.0860	0.0860	0.0860	0.0860	0.0860
L-Proline (Pro)	0.0917	0.0917	0.0917	0.0917	0.0917	0.0917	0.0917	0.0917	0.0917	0.0917	0.0917	0.0917	0.0917	0.0917	0.0917	0.0917	0.0917
L-Serine (Ser)	0.1634	0.1634	0.1634	0.1634	0.1634	0.1634	0.1634	0.1634	0.1634	0.1634	0.1634	0.1634	0.1634	0.1634	0.1634	0.1634	0.1634
L-Threonine (Thr)	0.0758	0.0758	0.0758	0.0758	0.0758	0.0758	0.0758	0.0758	0.0758	0.0758	0.0758	0.0758	0.0758	0.0758	0.0758	0.0758	0.0758
L-Tryptophan (Trp)	0.0164	0.0164	0.0164	0.0164	0.0164	0.0164	0.0164	0.0164	0.0164	0.0164	0.0164	0.0164	0.0164	0.0164	0.0164	0.0164	0.0164
L-Tyrosine (Tyr)	0.0295	0.0295	0.0295	0.0295	0.0295	0.0295	0.0295	0.0295	0.0295	0.0295	0.0295	0.0295	0.0295	0.0295	0.0295	0.0295	0.0295
L-Valine (Val)	0.1072	0.1072	0.1072	0.1072	0.1072	0.1072	0.1072	0.1072	0.1072	0.1072	0.1072	0.1072	0.1072	0.1072	0.1072	0.1072	0.1072
MINERAL SALTS																	
$CaCl_2$	0.0250 2x↓	0.0125 4x↓	0.0500	0.0500	0.0500	0.0063 8x↓	0.0050 10x↓	0.0500	0.0250 2x↓	0.0125 4x↓	0.0063 8x↓	0.0050 10x↓	0.0250 2x↓	0.0125 4x↓	0.0063 8x↓	0.0050 10x↓	0.0063 8x↓
$CoSO_4 \cdot 7H_2O$	0.0015 2x↓	0.0008 4x↓	0.0030	0.0030	0.0030	0.0004 8x↓	0.0003 10x↓	0.0030	0.0015 2x↓	0.0008 4x↓	0.0004 8x↓	0.0003 10x↓	0.0015 2x↓	0.0008 4x↓	0.0004 8x↓	0.0003 10x↓	0.0004 8x↓
$CuSO_4 \cdot 5H_2O$	0.0015 2x↓	0.0008 4x↓	0.0030	0.0030	0.0030	0.0004 8x↓	0.0003 10x↓	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0030	0.0015 2x↓
$FeSO_4 \cdot 7H_2O$	0.0007 2x↓	0.0004 4x↓	0.0014	0.0014	0.0014	0.0002 8x↓	0.0001 10x↓	0.0014	0.0007 2x↓	0.0004 4x↓	0.0002 8x↓	0.0001 10x↓	0.0007 2x↓	0.0004 4x↓	0.0002 8x↓	0.0001 10x↓	0.0002 8x↓
$MgSO_4 \cdot 7H_2O$	0.1000 2x↓	0.0500 4x↓	0.2000	0.2000	0.2000	0.0250 8x↓	0.0200 10x↓	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000	0.2000	0.1000 2x↓
$MnSO_4 \cdot H_2O$	0.0080 2x↓	0.0040 4x↓	0.0160	0.0160	0.0160	0.0020 8x↓	0.0016 10x↓	0.0160	0.0160	0.0160	0.0160	0.0160	0.0160	0.0160	0.0160	0.0160	0.0080 2x↓
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.0015 2x↓	0.0008 4x↓	0.0030	0.0030	0.0030	0.0004 8x↓	0.0003 10x↓	0.0030	0.0015 2x↓	0.0008 4x↓	0.0004 8x↓	0.0003 10x↓	0.0015 2x↓	0.0008 4x↓	0.0004 8x↓	0.0003 10x↓	0.0004 8x↓
NaCl	1.4500 2x↓	0.7250 4x↓	1.4500 2x↓	0.7250 4x↓	0.3625 8x↓	0.3625 8x↓	0.2900 10x↓	0.2900 10x↓	2.9000	2.9000	2.9000	2.9000	1.4500 2x↓	0.7250 4x↓	0.3625 8x↓	0.2900 10x↓	0.3625 8x↓
$ZnSO_4 \cdot 7H_2O$	0.0025 2x↓	0.0013 4x↓	0.0050	0.0050	0.0050	0.0006 8x↓	0.0005 10x↓	0.0050	0.0025 2x↓	0.0013 4x↓	0.0006 8x↓	0.0005 10x↓	0.0025 2x↓	0.0013 4x↓	0.0006 8x↓	0.0005 10x↓	0.0006 8x↓
VITAMINS																	
Thiamine-HCl (B_1)	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051	0.00051
Riboflavin (B_2)	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033
Nicotinamide (B_3)	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033	0.00033
Ca-pantothenate (B_5)	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065	0.00065
Pyridoxine (B_6)	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064	0.00064
Biotin (B_7)	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031	0.00031
Folic acid (B_9)	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121	0.00121
OTHER COMPOUNDS																	
Lipoic acid	0.00018	0.00018	0.00018	0.00018	0.00018	0.00018	0.00018	0.00018	0.00018	0.00018	0.00018	0.00018	0.00018	0.00018	0.00018	0.00018	0.00018

[1] In addition, the growth of *L. lactis* IL1403 on V28, V29, V30 and V31 media was monitored in test tubes and the results were, respectively, OD_{max} = 1.62±0.04, 1.68±0.03, 1.69±0.02 and 1.74±0.02; μ_{max} = 0.65±0.04, 0.61±0.05, 0.56±0 and 0.62±0.04 h^{-1}; lag-phase 4.5, 4.0, 4.0 and 5.0 hours; exponential phase 4.5, 5.0, 5.0 and 4.0 hours.