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Quantitative omics-level analysis of growth rate dependent energy metabolism in *Lactococcus lactis*

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Petri-Jaan Lahtvee

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LOODUS - JA TÄPPISTEADUSED B139

Kvantitatiivsetel oomika-meetoditel põhinev kasvuerikiirusest sõltuv *Lactococcus lactis'e* energiametabolismi analüüs

PETRI-JAAN LAHTVEE

TTÜ KIRJASTUS

CONTENTS

ABSTRACT

Unicellular organisms live under dynamic environmental conditions, where temperature, the availability of nutrients, and the presence of various chemicals vary. Adaptation is thus partially determined by substrate consumption rates, *e.g.* the specific growth rate (μ). Adaptation is a dynamic process and results in dynamic changes in the optimization of their metabolism. Metabolic shifts in cell size, ribosomal content and metabolic efficiency results in adapting patterns in the consumption of secondary substrates as well as in product formation. The aim of this study is to unravel the molecular mechanisms that explain the 30% increase in growth efficiency of the lactic acid bacterium *Lactococcus lactis* that occur when μ is increased from 0.1 to 0.5 h^{-1}. For this we applied metabolic modeling to analyze the intra- and extracellular metabolic fluxes using μ-dependent steady state cultivation data together with absolute measurements of the proteome and transcriptome.

Reorganization of amino acid consumption patterns were observed with the increase of the specific growth rate. An energy (ATP) producing arginine deiminase pathway was found to have a constant specific consumption rate ($mmol \cdot g_{DWT}^{-1} \cdot h^{-1}$) over the specific growth rates studied which is explained by the control of a catabolite repression regulator. At μ = 0.35 h^{-1} and above, we observed steady production of both the fermentation by-products formate, acetate, and ethanol, and the amino acids which are directed into the central carbon metabolism (asparagine, serine, threonine, alanine, and cysteine). This trend continued until almost all amino acids consumed were used exclusively for biomass synthesis. Near the maximum μ, only precursor amino acids for nucleotide synthesis (glutamine, serine and asparagine) were consumed in higher amounts than were required for cellular protein synthesis.

As μ was increased, our quantitative proteome measurements revealed an increased rate of translation. The most abundant and energetically expensive metabolic groups were glycolytic enzymes and ribosomal proteins (containing over 2000 copies per cell), followed by transporters. However, enzymes in nucleotide metabolism formed the most abundant biomonomer synthesis pathway in the cells with an expenditure of 5% of the total ATP spent for biosynthesis. At higher values of μ, metabolic fluxes increase along with an increase in the apparent catalytic activities of enzymes and ribosomes by an average of 3.3 times (comparing μ = 0.5 and μ = 0.1 h^{-1}). These observations suggest that the most prevalent regulation of cells is post-translational.

Change to more efficient metabolism at higher μ values was accompanied with a shift from mixed-acid to homolactic fermentation, although, extra ATP generated during mixed-acid fermentation exceeded the synthesis costs for enzymes of related pathways. More efficient metabolism was realized by a 50% decrease in non-growth energy demands with increasing μ, and this decrease is mainly due to the decrease in energy requirements for protein re-synthesis. Taken together, these observations regarding amino acid consumption strategies and regulation of energy metabolism provide valuable knowledge for bioprocess optimization and the construction of producer strains.

KOKKUVÕTE

Üheraksed mikroorganismid elavad muutuvates keskkonna tingimustes, kus temperatuur, toitainete kättesaadavus ning inhibeerivate kemikaalide olemasolu muutub pidevalt. Adapteerumine sellistes tingimustes määrab substraadi tarbimise kiiruse ehk kasvuerikiiruse, mis omakorda põhjustab dünaamilisi muutusi metabolismi optimeerimisel. Metaboolsed muutused rakkude suuruses, ribosoomide sisalduses ning metaboolses effektiivsuses põhjustavad muutusi sekundaarsete substraatide tarbimises ning produktide tootmismustrites. Käesolev töö on pühendatud molekulaarsete mehhanismide mõistmisele, mis kaasnevad piimahappebakter *Lactococcus lactis'e* adapteerumisel suureneva kasvuerikiirusega, millega kaasnes 30% tõus kasvueffektiivsuses (μ = 0.1 kuni μ = 0.5 h^{-1}). Seetõttu, kaksvuerikiirusest sõltuv statsionaarsest füsioloogilisest seisundist kogutud andmed kvantitatiivsest proteoomist ning transkriptoomist koos rakusiseste ja –väliste μ-sõltuvate voogude mustritega analüüsiti kasutades metaboolseid mudeleid.

Aminohapete metabolismi reorganiseerumine toimus koos kasvuerikiiruse muutmisega. Energiat (ATP-d) tootev arginiin deiminaasi rada leiti olevat kataaboolse repressiooni kontrolliva regulaatori mõju all, kuna selle eritarbimiskiirus ($mmol \cdot g_{DWT}^{-1} \cdot h^{-1}$) püsis uuritud kasvuerikiiruste vahemikus konstantsena. Koos fermentatsiooni lisaproduktide formiaadi, atsetaadi ning etanooli tootmiskiiruste stabiliseerumisega kasvuerikiirusel 0.35 h^{-1} ja kõrgemal, stabiliseerusid ka tsentraalsesse süsiniku metabolism suunatavate aminohapete (asparagiini, seriini, treoniini, alaniini ja tsüsteiini) eritarbimise kiirused. Maksimaalse kasvuerikiiruse lähistel toimus aminohapete ületarbimine vaid nukleotiidide biosünteesi suunatavate aminohapete arvelt (glutamiin, seriin, asparagiin).

Vaid minoorseid muutuseid oli võimalik tuvastada kvantitatiivses proteoomis kasvuerikiiruse tõustes (põhiline muutus leidis aset translatsiooni aparatuuris). Energeetiliselt kõige kulukamad metaboolsed grupid on glükolüütilised ensüümid ning ribosomaalsed valgud (sisaldades üle 2000 valgu molekuli raku kohta), millele järgnevad transporterid. Seevastu nukleotiidide metabolismi kuuluvad ensüümid moodustavad kõige kulukama biomonomeeride sünteesi raja, kasutades 5% kogu biosünteesile kuluvast ATP-st. Kõrgemad metaboolsed vood kõrgematel kasvuerikiirustel saavutatakse 3.3-kordse tõusuga näilistes ensüümide ja ribosoomide katalüütilistes aktiivsustes (μ = 0.5 vs μ = 0.1 h^{-1}). See omakorda viitab valdavale post-translatoorsele regulatsiooni mehhanismile.

Muutus anaboolselt effektiivsema metabolismi suunas kõrgematel kasvuerikiiruste väärtusetel kaasnes sega-happelise metabolism üleminekuga homolaktilisema suunas, kuigi ATP tootmine ületas mitmekümne kordselt enrgia kulu antud metabolismiradade ensüümide tootmiseks. Effektiivsem metabolism tagati 50% langusega mitte kasvuks kuluvale energiale (alalhoiu energia), mis vähenes põhiliselt valkude resünteesile tehtavate kulutuste arvelt. Kogu see eelnev informatsioon aminohapete tarbimise mustritest ning energiametabolismi regulatsioonist annab väärtuslikku informatsiooni bioprotsesside optimeerimisel ning tootjarakkude disainil.

LIST OF PUBLICATIONS

The following publications form the basis of this dissertation and are reproduced in the appendices with permission from the publishers.

I Adamberg K, Lahtvee PJ, Valgepea K, Abner K, Vilu R. **Quasi steady state growth of *Lactococcus lactis* in glucose-limited acceleration stat (A-stat) cultures.** *Antonie Van Leeuwenhoek*, 95(3):219-226, (2009)

II Lahtvee PJ, Valgepea K, Nahku R, Abner K, Adamberg K, Vilu R. **Steady state growth space study of *Lactococcus lactis* in D-stat cultures.** *Antonie Van Leeuwenhoek*, 96(4):487-496, (2009)

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

ADDITIONAL PUBLICATIONS

IV Nahku R, Valgepea K, Lahtvee PJ, Erm S, Abner K, Adamberg K, Vilu R. **Specific growth rate dependent transcriptome profiling of *Escherichia coli* K12 MG1655 in accelerostat cultures.** *Journal of Biotechnology*, 145(1):60-5, (2010)

V Valgepea K, Adamberg K, Nahku R, Lahtvee PJ, Arike L, Vilu R. **Systems biology approach reveals that overflow metabolism of acetate in *Escherichia coli* is triggered by carbon catabolite repression of acetyl-CoA synthetase** *BMC Systems Biology*, 4:166, (2010)

SUMMARY OF AUTHOR'S CONTRIBUTION

I In Publication I, the author carried out the experiments, analyzed the data, and participated in writing the manuscript.

II In Publication II, the author conceived the project, designed and carried out the experiments, analyzed the data, and wrote the manuscript.

III In Publication III, the author conceived the project, designed and carried out the experiments, aided in data analysis, and wrote the manuscript.

LIST OF PRESENTATIONS

I Lahtvee PJ, Arike L, Aller K, Adamberg K, Vilu R. **Absolute multi-omics analysis of energy metabolism in *Lactococcus lactis*.**
Poster presentation at Microbial Stress: from Molecules to Systems,
May 2012, Belgirate, Italy.

II Lahtvee PJ. **Systems biology approach for description of *Lactococcus lactis* amino acid and energy metabolism.**
Oral presentation at the FMTDK conference,
Feb 2012, Tartu, Estonia.

III Lahtvee PJ, Arike L, Aller K, Nahku R, Adamberg K, Vilu R. **Description of growth efficiency and amino acid metabolism in *Lactococcus lactis* at various specific growth rates at systems biology level.**
Oral and Poster presentations at the 10^{th} Symphosium on Lactic Acid Bacterium,
Aug 2011, Egmond aan Zee, The Netherlands.

IV Lahtvee PJ, Arike L, Aller K, Adamberg K, Vilu R. **Description of growth efficiency and amino acid metabolism in *Lactococcus lactis* at various specific growth rates at systems biology level.**
Poster presentation at the OpenGene Workshop,
Aug 2011, Tartu, Estonia.

V Lahtvee PJ, Arike L, Aller K, Nahku R, Adamberg K, Vilu R. **Growth Rate Dependent Dynamics in *Lactococcus lactis* Metabolism Studied by Omics and Metabolic Flux Analysis.**
Poster presentation at Systems Biology of Microorganisms,
May 2010, Paris, France.

VI Lahtvee PJ, Arike L, Aller K, Nahku R, Abner K, Adamberg K, Vilu R. **Adjustment of *Lactococcus lactis* metabolism in response to specific growth rate change studied by continuous cultivation (A-stat) and metabolic flux analysis.** *Poster presentation at RAFT VIII*, Nov 2009, San Diego, USA.

VII Lahtvee PJ. **Acid and temperature stress response of *Lactococcus lactis* in steady state D-stat culture.**
Oral presentation at Microbial Stress: from Molecules to Systems,
May 2009, Semmering, Austria.

VIII Lahtvee PJ. **Method for intracellular metabolome analysis of *Lactococcus lactis* and *Escherichia coli*.**
Poster presentation at the Waters MS users meeting,
Feb 2008, Helsinki, Finland.

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ACRONYMS

ABC	ATP-binding cassette (transporter)
ADI	arginine deiminase
ADS	arginine deiminase system
APEX	absolute protein expression
A-stat	accelerostat cultivation
BCAA	branched chain amino acid
CCM	central carbon metabolism
CDM	chemically defined medium
cDNA	complementary DNA
D-stat	dilutionstat cultivation
DWT	dry cellular weight
GEO	NCBI Gene Expression Omnibus
GRAS	generally regarded as safe
INRA	Institut national de la recherche agronomique
iTRAQ	isobaric tags for relative and absolute quantitation
K_{app}	apparent catalytic activity of enzyme
K_{cat}	the maximum number of enzymatic reactions catalyzed per second
K_m	measure of the substrate concentration required for effective catalysis to occur in reactions that obey Michaelis-Menten kinetics
KEGG	Kyoto encyclopedia of genes and genomes
LAB	lactic acid bacteria
LC	liquid chromatography
MFA	metabolic flux analysis
mRNA	messenger RNA
Mbp	10^6 base pairs
MS	mass-spectrometry
ORF	open reading frame
PPP	pentose phosphate pathway
PTS	phosphotransferase system
RNA	ribonucleic acid
rRNA	ribosomal RNA
SOM	Supplementary Online Materials
subsp.	subspecies
tRNA	transport RNA

CHEMICALS AND ENZYMES

AckA2	acetate kinase product, L.lactis: L0224
arcA	arginine deiminase, L.lactis: L0329
arcB	ornithine carbamoyltransferase, L.lactis: L0109
arcC	carbamate kinase, L.lactis: L93826
argF	ornithine carbamoyltransferase, L.lactis: L0108
ATP	adenosine-5'-triphosphate, KEGG: C00002
CarB	carbamoyl-phosphate synthase large subunit product, L.lactis: L198033
CcpA	catabolite control protein A product, L.lactis: L0143
CodY	transcriptional repressor CodY product, L.lactis: L0243
DHAP	dihydroxyacetone phosphate, PubChem: 668
DNA	deoxyribonucleic acid, PubChem: 44135672
E4P	erythrose 4-phosphate, PubChem: 122357
EutD	phosphate acetyltransferase product, L.lactis: L107797
FBP	fructose-1,6-diphosphate, PubChem: 10267
G6P	β-D-Glucose 6-phosphate, PubChem: 439427
GAP	L-glyceraldehyde 3-phosphate, PubChem: 25201921
GlcU	putative glucose uptake protein glcU product, L.lactis subsp. cremoris: llmg_2561
GlnR	glutamine synthetase product, L.lactis: L67158
ldh	L-lactate dehydrogenase, L.lactis: L0017
Ldh	L-lactate dehydrogenase product, L.lactis: L0017
NADPH	reduced dihydronicotinamide-adenine dinucleotide phosphate, PubChem: 5884
NADH	coenzyme I, reduced, PubChem: 439153
NAD+	coenzyme I, oxidized, PubChem: 439153
pepQ	proline dipeptidase, L.lactis: L96847
pfkA	6-phosphofructokinase, L.lactis: L0002
PfkA	6-phosphofructokinase product, L.lactis: L0002
Pfl	pyruvate-formate lyase product, L.lactis: L57408
PurR	pur operon repressor product, L.lactis: L134453
pyk	pyruvate kinase, L.lactis: L0003
Pyk	pyruvate kinase product, L.lactis: L0003
R5P	D-ribose-5-phosphoric acid, PubChem: 77982

THESIS

1

LITERATURE REVIEW

Lactic acid bacterium (LAB) *Lactococcus lactis* is a natural inhabitant of dairies and plants. Strains of this bacteria are widely used in the production of semi-hard cheeses and other fermented dairy, vegetable, fruit, cereal, and meat products. The widespread use of LAB can be partially attributed to the generally regarded as safe (GRAS) status it was given by The Food and Drug Administration of the United States of America.

1.1 *Lactococcus lactis* physiology and metabolism

Lactococcus lactis, and other LAB have good acid tolerance during the homofermentive conversion of sugars into lactic acid. These bacteria slow down and often prevent spoilage by acidifying its growth environment and producing growth-inhibiting bacteriocins [1]. In addition to extending the shelf life of food products, it contributes to their sensory enhancement. Because of its industrial importance, *Lactococcus lactis* is the most studied lactic acid bacterium.

Lactococcus lactis belongs to the *Bacilli* class, the *Lactobacillales* order, and the *Streptococcaceae* family. *L. lactis* has been further divided into five subspecies: *L. lactis* subsp. *cremoris*, *L. lactis* subsp. *hordniae*, *L. lactis* subsp. *lactis*, *L. lactis* subsp. *lactis* biovar *diacetylactis* and *L. lactis* subsp. *tructae* [2]. *Lactococcus lactis* subsp. *lactis* IL1403 was the first LAB whose complete genome was sequenced [3]. Today, the complete genomes of seven *L. lactis* subsp. (*lactis* and *cremoris*) are annotated in public databases (01.08.2012; Kyoto encyclopedia of genes and genomes (KEGG)) and many more industrial strains are being sequenced. However, classification of LAB has been accompanied with controversies because historical phenotypic classification does not match the rRNA-based phylogeny [4].

Historically, fermentative patterns were largely used to classify LAB and divide them into their various genera and species. The nomenclature of *L. lactis* changed several times and recent characterizations based on genotyping reveal some discrepancies between the genotype/phenotype classifications of some strains. It is now generally accepted that bacterial classification should reflect the natural, *i. e.* phylogenetic, relationships between bacteria, and species are more often defined by percentage similarities between the 16S or 23S rRNA sequences [5, 6]. The *L. lactis* genome is approximately 2.5 Mbp long and encodes more than 2300 proteins [3, 7]. This is about half the size of the *Escherichia coli* genome. This relatively short genome provides a relatively simple energy and carbon metabolism with only rudimentary parts of many of the biosynthetic pathways. This

property makes them promising targets for metabolic engineering strategies. Yet, in spite of its relative simplicity, *L. lactis* has acquired an amazing variety of adaptations and defense mechanisms that allow it to cope with its heterotrophic way of life and allow it to survive in continuously changing and often hostile environments.

L. lactis is a mesophilic bacterium with an optimal growth environment around 30°C and pH between 5.5 to 6.5. *L. lactis* is a nonsporulative, nonmotile, gram positive, oval shaped microorganism with a median diameter of 0.83 ± 0.08 μm in exponentially growing dairy isolates [8]. *Lactococci* are adapted to live in nutritionally rich environments such as milk, gastrointestinal tracts, plants, and is an auxotroph for several amino acids and vitamins. Additionally, *lactococci* are characterized as microaerophilic bacteria which multiply by fermentative metabolism. However, within the past few decades it has been shown that *L. lactis* may respire under specific conditions [9–17]. Specifically, *L. lactis* has the potential to establish an aerobic respiratory chain when provided with an exogenous source of heme. *L. lactis* encode the late genes that allow iron insertion in the protoporphyrin. This phenomenon may be inherited from their plant origin, where heme is present in the environment and thus there is no need to synthesize it. Respiration in *L. lactis* saves energy by not needing to extrude protons, which has the effect of increasing both the final biomass yield and long-term survival.

1.2 USE OF *Lactococcus lactis* IN BIOTECHNOLOGY

L. lactis is extensively used in food related biotechnology to prevent spoilage and add texture and flavour to various fermented products. It is a non-pathogenic bacterium that is critical for manufacturing dairy products such as buttermilk, yogurt, and cheese. In addition, several LAB possess health promoting characteristics (mainly from the genera *Lactobacillus*); *L. lactis* lacks these characteristics.

Recently, *L. lactis* has drawn attention as the first genetically modified organism to be used in a surviving state for the treatment of human Crohn's disease [18]. Several genetic tools have been developed to make the genetic manipulation of this organism straightforward, and has opened up the use of *L. lactis* as a cell factory and increase its industrial functionality [19, 20]. A number of useful tools available for the directed genetic manipulation of this bacterium include the nisin-controlled overexpression system [21], a toolbox that allows for the modulation of gene expression at a selected level [22], the pORI/pVE6007 two-plasmid system to obtain clean food-grade deletions of genes of interest [23], and oligonucleotide-mediated recombineering [24].

Because of its GRAS status, the availability of tools for genetic manipulation and their capacity to survive the passage through the gastrointestinal tract has rendered *L. lactis* and some *Lactobacillus* strains as excellent candidate hosts for the production and delivery of therapeutic proteins [25–27]. Interest in the use of LAB as delivery vehicles initially focused on the development of mucosal vaccines, because a delivery system is needed to avoid degradation and promote uptake of the antigen in the gastrointestinal tract, and stimulate adaptive immune responses, rather than the tolerogenic immune responses that are seen in feeding studies with soluble antigens [28, 29]. For vaccine delivery, it is easier to obtain immune responses by intranasal administration, and even killed LAB are effective in this administration route. The use of killed LAB would have distinct

advantages over live organisms with respect to the regulatory issues for the clinical use of genetically modified organisms, however, the safety of intranasal immunization with a bacterial carrier needs to first be addressed. Oral (intragastric) immunization remains a very attractive alternative, however, frequent doses over a period of several weeks are required to achieve protection. However, recent targeting strategies for LAB show that it is possible to enhance the immunogenicity and efficacy of LAB vaccines.

A recent development in the use of LAB as delivery vehicles has been in the field of DNA vaccination. The advantage of DNA vaccines lie in their ability to induce potent cellular immune responses in addition to antibodies and the flexibility to express multiple antigens or epitopes using one DNA vector. For viral antigens, the correct posttranslational modifications (*e.g.*, glycosylation) should be carried out by the host cell machinery. Despite the successful use of DNA vaccination in small animals, its translation to primates, humans, and other large animals has been beset with problems [30]. Excellent reviews about the various applications of LAB as a therapeutic host and delivery vehicles are available: [25–27]. The extensive and increasing use of this microorganism in biotechnological processes intensifies the need to better understand its metabolism and regulatory mechanisms. Such knowledge is useful for directed product formation or the creation of efficient cell factories.

1.3 ENERGY METABOLISM

Because oxidative phosphorylation does not occur under conditions where heme is absent, *L. lactis* produces the majority of phosphorylated metabolites during glycolysis. The glycolytic end product pyruvate is mainly directed to lactate to regenerate NAD+. This approach allows for an almost perfect splitting of the metabolism into an anabolic module where biomonomers are synthesised and assembled into macromolecules where ATP is consumed (ATP demand), and a catabolic module where ATP is generated (ATP supply). The anabolic and catabolic modules are linked mainly through the balance between energy supply and demand. A more detailed description of anabolic and catabolic reactions are given below.

1.3.1 *Biomass formation (anabolic reactions)*

Based on stoichiometric metabolic pathways and experimental data the expenditure of phosphorylated metabolites used in the production of biomass components in exponentially growing *Escherichia coli* has been estimated [31]. However, this information is lacking for *Lactococci*, especially information gathered under controlled physiological conditions. Existing information shows that protein polymerization is by far the highest cost in biomass formation, followed by amino acid transport. During protein synthesis and polymerization, 4 ATP equivalents per amino acid are spent activating the ribosomal site and incorporating them into the peptide chain, 0.2 for mRNA synthesis, 0.1 for proofreading, and 0.006 for assembly and modification [32, 33]. Because *L. lactis* is auxotrophic for several nutrients, the majority of biomonomers must be transported from the environment instead of *de novo* synthesis. The genome of *L. lactis* IL1403 encodes about 250

proteins that are directly involved in solute transport. This means that well over 10% of all genes in its genome encode transport proteins, which reflects the important role of transport processes in this bacterium. The largest class of transporters is the secondary transport systems followed by the ABC transporters [34]. Although, amino acid transport is an energy demanding process, the combination of ammonia transport and amino acid *de novo* synthesis results in a similar or even higher energetic demand [35]. In brief, it has been demonstrated that biomonomer synthesis is responsible for approximately 10% of energetic demand, transport for 20%, and polymerization for the remainder of the energy spent for biomass formation [35, 36].

It is assumed that all of the energy from catabolism can be used for growth, however, energy is also spent on functions that are not directly growth related. This non-growth related energy is also termed cell maintenance. There have been debates whether the maintenance is a constant value. Observations have shown that maintenance could vary from condition to condition [37–40], it has been assumed that under the same environmental conditions maintenance is μ independent [41–43]. Typically, a linear relationship is assumed and the growth rate dependent specific substrate consumption rate (q_s) is found from the intercept of this linear relationship with the y-axis. This provides a value for the maintenance coefficient. Bacterial maintenance energy has not been precisely defined, but at least three functions are known to contribute: (I) re-establishment of the ion gradients across the cell membrane, (II) turnover of macromolecules (*e.g.* protein) and sensory molecules, and (III) futile cycles [35, 36]. The exact shares of those components in the energy balance of cells has not been estimated nor has their growth rate dependence been analyzed. It is presumed that maintenance of the ion gradient might have the highest energetic demand among various functions of maintenance energy [35, 44]. The presence of futile cycles has been reported in *Escherichia coli* [45–47] and LAB, where ATP-cycling in glycolysis has been proposed [48]. The energy demand for macromolecule re-synthesis has not been calculated before, however, methods for detecting degradation rates are emerging [49–51]. A special cultivation method was developed to study the maintenance energy demand at very low specific growth rates under controlled environmental conditions [52], which was also used to study *Lactobacillus plantarum* [43]. Because constant maintenance costs were predicted (growth rate independent), maintenance costs were proposed to increase from 13 to 94% with the decrease of μ from 0.025 to 0.0006 h^{-1} [43].

1.3.2 *Phosphorylated metabolite generating pathways*

1.3.2.1 *Central carbon metabolism (CCM)*

The metabolic energy in anaerobic *lactococci* is generated *via* substrate level phosphorylation [53–55]. Glucose is usually the preferred source of carbon, however, *lactococci* are usually able to transport a variety of sugars. For the transport of glucose and other hexose sugars there are two distinct phosphoenolpyruvate:phosphotransferase systems (mannose-PTS and cellobiose-PTS). Recently, GlcU was identified as the sole non-PTS permease (proton motive force dependent) involved in the transport of glucose [56]. Glucose imported by PTSs is concomitantly phosphorylated and fed into glycolysis. Other hexose

sugars or disaccharides must first be converted and/or hydrolyzed to sugar-phosphate species that can be glycolysed. The conversion of lactose and sucrose into glycolytic intermediates has been extensively studied in *L. lactis* [57–62]. Galactose and lactose are converted to glycolytic intermediates in *L. lactis* subspecies *via* either the tagatose or Leloir pathways. The main difference between the two pathways are the mechanisms for sugar import. In the tagatose pathway, lactose and galactose are imported and concomitantly phosphorylated *via* the PTS_{lac} [60] while in the Leloir pathway the sugars are imported by dedicated permeases [58, 63, 64]. This influences the products formed, with the tagatose pathway forming G6P and the trioses, DHAP and GAP, and the Leloir pathway producing G6P and glucose. The use of the Leloir and/or the tagatose pathways for galactose utilization is strain dependent [63]. During glycolysis glucose is converted to pyruvate and the free energy is released in the form of ATP, a reaction which requires NAD^+.

The regulation of glycolysis and the shift between different fermentation modes of *L. lactis* has been extensively studied [12, 19, 57, 62, 65–70]. No single enzymatic step controls the rate of glycolysis in *L. lactis* and control appears to be distributed over many glycolytic steps, sugar uptake and lactate efflux [71]. The distribution of flux control over a number of enzymatic steps is partly regulated by catabolite control protein A (CcpA). In addition to controlling the transcription of sugar-specific operons, CcpA acts as an activator of transcription of the las operon genes *pfkA*, *pyk* and *ldh*, encoding PfkA, Pyk and Ldh, by binding to a *cre* site upstream of the operon [62]. CcpA and the metabolite fructose-1,6-diphosphate (FBP) mediate both transcriptional and allosteric activation of Ldh to ensure the rapid formation of lactate.

1.3.2.2 *Pyruvate metabolism*

During the growth under anaerobic conditions with high sugar uptake rates, pyruvate generated *via* glycolysis is predominantly converted to lactate by lactate dehydrogenase (Ldh). This reaction regenerates NAD^+ which is used in glycolysis and also to maintain the redox balance. Alternatively, pyruvate could be converted to acetyl-CoA *via* pyruvate-formate lyase (Pfl) and further into byproducts which can produce extra energy (acetate), regenerate NAD^+ (ethanol) or contribute to flavour production in dairy products (diacetyl, acetoin, acetate, acetaldehyde and ethanol) [72–74]. The shift towards energetically more efficient mixed-acid fermentation is observed at lower specific growth rates, at growth on less energetic substrates, or under aerobic conditions. However, the cause of the shift has not yet been determined. When respiratory metabolism is active, the switch to the less efficient (fermentative) overflow metabolism has been proposed to be a consequence of flux sensing mechanisms [75]. Although mixed-acid fermentation in *L. lactis* yields additional ATP, in comparison with lactic acid fermentation, the mixed-acid fermentation pathway requires, in addition to glycolytic enzymes, synthesis of at least five enzymes, whereas lactic acid fermentation requires only lactate dehydrogenase. Hence, it is proposed that the trade-off between investment in proteins of the mixed acid pathway and the benefit of additional ATP generation could play a role in determining the metabolic shift [76–79].

1.3.2.3 *Arginine deiminase pathway*

The arginine deiminase (ADI) pathway, which is also referred to as the arginine deiminase system (ADS) is a multi-enzyme pathway (encoded by the arc operon) through which arginine is degraded into ornithine, ammonium and carbon dioxide with concomitant production of ATP [80–82]. The ADI pathway is the only amino acid degradation pathway in LAB that is known to provide ATP *via* substrate level phosphorylation, and is therefore of major energetic importance in these organisms, which cannot gain energy through oxidative phosphorylation [83]. The ADI pathway consists of the three enzymes, arginine deiminase (*arcA*), catabolic ornithine carbamoyltransferase (*argF*, *arcB*) and carbamate kinase (*arcC*) [81]. The enzyme hydrolyzes arginine into citrulline and ammonium. The product of ADI, citrulline is metabolized by catabolic ornithine carbamoyltransferase or ornithine transcarbamoylase into ornithine and carbamoyl phosphate. Ornithine, a by-product, is excreted out of the cell *via* the arginine-ornithine antiporter [84]. Electroneutral exchange of equimolar amounts of arginine and ornithine takes place without the need for an external energy supply. The ADI pathway produces ATP and arginine and can support growth in *L. lactis* subsp. *lactis* strains as well as in another LAB [85–87]. Typically, *L. lactis* subsp. *cremoris* strains do not possess the ADI pathway but the effect on pH tolerance and/or ATP production have not been studied. The promoter regions of the ADI pathway gene cluster of *L. lactis* and *Streptococcus gordonii* contain a *cre* site, likely responsible for the repression of these genes by glucose *via* CcpA [82, 84].

1.3.2.4 *Nitrogen metabolism*

L. lactis consumes amino acids as the primary nitrogen source. Nitrogen is present in many cellular compounds, including amino acids, nucleotides, amino sugars, NAD^+, cofactors and vitamins, some of which are ultimately incorporated into macromolecules such as proteins, DNA, RNA and cell wall components. Hence, amino acid consumption is of high importance and a well balanced nitrogen metabolism could save additional energy and result in higher biomass yields. To deal with the fluctuation in extracellular as well as intracellular nitrogen availability, bacteria have evolved elaborate regulatory mechanisms controlling assimilation, utilisation and integration of nitrogen into the central metabolism. For amino acid transport, *lactococci* use mainly ABC transporters, which usually have very high affinities for their solutes and catalyze transport at high rates. This may allow *L. lactis* to efficiently scavenge essential solutes from the environment, giving it a competitive advantage [34]. *L. lactis* is multiple amino acid auxotrophic and, with some strain variation, leucine, valine, isoleucine, methionine, histidine, and glutamine or glutamate, are essential amino acids [88, 89]. The inability to produce some of these amino acids is caused by point-mutations or more severe lesions in the genes of biosynthetic pathways, which is attributed to the adaptation of this organism to nitrogen-rich environments such as milk [90].

During growth in milk *L. lactis* acquires free amino acids through degradation of extracellular proteins by a thoroughly characterized proteolytic system [91], which is controlled by the global regulator CodY [92–94]. CodY controls the major genes involved in peptide assimilation, including their transport and their further degradation by pepti-

dases [95]. Furthermore, CodY also controls the expression of five genes encoding transporters involved in the uptake of free amino acids and *de novo* biosynthesis of amino acids [96]. CodY regulation is mainly associated with branched chain amino acids (BCAAs), especially with the intracellular concentration of valine. High intracellular valine concentration is presumed to activate CodY repression which hinders the protein cleavage and peptide transport [92, 93, 96]. In addition to the uptake of sugars, carbon catabolite repressor protein CcpA is controlling the regulation of the ADI pathway and proline dipeptidase (*pepQ*). Direct or indirect influence of the CcpA deletion strain has been observed for gene expression of various amino acids [97]. The only known regulator in glutamine/glutamate metabolism is GlnR. GlnR is extensively studied in *Bacillus subtilis*, whereas GlnR is active during conditions of nitrogen excess, repressing the expression of the glutamine synthetase (*glnRA*) operon [98] and the urease operon [99, 100]. Despite the similarities in the genome, the only common GlnR target in both organisms is the *glnRA* operon [101].

As there is limited information regarding amino acid transporters and regulatory mechanisms, it follows that amino acid consumption patterns are not well characterized under controlled environmental conditions. When complex media is used for the growth of *L. lactis*, usually, amino acids are in large excess and precise absolute consumption is difficult to measure. Moreover, only a limited number of studies have reported consumptions patterns for some amino acids from experiments, where CDM is used [41–43, 89, 102]. Amino acid content in the biomass of *lactococci* has been measured by [103], however, it has not been compared to amino acid consumption patterns to detect the over or underconsumption of individual amino acids and their utilization pathways [103]. Additionally, a full amino acid consumption pattern would be necessary to describe the whole carbon and nitrogen balance. We have yet to reach a clear understanding of the amino acid (and peptide) consumption preferences for *lactococci*, despite its importance.

In addition to amino acids, nucleotides are nitrogen containing compounds, which are obligatory metabolites in all organisms. Nucleotides are substrates for RNA and DNA synthesis, and serve as the main energy donors for cellular processes. In *L. lactis* the *de novo* synthesis of nucleotides are conducted by pur operons and activated by PurR protein, when bound to a conserved PurBox motif present on the DNA [44, 104, 105]. For *Escherichia coli* it has been demonstrated that transport of nitrogenous bases have a growth advantage in front of their *de novo* synthesis, which results in a higher biomass yield [35].

1.3.2.5 *Pentose phosphate pathway*

The pentose phosphate pathway (PPP) is an alternative route for glycolysis, where G6P enters into the pathway and FBP and GAP are sent back into glycolysis for energy generation. There are three main products formed as the result of a functioning PPP. First, the generation of reducing equivalents in the form of NADPH are used in anabolic reactions in the cells (*e.g.*, fatty acid synthesis). Second, production of ribose-5-phosphate (R5P) used in the synthesis of adenine and guanine ribonucleotides, energy and redox carriers. Polymerized products of these compounds are genetic material for cells (DNA and RNA). Third, production of erythrose 4-phosphate (E4P) used in the synthesis of aromatic amino

acids – phenylalanine, tyrosine, and tryptophane. There are two distinct stages in the pathway. First, the oxidative branch of the PPP is used for NADPH formation, and the second, nonoxidative branch, is used for formation of pentose sugars.

Although extraordinary progress has been made in studying this extremely diverse group of LAB, systems level comprehensive methods are required to fully understand their metabolic regulation.

1.4 SYSTEMS BIOLOGY APPROACH

Many biological studies look only at specific aspects of metabolism. To gain a higher level systems understanding of metabolic regulation, both knowledge of the components in the system and the interactions between them must be elucidated [106]. Therefore, an integrative approach, where global analysis together with metabolic modeling is now being applied more frequently. Systems biology is an interdisciplinary field of study that focuses on the interactions of a large number of disparate biological processes with the aim of generating new insights into how organisms respond to different stimuli [107]. In recent decades we have witnessed the development of analytical techniques for global analysis of various cell components. However, these are scarcely applied in the same experiment under strictly controlled physiological states. Genome-wide absolute quantitative mRNA and proteome data are available for mammalian and yeast cells [49, 108, 109]. However, this data has yet to be collected for LAB. Relative measurements of the transcriptome, proteome, metabolome, and fluxome have been gathered separately or in partial combination, and this information provides valuable information about potential controlling mechanisms of metabolic regulation and energy metabolism [42, 43, 89, 110–113].

Applying a systems biology approach, together with absolute numbers for individual mRNA and protein levels, extracellular metabolite concentrations, and biomass composition measurements, allows one to find flux distributions and provides an excellent chance to understand cellular adaptation and estimate the level of metabolic regulations in different metabolic pathways [49, 50, 114]. Currently, most quantitative proteomics investigations are focused on the analysis of protein expression differences between two or more sample specimens under different physiological states. With each analysis a static snapshot of a cellular state is captured with regard to protein expression. However, a more dynamic and multi-layered approach is required to understand the interactions between metabolic and regulatory networks during cellular adaptation to environmental changes [115].

Although important, protein degradation is usually left unnoticed because information on protein turnover cannot be obtained using classical methods. Protein turnover that results from protein synthesis and degradation, is a dynamic process, and is of equal importance with other omics analysis methods in our quest to understand physiological processes. Methods employing isotopic tracers have been developed to measure protein turnover. However, protein degradation is not often experimentally measured [49, 50, 116–119]. Dressaire *et al.* [111] calculated degradation rates based on proteome and transcriptome results and is the first study where specific growth rate dependent protein degradation rates were estimated from a steady physiological state. Additionally,

protein degradation has been measured for *Corynebacterium glutamicum* from various batch phases (different growth rates) where constant degradation rates were observed [120].

Another scarcely measured yet important analysis technique is the measurement of enzymatic activities. The most prevalent approach has been *in vitro* measurements [121–123]. However, *in vitro* kinetics might not apply to *in vivo* conditions [124]. Furthermore, sources of kinetic parameters span studies investigating different strains under a variety of growth conditions. A more progressive method was taken into use by Goel *et al.* [125], where enzyme activity measurements were carried out under a standardized *in vivo*-like assay medium. A wide range of enzymes in the central metabolic pathways of *L. lactis* were measured under these standardized conditions[125]. However, this method cannot provide information about *in vivo* catalytic activities under various conditions. In general, it is observed that K_{cat} values are significantly higher for high-flux specialist enzymes than for all other enzymes and that essential enzymes have lower K_m values and therefore higher substrate affinity [126].

Computational modeling forms the essential backbone of systems biology. Depending on the proposed biological questions, the complexity of the model system can vary from small number of reactions in the main metabolic pathways [127] to comprehensive genome scale models [11, 128–131]. Using large data sets from various sources together with quantitative modeling allows one to find insight into the physiological system that intuition and deductive reasoning would perhaps never achieve.

This dissertation concerns *L. lactis* physiology, and consists of a series of studies that work together to further our understanding of this technologically important organism. First, continuous cultivation methods were developed and implemented to both observe dynamic changes in *L. lactis*, and optimize the environmental conditions under strictly controlled physiological states. Working in this strictly defined state is essential to collect quantitative multi-omics data that represents the physiological state of cells. Second, we analyzed the energetic burden and regulation of cell components to the adaptation of *L. lactis* cells with increasing specific growth rate using global multi-layer omics analysis. With this increase in specific growth rate we observe a 30% increase in biomass yield and a switch to a less energy efficient metabolism. Global quantitative measurements of the intra and extracellular metabolome, transcriptome, proteome, and cell component measurements, together with metabolic flux analysis, were carried out to elucidate the mechanisms behind this switch. Quantitative abundances of cell components allowed us, for the first time in a LAB, to precisely calculate the metabolic burden of cell replication and compare those values with the production of phosphorylated metabolites. Good coverage of the global quantitative proteome provided the basis for various insights into the characteristics of these cells. By combining specific growth rate dependent data, proteome abundance measurements, and metabolic fluxes allowed us to find the apparent catalytic activities of enzymes. Moreover, specific growth rate dependent abundance of mRNA and protein levels were used to estimate protein degradation rates for individual proteins and the energetic burden of maintenance costs. Although the latter is a putative analysis, this is the first attempt to characterize the specific growth rate dependent distribution of maintenance energy. This study provides new global-level information about this widely used industrial microorganism and furthers our understanding of why

published metabolic patterns operate as such. Moreover, new hypotheses were raised that may be tested in future experimental studies.

2

AIMS OF THIS DISSERTATION

The general aim of the present study is to gain insight into the metabolism and physiology of *Lactococcus lactis*. Specifically, the aims are as follows:

I Optimization of environmental conditions (pH, temperature, composition of the cultivation medium) to achieve the highest biomass yield of *L. lactis* growing in a steady physiological state.

II Application of relative and absolute omics analysis methods to study the specific growth rate dependent metabolism of *L. lactis*.

III Quantitative analysis of the utilization of amino acids by *L. lactis* using a systems biology approach that utilizes computational modeling.

IV Study the production of phosphorylated metabolites in *L. lactis*, and the efficiency of their utilization using multiple data sets and models, with specific focus on the estimation of maintenance energy and protein turnover.

3

MATERIALS AND METHODS

More detailed descriptions of the materials and methods applied are available in the publications. The following sections are provided to make this material more accessible.

3.1 *L. lactis* strain, inocula, and media used

The strain used in all experiments, *Lactococcus lactis* subsp. *lactis* IL1403, was kindly provided by Dr. Ogier from INRA (Jouy-en-Josas, France). Inocula were prepared from a lyophilized stock culture stored at -80°C which was pre-grown twice on the cultivation medium. The composition of the medium used for cultivation in Publication I and Publication II was taken from previous studies with only slight modification. In Publication III and thereon, an optimized medium composition was used, which supported growth without any nutritional limitations (except the primary carbon source - glucose). This optimized medium limited the excess of other nutrients to allow for more accurate determination of the consumption of medium components.

3.2 Bioreactor set-up and cultivation conditions

Cultivation experiments 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). Reactors were equipped with pH, temperature and gas control, and an *in situ* OD sensor (model TruCell2; Finesse, San Jose, CA; used only in Publication III). Cultivation experiments were carried out under anaerobic conditions (N_2-environment) with an agitation speed of 300 rpm at 34°C and pH 6.4, if not stated otherwise.

3.3 Cultivation techniques

All aspects of this study utilized changestat cultivation methods. Changestat cultivation techniques are derived from chemostat cultivation, where the cell culture is stabilized at an initial environmental condition. After reaching this steady physiological state, acceleration is applied consisting of a smooth change of a chosen environmental parameter: $Z = Z_o + a \cdot t$; where Z is an environmental parameter of interest, Z_o is initial value of the environmental parameter, a is the acceleration, and t is time. According to this

formula, the dilution rate (A-stat; [132, 133]), pH and temperature (D-stat; [134]) were changed in separate experiments in this current study. The aim is to stay as close to a steady physiological state as possible. To achieve this, the acceleration of environmental parameter must be carefully chosen.

3.4 ANALYTICAL METHODS AND GROWTH CHARACTERISTICS

3.4.1 *Biomass concentration, composition, and cell size*

Biomass was constantly monitored by measuring the optical density at 600 nm. The dry weight of the biomass was determined gravimetrically. The biomass correlation constant, K, was determined separately for each experiment and was found to be independent of specific growth rate, but changed with the medium composition used. An average K value was determined in Publication III to be 0.372 ± 0.005.

For measuring amino acid concentrations in the protein content, the biomass was hydrolysed. From the hydrolysate, amino acids were determined as free amino acids as described below (see Section 3.4.2). The protein content of the biomass dry cell weight was calculated based on the amino acid analysis and, additionally, measured using the Lowry method [135]. For measurement of DNA content in the biomass, genomic DNA was extracted and measured using instructions provided within the RTP® Bacteria DNAMini Kit (Invitec, Germany). The protocol applied for fatty acid quantification is described in [136]. Cell size was calculated based on unit mass using an in-house built model [Abner *et al.*, submitted]. The results are in concordance with published results for exponentially growing *L. lactis* [137]. The change in cell size with changing μ was confirmed using flow cytometry (Accuri).

3.4.2 *Metabolome analysis*

The concentration of glucose, lactate, formate, acetate, and ethanol in the culture media was measured using a liquid chromatograph (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 $ml \cdot min^{-1}$ and at 35°C. A refractive index detector (model 2414; Waters Corp.) was used to detect and quantify these substances. The detection limit for the analytical method was 0.1 mM. Samples from the culture media were centrifuged (14,000 × g, for 4 min); The supernatants were collected and analyzed immediately or stored at -20°C until analysis. Free amino acid concentrations were determined from the same sample (analyzing frequency of around 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 data processing. Additionally, UPLC®-MS (Waters Acquity UPLC® and LCT Premier ESI TOF, respectively) method, adapted from [138] was used for detection and quantification of a variety of metabolic by-products. MassLynx® software (Waters Corp.) was used for data manipulation. Detected products are listed in Publication III. It should be noted that the concentration of summed by-products formed less than 0.1% of all products determined.

3.4.3 *Transcriptome analysis*

Quasi steady state samples from A-stat experiments described in Publication III at specific growth rates of 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 at 0.1 h^{-1}. Total RNA was extracted and quantified, cDNA synthesized and labeled with Cy3 (CyTM3 Mono Reactive Dye Pack, Amersham, Buckinghamshire, UK) or Cy5 (CyTM5 Mono Reactive Dye Pack, Amersham). Hybridization, slide washing and scanning was performed using standard Agilent reagents and hardware http://www.chem.agilent.com. Microarray slides were scanned using an AgilentDNA Microarray Scanner (Santa Clara, CA). Spot intensities and corresponding background signals were quantified with Genepix Pro (version 6; Axon Instruments [http://www.moleculardevices.com/pages/software/gn_genepix_pro.html]). For data analysis, global lowess normalization was used. Spots with intensities lower than 100 units in both channels and outliers among technical replicates were filtered according to the method in [139]. After filtering, seven technical replicates showed average standard deviation <10%. Gene (and protein) expression measurement results are shown in Publication II Additional file 1, and Publication III Additional file 3. DNA microarray data is also available at NCBI Gene Expression Omnibus (GEO) (Reference series: GSE26536). For mRNA abundances, average intensities from seven different mRNA oligos from a single experimental point in the Agilent transcriptome array (GEO number GSE26536) were summed, excluding ribosomal RNA (rRNA) and transport RNA (tRNA) units. This sum corresponds to the total amount of mRNA. It was assumed that mRNA composed 5% of the total RNA (that was measured) and the intensity units of each mRNA were proportional to the total mRNA. The amount of molecules in each mRNA in the biomass were calculated taking into account the molecular weights of each mRNA. Absolute mRNA values can be found from Supplementary Online Materials (SOM) 1 (https://sites.google.com/a/tftak.eu/lahtvee-phd-thesis/)

3.4.4 *Proteome analysis*

For protein expression analysis, the steady state chemostat culture of *L. lactis* IL1403 was used as a 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 analyzed. Samples intended for proteome analysis were collected, washed with PBS, flash frozen in liquid nitrogen and stored at -80°C prior to protein extraction. Proteins were extracted in ice-cold SDS-buffer. Cells were disrupted as a result of agitating the suspension with glass-beads. Protein concentration in sample 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 Biosys- tems, Foster City, CA) according to manufacturer's instructions. The combined peptide mixtures were separated into 10 fractions with a cation exchange cartridge system (Applied Biosystems, Foster City, CA) and cleaned by StageTips [140]. All fractions were analyzed twice by LC-MS/MS using an Agi-

lent 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). The Data generated was searched against *L. lactis* IL1403 NCBI database (22092009) by MassMatrix search tool [141]. Proteomic analysis raw data is available at the PRIDE database [142] `http://www.ebi.ac.uk/pride` under accession numbers 13105-13162 (username: review17185, password: wyd*b6_6). The data was converted using the PRIDE Converter `http://code.google.com/p/pride-converter` [143]. Proteome data were recalculated into absolute values using the APEX algorithm, which assumes proportionality of spectral counts of peptides from each protein to be linearly correlated to abundance of this protein in the cell [144]. Absolute protein values can be found from SOM 1.

3.5 METABOLIC MODELING

3.5.1 *Metabolic flux analysis*

The network for metabolic flux analysis was adapted from (Adamberg *et al.*, unpublished). In brief, a simplified metabolic network of *L. lactis* subsp. *lactis* IL1403 under different growth conditions was constructed based on information from the BioCyc database (`http://biocyc.org`) and using a spreadsheet to perform the metabolic flux calculations. Only the reactions from the central pathways (glycolysis, pentose phosphate cycle, pyruvate metabolism), amino acid metabolism, and biomonomer synthetic fluxes with branch-point metabolites were taken into account to construct the network *i. e.*, all linear reaction chains were combined into a single flux (altogether 103 merged pathways or single reactions). This model calculates the intracellular fluxes and the balance of carbon, nitrogen, energy and redox metabolism. As an input data changing information from consumption/production patterns as well as biomass composition were used. Reactions of the metabolic model and calculated flux values can be found from SOM 2 and SOM 1, respectively.

3.5.2 *Protein degradation calculations*

For calculating protein degradation rates and their corresponding half-lives, a MATLAB® based model was adapted from Dressaire *et al.* [111]. Briefly, specific growth rate dependent individual absolute mRNA and protein levels were used to calculate translation and protein degradation rates according to:

$$\frac{d[\text{protein}]}{dt} = k'[\text{mRNA}] - \mu[\text{protein}] - k''[\text{protein}] \tag{3.1}$$

where [protein] is the absolute protein concentration ($\text{molecules}\cdot g_{DWT}^{-1}$), t is time (h), k' is the translation rate (h^{-1}), [mRNA] is the absolute mRNA concentration ($\text{molecules}\cdot g_{DWT}^{-1}$), and k'' is protein degradation rate (h^{-1}). This model estimates the specific growth rate dependent pattern of translation and degradation rates and calculates values for each mRNA-protein pair. Calculated half-lives for individual proteins can be found from SOM 1.

3.5.3 *Energetic burden of protein degradation*

Based on quantitative protein amounts (in molecules$\cdot g_{DWT}^{-1}$), the number of amino acids per protein, and a literature based value for the polymerization of one amino acid into the chain (4.306 ATPs [32, 33]), we calculated the total production cost for every measured protein (in molecules ATP spent per protein amount in g_{DWT}).

4

RESULTS AND DISCUSSION

THE RESULTS OF THIS DISSERTATION are presented and discussed in six sections organized by topic.

4.1 OPTIMIZATION OF GROWTH CONDITIONS

4.1.1 *Acceleration of dilution (Publication I)*

Lactococcus lactis is the most studied lactic acid bacterium and is widely used in industrial processes. However, there exists a lack of knowledge about the peculiarities of *L. lactis* metabolic regulation, especially regarding its energy regulation mechanisms and amino acid metabolism. Our interest was to characterize the dynamics of the metabolism of *L. lactis* at various growth rates because this is one of the most important parameters that regulate cellular physiology. Hence, accelerostat (A-stat) cultivation [132] was chosen as it allows for the acquisition of vast amounts of data on quasi steady state growing cells and precise determination of growth characteristics (including metabolic switch-points). A-stat has advantages compared with chemostat because it allows for the collection of data from multiple specific growth rates more rapidly. However, one must choose the acceleration of dilution carefully to ensure that the physiological state of the cells is comparable to that obtained in chemostat experiments conducted at the same specific growth rates. When this parameter is well chosen, quasi steady state data acquired from A-stat experiments and steady state data from chemostat experiments are comparable [133, 145, 146].

Because the acceleration of dilution rate is an important parameter in A-stat experiments, the impact of various acceleration values were studied and a suitable acceleration to obtain steady state representative data was determined. Suitable acceleration is a tradeoff between obtaining strictly steady state data (*i. e.* considering protein turnover) and avoiding mutations that might occur during long experiments. Another goal is to minimize the resources, labour, and consumables used. Therefore, a series of experiments was conducted with various acceleration values (0.06 to 0.003 h^{-1}). The presence of a quasi steady state was evaluated by (I) halting the acceleration and continuing the experiment in chemostat (while expecting constant yields), and (II) reaching the maximal specific growth rate pre-determined in batch cultivation. It is important to note that suitable acceleration is strain and environmental condition dependent and, hence, must be validated separately when changes are made in the cultivation setup.

4.1.2 *pH and temperature (Publication II)*

Next, we optimized the environmental conditions for the growth of *L. lactis* in terms of pH and temperature. The growth space of *L. lactis* subsp. *lactis* IL1403 was studied at a constant growth rate using D-stat cultivation. D-stat continuous cultivation was preferred to batch cultivation because a steady physiological state was required and biased results from batch cultivations were observed previously (data not shown). Starting from steady state condition in a chemostat culture ($D = 0.2\ h^{-1}$), the pH and/or temperature were continuously changed over the range of 5.4 to 7.4 and 26 to 34°C, respectively. As a result the optimal growth conditions that supported the highest biomass yield were determined to be pH 6.4 and 34°C for *L. lactis* subsp. *lactis* IL1403 (see Figure 1). These values were then used in all cultivation experiments throughout these studies.

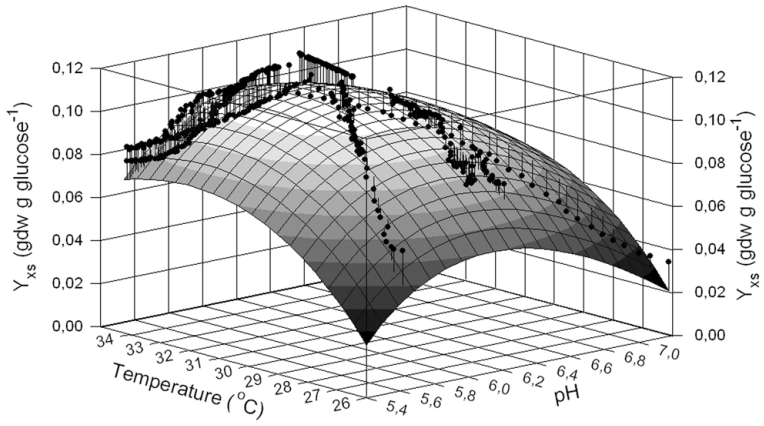

Figure 1 – *Lactococcus lactis* IL1403 quasi steady state growth surface describing the dependence of biomass yield (based on glucose consumption) on temperature and pH. Black dots represent quasi steady state data points from D-stat experiments and the surface represents the best fit. Y_{XS} – biomass yield calculated on glucose consumption ($g_{DWT} \cdot g_{glucose}^{-1}$)

4.1.3 *Medium composition (Publication III)*

Next, based on previously determined consumption patterns, we modified the composition of the cultivation medium to support higher biomass yield and reduce the concentration of individual compounds which were in excess. To obtain higher precision in the determination of amino acid consumption patterns, concentrations of most amino acids in the growth medium were reduced about three times compared with traditionally used chemically defined medium (CDM) [102]. Exceptions were arginine and glutamine, whose concentrations were increased in the medium to avoid amino group shortages during growth. Although the composition of the medium was improved, there is room for further optimization.

4.2 GROWTH RATE DEPENDENT CHARACTERIZATION OF *L. lactis* METABOLISM (PUBLICATION III)

4.2.1 *Biomass yield profile*

Optimized environmental conditions (medium, pH, and temperature) together with validated optimal acceleration of dilution (0.005 h^{-2}; data not shown) were used to study the growth rate dependent metabolic characterization of *L. lactis*. Five independent parallel experiments were carried out, where culture was stabilized in chemostat at a dilution rate of 0.1 h^{-1}. After achieving steady physiological state, smooth acceleration of dilution was applied and experiments were continued until the maximal specific growth rate of the culture was reached at 0.59 ± 0.02 h^{-1} (average value of five independent experiments $\pm$ standard deviation).

With an increase of dilution rate, which in steady state corresponds to specific growth rate, a remarkable 30% increase in biomass yield was observed (average from 0.127 ± 0.003 to 0.168 ± 0.008 $g_{DWT} \cdot g_{glucose}^{-1}$ when μ 0.1 h^{-1} was compared to μ_{max}, respectively; (see Figure 2). A similar increase in biomass yield was observed previously [42, 113], however, the mechanism behind this phenomenon were not discussed. One could hypothesize that this increase in biomass yield, based on glucose consumption, might have been caused by (I) more efficient use of amino acids; (II) optimized enzyme abundances or activities; or (III) more efficient use of phosphorylated metabolites. All of these aspects were considered in the following analysis.

4.2.2 *Biomass composition*

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}. RNA content increased from 6.5 ± 1.0% to 9.5 ± 1.5% in cell dry weight in between the latter μ values. DNA content was μ independent at 1.5 ± 0.5%. From the same experiments, the composition of fatty acids were determined by Špitsmaister *et al.* [136]. The greatest changes in biomass composition with changing growth rate were detected in the content of RNA, membrane, and polysaccharides (specific growth rate dependent composition of the biomass can be found from SOM 2).

4.2.3 *Product formation profiles*

The main fermentation by-product throughout the studied growth rate values was lactate, whereas 92 ± 2% of consumed glucose was constantly directed towards lactate production to regenerate NAD+ ($Y_{lg} = 1.83 \pm 0.03$ $mol_{lactate} \cdot mol_{glucose}^{-1}$). Although lactate production per consumed glucose remained constant, the production of other by-products (formate, acetate, ethanol) decreased with the increase of specific growth rate by about four times (from 0.22 to 0.05 $mol_{products} \cdot mol_{glucose}^{-1}$). The corresponding yield of these products (lactate, formate, acetate, ethanol) per consumed glucose decreased from 2.05 to 1.88 $mol_{products} \cdot mol_{glucose}^{-1}$. As by-product formation exceeded maximum possible

yield (2 mol·mol^{-1}) per consumed glucose at growth rates below 0.4 h^{-1}, it indicated that part of the amino acids should have been catabolized to pyruvate and eventually to by-products. Moreover, if biomass composition was taken into account, a need for additional carbon from amino acids was detected throughout the studied growth rate range. Ten percent flux from serine to the central carbon metabolism was measured using 14 C flux analysis in *L. lactis* batch cultivation [103]. In this dissertation, the calculated flux formed 8 to 6% of the total carbon influx, which is very close to the literature value. Observed results indicate that NAD+ regeneration through lactate production must remain constant at all growth rates studied, however, energy generation through acetate production and NAD+ regeneration through ethanol production may decrease with the increase of specific growth rate and must be balanced from other sources.

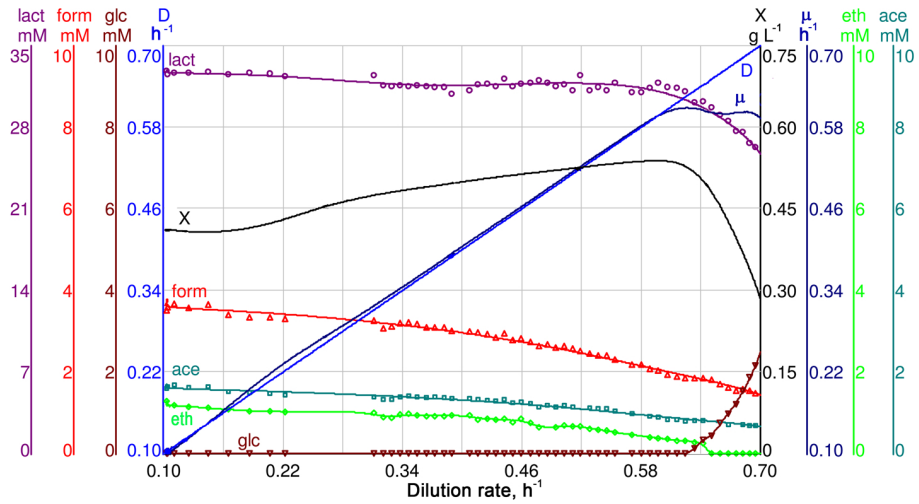

Figure 2 – Typical A-stat cultivation, where the dilution rate dependent metabolism of *L. lactis* is illustrated. Experiments were carried out under anaerobic conditions on a chemically defined medium containing amino acids, vitamins, minerals, and glucose (3.5 g·L^{-1}) as a limiting substrate. D – dilution rate h^{-1}; X – biomass concentration (g_{DWT}·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}.

4.2.4 *Substrate consumption profiles*

Because the primary carbon source (glucose) was depleted from the environment throughout the range of μ studied until μ_{max}, attention was paid to the amino acid consumption (in mmol·g_{DWT}^{-1}). Because all the amino acids in the medium were in excess, one would presume that the increase of biomass yield might be caused by the increased consump-

tion of amino acids. Surprisingly, with the increase of specific growth rate and increase in biomass yield, the overall consumption of amino acids decreased by more than 25% from 12.5 $\pm$ 0.5 mmol·g_{DWT}^{-1} to 9.3 $\pm$ 0.3 mmol·g_{DWT}^{-1} (see Figure 3). However, it is important to note that consumption of amino acids exceeded the requirements for the synthesis of proteins in the biomass by two to three times throughout the range of μ studied (4.2 $\pm$ 0.1 mmol·g_{DWT}^{-1}; see Figure 3). Over-consumed amino acids were mainly directed into pyruvate metabolism for fatty acid production or by-product formation.

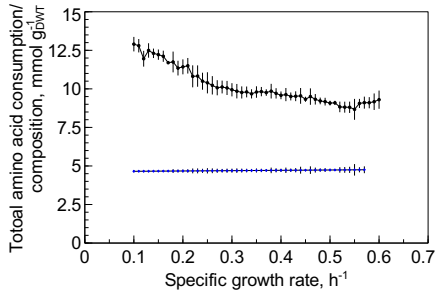

Figure 3 – *Lactococcus lactis* specific growth rate dependent total amino acid consumption profiles and total amino acid requirement for biomass synthesis. The black line represents average consumption (mmol·g_{DWT}^{-1}) from five independent A-stat experiments and error bars represent their standard deviation. Blue line represents total amino acid requirement for biomass synthesis (mmol·g_{DWT}^{-1}).

Based on amino acid concentrations in the cultivation broth and culture medium, specific consumption patterns (mmol·g_{DWT}^{-1}) for all 20 amino acids were calculated (Publication III, Supplemental Figure 2). The most abundantly consumed amino acid throughout the entire μ range studied was glutamine. Asparagine, arginine, serine, threonine, alanine, leucine, isoleucine, and cysteine were the next most intensively consumed amino acids whose consumption exceeded the amounts necessary for biomass formation. Lysine, phenylalanine, and valine were consumed in slightly higher amounts than were required for biomass production. Consumption of aspartate, histidine, and proline were all within the range of measurement errors, hence, their consumption can be considered minimal or nonexistent. It has been shown that the latter amino acids are nonessential for the growth of *L. lactis* [68]. In more detail, specific growth rate dependent consumption 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}. Consumption of arginine decreased rapidly in the μ range of 0.10 to 0.35 h^{-1} from 2.15 $\pm$ 0.04 mmol·g_{DWT}^{-1} and levelled at 0.44 $\pm$ 0.07 mmol·g_{DWT}^{-1} at higher growth rates – at an amount greater than was necessary for biomass production (0.20 $\pm$ 0.02 mmol·g_{DWT}^{-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 whose consumption increased during increasing μ, however, its consumption was always lower than was needed for biomass formation.

The specific flux, in $mmol \cdot g_{DWT}^{-1} \cdot h^{-1}$, of histidine, isoleucine, leucine, methionine, phenylalanine, serine, threonine, tryptophane, and valine increased until $\mu = 0.4\ h^{-1}$ and leveled off thereafter (see Figure 4). As these amino acids which, according to catabolic pathways, may be directed towards pyruvate, the question remains, whether there is a maximal limit of the flux towards pyruvate, which eventually limits by-product formation or there is a universal amino acid consumption regulator which limits their consumption. Exceptions were proline, whose specific flux rates leveled off at $\mu = 0.3\ h^{-1}$; asparagine, glutamine, glycine, and lysine whose fluxes increased continuously, and arginine consumption, and production of ornithine and glutamate, whose specific fluxes were constant (probably under CcpA control) throughout the μ range studied.

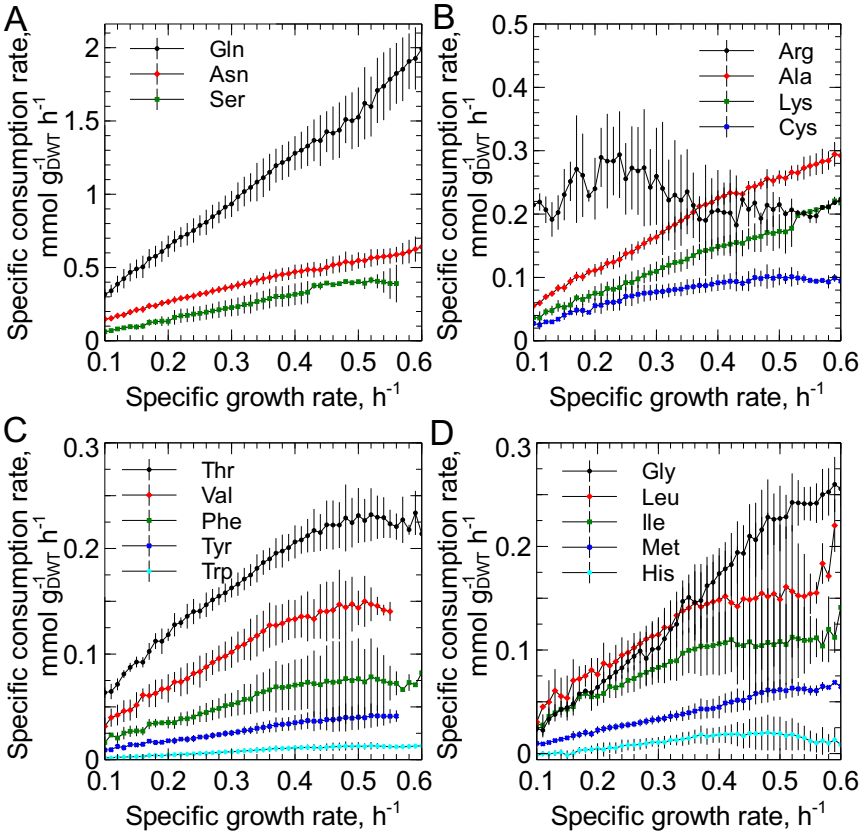

Figure 4 – Specific growth rate dependent specific consumption rates ($mmol \cdot g_{DWT}^{-1} \cdot h^{-1}$) of all consumed amino acids during the cultivation of *L. lactis* IL1403. Lines represent the average value of five independent experiments and error bars represent the standard deviation.

4.2.5 *Carbon balance (according to MFA)*

Glucose and amino acid consumption, product formation, and biomass composition were used as input data for metabolic flux analysis, and in this case also for carbon balance analysis. The calculated carbon recovery was well balanced (close to 100%) at lower μ values, however, at μ = 0.3 h^{-1} there is a 2 to 4% higher consumption of substrates which indicates the possible presence of a small amount of unmeasured products. Although amino acid consumption normalized to biomass reached a minimum near μ_{max}, amino acid flux formed 12 to 14% of the total consumed carbon with the highest proportion near μ_{max}. On the other hand, biomass formed 12 to 15% from the total products with increasing μ, indicating that the highest proportion of carbon is directed towards biomass near μ_{max} conditions. In accordance with previous findings, by-product ratios decreased with an increase in μ.

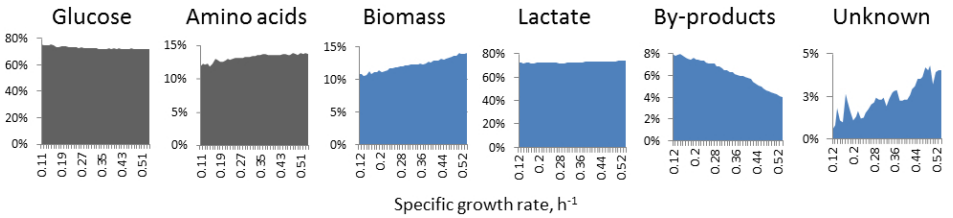

Figure 5 – Summarized carbon balance of *Lactococcus lactis* at various specific growth rates. Black areas represent consumption (in percentages from total carbon consumed), blue areas represent production (in percentages from total carbon produced). By-products represent the sum production of formate, acetate, ethanol and ornitine; Unknown group represents undetected products.

4.3 RELATIVE AND QUANTITATIVE OMICS ANALYSIS

4.3.1 *Characterization of main parameters*

DNA microarray analysis allowed us to measure mRNA expression levels for 2300 genes at four different quasi steady state μ values (0.17, 0.24, 0.44, 0.52 h^{-1}) and compare them to steady state value at μ = 0.1 h^{-1}. Relative ratios from DNA microarray experiments were discussed in Publication III. As the microarray spot intensities showed an error less than 10% between seven different oligos for the same gene, the same results were quantified into units of molecules per cell and molecules g_{DWT}^{-1}. Similarly, relative protein measurements from the same specific growth rate values were converted to absolute by using the APEX algorithm [144]. We were able to quantify 26% of the ORFs (570 proteins) at all specific growth rates studied. Moreover, it is estimated that these form 71% of the total proteome abundance. Absolute quantitative transcriptome and proteome data have enormous advantages compared with relative data when trying to understand cellular regulatory mechanisms. It is important to note that cell volume increased by 1.7 times (μ 0.5 compared to 0.1 h^{-1}) and this change is taken into account when copy numbers

per cell are calculated. However, it must be noted that the experimental data is based on a population of non-synchronized cells. Therefore, our estimated rates provide an average over the population and cell cycle and our data fails to characterize whether mRNA and protein synthesis occurs in bursts or maintains a constant level throughout the cell cycle.

4.3.2 *Absolute mRNA and protein copy numbers*

Absolute mRNA and protein copy numbers per cell spanned approximately two and four orders of magnitude, respectively (see Figure 6A). Median number of mRNA molecules per cell increased by 3.3 times with an increase of μ from 0.1 to 0.5 h^{-1}. At the same time, the median number of protein molecules per cell increased by only 1.7 times, meaning that the concentration of all proteins in the cell remained the same. On average, 1590 protein molecules were synthesised per mRNA at lower μ values (μ = 0.1 h^{-1}) and it decreased almost two times while reaching μ = 0.5 h^{-1} (860). Correlation between 570 identified protein and mRNA abundances, the most comprehensive to date for this lactic acid bacteria, has an R^2 value of 0.39 (see Figure 6B). However, similar correlation have been found for several bacteria, eukaryotes, and multicellular organisms. When correlation was analyzed for different metabolic groups, higher correlation values (R^2 > 0.5) were determined for biosynthesis of co-factors, transcription, energy metabolism, cellular processes, and the cell envelope (data not shown).

Additionally, it was possible to calculate how many proteins are synthesised from each mRNA per hour, *i. e.* the translation rate constant. The median translation rate constant appeared to be 159 proteins per mRNA per hour at μ = 0.1 h^{-1} and it increased 2.7 times (up to 432 proteins per mRNA per hour) until reaching μ = 0.5 h^{-1} (see Figure 6D). These values are about 4 to 10 times higher than reported for mammalian cells [49], however, are in the same range compared with *Escherichia coli* [Valgepea *et al.*, unpublished]. Saturation of translation rate constants was observed at higher growth rates for mammalian and *Escherichia coli* cells, no such phenomena was detected for *L. lactis*. In common with mammalian cells and *Escherichia coli*, plotting translation rate constants against protein levels revealed that abundant proteins are translated about 100 times more efficiently than low abundance proteins.

4.3.3 *Calculations of catalytic activities of enzymes*

Based on the metabolic fluxes found and enzyme abundances, our data allow us to calculate apparent catalytic activities of enzymes (K_{app} [s^{-1}]) for 179 proteins (see Figure 6C). Median K_{app} values increased linearly by 3.3 times from 2.2 to 7.3 s^{-1} with the increase of μ from 0.1 to 0.5 h^{-1}. The most efficient K_{app} values belonged to glycolytic enzymes and sugar transporters, which increased between 2.5 to 4 times with an increase in μ from 0.1 to 0.5 h^{-1}. The highest increase (up to 12 times) in K_{app} values with increase of μ was observed in the purine/pyrimidine metabolism. In contrast, decrease in K_{app} values was determined only for some enzymes in amino acid metabolism, and was in concordance with amino acid consumption data, which decreased with the increase of

μ. Median K_{app} values for ribosomes were more than two times higher than the overall median and increased from 5 to 17 s^{-1} with the increase of μ. Prevalent increase in K_{app} values allow us to presume that the metabolism is controlled by post-translational regulation.

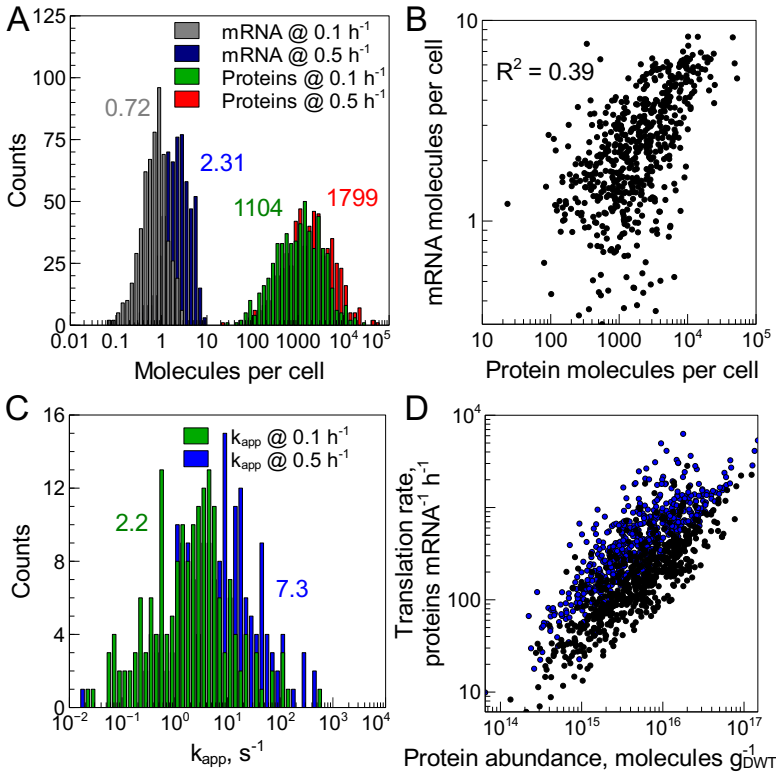

Figure 6 – mRNA and protein distribution, correlation, translation rate, and apparent catalytic activities of enzymes (K_{app}). **A**) mRNA and protein distribution at specific growth rates 0.1 and 0.5 h^{-1}; numbers on the chart represent according median values [molecules per cell]. **B**) Quantitative mRNA and protein correlation at specific growth rate 0.5 h^{-1}. **C**) Distribution of 179 calculated values for the apparent catalytic activities of enzymes at specific growth rates 0.1 and 0.5 h^{-1}. Numbers on the chart indicate median K_{app} values at respective specific growth rates [s^{-1}]. **D**) Correlation between protein abundances and translation rate at specific growth rates 0.1 h^{-1} (black dots) and 0.5 h^{-1} (blue dots).

Prevalent changes in enzyme catalytic activities rather than their concentrations seems to be a reasonable explanation of how cells cope with constantly varying environmental conditions. When metabolic fluxes are regulated by single enzymes in the pathway and most of the enzymes are in abundance, this situation allows the cell to quickly change its specific growth rate. This is in contrast to the situation where a cell must start synthesizing the entire proteome apparatus to grow faster. It has been demonstrated in

Escherichia coli, that cells need only 200 ms to increase its μ from 0.1 h^{-1} to its maximum [147], while another study demonstrated that it took about 15 seconds to achieve a μ 70% from the maximum [148]. Additionally, it has been demonstrated that the first enzymes of various pathways have a lower relative protein abundance (and higher K_{app} values) than others, and hence, could be controlling the actual flux through the pathway [Adamberg *et al.*, unpublished]. This kind of regulation could give an evolutionary advantage because in their natural habitats LAB often encounter drastic fluctuations in the composition of their environment. Consequently, LAB are often in a transient state with periods of abundant supply of energy followed by periods of starvation.

4.4 DESCRIPTION OF ENERGY METABOLISM

During the fermentative metabolism of anaerobically growing *L. lactis* there are three main pathways for ATP generation: glycolysis, acetate production, and the arginine deiminase (ADI) pathway. Growth rate dependent energy generation from these pathways were taken into account in the MFA calculations. With the increase of specific growth rate from 0.1 to 0.6 h^{-1}, ATP generation per biomass produced decreased from 89 to 62 $mmol_{ATP} \cdot g_{DWT}^{-1}$ (Figure 7). In glycolysis, only the net change (2 $mol_{ATP} \cdot mol_{glucose}^{-1}$) is taken into account. However, energy generation per cell increased from 50 to 61 $pmol_{ATP} \cdot cell^{-1}$. As predicted, the majority of energy was produced in glycolysis, forming 90 to 94% of ATP generation with an increase in specific growth rate from 0.1 to 0.59 h^{-1}. Acetate production and the ADI pathway contributed 10 to 6% of the total ATP produced.

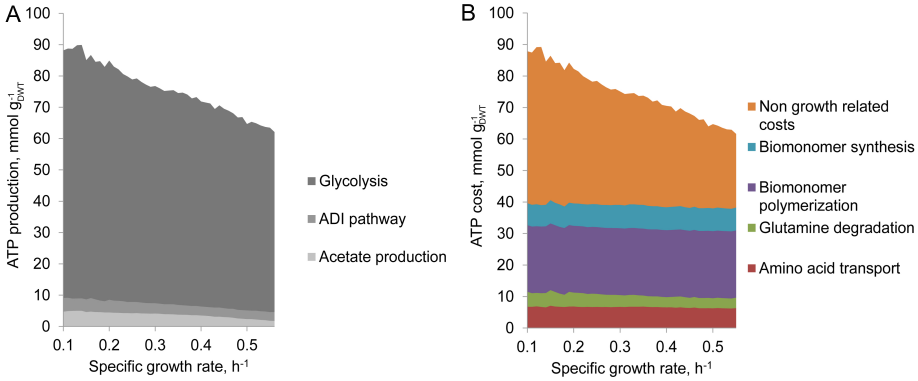

Figure 7 – Distribution of energy generation pathways (**A**) and energy demand (**B**) during fermentative growth of *L. lactis* IL1403. In glycolysis, only the net change (2 $mol_{ATP} \cdot mol_{glucose}^{-1}$) is taken into account.

From an anabolic point of view, the cost of biomonomer synthesis (DNA, RNA, fatty acids) slightly increases. Although RNA content increases with an increase of μ by 1.7 times, the content of fatty acids decreases (cell size increases while membrane surface

/ cell volume ratio decreases). Biomonomer polymerization has roughly three times higher energetic burden than biomonomer synthesis with an expenditure of 21.1 to 21.3 $mmol_{ATP} \cdot g_{DWT}^{-1}$. The majority of the burden comes from amino acid polymerization which is μ independent in *L. lactis* because there are only minor changes in the growth rate dependent proteome. Hence, the increase of the cost with an increase in μ and RNA content is marginal. Cost for amino acid transport also decreases with the increase in μ and takes roughly 5% of the total energy balance. Additionally, a small portion of ATP (2.5% of the total) is spent for glutamine degradation. The cost for biomonomer synthesis and polymerization have been previously calculated for exponentially growing *Escherichia coli* [31, 35, 36]. Despite the fact that *Escherichia coli* needs to synthesize all biomonmers, the cost for biopolymer synthesis is in the same range as that calculated for *L. lactis*.

The gap between produced energy and consumed energy for biopolymer synthesis and transport may be termed non-growth related energy or maintenance energy. Contrary to previous assumptions, we determined the maintenance energy to be μ dependent, even while growing on the same substrates under carbon limitation. Maintenance energy formed 53 to 23 $mmol_{ATP} \cdot g_{DWT}^{-1}$ (56 to 38% of the total ATP produced; see Figure 7B), with the increase of μ from 0.1 to 0.56 h^{-1}, respectively. A significant decrease by more than 50% in maintenance energy might have a strong effect for increasing biomass yield with the increase of μ. It is interesting to note that although total ATP production per cell increased (from 50 to 61 $pmol_{ATP} \cdot cell^{-1}$) the maintenance costs continuously decreased from 15 to 7.5 $pmol_{ATP} \cdot cell^{-1}$) when μ 0.1 was compared with 0.6 h^{-1}.

Because the maintenance energy may have a significant role in cell regulation, a subsequent aim was to analyze the distribution of various contributions towards the maintenance energy and describe the effect of each component compared with the whole. We presume that maintenance energy may consist of biomonomer resynthesis (with the highest expenditure for protein resynthesis), homeostasis (cell leakage), and futile cycles.

4.5 CALCULATION OF PROTEIN DEGRADATION RATES AND (RE)SYNTHESIS COSTS

Because amino acid polymerization has a high metabolic burden, it was presumed that protein resynthesis may also contribute significantly in the maintenance. Among microorganisms, protein turnover has been measured previously in yeast cells [116–118] and *Mycoplasma pneumoniae* [50] and calculated for *L. lactis* [111]. Model calculations have shown turnover rates in the same range as the measured values.

In the current study, we applied the method described in Dressaire *et al.* [111], and protein degradation rates were calculated for 541 individual proteins. The model predicted them to be μ independent. The degradation rates spanned over two orders of magnitude from 10 to 1000 minutes with a median value of 123 minutes (see Figure 8). No correlation was found between turnover rates and proteome abundances, however, the opposite was found for HeLa cells [119]. Between metabolic groups, only ribosomes were detected with almost two times lower median half-lives than the total. Similar results have also been observed in yeast [118].

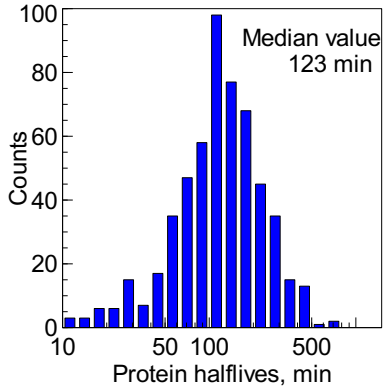

Figure 8 – Distribution of protein half-lives for 541 individual *L. lactis* IL1430 proteins calculated using the model described in Dressaire *et al.* [111]

4.5.1 *Protein abundance and cost distribution between metabolic groups*

The quantitative proteome data was divided into metabolic groups as in Bolotin *et al.* [3] and abundance of those groups were compared. By far the most abundant metabolic group was translation, forming 17 to 21% of the total proteome with increasing μ from 0.1 to 0.5 h^{-1} (see Figure 9A). This 26% increase between selected growth rates in this proteome group was the highest observed. In terms of abundance, metabolic groups such as unknowns, energy metabolism, transporters, and purine and pyrimidine metabolism were next in abundance following the translation group.

However, protein abundances may not provide adequate information when we are looking at the metabolic burden of different metabolic groups. Therefore, protein cost, *i. e.* amount of ATP spent for protein synthesis, was calculated taking into account protein abundance, length, and literature based values for the synthesis of one peptide bond. As metabolic group translation mainly consists of relatively short ribosomes, its burden to the cell was lower (forming 14 to 16%) and at lower growth rates, energy metabolism was the most ATP demanding metabolic group (Figure 9B). These two groups were followed by purines/pyrimidines and transporters with the burden around 5% out of the total proteome over the growth rates studied.

Subsequently, calculated protein half-lives were taken into account when the metabolic burden of metabolic groups was calculated. Although protein content only marginally changed during the increase of μ, the total cost for protein synthesis (taking into account costs for protein re-synthesis) decreased by more than two times with increasing μ from 0.1 to 0.5 h^{-1} (Figure 9C). The most energetically expensive pathway at low growth rates was energy metabolism (μ independent) 15.5 $\pm$ 0.5% of the total energetic burden on proteome synthesis. Energy metabolism was followed by translation whose contribution to the energetic burden increased with the increase in μ from 14.5 to 17.0%. Other significant increases were detected among the purines/pyrimidines (3.9 to 4.7%) and amino acid biosynthesis (1.7 to 2.3%) proteins. Increases in the relative contribution of those groups occurred due to a decrease in the unknowns/unmeasured groups.

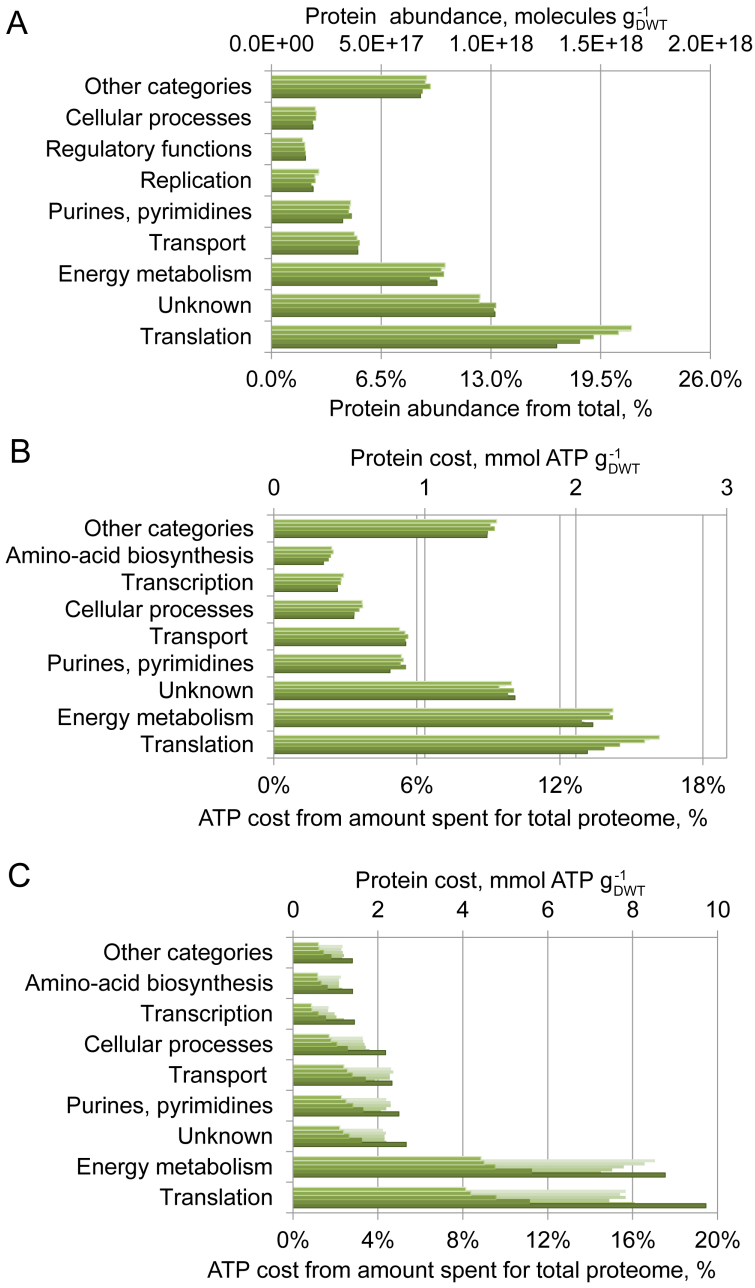

Figure 9 – Abundances and protein cost for *L. lactis* metabolic groups together and without taking into account protein half-lives. Bar colors from darker to lighter represent specific growth rates from 0.1 to 0.5 h^{-1}, with a 0.1 h^{-1} step. **A**) Protein abundances. **B**) Protein cost without taking protein half-lives into account. **C**) Protein cost while protein half-lives are taken into account. Lighter bars on the chart represent the percentage at the respective μ.

4.6 DISTRIBUTION OF MAINTENANCE COST

In addition to total cost for protein polymerization, cost for protein re-synthesis was calculated. Presuming μ is independent of degradation rates for proteins, the cost for protein re-synthesis was more than fourfold higher at low specific growth rates compared to the highest one studied. ATP cost for protein re-synthesis ranged from 44.2 to 9.8 $mmol_{ATP} \cdot g_{DWT}^{-1}$ with the increase of μ from 0.1 to 0.5 h^{-1}, respectively. It is important to note that cost for protein re-synthesis were constantly lower than calculated total maintenance cost and therefore in accordance with energetic point of view. However, protein re-synthesis formed 90 to 35% of the total maintenance with increase of μ from 0.1 to 0.5 h^{-1}, respectively. To estimate the rest of the maintenance, next, protein abundances of ion channels were taken into account. By applying average enzyme catalytic activities and presuming the wastage of half an ATP per reaction, ion leakage showed the major ATP cost at specific growth rate values of 0.2 h^{-1} and higher forming 30 to 66% of the total maintenance with increasing μ from 0.1 to 0.5 h^{-1}. Additionally, the ATP cost for a potential futile cycle in glutamine metabolism was taken into account. Based on the protein abundance of enzymes **CarA!** and CarB and median catalytic activity of enzymes, ATP cost for a futile cycle in glutamine metabolism was calculated.

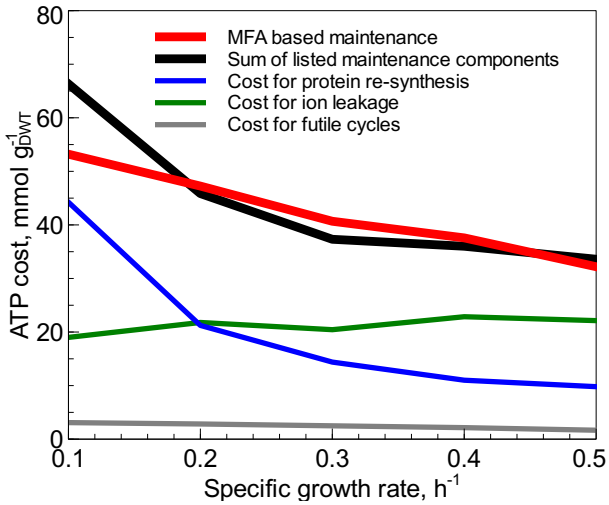

Figure 10 – Specific growth rate dependent distribution of maintenance costs in *L. lactis*. The blue line represents calculated non growth related energy (maintenance); the red line is the ATP cost for protein resynthesis; the green line is the ATP cost for ion leakage; the purple line is the ATP cost for futile cycles (glutamine regeneration); and the black line represents the sum of mentioned maintenance energy components.

It has been previously hypothesized that ion transport could be the highest maintenance cost in exponentially growing bacteria [36], which is in accordance with our data. According to our calculations, futile cycles had only a minor role in maintenance, forming 6 ± 1% of the total maintenance. Summing up these μ dependent non-growth related energy costs, we were able to precisely describe the entire maintenance costs in

the specific growth rate range of 0.2 to 0.5 h^{-1}. This indicates that costs for resynthesis of other biopolymers such as mRNA and fatty acids should be marginal in this μ range. These are estimations, however, they may provide valuable insight into potential energetic costs. However, calculated maintenance costs exceeded the MFA based calculations in the μ range < 0.2 h^{-1}. This was due to a rapid increase in protein re-synthesis costs if protein degradation rates were considered constant. Those results may indicate that at lower specific growth rates protein half-lives should decrease, otherwise growth at low rates would be energetically too expensive. Real growth rate dependent measurements of protein half-lives should provide a better answer to this question.

Up until now, we have determined that due to increased apparent catalytic activities of enzymes and decreased maintenance energy during the increase of specific growth rate, there is a possibility for bacteria to increase their biomass yield. However, the question remains, why are alternative energy generation pathways inhibited at higher μ and not used to achieve even higher specific growth rates? It has been proposed that metabolic shifts from energetically efficient mixed acid fermentation to catabolically efficient homolactic fermentation in *L. lactis* might be caused by trade-offs between the energetic burden of biosynthesis of alternative pathways compared to the ATP generation efficiency [78]. Availability of absolute protein abundances of those pathways, protein degradation rates and MFA-based ATP generation coefficients allowed us to calculate the energetic efficiency of various energy generation pathways. We can conclude that all energy generation pathways seem to be efficient and even improve with increasing μ (except the ADI pathway which is clearly repressed; see Figure 11). A decrease in protein abundance in acetate formation pathways (AckA2 and EutD) by two times may be related to limited carbon flux from pyruvate. Because a constant amount of carbon is directed to lactate (μ independent), the carbon flux from pyruvate decreases and there may be no reason to keep constant protein levels in the cell. Whether the decrease in flux from pyruvate is regulated through CodY or by a more balanced nitrogen metabolism is a question to be addressed in future studies. Downregulation of the ADI pathway is most probably controlled by CcpA because a constant specific flux of arginine (0.23 ± 0.03 $mmol \cdot g_{DWT}^{-1} \cdot h^{-1}$) was monitored throughout the range of μ studied.

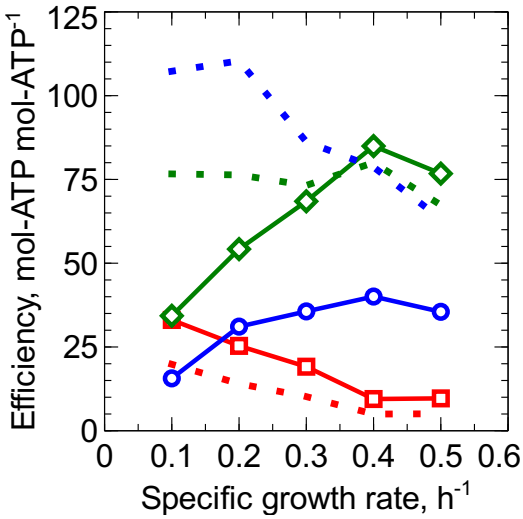

Figure 11 – Specific growth rate dependent efficiencies of *L. lactis* energy generation pathways (mol ATP produced in each pathway per mol ATP spent for producing their respective enzymes) during fermentative metabolism. Dashed lines represent the efficiency without taking protein half-lives into account; solid lines (with markers) represent the efficiency with protein half-lives taken into account. Blue lines represent glycolysis; green lines acetate pathway; and red lines the ADI pathway.

SUMMARY

5

CONCLUSIONS

First, continuous cultivation methods were developed and implemented to study dynamic changes in the metabolism of *L. lactis* under strictly controlled physiological states and optimized environmental conditions:

I Changestat cultivation methods (A-stat and D-stat) were proven to provide reproducible and chemostat representative data from strictly controlled physico-chemical conditions if acceleration was correctly chosen.

II By using changestat cultivation methods optimization of pH and temperature to achieve highest biomass yield were carried out for *L. lactis* in steady physiological state.

Second, a detailed multi-omics analysis was carried out to describe the adaptation of energetic burden and regulation of cell components in *L. lactis* as specific growth rate is increased from 0.1 to 0.5 h^{-1}:

III A good correlation (R^2 = 0.39) was found between quantitative transcriptome and proteome data. However, regulation of *L. lactis* metabolism was found to be mainly controlled at the level of enzyme activities (post-translational control). With the increase of μ by five times, enzyme activities increased by 3.3 times, on average.

IV The main reasons for the significant increase in biomass yield in *L. lactis* as μ is increased are decreased energetic demand for maintenance (mainly for protein re-synthesis) and increased enzyme catalytic activities (both at higher μ values).

V Increased energetic demands at lower μ values were compensated by a switch to an energetically more efficient metabolism and activation of alternative energy generation pathways (ADI pathway and acetate production), all of which had a positive influence on energetic efficiency.

VI The specific consumption rate of arginine (in $mmol \cdot g_{DWT}^{-1} \cdot h^{-1}$) was constant over the range of μ studied, indicating a potential control of catabolite repression regulator. The specific consumption rates of the amino acids directed into the central carbon metabolism correlated with formation of the by-products acetate, formate, and ethanol, indicating that acetate generation could be controlled by nitrogen metabolism.

VII Calculated protein half-lives for individual proteins were μ independent and spanned over two orders of magnitude with a median value of 123 minutes. This allowed us to calculate the energetic burden for protein re-synthesis as well as estimate the efficiency of energy generation pathways.

VIII This study demonstrates the usefulness of genome-scale quantitative data, including absolute mRNA and protein concentrations and half-lives. The systems biology approach employed in this dissertation may be applied in other biological systems to understand the complex relationships between thousands of genes and their products.

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- Co-ordination of industrial project with DuPont Nutrition and Health.
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APPENDICES

PUBLICATION I

Adamberg K, Lahtvee PJ, Valgepea K, Abner K, Vilu R.

Quasi steady state growth of *Lactococcus lactis* in glucose-limited acceleration stat (A-stat) cultures.

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ORIGINAL PAPER

Quasi steady state growth of *Lactococcus lactis* in glucose-limited acceleration stat (A-stat) cultures

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Abstract Quasi steady state growth of *Lactococcus lactis* IL 1403 was studied in glucose-limited A-stat cultivation experiments with acceleration rates (a) from 0.003 to 0.06 h^{-2} after initial stabilization of the cultures in chemostat at $D = 0.2$–0.3 h^{-1}. It was shown that the high limit of quasi steady state growth rate depended on the acceleration rate used—at an acceleration rate 0.003 h^{-2} the quasi steady state growth was observed until $\mu_{crit} = 0.59$ h^{-1}, which is also the μ_{max} value for the culture. Lower values of μ_{crit} were observed at higher acceleration rates. The steady state growth of bacteria stabilized at dilution rate 0.2 h^{-1} was immediately disrupted after initiating acceleration at the highest acceleration rate studied—0.06 h^{-2}. Observation was made that differences [$\Delta(\mu - D)$] of the specific growth rates from pre-programmed dilution rates were the lowest using an acceleration rate of 0.003 h^{-2} ($< 4\%$ of preset changing growth rate). The adaptability of cells to follow preprogrammed growth rate was found to decrease with increasing dilution rate—it was shown that lower acceleration rates should be applied at higher growth rates to maintain the culture in the quasi steady state. The critical specific growth rate and the biomass yields based on glucose consumption were higher if the medium contained $S_0 = 5$ g L^{-1} glucose instead of $S_0 = 10$ g L^{-1}. It was assumed that this was due to the inhibitory effect of lactate accumulating at higher concentrations in the latter cultures. Parallel A-stat experiments at the same acceleration and dilution rates showed good reproducibility—$\Delta(\mu - D)$ was less than 5%, standard deviations of biomass yields per ATP produced (Y_{ATP}), and biomass yields per glucose consumed (Y_{XS}) were less than 15%.

Keywords A-stat · Continuous culture · *Lactococcus lactis* · Quasi steady state

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Introduction

Lactic acid bacteria (LAB) are industrially important microorganisms widely used as dairy starters. Their relatively simple metabolism makes them also favourable objects for metabolic studies and for the development of tools for modelling (Oliveira et al. 2005). *Lactococcus lactis* IL1403 was the first LAB to be sequenced and it is also the most studied strain,

thus providing a good case for understanding physiology of the lactic acid bacteria in both natural and laboratory environments (Bolotin et al. 2001). Generally, metabolic studies of LAB are quite often performed using batch cultivations, continuous (chemostat) cultivations are used only in special cases (Thomas et al. 1979; Jensen et al. 2001; Even et al. 2003). Continuous cultivations are more time-consuming and require complex equipment, however, continuous cultures should be preferred if quantitative data on cell metabolism is required.

Steady state, quasi steady state, and non-steady state growth of *Lactococcus lactis* IL1403 using the A-stat (Paalme et al. 1995) was studied in this work. A-stat was chosen as a more convenient and less time—and medium-consuming cultivation method than chemostat in physiological studies of bacteria. As is well-known, after each stepwise change of dilution rate in chemostat the culture should be restabilised, should be brought back to the steady state pumping through 4–5 culture volumes of feeding medium. It has been shown that stepwise change of dilution rate in chemostat can disrupt the steady physiological state of cells especially close to the critical or maximal specific growth rates and cause earlier washout of cultures (Paalme et al. 1995). A-stat with its smooth, continuous change of the dilution rate is devoid of the negative sides of the chemostat mentioned. In addition, A-stat enables also to study adaptability of cells in the controlled changing growth conditions. The basic idea of A-stat cultivation method is that it is possible to maintain the specific growth rate (μ) equal to the changing dilution rate (D) if appropriate low acceleration rates have been applied. It has been shown that in these continuously changing growth conditions the bacteria are in the quasi steady state physiological state (Paalme et al. 1995). However, high acceleration rates disturb the quasi steady state of the A-stat culture, and might lead to wash-out conditions at dilution rates lower than μ_{max} (Barbosa et al. 2003; van der Sluis et al. 2001).

A-stat has been used in studies of *Escherichia coli* (Paalme et al. 1995), yeast (Paalme et al. 1997; van der Sluis et al. 2001), *Lactobacillus plantarum* (Kask et al. 1999) and photobacteria (Barbosa et al. 2003). In addition to A-stat, several other cultivation methods using similar approach have been developed (Kasemets et al. 2003).

As mentioned, *Lactococcus lactis* has been studied in the chemostat experiments—Jensen et al. (2001) studied the effect of oxygen and Even et al. (2003) the effect of pH on the metabolic characteristics of bacteria at different dilution rates. Thomas et al. (1979) investigated the effect of growth rate on the growth characteristics (Y_{XS}, Y_{ATP}, etc.) in chemostat cultures of *Lactococcus lactis*.

The growth characteristics of *Lactococcus lactis* IL1403—adaptability, yield coefficients, etc. were studied in glucose limited A-stat cultivation experiments in this work. The A-stat method was chosen as a fast and efficient cultivation method enabling to study adaptability of cells to changing environment, and to detect limits of quasi steady state growth space of bacteria. The results obtained showed clearly that the A-stat is an efficient method of cultivation allowing to obtain reproducible quasi steady state growth characteristics of bacteria, making possible also systematic studies of non-steady state growth.

Materials and methods

Bacterial strains and culture media

Lactococcus lactis IL1403 was kindly provided by Dr. Ogier INRA (France). Inoculum was prepared using a single colony from a MRS plate and grown up twice in the cultivation medium (30°C, 20 h). The cultivation medium composition was used as follows (g L^{-1}): alanine, 0.24; arginine, 0.125; asparagines, 0.21; aspartate, 0.21; cysteine, 0.13; glutamate, 0.2; glutamine, 0.1; glycine, 0.175; histidine, 0.15; isoleucine, 0.33; leucine, 0.66; lysine, 0.44; methionine, 0.125; phenylalanine, 0.175; proline, 0.225; serine, 0.52; threonine, 0.225; tryptophane, 0.05; tyrosine, 0.08; valine, 0.33; K_2HPO_4, 3; KH_2PO_4, 2.5; NaCl, 2.9; $MgSO_4$ 7H2O, 0.2; CaCl $2H_2O_2$, 0.05; $MnSO_4$ H_2O, 0.016; $ZnSO_4$ $7H_2O$, 0.005; $CoCl_2$ $5H_2O$, 0.003; $CuSO_4$ $5H_2O$, 0.003; $CuSO_4$ $5H_2O$, 0.003; $(NH_4)_6$ Mo_7O_{24} $4H_2O$, 0.003; $FeSO_4$ $4H_2O$, 0.0014; pyridoxine, 0.002; biotin, 0.001; folic acid, 0.001; niacin, 0.001; pantothenic acid, 0.001; riboflavin, 0.001 and thiamine, 0.001. Glucose concentration S_0 was 3, 5 or 10 g L^{-1}.

Cultivation system

The A-stat cultivation system was built around a 1.25 L Biobundle fermenter (Applikon, The Netherlands)

controlled by an ADI 1030 biocontroller (Applikon) and a cultivation control program "BioXpert" (Applikon). The system was equipped with pH, pO_2, and temperature sensors. Two variable speed pumps (feeding and out-flow) were controlled using "BioXpert" control software. The fermenter was set on a balance whose output was used as the control variable to ensure the constant culture volume (300 ± 1 mL). Similarily, the inflow was controlled through measuring the mass of the fresh culture media. Fermentations were carried out under anaerobic conditions (N_2-environment) at a temperature of 30°C and with an agitation speed of 300 rpm. The pH of the culture was kept at 6.5 ± 0.1 using 2 M NaOH.

A-stat cultivation

The principle of A-stat cultivation was previously described by Paalme et al. (1995). In our experiments, 300 mL of cultivation medium was inoculated with 6 mL of an overnight culture (20–24 h). After the optical density (OD_{600}) reached 1.5–2 the dilution rate (D) was increased up to 0.2 or 0.3 h^{-1} and the culture was stabilized at a constant dilution rate. After a steady state culture (OD = const.) was obtained pumping through at least 5 culture volumes of fresh medium, acceleration of dilution rate was switched on, and it was changed according to the equation $D = D_0 + a \times t$, where D_0 is the initial dilution rate, h^{-1}, and "a" in the equation is the acceleration rate (h^{-2}), and t is the time from the start of acceleration (h). The acceleration rates used in different experiments were 0.003, 0,005, 0.01, 0.015, 0.02, 0.03 and 0.06 h^{-2}, respectively. In some of the experiments the acceleration rate was decreased step-wise several times (while keeping the dilution rate increasing) to avoid transition to the non-steady state growth conditions at high growth rates. To check the steady state conditions, acceleration was occasionally stopped and the experiment was continued for a certain time as chemostat. After checking the steady state conditions in chemostat, acceleration of the dilution rate was started again.

Analytical methods

The concentrations of organic acids (lactate, acetate and formate), ethanol and glucose in the culture media were analysed by liquid chromatography (HPLC, Waters, UK), using a BioRad HPX-87H column, and isocratic elution at a flow rate of 0.6 mL min^{-1} with 0.009 NH_2SO_4 at room temperature. UV (210 nm, Waters 2487) and refractive index detectors (Waters 2414) were used for detection and quantification of the substances. The samples of culture media of 1.5 mL were centrifuged (14,000g 1 min), 0.5 mL supernatant was mixed with 0.125 mL 10% sulfosalicylic acid and stored at −20°C before HPLC analyses. Data manipulation was performed using Empower software (Waters).

The concentration of amino acids was determined with an amino acid analyser (Alliance, Waters) using precolumn derivatization with an AccQ-Fluor reagent (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate). 10 μL of 100x diluted sample supernatants were mixed with 70 μL phosphate buffer and 20 μL AQC reagent, (Waters). After incubation for 10 min at 55°C, the mixture was ready for analyses. Derivatized samples were stored at 4°C and analysed within one week. Sample supernatants were stored at −20°C before analysis. Empower software (Waters) was employed for the data manipulation.

The biomass concentration was determined by measuring the optical density at 600 nm using a biomass conversion factor $\gamma = 0.32$ g L^{-1} $OD_{600}{}^{-1}$ ($\gamma = \frac{X}{OD_{600}}$, g L^{-1}, where X is g dry weight of biomass in 1 L).

Dry weight of bacteria was determined by centrifuging the biomass from a 10 mL culture, washing quickly twice with distilled water and drying at 100°C for 24 h in an oven.

Calculation of growth characteristics

The growth characteristics of the bacteria in A-stat experiments were calculated on the basis of OD, total volume of medium pumped out from fermenter (V_{OUT}, L) and organic acid concentrations in culture medium (mol L^{-1}) as follows:

$$\mu = \frac{d(V_{OUT})}{V \times dt} + \frac{d(OD)}{dt \times OD} \quad (1)$$

$$Q_{GLC} = \frac{S_{GLC} \times d(V_{OUT})}{V \times OD \times \gamma \times dt} - \frac{d(S_{Glc})}{dt \times OD \times \gamma} \quad (2)$$

$$Y_{XS} = \frac{\mu}{Q_{Glc}} \quad (3)$$

$$Y_{lg} = \frac{Q_{LAC}}{Q^{M}_{GLC}} \quad (4)$$

$$Q_{LAC} = \frac{LAC \times d(V_{OUT})}{V \times OD \times \gamma \times dt} + \frac{d(LAC)}{dt \times OD \times \gamma} \quad (5)$$

$$Y_{ATP} = \frac{\mu}{Q_{ATP}} \quad (6)$$

where μ is the specific growth rate (h^{-1}); LAC is concentration of produced lactic acid (mol-Lac L^{-1}); Q_{GLC} and Q^{M}_{GLC} are glucose consumption rates (g-glc g-X^{-1} h^{-1} and mol-glc g-X^{-1} h^{-1}, respectively); S_{GLC} is the concentration of consumed glucose (g-glc L^{-1} or mol-glc L^{-1}); Y_{XS} is the growth yield based on glucose consumption (g-X g-glucose^{-1}); Y_{lg} is the lactate yield based on glucose consumption (mol-Lac mol-glc^{-1}); Q_{LAC} is the specific production rate of lactate (mol-Lac g-X^{-1} h^{-1}); V is the current fermenter volume (L^{-1}); OD is the optical density at 600 nm; γ is the conversion factor (0.32 g OD_{600}^{-1}) and t is the cultivation time (h) and Q_{ATP} is the specific ATP production rate based on theoretical estimation assuming that the production of 1 mol lactate is accompanied by the synthesis of 1 mol ATP, and the production of 1 mol acetate leads to the synthesis of 2 mol of ATP.

Results

A-stat experiments

Results of a typical A-stat experiment with *Lactococcus lactis* IL1403 are shown on Fig. 1a. After stabilization of the culture in chemostat at 0.2 h^{-1}, increase of dilution rate with the acceleration rate 0.003 h^{-2} was switched on at the 60th h. Stabilization of the culture taking place before 60 h from the beginning of cultivation is not fully shown on the Figure. As seen from the Fig. 1 growth rate determined experimentally followed very precisely the changing dilution rate, deviations of the μ from D [$\Delta(\mu - D)$] being less than 5%. The increase of the dilution rate was stopped at the 142th h of cultivation, and the culture was transferred without any transition processes into chemostat at $D = 0.47$ h^{-1}. After verifying that the culture was in steady state during the chemostat cultivation (142–158 h), the acceleration was switched on again, and the quasi steady state growth, where $\mu = D$, proceeded up to $D = 0.59$ h^{-1}. At $D = 0.59$ h^{-1}, which is also the μ_{max} value of the culture, wash-out of the culture and increase of glucose concentration from 0.1 to 6.2 mM was observed. μ_{crit}, the growth rate at which departure from the quasi steady state was observed was defined as the dilution rate at which μ differed from D more than 5%. It should be emphasized that until the critical growth rate ($D = \mu_{crit}$) the A-stat culture was maintained in a quasi steady glucose-limited physiological state (the glucose concentration was maintained at less than the detection limit of the HPLC method (0.1 mM). At $\mu = D = \mu_{crit}$ biomass, substrate and product concentrations started to change—the culture was transferred to non-steady state. It should be emphasized also that using the $a = 0.003$ h^{-2} the $\mu_{crit} = \mu_{max}$, which indicated that the culture was maintained in the quasi steady state in the full range of growth rates in this A-stat experiment.

The effect of acceleration rate on the critical growth rate

The results obtained in different A-stat experiments showed that with the increase of acceleration rate, μ_{crit} value determined decreased (Fig. 1b). As seen from the Fig. 1b, in this case where $a = 0.15$ h^{-2}, the quasi steady state growth of the bacteria was observed after the initial stabilization of the culture in chemostat at $D = 0.2$ h^{-1} only up to the $\mu_{crit} = 0.3$ h^{-1} (64th h of cultivation). The wash-out of the culture was started at $\mu_{crit,}$ biomass, and lactate concentration were decreasing and the residual glucose concentration was increasing. However, despite the lagging behind D, μ was increasing during the non-steady-state growth, and eventually even caught up with the changing D. The calculated $\mu_{max} = 0.58$ h^{-1} was nearly the same (0.59 h^{-1}) determined for the bacteria using low acceleration rates (see above). As seen from Fig. 1b, despite stabilization at $D = 0.57$ h^{-1} the washout of the culture started immediately after acceleration of D was switched on anew at 98th h of cultivation. Needless to say that the non-steady state growth data should be interpreted carefully, taking into account all the relevant data, especially data on the state of the intracellular metabolism which was not obtained in our experiments.

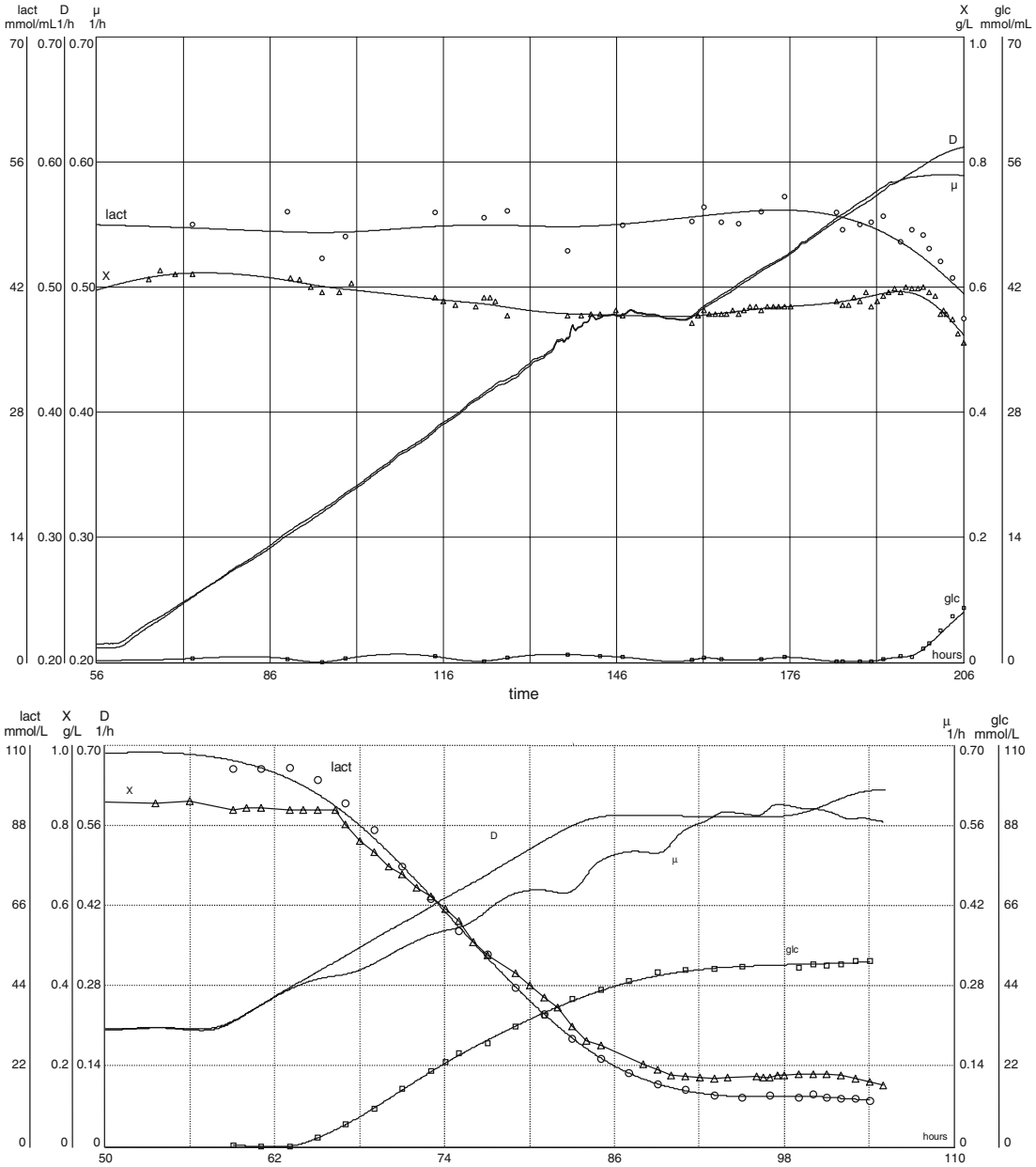

Fig. 1 A-stat cultivation with *Lactococcus lactis* IL1403. OD, optical density at 600 nm; ace, acetate concentration in the fermenter, mmol L^{-1}; μ, specific growth rate, h^{-1}; D, dilution rate, h^{-1}; glc, glucose concentration in the fermenter, mmol L^{-1}; lact, lactate concentration in the fermenter, mmol L^{-1}. In **a** $a = 0.003$ h^{-2} and $S_0 = 5$ g L^{-1} and **b** $a = 0.015$ h^{-2} and $S_0 = 10$ g L^{-1}

The μ_{crit} values determined from the experiments with different acceleration rates are given on Fig. 2. As seen from the Figure, the values of μ_{crit} determined depended remarkably on the acceleration rates used. The highest μ_{crit} values were observed in the experiments with low ($a < 0.01$ h^{-2}) acceleration

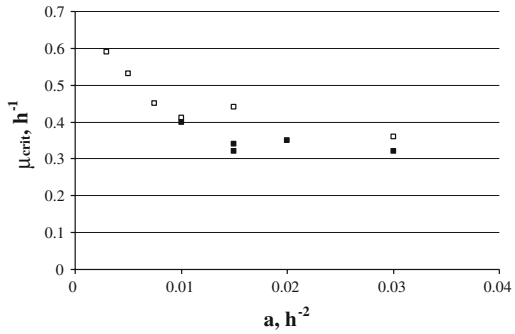

Fig. 2 Critical specific growth rates (μ_{crit}) of *Lactococcus lactis* IL1403 obtained in A-stats with different acceleration rates. *Open squares* indicate the experiments with 5 g L^{-1} and *closed squares* 10 g L^{-1} glucose in the medium

rates used. As noted already above, it was possible to maintain the culture in the quasi steady state of growth in the whole range of dilution rates up to $\mu_{max} = 0.59\ h^{-1}$ if $a = 0.003\ h^{-2}$.

As also seen in Fig. 2 μ_{crit} values determined depended on the concentration of glucose in the medium: the μ_{crit} values determined in the experiments with $S_0 = 10$ g L^{-1} of glucose were somewhat lower than the respective values determined in the case of $S_0 = 5$ g L^{-1} (glucose). The noted higher μ_{crit} values could be explained by the lower concentration of lactate in the fermenters in the case of $S_0 = 5$ g L^{-1}.

On the basis of the data obtained it was assumed that the adaptability of bacteria decreases at higher growth rates, and that lower acceleration rates should be used to ensure that the cells remain growing in quasi steady state at higher growth rates. To test this hypothesis experiments with step-wise decrease of acceleration rates at higher dilution rates were carried out. In one of these experiments acceleration rate was decreased step-wise from 0.03 h^{-2} (at $D = 0.3\ h^{-1}$) to 0.003 h^{-2} (close to μ_{crit}) and the results similar to those obtained using only acceleration rate 0.003 h^{-2} were obtained—see Fig. 1a above (data not shown).

Our experiments showed that acceleration rate of 0.06 h^{-2} was too fast to keep culture in quasi steady state growth. Applying this acceleration rate caused the deviation of the specific growth rate (μ) from the pre-set profile of D already at stabilization dilution rate D_0 (immediately after the acceleration was switched on after the stabilization).

Growth characteristics

The variability of the yield coefficient values did not exceed 10% within the growth range studied. This observation is in full accordance with the theory of chemostat. However, biomass yields were dependent on glucose concentration in the feeding medium (S_0). In experiments with $S_0 = 5$ g L^{-1} of glucose in the medium, Y_{ATP} varied between 12 and 14 g-dwt mol-ATP^{-1}. In analogous experiments with $S_0 =$ 10 g L^{-1} glucose in the medium the values of the Y_{ATP} were in the range of 9–11 g-dwt mol-ATP^{-1}. Similarly values of Y_{XS} were in the range 0.12–0.14 g-dwt g-glc^{-1} and 0.08–0.12 g-dwt g-glc^{-1}, at lower and higher initial glucose concentrations, respectively.

Very good reproducibility of the measured and calculated data was observed in the parallel A-stat experiments over the entire range of growth rates studied—variance of growth yields was less than 5% between experiments. It was also shown that in quasi steady state growth conditions standard deviations of the growth characteristics were lower than in non-steady state. The average standard deviations of Y_{XS} were 0.007 in quasi steady state and 0.013 g-dwt g-glc^{-1} in non-steady state. Similarly standard deviations of Y_{ATP} were 0.9 g-dwt g mol^{-1} in quasi steady state and 1.2 g-dwt g mol^{-1} in non-steady state.

Fermentation patterns

In quasi steady state conditions the cultures of *Lactococcus lactis* were glucose-limited as cell concentrations were proportional to the amounts of consumed glucose and no change of biomass yield (Y_{XS}) was observed. Residual glucose concentration was less than the detection limit of HPLC used (0.1 mM). However, during the non-steady state growth beyond μ_{crit} glucose started to accumulate in the culture media, biomass and lactate concentrations started to decrease (see Fig. 1b), patterns of changes depending on the acceleration rates used. There is a distinctive need for further systematic study of dynamic responses of the bacteria to the changing environments in A-stat which is clearly beyond the scope of the present study.

The glucose fermentation was essentially homolactic over the entire range of growth rates studied ($Y_{lg} > 1.8$ mol-Lac mol-glc^{-1}). Acetate and formate

production remained clearly below 6 mol% of total fermentation end-products. Lactate (and acetate) yields per glucose were close to 2 mol-Lac mol-glc^{-1} or higher at high growth rates and $S_0 =$ 10 g L^{-1}. Values of $Y_{lg} > 2$ could be realized only if additional to glucose substrates, amino acids in our case were also consumed.

It was observed that the use of amino acids did not exceed 10 mol% of glucose consumption. Serine, glutamate and arginine were preferably consumed during the growth—2.2, 0.8, and 0.7 mmol g-dwt^{-1}, respectively. Arginine consumption decreased with the increase of dilution rate being negligible at maximal growth rates indicating that no functional arginine deiminase pathway was present in the bacteria. Consumption of all the other amino acids remained below 10% from the initial amounts in the feeding, and did not change significantly with the increase of the dilution rates.

Discussion

Similarly to earlier studies (Barbosa et al. 2003; van der Sluis et al. 2001) it was shown also in our experiments that the limit of the quasi steady state growth is depending on the acceleration rates used—the higher the acceleration rate used, the lower the value of μ_{crit} determined (Fig. 2.). The results obtained with step-wise decreasing of the acceleration rates at higher growth rates showed that the cells have different adaptive capacity at different growth rates. It is possible to operate at lower dilution rates without disturbing the steady state using higher acceleration rates, i.e. in the dilution range of 0.2–0.35 h^{-1} acceleration rates up to 0.03 h^{-2} could be applied, whereas at higher dilution (growth) rates ($D > 0.35$ h^{-1}) acceleration rates not exceeding 0.003 h^{-2} should be used. This interesting observation could be tentatively explained assuming that the regulation of short cell cycles at high growth rates requires more constant environmental conditions than regulation of longer cell cycles where the DNA replication cycles are better separated in time.

The choice of acceleration rate is important in carrying out A-stat cultivations—optimisation of the acceleration rates saves experimental time while providing important information about the culture's adaptability to the conditions of changing environment.

It was shown that optimal choice of the acceleration rates is also important in determining accuracy with which the growth of the culture is following the computer controlled dilution rate changes. Observation was made that the reproducibility of the A-stat experiments was remarkably better at lower acceleration rates. Quasi steady state growth in the full range of growth space was realized using a low acceleration rate of 0.003 h^{-2}—in this case $\mu_{crit} = \mu_{max}$.

The growth beyond μ_{crit} can be characterised as non-steady-state growth. The growth of the bacteria in non-steady state is depending on the growth history of the culture. Systematic study of the non-steady-state growth of bacteria is only beginning. These studies should supply important information on the dynamic responses of bacteria to the changing with different rates environmental conditions.

Growth of *Lactococcus lactis* was essentially homolactic in the full range of growth conditions studied, however, at higher growth rates value of Y_{lg} exceeded 2 indicating that in addition to glucose other substrates (amino acids) were consumed in notable amounts. Indeed, as shown, this was the case. Serine, and glutamate and arginine in lesser extent were consumed by the bacteria. It should be noted, however, that details of the metabolism of amino acids require further elaboration. Noted serine consumption might lead to the formation of additional pyruvate. Additional pyruvate might be needed for regeneration of NADH and synthesis of additional lactate leading to the increase of the value of Y_{lg}. NADH regeneration through amino acid metabolism can be also needed for NADPH synthesis as shown by Teusink et al. (2006) for *Lb. plantarum* culture. To prove the hypothesis catabolic products of amino acid metabolism need to be analysed.

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PUBLICATION II

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Steady state growth space study of *Lactococcus lactis* in D-stat cultures.

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ORIGINAL PAPER

Steady state growth space study of *Lactococcus lactis* in D-stat cultures

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Abstract Growth space of *Lactococcus lactis* subsp. *lactis* IL1403 was studied at constant growth rate using D-stat cultivation technique. Starting from steady state conditions in a chemostat culture ($\mu = 0.2\ h^{-1}$), the pH and/or temperature were continuously changed in the range of 5.4–6.4 and 26–34°C, respectively, followed by the return to the initial environmental conditions. Based on substrate consumption and product formation yields and expression changes of 1,920 genes, it was shown that changes of physiological state were not dependent on the direction of movement (from pH 6.3 to 5.4 or from 5.4 to 6.3), showing that quasi steady state values in D-stat corresponded to the steady state values in chemostats. Relative standard deviation of growth characteristics in triplicate D-stat experiments was below 10%. Continuing the experiment and reestablishing initial growth conditions revealed in average 7% difference (hysteresis) in growth characteristics when comparing chemostat steady state cultures prior and after the change of environmental conditions. Similarly, shifts were also seen at gene expression levels. The large amount of quantitatively reliable data obtained in this study provided a new insight into dynamic properties of bacterial physiology, and can be used for describing the growth space of microorganisms by modeling cell metabolism.

Keywords Continuous culture · *Lactococcus lactis* · Acid stress · Growth space · Transcriptome

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Introduction

We have entered the era of synthetic biology with high throughput DNA sequencing (genetic) and omics methods, which in principle should make comprehensive quantitative characterization of molecular physiology of cells possible. However, cultivation methods where cells are in defined physiological states (Hoskisson and Hobbs 2005) and thus quantitatively

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reliable data on cell metabolism could be obtained, have found little use in practice. Until present, multidimensional growth space has been systematically determined, and studied in batch cultures (Le Marc et al. 2005, see also http://www.bioprocessors.com/). However, batch experiments do not supply information about the growth space dynamics. For obtaining data on dynamic properties of the bacteria, they have to be cultivated preferably in continuous cultures in defined physiological states. Defined physiological states could be most readily realized in chemostat cultures. In spite of that, chemostat has not been used in bacterial physiology studies as widely as expected because the method is quite complex, laborious and time-consuming.

Due to its industrial importance, *L. lactis* is one of the best-studied microorganisms. However, a limited number of studies about growth dynamics—especially about dynamics of change of physiological state of cell on the change of growth conditions, could be found in the literature. Effects of pH, oxygen and dilution rate on the growth of *L. lactis* in chemostat have been studied by Thomas et al. (1979), O'Sullivan and Condon (1999), Even et al. (2001, 2003), Jensen et al. (2001), Dressaire et al. (2008). Dressaire et al. have made the first attempt to characterize the growth of *L. lactis* at the whole genome level using global transcriptome profiling.

We have previously shown that A-stat (chemostat with smooth change of dilution rate) is reproducible, informative and efficient cultivation method for studying physiology of cells at different specific growth rates in (quasi) steady state (Adamberg et al. 2009), provided that the change of growth conditions does not disrupt the quasi steady state of growing cells. Results of the *L. lactis* D-stat cultivation experiments (Kasemets et al. 2003) with smooth change of pH and/or temperature are reported in this paper. The rates of pH and temperature change sufficient for maintaining the quasi steady state growth of the culture during the transitions were determined, and the results obtained showed that D-stat cultures are reproducible tools for scanning the growth space of growing cells. In addition to the more conventional growth characteristics (Y_{xs}, Y_{lact} etc.) measured, also changes in transcriptome patterns of *L. lactis* IL1403 were investigated in different growth conditions. To our knowledge, this is the first attempt made to characterize quasi steady state growth in multidimensional growth space of *L. lactis*.

Materials and methods

Bacterial strain and culture medium

The cultivated strain *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. The composition of the cultivation medium used was as follows (g L^{-1}): limiting substrate glucose—5, alanine—0.24, arginine—0.125, asparagine—0.21, aspartate—0.21, cysteine—0.13, glutamate—0.2, glutamine—0.1, glycine—0.175, histidine—0.15, isoleucine—0.33, leucine—0.66, lysine—0.44, methionine—0.125, phenylalanine—0.275, proline—0.225, serine—0.52, threonine—0.225, tryptophan—0.05, tyrosine—0.08, valine—0.33, K_2HPO_4—3, KH_2PO_4—2.5, NaCl—2.9, $MgSO_4 \cdot 7H_2O$—0.2, $CaCl_2 \cdot 2H_2O$—0.05, $MnSO_4 \cdot H_2O$—0.016, $ZnSO_4 \cdot 7H_2O$—0.005, $CoCl_2 \cdot 5H_2O$—0.003, $CuSO_4 \cdot 5H_2O$—0.003, $(NH_4)_6 Mo_7O_{24} \cdot 4H_2O$—0.003, $FeSO_4 \cdot 4H_2O$—0.0014, pyridoxine—0.002, biotin—0.001, folic acid—0.001, niacin—0.001, pantothenic acid—0.001, riboflavin—0.001 and thiamine—0.001.

Cultivation system

The D-stat cultivation system consisted of a 1.25 L Biobundle bioreactor (Applikon, Schiedam, the Netherlands) controlled by an ADI 1030 biocontroller (Applikon) and a cultivation control program "BioXpert NT" (Applikon). The system was equipped with pH, pO_2 and temperature sensors. Two variable speed pumps (feeding and out-flow) were controlled using "BioXpert NT" control software. The bioreactor was set on a balance, which output was used as the control variable to ensure constant culture volume (300 ± 1 mL). Similarly, the inflow was determined by measuring the mass of fresh culture medium added. Cultivations were carried out under anaerobic conditions (N_2-environment) with an agitation speed of 300 rpm at 34°C and pH 6.4, unless otherwise stated.

pH of the culture was controlled using 2 M NaOH and temperature with a heating blanket.

D-stat cultivation

The D-stat cultivation technique has been previously described by Kasemets et al. (2003). The principle is similar to chemostat where the culture is initially "stabilized" in a steady state at a chosen dilution rate (D). After a steady state is obtained, smooth change(s) of environmental parameter(s) (e.g. pH, temperature, substrate concentration) is (are) started while keeping the dilution rate constant. In our experiments, 300 mL of cultivation medium was inoculated with 5 mL of an overnight grown culture. When optical density (OD_{600}) had reached 1.5–2, dilution rate was slowly increased up to the desired dilution rate where the culture was stabilized. Constant dilution rate $D = 0.2\ h^{-1}$ was maintained throughout all experiments. After pumping through at least five working volumes of fresh medium for obtaining a steady state chemostat culture (OD = const.), smooth change of temperature and/or pH was switched on (Figs. 1, 3) at the changing rates presented in Table 1. After reaching the desired pH or temperature, the change of environmental conditions were reversed, and pH and/or temperature were changed back to the initial values, either immediately (Fig. 1) or after a period during which the

Table 1 Overview of *L. lactis* IL1403 D-stat experiments at dilution rate 0.2 h^{-1}

	Parameter(s) changed	Acceleration rate	Range	Units
Expt. 1	pH	0.1 U h^{-1}	6.4–7.2–6.4	pH
	Temp.	1°C h^{-1}	34–26–34	°C
Expt. 2	pH	0.1 U h^{-1}	6.4–5.6–6.4	pH
	Temp.	1°C h^{-1}	34–26–34	°C
Expt. 3	pH	0.025 U h^{-1}	6.4–5.6–6.4	pH
	Temp.	0.25°C h^{-1}	34–26–34	°C
Expt. 4	pH	0.02 U h^{-1}	6.3–5.4–6.3	pH
Expt. 5	pH	0.0125 U h^{-1}	6.1–5.4–6.1	pH
Expt. 6	pH	0.02–0.006 U h^{-1}[a]	6.4–5.6–6.4	pH
Expt. 7	pH	0.02 U h^{-1}	5.4–6.1–5.4	pH
Expt. 8	Temp.	0.25°C h^{-1}	34–27–34	°C

[a] Acceleration of pH was decreased gradually based on biomass formation and lactate production

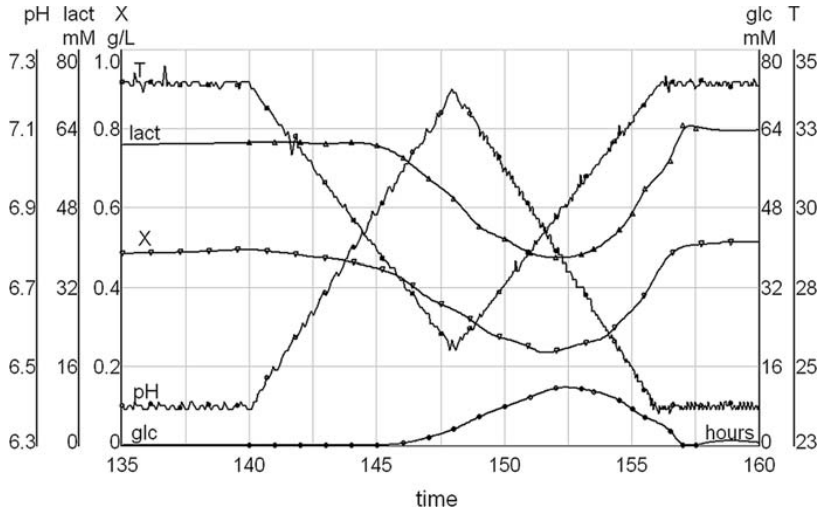

Fig. 1 *Lactococcus lactis* IL1403 D-stat exp. 1 with simultaneous two-parameter change (pH and temperature). glc—glucose concentration in bioreactor (mM); lact—lactate concentration in bioreactor (mM); T—temperature (°C), X—biomass concentration in bioreactor (g L^{-1})

culture was "stabilized" in chemostat (Fig. 2). Stability of the culture growth characteristics in chemostat was used to validate quasi steady state growth of the bacteria during the change of pH and/or temperature.

Analytical methods

The concentrations of organic acids (lactate, acetate and formate), ethanol and glucose in the culture media were analyzed by liquid chromatography (Alliance 2795 system, Waters Corp., Milford, MA), using a BioRad HPX-87H column (Hercules, CA) with isocratic elution of 0.005 M H_2SO_4 at a flow rate of 0.6 mL min^{-1} and at 35°C. UV (210 nm; model 2487; Waters Corp.) and refractive index (RI) detectors (model 2414; Waters Corp.) were used for detection and quantification of the substances. Detection limit for the analytical method was 0.1 mM. Samples from culture media were centrifuged (14,000*g*, 4 min), supernatants were collected and analyzed immediately or stored at −20°C until analysis. Amino acid concentrations were determined from the same sample with an amino acid analyzer (UPLC; Waters Corp.) according to the manufacturer's instructions. Empower software (Waters Corp.) was used for the data processing.

Biomass concentration was calculated by measuring the optical density at 600 nm using a biomass

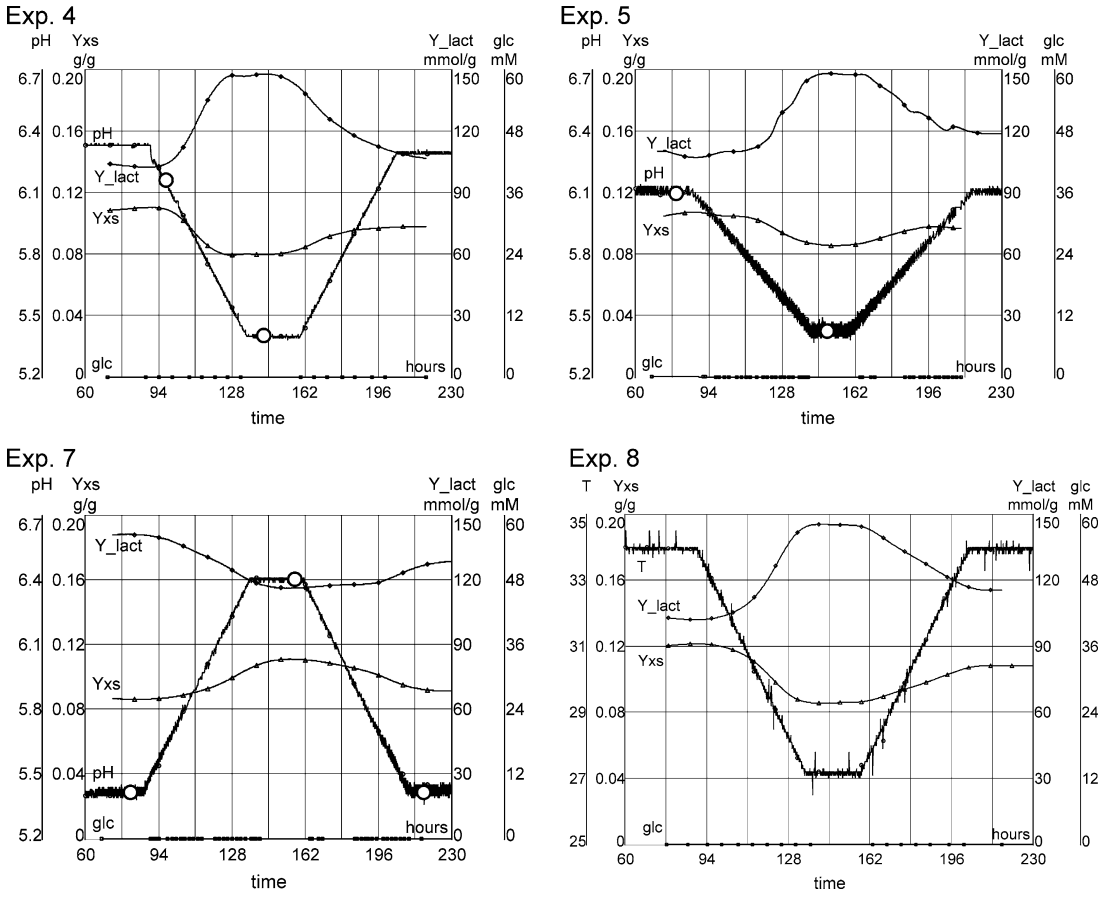

Fig. 2 *Lactococcus lactis* IL1403 D-stat experiments with the change of pH or temperature. Descriptions of the experiments can be seen in Table 1. *Circles* indicate sampling points for transcriptome analysis which in each experiment were compared to one another. glc—glucose concentration in bioreactor (mM); Y_{lact}—lactate yield based on biomass production (mmol lact gdw^{-1}); T—temperature (°C); *Y*xs—biomass yield based on glucose consumption (g gdw^{-1})

conversion factor $K = 0.30 \pm 0.02$ g L^{-1} OD_{600}^{-1} ($K = X/OD_{600}$, where X is in g dry weight [gdw] L^{-1}). Dry weight of bacteria was determined gravimetrically by centrifuging biomass from 15 mL of culture, washing it multiple times with distilled water and drying at 100°C for 24 h.

Global transcription profiling

Microarrays used in this study were purchased from Eurogentec (Seraing, Belgium) and contained 1,920 open reading frames (ORF) of *L. lactis* spotted in duplicates on standard 2.5 × 7.5 cm glass slides.

Samples of biomass were fixed with RNAprotect solution (QIAGEN, Valencia, CA)—the samples of culture media were mixed with RNAprotect solution (1:1 ratio), incubated for 5 min at room temperature and centrifuged for 10 min at 8,000*g*. Supernatant was discarded and the pellet stored at −80°C. Total RNA was extracted with RNeasy Mini Kit (QIAGEN) and genomic DNA was removed using RNase-Free DNase Set (QIAGEN). After addition of 500 µL lysozyme (10 mg lysozyme in 1 mL TE pH 8; Amresco Inc., Solon, OH), three freeze-thawing cycles were carried out followed by incubation at 37°C for 1 h. 20 µL of proteinase K solution (20 U proteinase K in 1 mL; Amresco Inc.) was added and incubated for 1 h at 37°C before RNA extraction.

cDNA was synthesized from 15 µg of total RNA at 46°C overnight using the following reagents per reaction: 1 µg random primers (Random decamers; Invitrogen, Carlsbad, CA), 6 µL five times concentrated first strand buffer (Invitrogen), 3 µL 0.1 M DTT (Invitrogen), 0.9 µL dNTPs (final concentrations: dATP 0.5 mM, dCTP 0.5 mM, dGTP 0.5 mM, dTTP 0.3 mM, aminoallyl-dUTP 0.2 mM), 1 µL RNase Inhibitor (Bioron, Ludwigshafen, Germany), 2 µL Superscript III (Invitrogen). RNA strands were hydrolyzed in the samples by adding 4.5 µL 1 M NaOH and incubating at 70°C for 10 min, after incubation the samples were neutralized with 4.5 µL 1 M HCl. cDNA was purified with MinElute PCR purification kit (QIAGEN) and labeled with Cy3 ($Cy^{TM}3$ Mono Reactive Dye Pack, Amersham, Buckinghamshire, UK) or Cy5 ($Cy^{TM}5$ Mono Reactive Dye Pack, Amersham). Staining was carried out in 10 µL of $NaHCO_3$ (pH 9) at room temperature in dark for 1 h and the samples were purified again with MinElute PCR purification kit. Subsequently, the following hybridization master mix was used (per slide): 20 × SSC 16.3 µL, 100% formamide 32.5 µL, 10% SDS 0.65 µL. The hybridization was carried out at 42°C for 16 h.

Analysis of gene expression data

Microarray slides were scanned using an Agilent DNA Microarray Scanner (Santa Clara, CA). Spot intensities and corresponding background signals were quantified with Genepix Pro (version 6; Axon Instruments [http://www.moleculardevices.com/pages/software/gn_genepix_pro.html]). Spots which had a signal-to-noise ratio less than three or intensities of both, 635 and 532 nm channels lower than 500 units were filtered. Further analysis was carried out in R environment (version 2.6.1; R Development Core Team [http://www.r-project.org/]) using KTH package (KTH Microarray Center [http://www.biotech.kth.se/molbio/microarray/dataanalysis/index.html]). Flagged spots and background were extracted before "printTipLoess" normalization.

Calculation of growth characteristics

Growth characteristics of bacteria were calculated based on OD of the culture, total volume of medium pumped out from bioreactor (L) and lactate or glucose concentrations in culture medium (mol L^{-1}) as follows:

$$\mu = \{[d(V_{\text{out}})]/(V \times dt)\} + \{[d(\text{OD})]/(dt \times \text{OD})\}$$

$$Q_{\text{glc}} = \{[\Delta S_{\text{glc}} \times d(V_{\text{out}})]/(V \times \text{OD} \times K \times dt)\} - \{[d(\Delta S_{\text{glc}})]/(dt \times \text{OD} \times K)\}$$

$$Y_{\text{XS}} = \mu/Q_{\text{glc}}$$

$$Q_{\text{lact}} = \{[\text{lact} \times d(V_{\text{out}})]/(V \times \text{OD} \times K \times dt)\} - \{[d(\text{lact})]/(dt \times \text{OD} \times K)\}$$

$$Y_{\text{lact}} = Q_{\text{lact}}/\mu,$$

where μ is specific growth rate (h^{-1}); lact is concentration of produced lactic acid (mmol L^{-1}); Q_{glc} is specific glucose consumption rate (g glc gdw^{-1} h^{-1} or mmol glc gdw^{-1} h^{-1}); ΔS_{glc} is amount of consumed glucose (g glc L^{-1} or mmol glc L^{-1}); Y_{XS} is biomass yield calculated on glucose consumption (gdw g $glucose^{-1}$); Q_{lact} is specific production rate of lactate (mmol lact gdw^{-1} h^{-1}); V is bioreactor volume (L); Y_{lact} is lactate yield based on biomass

production (mmol lact gdw^{-1}); OD is optical density at 600 nm; V_{OUT} is total volume of medium pumped out from bioreactor (L); K is biomass conversion factor (see above for equation) and t is running cultivation time (h).

Growth space visualization

3-D growth space was visualized using Datafit program (version 9.0; Oakdale Engineering [http://www.curvefitting.com]) with polynomial fit $[Y_{XS} = a + b/\mathrm{pH} + c/\mathrm{pH}^2 + d \times \ln T + e \times (T)^2]$ and quasi steady state data points (the chosen regression represented our data points the best).

Results

D-stat cultivation experiments with continuous change of one parameter (pH or temperature) or simultaneous change of two parameters (pH and temperature) were carried out, and growth characteristics of *L. lactis* IL1403 in different pH and/or temperature conditions at dilution rate 0.2 h^{-1} were studied (see Table 1). Thus continuous moving in two-dimensional bacterial growth sub-space was performed. As can be seen on Figs. 1 and 2, environmental conditions were changed in D-stat experiments back and forth, where initial growth conditions were restored in the end of the experiments. Special attention was paid to make sure whether the initial physiological states (steady states) were reinstated after the back and forth change of growth conditions.

Experiments with two-parameter change (exp. 1, 2, 3 in Table 1; Fig. 1) showed a possibility for culture characteristics to return to their initial values after initial optimal growth conditions were reestablished. However, it has to be noted that the culture was not in quasi steady state throughout the whole experiment (see transient increase of residual glucose concentration on Fig. 1). This indicated that changing rates of pH and temperature, 0.1 U h^{-1} and 1°C h^{-1} respectively, were too fast to enable the culture to adapt to the changing environmental conditions, and maintain steady state growth in the glucose limited state. Hence, single parameter change experiments with slower changing rates (see Table 1) were carried out subsequently to make sure that the bacteria were in the glucose limited quasi steady state during the full course of the experiments.

No glucose was observed in the culture media during the transition from the initial stabilization value of pH (pH 6.1–6.4) until pH 5.4–5.6 (exp. 4–6; Table 1), indicating maintenance of quasi steady state growth in these conditions (for exp. 4 and 5, see also Fig. 2). To prove the maintenance of the steady state, D-stat experiments were switched into chemostat at the lowest pH values and kept in constant environmental conditions for over five generations. Results obtained in latter chemostat showed that production rates of biomass and main growth by-products were maintained constant, varying only in the range of measurements error (<5%) (Fig. 2). In addition, Y_{lact} and Y_{XS} values at pH 5.4 in exp. 7 (initial chemostat) and quasi steady state values at pH 5.4 in exp. 4 and 5 (after the first transition) were similar in the range of standard deviation. Furthermore, it was observed that growth characteristics were not dependent on the direction of pH movement (from pH 6.3 to 5.4 in exp. 4 or from 5.4 to 6.3 in exp. 7), showing that quasi steady state values in D-stat were identical to steady state values in chemostat. Hence, it can be assumed that the bacteria were in (quasi) steady state during the entire transition. Maintenance of quasi steady state growth during the change of temperature from 34 to 27°C was proved also in exp. 8.

Growth characteristics

For the comparison of four D-stat cultivations with pH changing rates below 0.02 U h^{-1} (quasi steady state growth; exp. 4–7), biomass yield per consumed glucose (Y_{XS}) and lactic acid yield per biomass produced (Y_{lact}) were calculated. Y_{XS} was 0.108 ± 0.003 gdw g $glucose^{-1}$ at optimal pH, and decreased to 0.084 ± 0.003 gdw g $glucose^{-1}$ at pH 5.4. Small relative standard deviation (6%) of the Y_{XS} values determined in four experiments showed very good reproducibility of D-stat method. The value of Y_{lact} decreased with the decrease of pH from 144 ± 11 mmol gdw^{-1} at optimal pH to 106 ± 14 mmol gdw^{-1} at pH 5.4 with the average standard deviation value of 9%. Similarly, changes in Y_{lact} and Y_{XS} were observed in the experiments with simultaneous pH and temperature change (exp. 1, 2, 3 in Table 1) as well. However, biomass yield (Y_{XS}) coefficients values at pH 5.6 and temperature 26°C were about 15% lower in

comparison with those observed in the case of only changing pH or temperature—see exp. 4–7. This can be explained by applying higher adaptive stress on the metabolism in the case of simultaneous pH and temperature change in double-parameter experiments than in the single-parameter experiments, and by the fact that changing rates used were higher ($a_{pH} = 0.025$ U h^{-1} and $a_T = 0.25$°C h^{-1}) in the double-parameter experiments.

All described cultivations could be considered as homolactic—lactate production comprised more than 90% of all products observed, and the share of lactate slightly increased in higher stress conditions.

Transcriptome

To characterize the effects of changing pH on the physiological state of the bacteria more thoroughly, global transcriptome measurements were carried out at different quasi steady state growth conditions. Samples taken for comparison are shown in Fig. 2 and the list of measured genes which expression changed more than 1.8 times in different environmental conditions may be found in supplementary materials (http://www.tftak.eu/?id=69).

Forty-one genes which expression changed more than 1.8 times were observed as the result of the first pH change, i.e. as the result of moving away from the initial chemostat conditions in exp. 4, 5 and 7 (Fig. 2 exp. 4, 5 and 7). The most remarkable change at transcriptional level took place in arginine (urea cycle) metabolism, where expression of genes *arcA*, *arcC1*, *arcC2*, *arcD1*, *argE* and *argR* increased 2- to 6.25-fold on lowering the pH. When moving from acidic conditions to optimal pH values, expression levels of the same genes decreased (in exp. 7) as a response to the change of pH—independent of the movement direction in growth space. It must be noted that the concentration of arginine in the culture media was below the detection limit (0.01 mM) during the whole experiment. Citrate cycle genes *citC*, *citD* and *citE* were down-regulated when lowering the pH and up-regulated when the pH was returned to the initial value. Arginine and citrate formation pathways are well-known as taking part in the regulation of survival against acid stress by increasing internal pH or generating additional metabolic energy (Konings 2002). In the case of *lysQ*, which encodes a lysine/histidine specific transport permease protein (Vitreschak et al. 2004), a significant fivefold up-/down-regulation was observed while pH was decreased/increased, respectively. As the overall consumption of lysine and histidine were less than the error of the measurement (5%), we can only assume that shifts in the expression of the genes responsible for lysine/histidine transport were necessary to adapt to the varying pH conditions. Moreover, based on homology analysis, genes *yxbE* and *yxbF*, which are known to encode universal stress proteins, were strongly up-regulated at lower pH conditions. All other detected major changes in transcriptomes concerned hypothetical or poorly studied proteins (see supplementary materials).

Hysteresis

As can be seen from Fig. 2, biomass concentration in chemostat in the beginning of the experiments and after the return to the initial conditions in the end of the experiments differed by 8 ± 3%, indicating hysteresis in the physiological states of the culture. Production of lactate per biomass and glucose consumption per biomass was lower in the end of the experiments (exp. 4–8) by 6–13%, in comparison with the beginning of the experiments. Microarray analysis revealed that the expression levels of certain arginine and citrate metabolism genes—*arcA*, *arcC1*, *arcC2*, *arcD1*, *argE*, *argR* and *citC*, *citD*—changed during the first pH shift. However, instead of recovering their expression after moving back to initial environmental conditions, these genes remained expressed at the level attained during the first pH shift (exp. 7 in Fig. 2). See also supplementary materials for more information about transcription differences during the back and forth changes of the growth conditions (hysteresis).

It was shown in three additional parallel chemostat experiments with *L. lactis* that no changes, neither in biomass concentration, nor in the levels of main metabolites were observed at optimal growth conditions during 70 generations (data not shown). However, as mentioned above, after reducing of the changing rates used in the experiments 4–8 which led to the remarkable prolongation of the D-stat experiments, and the time while the bacteria were exposed to the changed environmental conditions (mild stress), hysteresis of the gene expression and growth parameters were observed in our experiments. Although significant, further characterization and

study of the hysteresis was out of the scope of the present study, and was postponed to the future.

Discussion

The results obtained showed that D-stat cultivation method made it possible to reproducibly scan relatively large growth space areas of *L. lactis* in reasonable time in quasi steady state at fixed dilution rate. 3D graph describing the dependence of biomass yield (based on glucose consumption) on temperature and pH for the bacteria is shown on Fig. 3. The differences between modelled surface and actual data points were less than 10%. The region of the optimal growth conditions in the studied range is indicated by the lightest area on the surface on Fig. 3. Simultaneous change of pH and temperature led to a more noticeable change of growth parameters in comparison with a single parameter change, as expected.

Important issues in planning and carrying out D-stat (changestat) experiments are duration of cultivation experiments, range of growth conditions scanned and choice of changing rates, which should enable to maintain quasi steady state. Duration of the cultivation experiments might be a crucial factor in prolonged experiments. It has been shown previously that extended chemostats (100–1,000 generations) could be used for studying culture adaptation and selecting mutated strains with special characteristics (Francis and Hansche 1973; Maharjan et al. 2007). The observation that bacteria can be found in different physiological states in the same environmental conditions before and after applying mild stress in D-stat experiments during 60 generations was made in the present study. The data obtained in our experiments indicated that the changed environmental conditions applied in the D-stat experiments might lead to the onset of the adaptive mechanisms in shorter time than in the constant environmental conditions in chemostat.

The hysteresis of physiological states of the D-stat cultures observed seemed to be phenomenologically similar to the temporary application of the sub-lethal stress after which the vitality of microorganisms have been improved and maintained even after the cessation of stress conditions (Sánchez et al. 2007). Even though sub-lethal stress is still not sufficiently studied at molecular level, three general hypotheses for the explanation of this phenomenon have been formulated: (1) some stress response proteins are expressed in stress conditions and maintained expression of these genes leads to the increase of the survival even long after the cessation of the stress; (2) mutations take place during the sub-lethal stress, or (3) some mutated sub-populations could survive with more ease than the main population. Taking into account these possibilities, more detailed analysis of stress and hysteresis should be carried out in the future, investigating especially possible mutations—e.g. sequencing genomes of the culture during the cultivation.

Another important factor in carrying out changestat experiments is the changing rate of environmental parameters. It has been previously shown that the

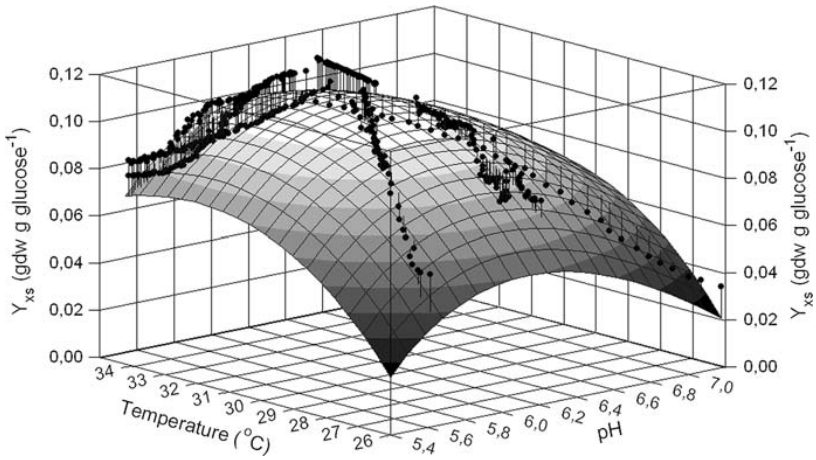

Fig. 3 *Lactococcus lactis* IL1403 quasi steady state growth surface describing the dependence of biomass yield (based on glucose consumption) on temperature and pH for the bacteria. The surface is based on all D-stat experiments. Y_{xs}—biomass yield calculated on glucose consumption (gdw g glucose^{-1}). Black dots represent quasi steady state data points from exp. 1–8

farther the culture is moved from optimal growth conditions, the lower changing rates have to be applied in order to maintain quasi steady state (Adamberg et al. 2009; O'Sullivan and Condon 1999; van der Sluis et al. 2001). Therefore, identification of physiological parameters, which would indicate the (forthcoming) loss of quasi steady state, is a critical issue in carrying out changestat experiments in practice, especially when developing and applying adaptive algorithms. The most suitable indicators of physiological states are the parameters, which could be measured online—e.g. optical density, titration rate, gas production rate etc. In addition, substrate consumption and product formation can be used. Indeed, it has been shown that online measurements of carbon dioxide evolution could be successfully used for the elucidation of the response of the metabolism to the change of the growth conditions (Vemuri et al. 2006). Additionally, indicator molecules of overflow metabolism (acetate, ethanol) in microorganisms having respirofermentative growth like *E. coli* and yeast could be used for adaptive control (Jobé et al. 2003). However, the changes of these metabolic characteristics can also be a "normal" quasi steady state physiological response of the cell metabolism to the change of the environmental conditions. Therefore, the most reliable, though time-consuming way to check the maintenance of steady state growth in changestat experiments is the "chemostat control", i.e. halting the changing rate and continuing the experiment in chemostat. If all physiological parameters (production or consumption rates of metabolites per biomass etc.) remain constant, it can be concluded that the changestat (D-stat, etc.) culture was in a quasi steady state before the acceleration was stopped. This kind of control was also routinely used in our experiments.

A method for scanning the growth space of microorganism was tested in this paper. Use of D-stat cultivation method allowed obtaining a large amount of steady state data in multidimensional quasi steady state growth space of *L. lactis* in reasonable time and effort. It was possible to study reproducibly and quantitatively adaptive responses of growth characteristics as well as gene expression at transcriptome level while changing the pH and temperature in D-stat cultures.

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PUBLICATION III

Lahtvee PJ, Adamberg K, Arike L, Nahku R, Aller K, Vilu R.

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

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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}

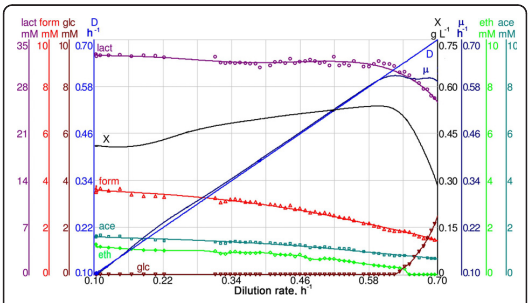

Figure 1 Typical A-stat cultivation, where dilution rate dependent metabolism of *L. lactis* is illustrated. D - dilution rate (h^{-1}); X - biomass concentration (g (dry cellular weight) L^{-1}); μ - specific growth rate (h^{-1}); lact, form, glc, eth, ace - lactate, formate, glucose, ethanol, acetate 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,

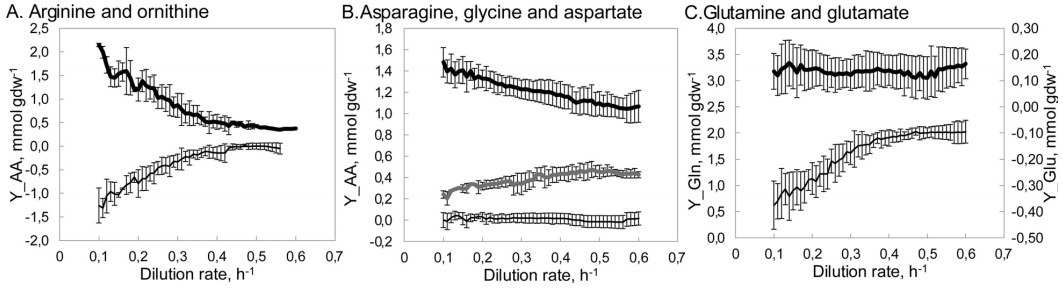

Figure 2 *L. lactis* dilution rate dependent amino acid consumptions (mmol gdw^{-1}) for (A) arginine (thick line) and ornithine (thin line); (B) asparagine (thick line), glycine (dashed line) and aspartate (thin line); (C) glutamine (thick line) and glutamate (thin line). Negative numbers on chart represent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

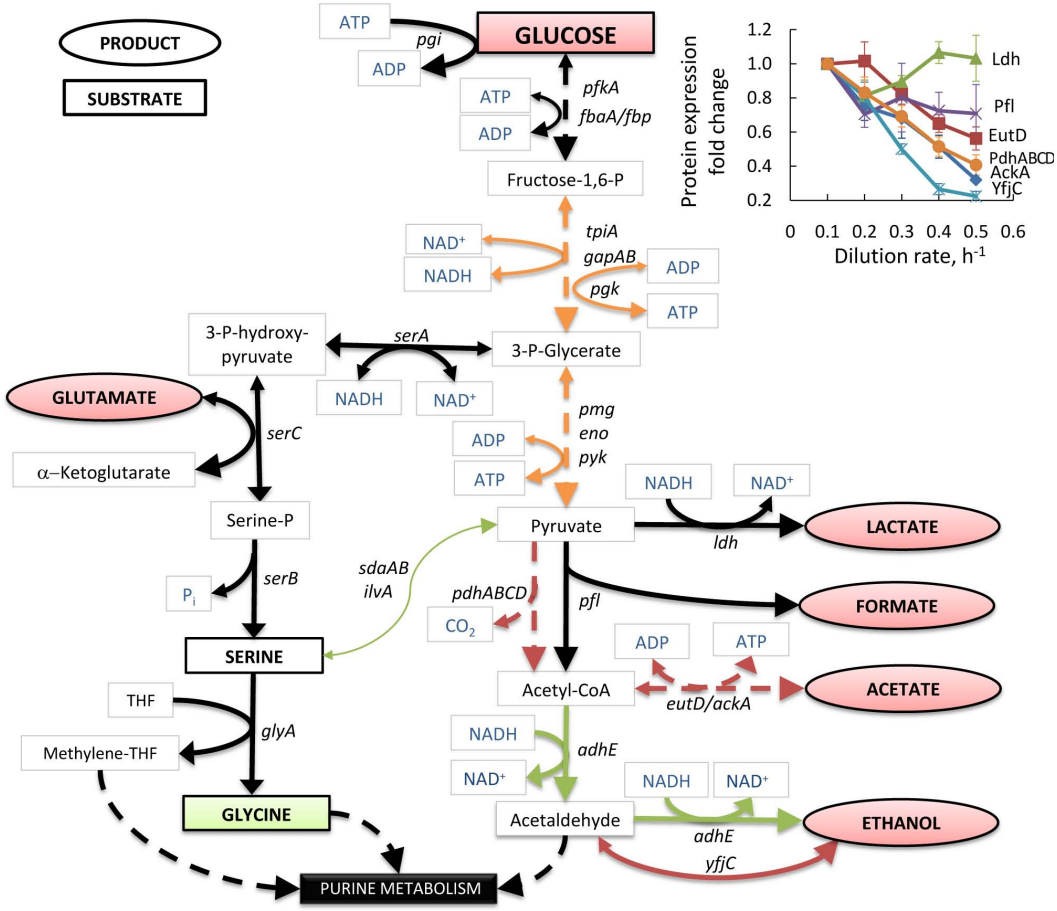

Figure 3 Overview of central carbon metabolism in *L. lactis* at various specific growth rates (μ). Black and capitalised metabolites were measured extracellular. Measured metabolites in boxes/ellipses were consumed/produced, respectively. Red/green/white background represents decrease/increase/no change, 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

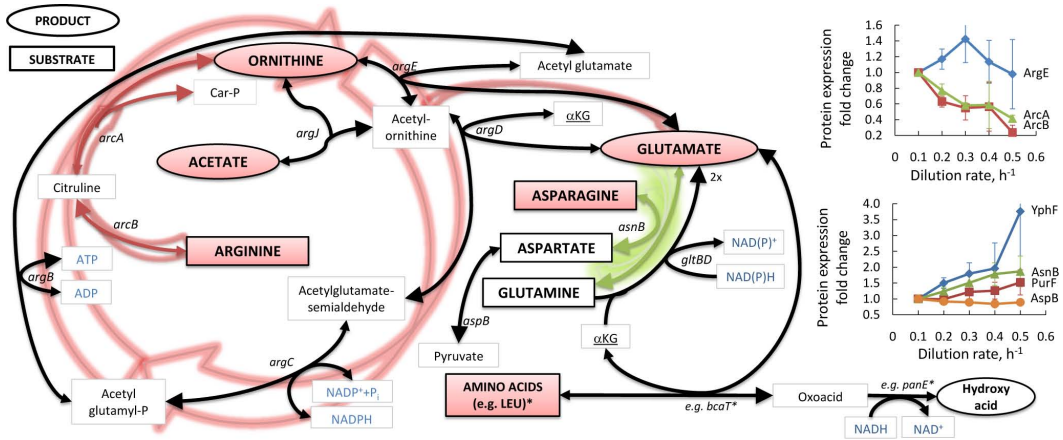

Figure 4 Overview of arginine and glutamine metabolism in *L. lactis* at various specific growth rates (μ). Black and capitalised metabolites were measured extracellular. Measured metabolites in boxes/ellipses were consumed/produced, respectively. Red/white background represents decrease/no change, respectively, 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

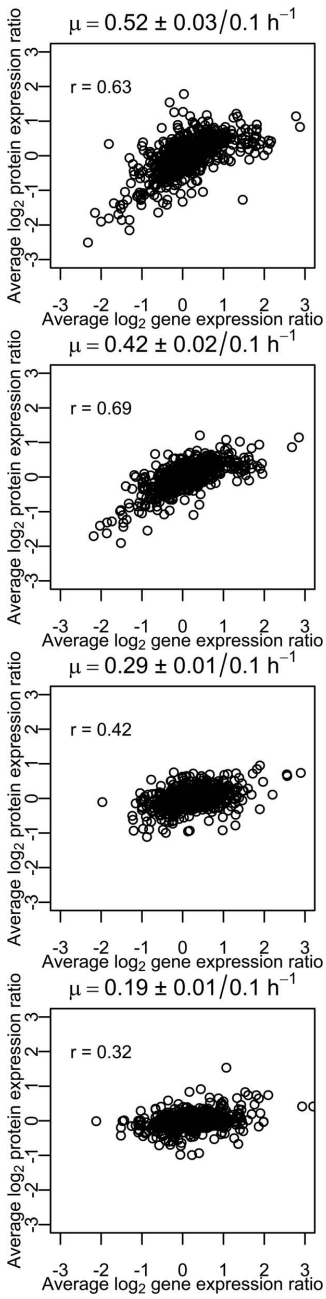

Figure 5 *L. lactis* transcriptome and proteome correlation at various specific growth rates. "R" value on chart represents Pearson coefficient. Six hundred proteins, with a standard deviation less than 30% and their corresponding genes are indicated on a graph.

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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