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Between *S. cerevisiae* and Lactic Acid Bacteria

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SUMMARY

Microorganisms in nature live in interconnected communities, where the language of biochemistry creates means for communicating, fighting, and cooperating with each other. This work investigates one of the ways for microbial interactions - nutrient exchange. It is focused primarily on co-metabolism of *Saccharomyces cerevisiae* and lactic acid bacteria - *Lactococcus lactis* and *Lactobacillus plantarum* - community whose composition was inspired by co-occurrence of yeast and LAB in a multitude of naturally fermented foods. Specifically, I was interested in detecting metabolic interactions between budding yeast and lactic acid bacteria, identifying transferred molecules, exploring metabolic mechanisms of their biosynthesis and excretion, and understanding possible causes behind them.

A combination of experimental and computational methods was used to understand how nutritional dependencies shape communities of microorganisms. First step involved composing a synthetic community of common laboratory strains of yeast and lactic acid bacteria. Following a series of experiments with chemically defined media, LAB revealed their metabolic dependency on yeast for growth and survival. This mixed species community appears to be stable and is sustained through the flow of small nitrogenous molecules from yeast to bacteria. Nutrient cross feeding was found to be a result of overflow metabolism in yeast, which release excess catabolites under particular combinations of available nitrogen sources. The observed nutrient excretion involves a set of genes that regulate yeast nitrogen metabolism when depleted of preferred nitrogen sources. We have recreated co-metabolism of yeast-LAB community, as well as multiple natural bacterial communities, with multi-species genome-scale metabolic modeling. Simulation results demonstrated a link between metabolic cross-feeding and species co-occurrence, and proved its high potential of the method for predicting metabolite exchange in microbial communities.

In this project, the inter-kingdom model community of wild type microorganisms has been established and characterized. Peculiarities of yeast regulatory network, in certain media compositions, cause “wasteful” excretion of amino acids and other metabolites. This in turn creates a stable niche for growth of lactic acid bacteria, which are auxotrophic for multiple amino acids. Described scenario of metabolic dependency between yeast and lactic acid bacteria demonstrates how survival of one species can be driven by metabolic idiosyncrasy of the other. The yeast-LAB interaction is established instantly, and thus can serve as a first step in evolution of cooperation.

ZUSAMMENFASSUNG

In ihrer natürlichen Umgebung leben Mikroorganismen in vernetzten Gemeinschaften, wo die Sprache der Biochemie die Grundlage für Kommunikation, Verteilungskämpfe und Kooperation ist. Diese Arbeit untersucht einen der vielen Wege mikrobieller Interaktionen – Nährstoffaustausch. Sie beschäftigt sich vorranging mit dem gemeinschaftlichen Stoffwechsel (Kometabolismus) von *Saccharomyces cerevisiae* und den Milchsäurebakterien (MSB) – *Lactococcus lactis* und *Lactobacillus plantarum*. Die Zusammensetzung ist inspiriert durch das gemeinschaftliche Auftreten von MSB und Hefe in einer Vielzahl natürlich fermentierter Nahrungsmittel. Ins besondere war ich daran interessiert die Stoffwechselinteraktionen zwischen Backhefe und Milchsäurebakterien zu entdecken, die ausgetauschten Moleküle zu identifizieren, die Stoffwechselmechanismen der Biosynthese und Ausscheidung zu erforschen, und die möglichen Gründe dafür zu verstehen.

Eine Kombination aus experimentellen und theoretischen Methoden wurde benutzt um zu verstehen wie Nährstoffabhängigkeiten eine Gesellschaft von Mikroorganismen formen und prägen. Der erste Schritt war eine künstliche Gesellschaft aus laborüblichen Hefe- und MSB-Stämmen zu etablieren. Durch eine Reihe an Experimenten mit chemisch definierten Medien zeigten MSB ihre metabolische Abhängigkeit von Hefe für Wachstum und Überleben. Diese zusammengesetzte Gesellschaft scheint stabil zu sein und wird durch den Fluss von kleinen stickstoffhaltigen Molekülen von Hefe zu MSB aufrechterhalten. Es zeigte sich, dass der Nährstoffaustausch eine Folge von Stoffwechselüberfluss in Hefe ist. Diese scheiden unter bestimmten Kombinationen von Stickstoffquellen überschüssige Stoffwechselabbauprodukte aus. Die beobachteten Stoffwechselausscheidungen erfordern einen Satz an Genen, die den Hefestickstoffstoffwechsel regulieren wenn präferierte Stickstoffquellen erschöpft sind. Mit Hilfe von Multi-Spezies genomumfänglichen Stoffwechsel-Modellierung haben wir den Kometabolismus von Hefe-MSB Gemeinschaften und vielen anderen natürlichen Bakteriengemeinschaften nachgebildet. Die Ergebnisse der Simulationen zeigen eine Verbindung zwischen Nährstoffaustausch und gemeinsamen Auftreten der Bakterienarten. Die entwickelte Methode hat sich bewährt für die Vorhersage von Stoffwechselproduktaustausch in mikrobiellen Gemeinschaften.

In diesem Projekt wurde eine domänenübergreifende Modellgesellschaft aus Wildtyp-Mikroorganismen etabliert und charakterisiert. In bestimmten Mediazusammensetzungen verursachen Besonderheiten im regulatorischen Netzwerk der Hefe die verschwenderische Ausscheidung von Aminosäuren und anderen Stoffwechselprodukten. Dies schafft eine stabile Nische für das Wachstum von Milchsäurebakterien, die auxotrophisch für verschiedene Aminosäuren sind. Das beschriebene Szenario von metabolischen Abhängigkeiten zwischen Hefe und Milchsäurebakterien zeigt, wie das Überleben einer Art von den Eigentümlichkeiten des Stoffwechsel einer anderen Art beeinflusst wird. Die Interaktion zwischen Hefe und MSB stellt sich sofort ein, und kann deswegen als erster Schritt in Richtung der Evolution von Kooperation dienen.

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ABBREVIATIONS

2-OG	2-oxoglutarate
EGO	Exit From G_0
EUROSCARF	EUROpean *Saccharomyces cerevisiae* ARchive for Functional Analysis
FBA	Flux Balance Analysis
FISH	Fluorescent In Situ Hybridization
GABA	gamma-Aminobutyric acid
GM17	M17 medium with glucose
HSV	hue-saturation-value (color model)
KEGG	Kyoto Encyclopedia of Genes and Genomes
LAB	Lactic Acid Bacteria
LC-MS	Liquid chromatography- mass spectrometry
MRS	de Man, Rogosa and Sharpe medium
mTORC1	mammalian Target of Rapamycin Complex 1
nano-SIMS	Nanoscale Secondary Ion Mass Spectrometry
NCR	Nitrogen Catabolite Repression
OD	Optical Density
ORF	Open Reading Frame
PCR	Polymerase Chain Reaction
PEG	Polyethylene glycol
TF	Transcription Factor
TOR	Target of Rapamycin
TORC1(2)	Target of Rapamycin Complex 1(2)
VPS	Vacuolar Protein Sorting
YPAD	Yeast extract-peptone-dextrose medium with adenine

1 INTRODUCTION[1]

1.1 Metabolic interactions in microbial communities

Microbial communities are intertwined with metabolic exchanges, whether viewed as narrowly as a pair of symbionts, or as generally as earth-wide ecosystem lined up with trophic chains. Understanding metabolic interactions at global level is indispensable in microbial ecology and evolution. Seeing microbial metabolism in the community context (as opposed to pure cultures) reveals new phenotypes (Jarosz et al., 2014), helps designing synthetic communities for biotechnology (Santala et al., 2014; Zhou et al., 2015), and enables cultivating the 'uncultivables' (Ling et al., 2015). Accumulating examples of metabolic cross-feeding (Morris et al., 2013; Seth and Taga, 2014) and evidence from metabolic modeling (Zelezniak et al., 2015) create an anticipation of many more to be discovered. This work is focused on nutrient exchange between microorganisms, arguably the most common metabolic interaction in microbial communities.

Multiple studies show that metabolite exchanges form a strategy for group success (Lawrence et al., 2012; McNally et al., 2014; Morris et al., 2013; Pande et al., 2014; Ren et al., 2015). Metabolic interactions frequently contribute, through division of labor, to the emergent abilities at community level, such as biodegradation (Fowler et al., 2014; Lykidis et al., 2011), faster growth (Pande et al., 2014) or increased virulence (Alteri et al., 2015; McNally et al., 2014). Outsourcing metabolic functions to fellow members embeds each pathway in a specialized microenvironment, hence avoiding biochemical conflict (Johnson et al., 2012). Moreover, under nutrient-poor conditions species can be prompted to share metabolites and thus complement each other's biosynthetic capabilities (Harcombe, 2010; Hom and Murray, 2014; Wintermute and Silver, 2010). Metabolic specialization can be found even within the same species, e.g., filamentous cyanobacteria with specialized heterocyst cells for nitrogen fixation (Kumar et al., 2010).

[1] The text in this section is written by myself and reproduced with some modifications from Ponomarova and Patil, 2015.

1.2 Evolution of microbial interactions

Despite benefits associated with cross-feeding, its evolution remains controversial, especially in case of metabolic cooperation (Foster and Bell, 2012; Oliveira et al., 2014). Emerging and maintenance of metabolic exchanges depends on particular circumstances, such as spatial structure of microbial community, nutrient availability, diffusion constraints and cost effectiveness of concerned biosynthetic processes (Allen et al., 2013; Hol et al., 2013; Morris et al., 2012). For example, aggregating or forming a biofilm maximizes efficiency of nutrient transfer and stimulates otherwise thermodynamically unfavorable metabolic processes (Agapakis et al., 2012). In extreme cases, metabolic dependency results in endosymbiotic relationship, a popular solution for hydrogen-producing ciliates that harbor methanogenic archaea for H_2 outflow (Fenchel and Finlay, 2010).

1.3 Experimental methods to study metabolic interactions in microbial consortia

Metabolic exchanges are ubiquitous in microbial communities. However, detecting metabolite cross-feedings is difficult due to their intrinsically dynamic nature and community complexity. Thus, while exhaustive description of metabolic networks operating in natural systems is a task for the future, the battle of today is divided between detailed characterizations of small, reduced complexity microbial consortia, and focusing on particular metabolic aspects of natural ecosystems.

Shifting attention from isolated metabolism of pure cultures to that of microbial communities is challenging and requires new tools and methods. And, as in case of any complex network, when choosing a focus point in the large web of metabolic interactions, we have to compromise between resolution of detail and coverage. Detecting metabolic interactions requires methodological blend able to capture species identity, dependencies and the nature of exchanged metabolites. Multiple combinations of diverse techniques, from metagenomics to imaging mass spectrometry, offer solutions to this challenge, each combination being tailored to the community at hand.

Enumerating metabolic exchanges, being difficult even for small communities, becomes overwhelming for natural communities with hundreds of species living in fluctuating environment. One of the main underlying reasons for this difficulty is that metabolites cannot be

directly attributed to a particular species or abiotic source. Furthermore, a large fraction of microbial diversity still remains largely undiscovered or uncharacterized for their metabolic needs and biosynthetic capabilities. These composite problems necessitate a trade-off between resolution and coverage (Fig. 1.1).

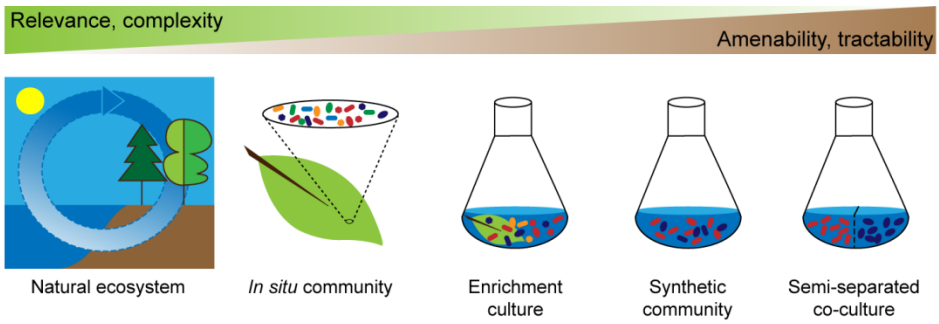

Figure 1.1: Spectrum of microbial community study-systems directed by trade-off between complexity and tractability. Microbial interactions play a central role in biogeochemical cycles in numerous ecosystems, yet are difficult to investigate in molecular details. In contrast, synthetic communities allow controlled environment and ease of interpretation. Each study-system in this spectrum offers a choice of resolution to view microbial interactions.

An attractive way to achieve increased resolution of metabolic dependencies is through constructing a smaller manageable model system or by focusing on a particular interaction within a large network. On the other end of spectrum, one can cover large system by grouping individual players into higher order units – guilds (e.g. methanotrophs, sulfur-reducers) or metabolite classes (e.g. electron equivalents, fixed nitrogen). Balancing between these two strategies can loosen the tangle and trace the main threads in the metabolic knot of interspecies interactions.

1.3.1 Meta-omics methods

Meta-omics analyses guide interaction discovery. Meta-omics technologies are culture independent and scalable in space/time. Metagenomics is a particularly powerful tool for discerning species identity and for detecting patterns of interspecies associations. These in turn can generate verifiable hypotheses about metabolic (and other) interactions between community members. Genotyping of associated microbes can reveal their functional palettes (Shafquat et al., 2014) and task distribution among community members (Lykidis et al., 2011). For example, individual genomes of co-aggregated pair of archaea showed that one of the symbionts is

dependent on another for lipid, cofactor, amino acid, and nucleotide biosynthesis (Waters et al., 2003). Following a specific community over time can also reveal metabolic dependencies as one species dynamically responds to change in abundance of the other, e.g. as shown in a activated sludge community (Ju and Zhang, 2015). Overlaying taxonomic data with other information, such as spatial distribution and geochemical profiles (Fuchsman et al., 2011) or specific enzymatic function (Bailey et al., 2013) can deepen insight into community co-metabolism. Beyond individual communities, metagenomics has allowed identifying species co-occurrence structure across different habitats/samples (Faust et al., 2012; Friedman and Alm, 2012) – associations that hint at interspecies interactions (Berry and Widder, 2014; Zelezniak et al., 2015).

Transcriptomics and proteomics are commonly used to complement metagenomics, to deduce what genome encoded metabolic potential is being used (Durham et al., 2015; Embree et al., 2014). For instance, analysis of transcriptional patterns in co-culture of marine bacterium and diatom, as well as ocean samples, pinpointed cross-feeding of 2,3-dihydroxypropane-1-sulfonate, a new link in marine microbial food web (Durham et al., 2015). Metabolic applications of meta-proteomics are more commonly used for relatively simple systems – it was used to demonstrate metabolic adjustments made by three species comprising model oral biofilm (Hendrickson et al., 2014) or to show how a presence/absence of *Aggregatibacter actinomycetemcomitans* modulates metabolism of other bacteria in 10-species biofilm (Bao et al., 2015). Although not distinguishing between species, these results give a sense of complexity and scale of metabolic adjustments that happen in "real-world" communities. On a larger scale, meta-proteomics, in combination with meta-genomics, allowed proposing differential flow of nitrogen, sulfur and hydrogen among the abundant taxa of marine microbial communities in response to oxygen availability (Hawley et al., 2014).

1.3.2 Isotope labeling for tracing community-scale pathways.

Tracing of isotope labeled substrates, a standard approach in pathway discovery, can also be adapted to reveal flow of metabolites in microbial consortium. Although this is the most conclusive method for showing metabolite exchange, the major challenge is to distinguish labeling fingerprints of different populations. To do so, one can use artificially expressed reporter protein (Ruhl et al., 2011), species-specific peptides (Ghosh et al., 2014), or detect

labelled DNA or RNA in conjunction with metagenomics analysis (Verastegui et al., 2014). To give some examples, 13C labeling served to experimentally prove bacterial feeding on fungal exudates (Pion et al., 2013), to suggest a chain of toluene degraders in methanogenic enrichment culture (Fowler et al., 2014) and to identify key naphthalene-degrading bacteria in situ (Herbst et al., 2013).

1.3.3 Imaging community structure – clues from the neighbors.

Efficient mass transfer between organisms is a prerequisite of successful metabolic interaction, therefore it is not uncommon for microbial partners to form tight aggregates and develop special structures that facilitate metabolite exchange. Microscopic detection of these structures can be a powerful tool in identifying interacting microorganisms. Illustrative is an example of nanotubes formed by cross-feeding E. coli auxotrophs (Pande et al., 2015) or variety of formations in acid mine drainage community, such as cytoplasmic bridges, pili, and "synaps like connections" (Comolli and Banfield, 2014).

Fluorescence in situ hybridization (FISH) based methods reveal spatial distribution of interacting partners, for instance showing stratification and co-aggregation patterns in biofilms (Almstrand et al., 2013) or bacterial groups attached to phytoplankton host (Cruz-López and Maske, 2014). In addition to resolving spatial structure, imaging, e.g. based on fluorescent dyes, can be used to assess general metabolic state of community members (Maurice and Turnbaugh, 2013; Vila-Costa et al., 2012).

1.3.4 Unbiased exploration using metabolomics.

Mass spectrometry (MS) based methods can detect a broad spectrum of compounds and are being developed by day. This technique has a wide range of modifications, varying in application from a single cell to multiple colonies on a petri dish (reviewed by Watrous et al. (Watrous et al., 2011)). MS can be used in an imaging set-up to study metabolic interactions (Shih et al., 2014). Potential of imaging-MS unfolded, for example, in a study of chemical interactions on actinomycete bacteria, showing interactions through spectra of secondary metabolites (Traxler et al., 2013). Application of MS to microbial interactions is, however, currently limited by various challenges in data analysis and compound identification (Chen et al., 2002; Garg et al., 2015; Jarosz et al., 2014; Traxler et al., 2013). Other methods that can facilitate interrogation of

metabolic space of the community are reviewed by Maurice et al. (Maurice and Turnbaugh, 2013) and Wessel et al. (Wessel et al., 2013).

Metabolomics alone usually does not provide sufficient resolution to pinpoint exchanged molecules. Elucidating cross-feeding in a complex nutritional environment is possible only in combination with other techniques such as stable isotope labeling and FISH. Such methodological blend allowed pinpointing nitrogen transfer from cyanobacteria to their symbiotic diatoms (Foster et al., 2011) or from methane-oxidizing archaea to sulfate-reducing bacteria in marine seeps (Dekas et al., 2009; Green-Saxena et al., 2014). Several examples of metabolic interactions detected through combination of different methods are described in Table 1.1

1.3.5 Divide and conquer through temporal / spatial compartmentalization.

Using a defined assembly of microorganisms opens opportunities to employ methods inapplicable to complex systems. For example, species quantification can be easily done with selective plating, quantitative PCR or flow cytometry. However, for better control over metabolite production and consumption, as well as for discerning metabolic roles of different populations, modification to mixed cultures can be made. One of the simplest techniques is based on the cell-free culture filtrate – the so-called conditioned or spent medium. This approach is frequently used to assay activity of secretome of the donor microorganism(s) by adding its conditioned medium to the recipient culture. This allows identifying non-induced dependencies such as interaction network between gut symbionts knitted by polysaccharide degradation products (Rakoff-Nahoum et al., 2014).

Other approaches try to preserve real time molecule diffusion between species, but keep symbionts physically separated, e.g. by means of semi-permeable membrane (Stadie et al., 2013), structuring microenvironment in microfluidics device (Kim et al., 2008), encapsulating cells in hydrogels (Connell et al., 2013), or co-culturing in a Petri dish (Kerr et al., 2002). Artificial barriers provide better control over conditions and more convenient quantification, separation and analysis of interacting populations, also in a high-throughput manner (Leung et al., 2012). It is important to note that the co-culture conditions can have a profound impact on community metabolism (Goers et al., 2014) and hence caution is warranted when extrapolating the conclusions to other contexts.

Table 1.1: Examples of metabolic exchanges in microbial communities

Community type	(Eco-) system	Interacting taxa	(Potentially) exchanged metabolite(s)	*Methods used to detect / infer –*			Reference
				Species identity	*Inter-species dependency*	*Exchanged metabolite(s)*	
Natural	Anoxic marine sediments	*Thioploca* (sulphur-oxidizing bacteria), anaerobic ammonium-oxidizing bacteria	NH_4, NO_2	FISH[a], 16S rDNA sequencing	FISH analysis of spatial association	Inference from N isotope distribution	(Prokopenko et al., 2013)
Natural	Deep-sea sediments	ANME-2 archaea group (anaerobic methane-oxidizing archaea), *Desulfosarcina* / *Desulfococcus* (sulfate-reducing bacteria)	Reduced N species	FISH	Observed co-aggregation, previously described syntrophic relationship	FISH-coupled nanoSIMS[c] showing ^{15}N incorporation across aggregates	(Dekas et al., 2009)
Natural	Ocean plankton	*Thalassiosira pseudonana* (diatom), *Roseobacter* clade bacteria	2,3-dihydroxypropane-1-sulfonate (DHPS)	Metatranscriptome analysis, fractionation of marine biomass	Metabolic exchanges in model bacterial-phytoplankton system	Metatranscriptome analysis, targeted MS metabolomics of the eukaryotic plankton size fraction	(Durham et al., 2015)
Enrichment culture[b]	Alkane-degrading methanogenic community	*Smithella* (bacteria); *Methanosaeta* and *Methanocalculus* (methanogenic archaea)	Acetate, electrons	Single-cell genome sequencing, community 16S rDNA analysis	Substrate dependent changes in community composition, *a priori* knowledge	Analysis of genome sequence and community metatranscriptome	(Embree et al., 2014)
Enrichment culture	Anaerobic terephthalate-degrading consortium	*Pelotomaculum* (anaerobic bacteria), *Methanosaeta* and *Methanolinea* (hyper-mesophilic methanogens)	CO_2, H_2, acetate	16S rDNA profiling, shotgun sequencing	FISH analysis of spatial association, *a priori* knowledge	Metagenome analysis, thermodynamic considerations	(Lykidis et al., 2011)
Synthetic	Isolates from a cellulose-degrading community	*Pseudoxanthomonas*, *Brevibacilllus*, *Clostridium*	Acetate, ethanol, saccharides	Known; assessed by real-time PCR	Mixed culture dynamics, conditioned medium experiments	Targeted quantification of cellulose and cellulose degradation products	(Kato et al., 2005, 2008)
Synthetic	Isolates from water kefir	*Z. florentina*, *S. cerevisiae*, *L. hordei*, *L. nagelii*	Amino acids, vitamin B6, unknown factors	Known	Co-culture in transwell plates	Single component exclusion, growth in pairwise cultures	(Stadie et al., 2013)
Synthetic	Human intestinal symbionts	*Bacteroides caccae*, *B. fragilis*, *B. ovatus*, *B. thetaiotaomicron*, *B. uniformis*, *B. vulgatus*, *Parabacteroides distasonis*	Polysaccharide degradation products (fructose, glucose, etc.)	Known	Analysis of species growth in defined media, conditioned media and co-cultures	Assessment of carbohydrate breakdown products released by donors and consumed by recipients	(Rakoff-Nahoum et al., 2014)

[a] Fluorescence *in situ* hybridization.
[b] Culture obtained from natural sample by promoting growth of organisms of interest, typically by manipulating medium composition.
[C] Nanoscale secondary ion mass spectrometry

1.3.6 In-silico methods to study metabolic exchanges

Average microbial genome contains around thousand enzymatic reactions, making computational approach necessary to achieve holistic view of cellular metabolism. Genome scale metabolic modeling typically starts with genome sequencing and gene annotation, which provides a list of (presumably) functional metabolic reactions that represent microbial cell. This model reconstruction can either be fully automatic through pipeline resources, such as Model SEED (Henry et al., 2010), GEMSiRV (Liao et al., 2012), *merlin* (Dias et al., 2015) etc., or contain manual post-processing and curation steps (Hamilton and Reed, 2014). Both number and quality of model continuously grows, providing more opportunities for computational experiments.

In silico studies can be extended to analyze multiple species interspecies interactions in microbial communities (Mahadevan and Henson, 2012), and steady state modeling can be scaled up to ecosystem level (Klitgord and Segre, 2010), revealing a coarse-grained, but often fundamental principles (Freilich et al., 2011). Quantitative predictions and dynamic simulations require more detailed input and thus usually applied to small, well described communities. Pairwise modeling has shown emergent biosynthetic abilities in co-cultures (Chiu et al., 2014), demonstrated that oxic/anoxic conditions stimulate mutualistic cross-feeding among gut bacteria (Heinken and Thiele, 2015). Quantitative effect of metabolite cross-feeding can also be calculated, as it was done using dynamic modeling (Song et al., 2014; Zomorrodi et al., 2014) or diffusion simulation of secreted nutrients between colonies in 2-3 species communities (Harcombe, 2010). Overlaying metabolic modeling with metagenomics data infers inter-species interactions and allows to make suggestions about ecological forces at play, e.g. habitat filtering and species assortment (Levy and Borenstein, 2014). Modeling sheds light on competition versus cooperation dilemma, showing that decision in favor of one or another strategy depends on intrinsic metabolic potential (Zelezniak et al., 2015), nutrient availability (Heinken and Thiele, 2015; Klitgord and Segre, 2010) and spatial distribution (Allen et al., 2013). Mathematical models of community metabolism hold a great potential to suggest cross-feeding scenarios and thus to narrow down the search space for experimental studies.

1.3.7 Synthetic communities as model systems.

While natural consortia are still difficult to scrutinize, enrichment cultures compromise between natural and synthetic communities (Fig 1.1). These are cultures obtained from natural samples by promoting growth of organisms of interest, typically by manipulating medium composition. Synthetic microbial communities provide further reduction in the complexity, creating a more tractable system for discovering metabolic exchanges (Grosskopf and Soyer, 2014; Song et al., 2014). Communities constructed with the isolates from the same environment maximize resemblance to the natural community and preserve indigenous interactions shaped by co-adaptation/evolution (Stadie et al., 2013).

The pre-requisite for common history of member species can be relaxed when addressing fundamental questions like emergence and evolution of metabolic interactions (Andrade-Dominguez et al., 2014; Hom and Murray, 2014). To this end, one might also turn to engineered dependencies through genetic manipulation and / or laboratory evolution (Harcombe, 2010; Mee et al., 2014; Summers et al., 2010; Wintermute and Silver, 2010). Despite being less "natural", engineered interactions have obvious advantage of known identity of transmitted metabolite (or at least of the involved pathways), as well as being easier to obtain, monitor, and control. Engineered communities are most common object to study synergistic growth effects of metabolic cross-feeding (Mee et al., 2014; Pande et al., 2014).

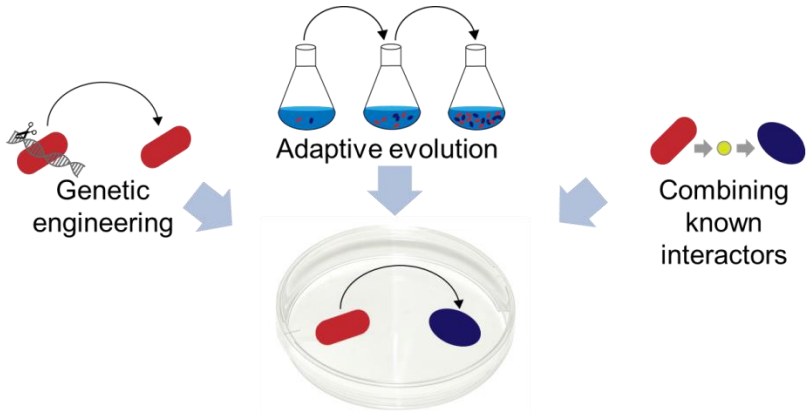

Figure 1.2: Most common means to create a synthetic microbial community.

Another group of model systems for microbial interactions emerge from microbiota of fermented food (Wolfe and Dutton, 2015). These associations typically have reduced complexity compared with most environmental or host-associated systems, and can be grown in well controlled

environment without loss of tractability, e.g. chees rinds (Wolfe et al., 2014). Spatial organization, species succession, stability, resilience, and co-evolution history, such as those of water and milk kefir grains (Laureys and De Vuyst, 2014; Marsh et al., 2013), create a rich ground in search of metabolic interaction mechanisms.

1.4 Synthetic community of yeast and lactic acid bacteria: motivation and design

Yeast and lactic acid bacteria go side by side in their habitats, particularly in nutrient rich human-influenced environments. This inter-kingdom symbiosis is a common recipe for naturally fermented foods all over the world, including but not limited to kefir, kimchi, airag, togwa, sourdough, and sour beer. Yeast-LAB co-fermentations have occurred spontaneously and have been sustaining nutritional needs of our ancestors long before the science of microbiology has emerged. Growth of yeast and LAB in fermented products not only created a unique flavor profile, but also ensured food safety by outcompeting food spoilage microbiota and served as a source of probiotics. First proteomic evidence for yeast-LAB symbiosis in food dates back to 3800 years (Yang et al., 2014). Despite such a long history our understanding of interactions between yeast and their bacterial neighbors is limited.

Re-occurring symbiosis of yeast and lactic acid bacteria inspired the composition of synthetic microbial community used to study interspecies interactions in this work. Budding yeast *Saccharomyces cerevisiae* S90, together with representatives of two lactic acid bacteria families, viz., *Lactococcus lactis* subsp. *lactis* (Streptococcaceae) and *Lactobacillus plantarum* (Lactobacillaceae) were selected to assemble a synthetic community. All three species are prototrophic (without introduced auxotrophy markers). Working with these well-characterized strains provided methodological flexibility, such as host independence, ease of cultivation, and the availability of diverse molecular biology tools and manually curated genome scale metabolic models.

The topic of interactions between yeast and lactic acid bacteria is not new, but studies to date are limited. Available data is gathered in the context of food microbiology, with an emphasis on co-fermentation in sourdough (Gobbetti et al., 2005; Minervini et al., 2012) and dairy products (Álvarez-Martín et al., 2008; Narvhus, 2003; Sudun et al., 2013). These studies discuss how metabolic composition, such as flavor and aroma compounds, change with variation in microbial community compositions. Direct metabolic exchanges, due to a complex matrix composition of

fermented food, are typically not addressed. Suggestion of metabolic interactions, however, appear since 90's (Gobbetti et al., 2005), leaving a lot of room for investigation. With development of new methods and approaches, we soon will know much more about yeast-LAB symbiosis. Such an example is provided in a recent study where authors demonstrate cross-feeding (summarized in Table 1.1) between yeast and LAB isolated from water kefir (Stadie et al., 2013).

1.5 Nitrogen metabolism and its regulation in *S. cerevisiae*

1.5.1 TOR pathway

Growth rate and behavior of the yeast cell is a function of the environment, which by large is defined through nutrient quantity and quality. Necessity to tune growth and regulation according to conditions resulted into dual role of nutrients: on one hand as biomass building blocks and energy sources, and on the other hand as signaling factors (Broach, 2012).

In *S. cerevisiae* multiple interconnected signaling networks work in concert to sense nutritional status and optimize resource utilization accordingly. One of them, TOR (Target of Rapamycin) network, primarily reacts to the quality and quantity of nitrogen sources (Zaman et al., 2008). TOR was discovered through specific inhibition by macrolide drug rapamycin, hence the name (Loewith and Hall, 2011). Rapamycin treated yeast cultures display numerous phenotypic changes: halt in G_0 phase, inhibition of protein synthesis, elevated autophagy, upregulation of stress response, accumulation of glycogen and trehalose, and induction of nitrogen catabolism (De Virgilio and Loewith, 2006b). These changes resemble the way yeast cell reacts to nitrogen deprivation, exposing the involvement of TOR in the nitrogen metabolism (Zaman et al., 2008).

This complex signaling system is conserved among many eukaryotes, but unlike most mammals, *S. cerevisiae* possesses two TOR complexes. TORC1, the only one responsive to rapamycin treatment, mainly controls growth, protein synthesis, nitrogen metabolism and autophagy in response to nutritional cues; and TORC2, a lesser-studied TOR complex, regulates actin polarization and overcoming DNA damage (Weisman et al., 2014).

TORC1 is found to be associated with membranes: vacuolar (thus in proximity to nutrient storage), endosome or plasma membranes (de Virgilio, 2006a). Coincidentally, this location places TORC in vicinity to its upstream regulators, such as vacuolar membrane associated EGO

complex (Fig. 1.3). EGO couples TORC1 activity to signals from amino acids (Binda et al., 2009; Kim and Guan, 2011). New studies also show that TORC1 depends on cellular trafficking, as was demonstrated through interaction with mutations of vacuolar protein sorting (VPS) complex, especially in class C genes (Fayyadkazan et al., 2014; Zurita-Martinez et al., 2007).

Rapamycin inhibits TOR signaling by binding to FKBP12 (FK506-binding protein), which then attaches to TORC1 complex, assumingly blocking phosphorylation of its downstream targets (Shimobayashi and Hall, 2014). The latter are better represented in literature, although, due to multiplayer regulation and interconnection with other signaling networks, assigning a target to that or another pathway can be tentative.

Multiple effects TORC1 enforces through activity of PP2A-like phosphatases – Sit4, Pph21, and Pph22 (Fig. 1.3). TOR1 complex phosphorylates Tap42 protein, which then binds to the phosphatases and modulates their specificity to certain substrates (Fig.1.3).

Another TORC1 effector is Sch9, PKA-like kinase with TORC1-dependent phosphorylation. Through phosphorylating other intermediate regulators it eventually causes activation of genes responsible for ribosomal biogenesis (and initiation of translation) (Hughes Hallett et al., 2014). Similar role has a transcription factor Sfp1. Phosphorylated by TORC1, it then stimulates expression of ribosomal protein genes (Loewith and Hall, 2011).

TORC1 also affects amino acid biosynthesis through upregulating RTG (retrograde signaling) pathway. Specific mechanism of TORC1 involvement is unknown, but resulting effect resembles response to dysfunctional mitochondria: upregulated transcription of anapleurotic enzymes refuels TCA cycle and thus replenishes carbon backbones (primarily 2-oxoglutarte) required for biosynthesis of glutamine and glutamate (Fig. 1.3 and 1.4).

TOR signaling negatively regulates amino acid biosynthesis through indirect phosphorylation and thus deactivation of Gcn2. Active Gcn2 activates translation of Gcn4 that in turn activates transcription of amino acid biosynthesis genes (Smets et al., 2010).

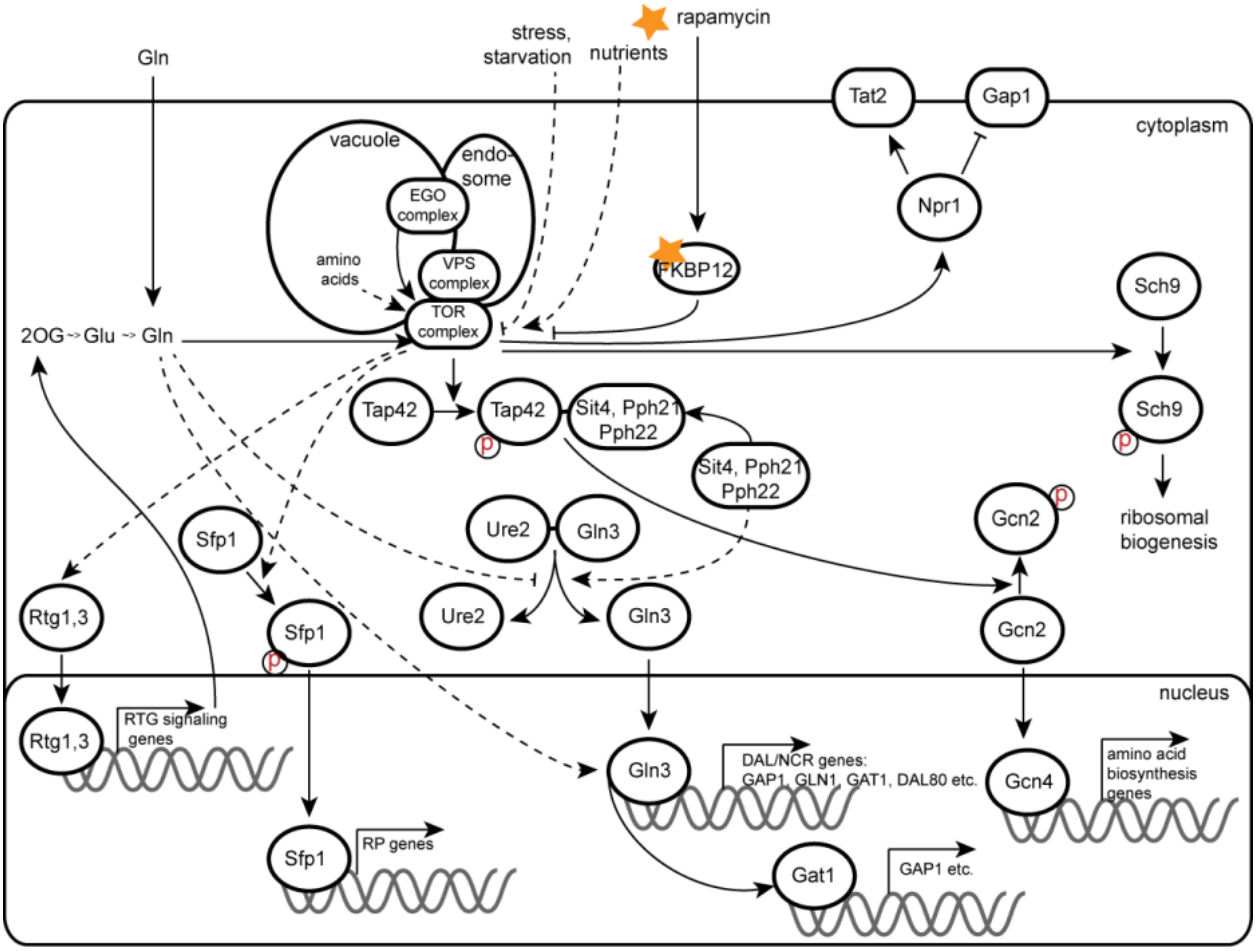

Figure 1.3: Major TORC1-related signaling regulators. RTG – retrograde signaling; RP – ribosomal protein (genes). Connections include indirect interactions. Dotted lines show interactions with less understood/putative mechanism/mediators. Compiled based on (Ljungdahl and Daignan-Fornier, 2012; Loewith and Hall, 2011; Smets et al., 2010; Zaman et al., 2008)

Sorting of amino acid permeases is controlled by TORC1 through Npr1 kinase in a way that at nutrient rich conditions constitutive permeases (like Tat2) are directed to the plasma membrane, and under nitrogen replete conditions NCR-controlled permeases like Gap1 are favored.

1.5.2 Nitrogen source discrimination

Nitrogen, making ~10% of *S. cerevisiae* cell by weight (Fraenkel, 2011), is an essential component of yeast nutrition. *S. cerevisiae* can assimilate around 30 different nitrogenous compounds (Godard et al., 2007), however "value" of these compounds to the yeast varies. Value of the compound depends on its fluidity as metabolic currency: while some metabolites can be easily incorporated into metabolic flow and are closer to the central pathways of the cell,

others require more enzymes, cofactors etc. for assimilation. Central to nitrogen metabolism are reactions that interconvert 2-oxoglutarate, ammonium, glutamine and glutamate (Figure 1.4). External nitrogen has to pass through this path to be further incorporated into biosynthetic reactions, typically transfer of amino/amide group from glutamate/glutamine to newly forming amino acids (Ljungdahl and Daignan-Fornier, 2012). Glutamate is responsible for 85% of total incorporated nitrogen, and glutamine makes up the other 15% (Cooper, 1982). In addition, intracellular pool of glutamine indicates nitrogen status of the cell and initiates complex nutrient response programs (Crespo et al., 2002; Murray et al., 1998). Although usually less potent than glutamine, 2-OG pool can also regulate nitrogen metabolism (Leigh and Dodsworth, 2007). Amino acids other than glutamine can also convey global regulatory signals, for example arginine, and leucine were shown to regulate TORC1 activity in yeast and mammals as well (Jewell et al., 2015).

Amino acids can be classified according to their "value". The first group, supporting the highest growth rates, includes ammonium, glutamine, asparagine, and is shortly followed by serine, arginine, aspartate, glutamate, and alanine (Godard et al., 2007). These amino acids are considered preferable due to their proximity to TCA cycle and absence of non-metabolizable catabolites. The least favorite sources of nitrogen are such compounds as proline, valine, phenylalanine, and ornithine. Preferred nitrogen sources induce inhibition of genes responsible for utilization of other nitrogen sources. Strain S288c (whose prototrophic version we use in our experiments) has somewhat exceptional nitrogen preferences – it does not show repression in presence of ammonium and prefers, unlike other strains, glutamine to inorganic nitrogen, (Godard et al., 2007; Zaman et al., 2008).

Pathway responsible for selective utilization of nitrogen sources is generally referred to as a nitrogen catabolite repression (NCR) pathway, or, less frequently, as nitrogen discrimination pathway (NDP). It governs expression of approximately 90 metabolic genes (Zaman et al., 2008). Other publications have used as many as 390 genes to assess NCR activity (Godard et al., 2007). Analogously to repressing the uptake of other carbon sources in presence of glucose, whenever a good source of nitrogen is present (such as glutamine) yeast cell focuses on its consumption and shuts down uptake and processing of other, suboptimal compounds. At this state NCR-sensitive genes are repressed. However, when yeast is grown on less favorite nitrogen source (such as proline), cell activates alternative metabolic routes to process the nitrogen which

is currently available. At this stage NCR controlled genes are activated. Of course, this is a simplified binary picture, and reality is much more of a "gray zone" between *on* and *off* states, involving multiple regulatory pathways.

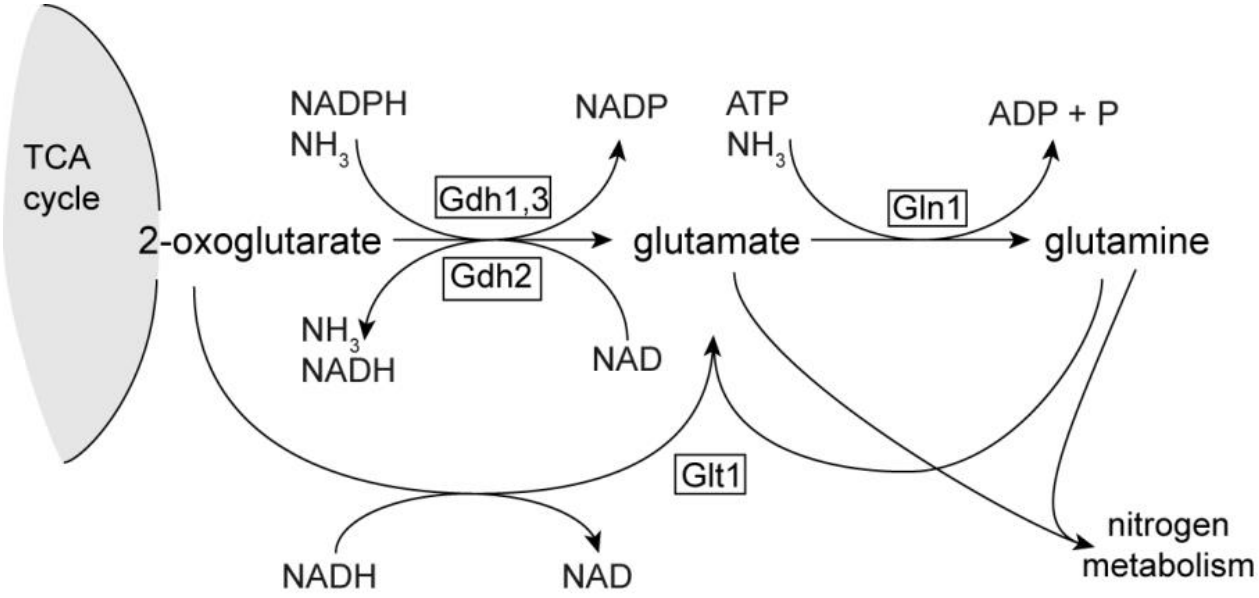

Figure 1.4: 2-oxoglutarate joins central carbon with central nitrogen metabolism through glutamate and glutamine. Ammonium (ammonium cation) is used by Gln1 and Gdh enzymes.

NCR is governed by several transcription factors: activators Gln3, Gat1, Dal81, Dal82 and repressors Gzf3, and Dal80 (Bertram et al., 2000). These effectors cross-regulate each other (Ljungdahl and Daignan-Fornier, 2012), and combinatorially control downstream targets that direct metabolism of nitrogenous components, e.g. amino acids, allantoin, ammonia and urea (Bertram et al., 2000). Gln3 is a GATA-type transcriptional activator controlling the majority of NCR-sensitive genes. Gln3 is one of the most studied TFs, as the first sequenced and cloned NCR activator (Minehart and Magasanik, 1991). It activates transcription of other NCR transcription factors (GAT1, DAL80, GZF3), but also enzyme-encoding genes, such as arginase *CAR1*, glutamate dehydrogenase *GDH2*, and glutamine synthase *GLN1* (Courchesne and Magasanik, 1988). Furthermore genes *UGA1*, *CAN1*, *GAP1*, *PUT4*, *ASP3*, *GDH1*, *DAL1-2*, and *DAL4* are dependent on *GLN3* (ter Schure et al., 2000). *GAP1* is a general amino acid permease, one of the multiple nitrogen metabolite transporters whose induction is a hallmark of nitrogen

catabolite derepression. Overexpression of Gln3, however, is toxic for the cell, which gives one of the possible reasons for nitrogenous metabolite excretion.

TORC1, as a global nutrient controller, also affects (activates) NCR (Loewith and Hall, 2011). Mechanistic details of NCR regulation by TORC1 are beginning to emerge, to date highlighting the link through Gln3. TORC1, through a complex of phosphatases, modulates binding of Gln3 to Ure2 (Fig. 1.3). This binding sequesters Gln3 in the cytoplasm and prevents it from activating transcription in the nucleus (Bertram et al., 2000; Tate et al., 2010). However, Gln3 can also be controlled through nitrogen limitation (likely by sensing the level of intracellular glutamine), in a TORC1-independent manner (Georis et al., 2011; Rai et al., 2013; Zaman et al., 2008). Together with transcription factors Rtg1 and Rtg3, Gln3 can be activated directly by intracellular levels of glutamine (Crespo et al., 2002).

1.6 Metabolism and nutrient requirements of LAB

Lactic acid bacteria taxonomically constitute an order Lactobacillales that includes families Aerococcaceae, Carnobacteriaceae, Enterococcaceae, Lactobacillacea, Streptococcaceae, and Leuconostocaceae. As the name suggests, one of the uniting factors for these bacteria is the production of large amount of lactic acid as the main end-product of carbohydrate fermentation. Other unifying features include positive Gram staining, resistance to acidity, absent sporulation, low GC content, and deficiency in respiration.

Lactic acid bacteria are fastidious organisms with limited biosynthetic capacity, that for stable growth require a number of amino acids, vitamins, and other nutrients (Hayek and Ibrahim, 2013; Snell, 1945; van Niel et al., 1999; Wegkamp et al., 2010). Amino acids are usually classified as essential (absolutely required), stimulatory, and non-essential. Requirements for essential amino acids varies largely between species (and strains) of lactic acid bacteria, estimated to fall somewhere between 3 to 14 amino acids (Hayek and Ibrahim, 2013).

High level of auxotrophy among LAB is in accord with their protein-rich natural habitats. These bacteria possess an arsenal of proteolytic machinery together with developed system of transporters and enzymes oriented on peptide catabolism. Although minimal/defined media are prepared with individual amino acids for the sake of simplicity and tractability, there is evidence that short peptides would be a better nitrogen source, for example for *L. lactis* (van Niel et al., 1999).

TCA cycle is not completely functional in lactic acid bacteria. Before the sequencing era (instead of common but rather uncertain inference from genome) activity of TCA enzymes has been directly estimated in cell-free extracts of several lactobacilli (Morishita and Yajima, 2014). The authors have found that none of the tested species possessed active isocitrate dehydrogenase, 2-oxoglutarate dehydrogenase, or succinate dehydrogenase (Fig. 1.5). Activity of other enzymes in the cycle is species specific. Additional common feature of tested lactobacilli was operation of citric acid cycle fragment in the reductive mode. Resulting deficiency in production of 2-oxoglutarate thus causes glutamine auxotrophy, which is very common among LAB. However, glutamine in the medium cannot be substituted for 2-oxoglutarate without growth arrest, presumably due to metabolite import issues (Morishita and Yajima, 2014).

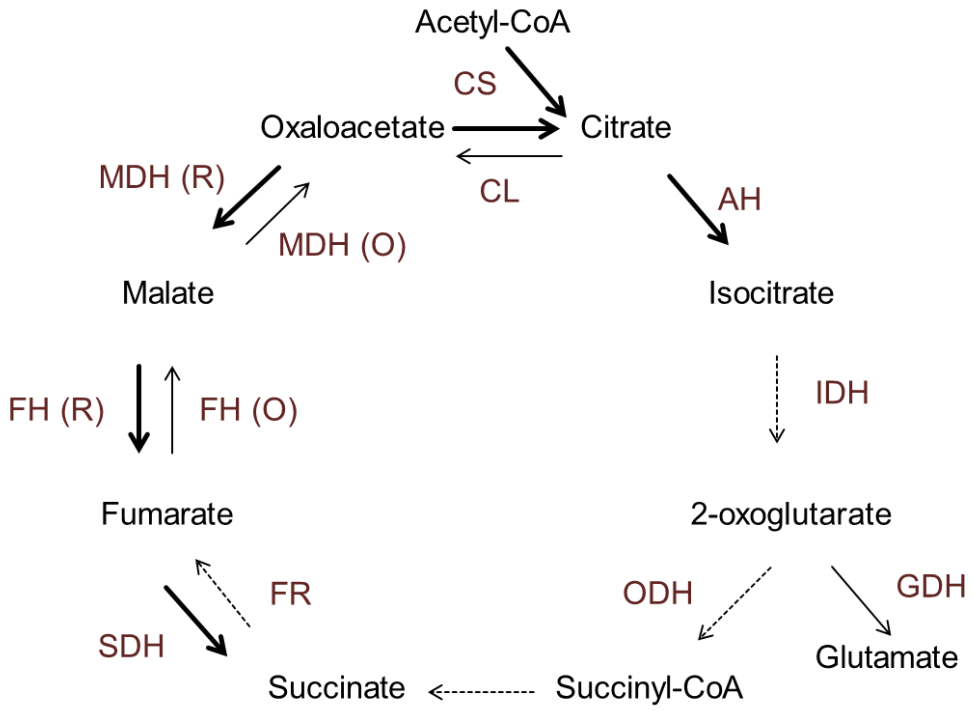

Figure 1.5: Tricarboxylic acid pathway in lactobacilli. Based on (Morishita and Yajima, 2014). Dotted/bold arrows show consensually undetected/present reactions for all tested species of lactobacilli. Enzymes: MDH – malate dehydrogenase, CS – citrate synthase, Cl – citrate lyase, AH – aconitase, IDH – isocitrate dehydrogenase, ODH – 2-oxoglytarate dehydrogenase, GDH – glutamate dehydrogenase, SDH – succinate dehydrogenase, FR – fumarate reductase, FH – fumarase. (O) and (R) denote oxidative and reductive reaction directions respectively.

In addition to incomplete TCA cycle, respiratory chain of lactic acid bacteria is also non-functional due to the absence of the pathway for biosynthesis of heme, which is necessary for function of cytochrome oxidase. In some LAB menaquinone is also missing. However, it has been shown that respiration can be restored in many species with addition of exogenous heme (e.g. in *L. lactis*), or with the combination of heme and menaquinone (e.g. for *L. plantarum*) (Yamamoto et al., 2005). *L. lactis* shows increased survival when grown in co-culture with respiring strain (Rezaiki et al., 2004). Despite the lack of full TCA cycle, NADH for donation of electrons is generated in alternative way via glycolysis (Pedersen et al., 2012).

1.7 Nutrient excretion

Microorganisms can often utilize and secrete a large number of metabolites (Barve and Wagner, 2013; Paczia et al., 2012). Plastic metabolic network is readily adapted and regulated in response to nutrients, e.g. to optimize resource allocation (Gallie et al., 2015; Xavier et al., 2011), but also in response to cues from other microorganisms (Estrela et al., 2015). For instance, certain bacterial species can modulate yeast metabolism, to reduce secretion of toxic ethanol, by deploying chemical signaling (Jarosz et al., 2014). Transcriptional response of Streptococcus species shows metabolic adaptations to other members of community (Liu et al., 2011).

While many secreted metabolites were linked to particular function, e.g. communication, many exometabolome components remain unexplained. It is tempting to link excreted molecules to exogenous reasons, such as stress or response to other organisms. However, some of these overproduction phenomena - unexpected, and, at first glance, wasteful - could be explained by processes and constraints inside the cell itself.

Overflow metabolism, when cells display seemingly inefficient metabolic strategy by releasing, for example, incompletely oxidized products is common in yeast, bacterial and mammalian cells. In exponentially growing *E. coli* culture 2-OG accumulates in the culture medium, assumingly by passive release (Yan et al., 2011). At later growth stages, partial re-uptake of 2-OG is conducted by active transport. Similar switch has also been described in *E. coli* for production and later consumption of acetate (Wolfe, 2005). In yeast, most commonly observed overflow metabolites are aerobically produced ethanol and glycerol, however overflow was also demonstrated for many other small metabolites, such a weak acids, lactate, pyruvate, fumarate, etc. Reasons for overflowing are not completely understood, and combine explanations as faster

growth strategy (Molenaar et al., 2009), limited respiratory capacity, and balancing redox ratio (NADH/NAD) (Vemuri et al., 2007). As for the mechanism, transposers can be involved, but also a "leakage" through lipid bilayer is a common process. The degree of metabolite seeping out of the cell will depend on intracellular pool, and extracellular nutrient availability (Dauner et al., 2001; Romano et al., 2014; Yan et al., 2011).

For a long time microbial exo-metabolome stayed out of focus, or was limited to relatively highly concentrated compounds. In recent years mass-spectrometry based methods allowed uncovering the unexpected diversity in exometabolome species (Paczia et al., 2012; Romano et al., 2014; Traxler et al., 2013). Besides commonly known and produced in relatively high amounts fermentation byproducts, such as ethanol and CO_2, yeast can produce a huge variety of metabolites: esters, short-chain fatty acids, organic acids, phenols, alcohols, sulfurous compounds, etc. Yeast cells were shown to leak phosphate and phosphate-containing metabolites (Robertson and Button, 1979), amino acids (Paczia et al., 2012).

Some of the metabolites are released as a direct consequence of primary metabolism. For example, fusel esters and fusel alcohols are secreted as unmetabolizable end products of amino acid degradation (Hazelwood et al., 2008); acetolactate and acetohydroxybutyrate are amino acid biosynthesis intermediates that leak out of the cell during fermentation (Bokulich and Bamforth, 2013). Others have been linked to potential evolutionary function, for instance, yeast releasing volatile acetate esters attract fruit flies and thus disperse better (Christiaens et al., 2014). Another example involves quorum sensing, where yeast excrete tryptophol and phenylethanol to signal transition to filamentous growth (Sprague and Winans, 2006). Altogether, however, physiological reasons behind production of many compounds are still unexplored.

When amino acids are used as a nitrogen source, most commonly they first undergo transamination, donating nitrogen to the 2-oxoglutarate or other keto-acid. In this way nitrogen is being centralized to the yeast metabolism in the form of glutamine, and remaining keto-acid either directed to the carbon processing pathways, excreted, or modified and then excreted. Branched-chain amino acids (valine, leucine, isoleucine), as well as methionine and phenylalanine, are usually catabolized through Ehrlich pathway. After transamination they undergo decarboxylation, and then, depending on redox state of the cell, are being converted into either fusel alcohol or acid and excreted (Hazelwood et al., 2008).

Expulsion of amino acids in yeast, contrary to amino acid uptake, was not properly researched due to unclear physiological role. Scarce data suggests that excretion of amino acids can occur with imbalanced central metabolism, for example in case of certain auxotrophies or when some nutrients are limiting (Velasco et al., 2004). Importance of amino acid release is indicated by presence of specific yeast proteins responsible for amino acid excretion, likely involving vesicular transport (Velasco et al., 2004).

1.8 Aims of the study

The goal of this work was to investigate principles of metabolic interactions in microbial communities with focus on interaction between yeast and lactic acid bacteria. Mechanisms and consequences of metabolic cross-feeding in this synthetic system were scrutinized by using an interdisciplinary blend of methods. Specifically, aims were to:

- Detect metabolic interaction between microorganisms;
- Identify metabolites exchanged between yeast and LAB;
- Explore the predictive power of simulations of multi-species metabolism;
- Verify stability of interacting microbial community;
- Examine prerequisites for metabolite exchange in yeast-LAB community;
- Reveal genetic determinants of metabolite extrusion by S. cerevisiae;
- Determine environmental perturbations and intrinsic regulatory factors that alter the interaction strength.

2 METHODS

2.1 Strains, media, and growth conditions

Strains used in the experiments*:* *Saccharomyces cerevisiae* S90 (*MATα, GAL2,* S288c background, S1 parent strain), *Lactobacillus plantarum* WCFS1, *Lactococcus lactis* IL1403. Yeast were routinely cultured in YPAD medium, GM17 and MRS were used to cultivate *L. lactis* and *L. plantarum* respectively. All cultures were grown statically at 30˚C. Chemically defined medium CDM47 was developed in this study for conditioned medium and co-culture experiments (Table 2.1).

For co-culture stability testing all combinations of three species were inoculated in starting amount of 0.01 (OD600) in 2 ml CDM47 cultures. Daily throughout 2 weeks 20 µl of stationary phase culture were transferred into fresh medium.

Same conditions were used for adaptive evolution experiment that included yeast-LAB pairwise cultures and monocultures with and without rapamycin (20 nM) and lasted for approximately 2 months.

2.2 Quantification of species in co-cultures

2.2.1 CFU count.

CFU (colony forming units) were counted after plating 3 different culture dilutions on selective media, each in 3 technical and 3 biological replicates. MRS or GM17 agar plates supplemented with 10 µg/ml cycloheximide were used to estimate quantities of bacteria, and SD minimal agar to count yeast CFUs.

2.2.2 Quantitative PCR.

Alternatively, in order to quantify species in microbial co-cultures and follow their temporal dynamics we adopted a real-time quantitative PCR assay. Species specific primers (yeast primers only within experimental co-cultures) were designed (Table 2.2) and verified for selectivity with BLAST and cross amplifications from the other species used in our co-culture experiments.

Table 2.1: Chemically Defined Medium (CDM47) for co-cultivation of yeast and LAB.

Component	Amount/L
Glucose	15 g
L-Histidine	0.17 g
L-Isoleucine	0.24 g
L-Leucine	1 g
L-Methionine	0.125 g
L-Valine	0.7 g
L-Arginine	0.72 g
Inositol	2 mg
KH_2PO_4	3.1 g
K_2HPO_4	6.48 g
Biotin	6 mg
Pantothenate hemicalciumsalt	1.2 mg
Nicotinic acid	0.9 mg
Pyridoxine HCl	4.8 mg
$MgCl_2$	0.386 g
$FeSO_4 \cdot 7H_2O$	4 mg
$ZnSO_4 \cdot 7H_2O$	5 mg
Folic acid	0.56 mg
p-Aminobenzoic acid	0.056 mg
Potassium acetate	0.9 g
Lipoic acid	1 mg
Tween 80	0.5 g
Adenine	11 mg
Guanine	5.6 mg
Uracil	23 mg
Xanthine	3.8 mg
MOPS	15 g
Tricine	1.5 g
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.19 mg
$MnSO_4 \cdot H_2O$	0.288 mg
$CaCl_2$	30.2 mg
$CoCl_2 \cdot 6H_2O$	0.19 mg
$CuSO_4$	0.12 mg
H_3BO_3	0.75 mg
KI	0.11 mg
K_2SO_4	23 mg
EDTA	7.34 mg
Nitrilotriacetic acid	7.34 mg
L-Glutathione reduced	15 mg
Ammonium citrate dibasic	1.7 g
NaCl	3 g
L-Tyrosine	0.3 g
Thiamine HCl	0.56 mg
Riboflavin	0.9 mg
Ascorbic acid	0.5 g
Pyridoxamine·2HCl	5 mg
$FeCl_3$	3 mg

Templates for qPCR analysis were prepared as described below. In brief, 1 ml of (co-)culture was pelleted and frozen until analysis. Frozen pellets were re-suspended in sterile PBS, 400 µl of mixture transferred into polypropylene screw cap tube with O-ring, previously filled with 0.5 ml of acid washed glass beads (0.2-0.3mm), kept on ice. Homogenization was done in FastPrep-24 bead beater (4 m/s setting, for 2 min with intermittent cooling tubes on ice). Samples were briefly vortexed, diluted 1000x with PEG reagent (Chomczynski and Rymaszewski, 2004), vortexed again and incubated for 10 min at 95 ˚C. Obtained lysate was used directly for qPCR reaction.

qPCR reaction mix was prepared using 1 µl of sample lysate and 19 µl of SYBR Green RT-PCR master mix (Life Technologies). Reaction conditions set to 40 cycles, 60˚C annealing temperature. Primers were synthesized and purified (desalting) by Sigma-Aldrich. Standard curve was made with serial dilutions of corresponding monocultures and generated on every plate. Data analysis was done using StepOne software (Applied Biosystems).

Table 2.2: Species specific primers used for microorganisms quantification in co-cultures

Primer sequence		**Target species**	**Target gene**	**Amplicon size**
Forward	**Reverse**			
AGTGGCCTACCATGGTTTCA	CTTGGATGTGGTAGCCGTTT	*Saccharomyces cerevisiae*	18S rRNA	86
GCAGGCGTAACTAAAGCAGC	AAGCGTTTCAGCAGGGGTAA	*Lactobacillus plantarum*	HAD (HAD superfamily hydrolase)	72
GGCGCTCTAAATCGAGTCGA	GCAAAGCCTGACTTGCTGTC	*Lactococcus lactis*	dnaA	82

Here we would like to note that finding a reproducible DNA extraction method that would give yields proportional to amount of microorganisms, irrespective of experimental conditions or target organism, presented an unexpected challenge. Type of medium used and cell culture age,

factors often not considered to be critical in microbial quantification experiments, turned out to be important. For example, most of the tested DNA preparation methods, including commercial DNA extraction kits, failed to reproduce a growth curve. Ultimately, supplementing chemical extraction step with mechanical cell disruption (described above) became a method of choice. This technical issue may in fact have serious implications for metagenomic samples preparation, especially when quantitative data is concerned. Many metagenomic DNA extraction methods rely on chemical cell lysis, which, depending on experimental conditions, can skew final counts by, for instance, under-representing lactobacillus.

2.3 Yeast genome-wide deletion library screening

Prototrophic haploid yeast collection of genome-wide single gene knockout library (Mulleder et al., 2012) of BY4741 background was used for this experiment (kindly provided by Dr. Markus Ralser, Cambridge University, UK). Library glycerol stock was thawed, pinned with a Singer Rotor robot in 96-pin format on YPAD agar plates and grown overnight (Fig. 2.1A). All cultivation was done statically at 30°C unless other conditioned are specified. From agar yeast were inoculated into liquid CDM47 medium in 96-well microtiter plates for overnight pre-culture. Next, *L. plantarum* overnight culture, yeast pre-culture and CDM47 medium containing 200 ug/ml of X-gal were mixed in a flat bottom 96-well plate with liquid handling robot (Biomek FXP, Beckman Coulter). Pipetting protocol was adjusted to yield plates with 120 µl of medium containing inocula of *L. plantarum* with final OD600 = 0.01, and knockout yeast strain with OD600 = 0.1 (on average across library). Resulting co-culture plates were used to take OD measurements every 2 hours during first 18 hours (before X-gal colored derivative starts to emerge). OD600 reading at this phase can be used to estimate yeast growth (we tested that mixing bacteria into yeast culture does not significantly influence OD readings). After 18 hours, every 6 hours plate cultures were re-suspended on orbital shaker for 5 min and imaged with a flatbed book scanner (CanoScan 9000F) until color had saturated. In this set up color development was used as a proxy for *L. plantarum* growth, since it has active beta-galactosidase genes (lacLM) and used yeast strain is galactosidase negative. Although co-culture of two species was analyzed, readouts of their growth were separated in time: initial phase before color development allowed reading OD roughly corresponding to yeast culture, and later stages of X-gal conversion approximated bacterial growth (example outcome Fig.2.2). Screening was replicated in a three independent experiments.

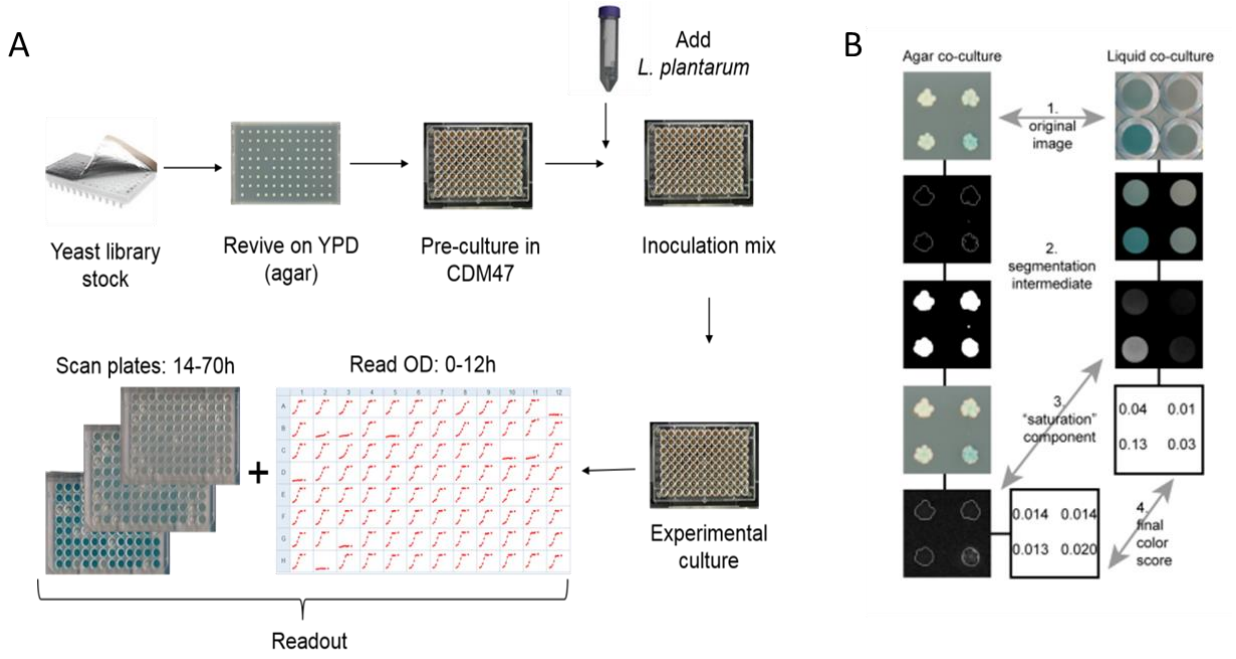

Figure 2.1: Yeast knockout library screening for interactions with *L. plantarum*. A. Screening co-culture set-up and workflow. B. Intermediate steps of image analysis for colonies recognition and scoring of color development.

2.4 Image analysis

MATLAB script was created to process scanned images. For analyzing images of plates with liquid cultures, well areas were first cropped accordingly to the 96-well plate geometry. For analysis of mixed culture colonies, separating colonies from the background was done by multi-step processing (Fig. 2.1B), using functions from MATLAB Image Processing Toolbox. Cultures were then scored proportional to "Saturation" component of image in HSV (hue-saturation-value) color space, averaged across culture image area. Score dynamics for individual wells/colonies across measured time-points were used to rank the ability of each yeast mutant strain to support growth of *L. plantarum*.

2.5 Flow cytometric assessment of yeast cell damage

LIVE/DEAD® FungaLight™ Yeast Viability Kit (Life Technologies) was used in compliance with manufacturer instructions to access fraction of dead yeast cells and/or cells with compromised membranes. Five strains of S90 background with highly varying levels of interaction with LAB were tested (deletions of *GLN3*, *GTR1*, *DAL81*, *URE2* and the wild type).

Exponentially growing cells were washed and carefully re-suspended in Tris buffered saline, pH 7 to produce a suspension of optical density equal to 0.4. Cells were stained with propidium iodide (PI) and SYTO® 9 fluorescent dyes and counted with LSR-Fortessa analyzer (Beckton Dickinson). Fluorescent events were recorded at excitation/emission 480/500 nm for SYTO9 and 490/635 nm for PI (Fig. 2.3). Gating was adjusted to exclude debris and cell duplicates. Each sample was measured until the minimum of 2000 of dead/damaged cells was reached.

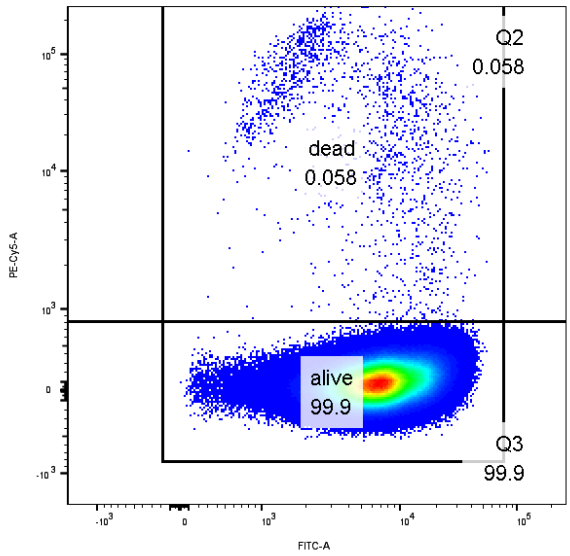

Figure 2.3: Separation of events of live cells (SYTO9 recorded in FITC channel) from dead/membrane-compromised (PI recorded in Cy5 channel).

2.6 Yeast strain construction

Five strains of existing gene knockout strains (deletions *GLN3*, *PEP3*, *DAL81*, *URE2* and *GTR1*) were taken from genome-wide deletion collection (Mulleder et al., 2012) for target DNA fragment amplification. Purified genomic DNA was used as a target in PCR reaction with previously described A-D primers (Winzeler, 1999). Resulting DNA amplicons contained kanMX4 with 200-400 bp of flanking genomic regions. These PCR products were used to transform WT *S. cerevisiae* S90 as described below.

2.7 Yeast transformation

Yeast transformation was done as described before (Gietz and Schiestl, 2007) with some modifications. Yeast mid-log culture (OD600=0.7, 50 ml) was centrifuged, washed, and re-

suspended in 1 ml of sterile water. Then 100 µl of cell suspension was topped with transformation mix (240 µl PEG 3500, 50% w/v; 36 µl LiAc, 1.0 M; 50 µl boiled single stranded carrier DNA, 2mg/ml; 34 µL of PCR amplification product described above) and resuspended. After incubation for 40 min on 42°C water bath, cells were re-suspended in YPAD and incubated for 3-4 hours to allow for expression from integrated antibiotic marker. Clones were selected on YPAD medium with G418 antibiotic (300 ug/ml). Success of homologous recombination was verified by colony PCR using A-D, A-KanB, and C-KanD primer pairs as described in (Winzeler, 1999).

2.8 Yeast exometabolome analysis[2]

In order to identify secreted by *S. cerevisiae* and uptaken by LAB, samples of supernatant were taken continuously during yeast growth in monoculture, and later during LAB cultivation in yeast conditioned medium (Fig.2.4). Conceptual set-up of this experiment relied on the fact that concentration of metabolites secreted by yeast should increase with yeast growth, and later, when yeast conditioned medium is used for LAB cultivation, concentration of important for bacteria nutrients should decrease. Thus, when analyzing dynamic concentration profiles of individual ions, we can identify metabolites of interest by bell-shaped profiles.

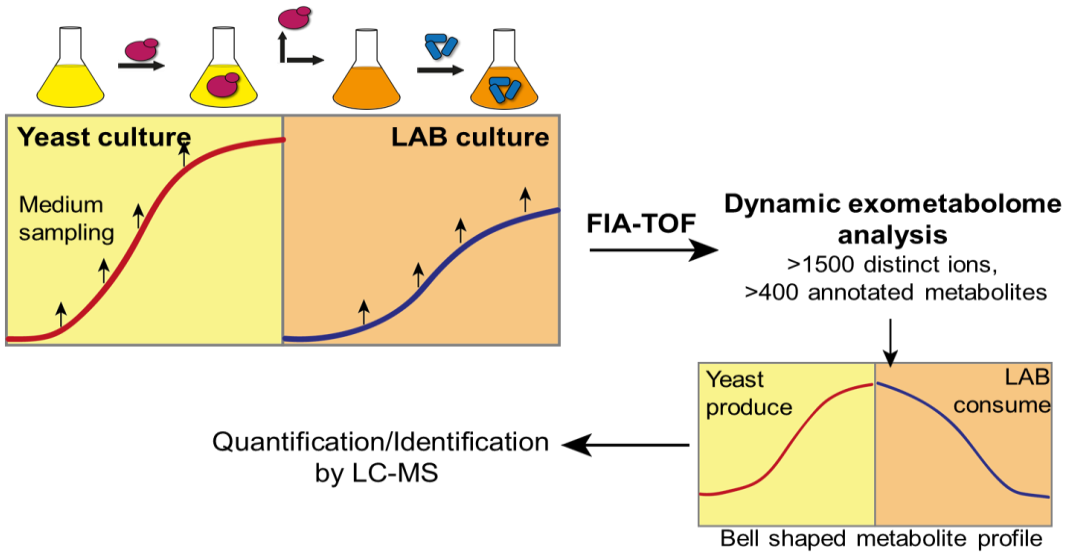

Figure 2.4: Yeast-LAB exometabolome analysis workflow.

[2] In collaboration with Uwe Sauer lab, ETH Zurich.

Supernatant samples were filtered through 0.2 µm syringe filters, then additionally through 3 kDa cut-off centrifugal filters, and stored at -80°C until analysis. For assessing dynamic changes in metabolite concentrations we established collaboration with Uwe Sauer and Daniel C. Sevin, ETH Zurich. Non-targeted flow injection time-of-flight mass spectrometry (FIA-TOFMS) was implemented by our collaborators as described in (Fuhrer et al., 2011). Platform used: Agilent 6550 ionFunnel QqTOF mass spectrometer, coupled to a Gerstel MPS2 autosampler and a Hitachi HPLC pump. Samples were diluted 10x and measured in technical duplicates. Flow injection analysis allowed omitting chromatographic separation step. Obtained m/z values were annotated using KEGG reference database (3225 unique compounds). Ion annotation settings allowed mass deviation 0.005 Da, -H(+) and +F(-) ions, neutral gains/losses: .H/K, .H/Na, .NaCl.

2.9 Amino acid quantification[3]

Amino acids in yeast conditioned medium were quantified in collaboration with Markus Ralser group using liquid chromatography (Agilent 1290 Infinity) and tandem mass spectrometry (Agilent 6460). Hydrophilic interaction chromatography was done using ACUITY UPLC BEH amide column with gradient elution, using mobile phases A (50:50 water:acetonitrile) and B (90:5:5 acetonitrile:methano:water), both containing 10 mM ammonium formate and 0.176% formic acid. Initial conditions were 75% solvent B at flow rate 0.7 ml/min, next over 2.55 min gradient descends until 5% eluent B, finishing with 5 sec isocratic 5% B, returning to initial conditions and equilibrating until 3.25 min before next injection.

Triple quadrupole mass spectrometry was set in selected reaction monitoring (SRM) mode, with settings as follows: 7 V cell acceleration voltage, nebulizer pressure 50 psi, negative capillary voltage 3000 v, nozzle voltage 500 V, gas flow 8 L/min 300 C. Quantification was done in relation to external standard dilution series, individual amino acid identification was done by retention time and fragmentation pattern.

2.10 Conditioned medium assay

Yeast culture has been grown in CDM47 medium until exponential phase (OD600~1), centrifuged, supernatant was filtered through 0.2 µm filter and used for cultivating of lactic acid bacteria. Resulting filtrate was used to culture LAB as is, or supplemented with CDM47 (1:1

[3] Data were obtained during collaboration visit to Markus Ralser lab, University of Cambridge.

mix) or glucose stock solution depending on application. For comparisons of conditioned media prepared with different strains/mutants or in different media etc., cultures were harvested at similar OD600 (~1).

2.11 Data analysis

All data handling, was done using R software. Statistics to estimate correlation between untargeted metabolomics ion data and growth of microorganisms on few data points was calculated as F-score of Pearson's correlation, or by overall distance correlation (R package 'energy'). Statistical significance analysis was done by t-test (paired for complete cases and unpaired in case of missing values) with multiple-testing correction. GO enrichment analysis was performed using "GOstats" R package and visualized by using GOrilla webtool (Eden et al., 2009).

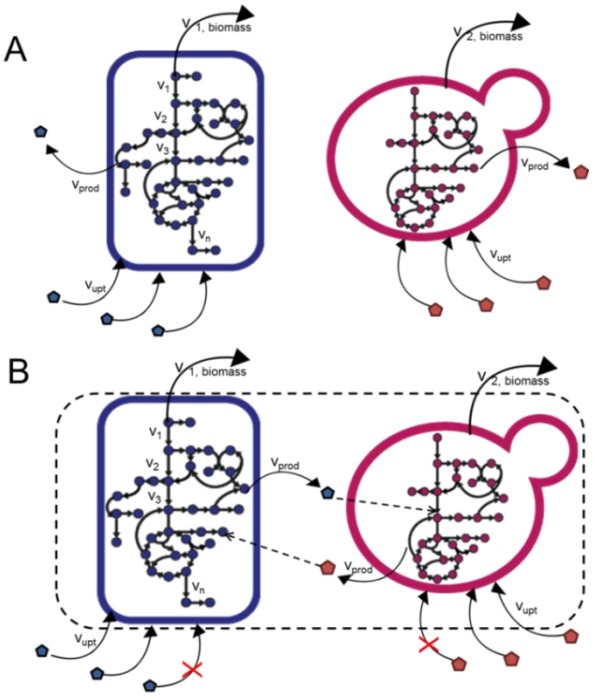

Figure 2.5: Schematic representation of creating a multi-species model for enumerating metabolite cross-feedings. **A.** Models of separate species have each a separate metabolic space. **B.** Models are united by shared level of metabolic species, allowing exchange of products. This joining reduces the total number of metabolites required for growth of all species in the community.

2.12 Simulations of metabolic exchanges in microbial communities

Genome-scale metabolic models for individual species were obtained from ModelSEED database (automatic model reconstruction) or from published (Oliveira et al., 2005; Teusink et al., 2005; Zomorrodi et al., 2014) sources (manually curated). Additional curation steps to remove spurious inter-models exchanges (replacing dipeptide transporters with single amino acids, limiting spermidine-based growth etc.) were performed when necessary. Obtained models were joined into one in a way that allowed them to freely exchange each other's metabolites that can cross the cell membrane/wall (Fig. 2.5). As a result, cross feeding community gets a chance to survive on fewer nutrients by supplementing each other's biosynthetic capabilities (Fig. 2.5 A vs B). Flux simulations were done by using in-house software (by S. Andrejev and A. Zelezniak) and SMETANA (Zelezniak et al., 2015), solving linear programming problem using IBM ILOG CPLEX solver.

3 YEAST EXOMETABOLOME CREATES A STABLE NICHE FOR LAB

3.1 *S. cerevisiae* sustains growth of lactic acid bacteria in stable mixed cultures

In order to reveal potential exchange of nutrients between yeast and LAB one should consider carefully the chemical composition of the experimental medium. For growth-based detection of interspecies metabolic exchanges balancing medium composition is key - it should lack components that could be supplied by cross-feeding between community members, and at the same time be nutritionally rich enough to provide missing nutrients and support growth of all species (Fig. 3.1A). Community minimal medium design can be a successful strategy for discovering positive metabolic interactions between microorganisms. However, extensive search of binary interactions with culture methods (even for a small three species community addressed here) would involve numerous combinations, and thus remains infeasible.

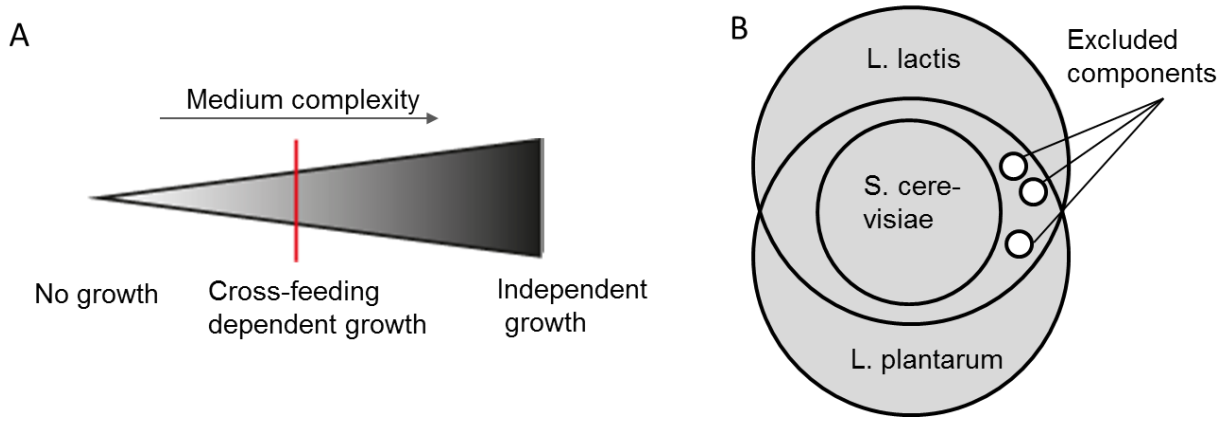

Figure 3.1: Medium design for revealing inter-species metabolic interactions. **A.** Balance of medium components dictates the best range to probe for metabolic dependencies. **B.** Medium design for optimal detection of cross-feeding from yeast to LAB. Areas represent compounds needed by each species. Excluding metabolite from one or another zone will define the direction of cross-feeding that can be observed.

L. plantarum and *L. lactis* are fastidious bacteria with extensive nutritional requirements that include and surpass those of *S. cerevisiae*. We composed community medium by joining together recipes of previously described chemically defined media (Verduyn et al., 1992; Wegkamp et al.,

2010; Zhang et al., 2009) created for independent growth of all three organisms. Then several variations of this universal medium were prepared by excluding groups of selected nutrients (Fig. 3.1B). Each resulting medium was used to compare growth of microorganisms in co-cultures and monocultures, observing whether any species can benefit from presence of others. In some media we observed interspecies dependencies, and finally selected medium CDM47 (composition shown in Table A1) for further experiments.

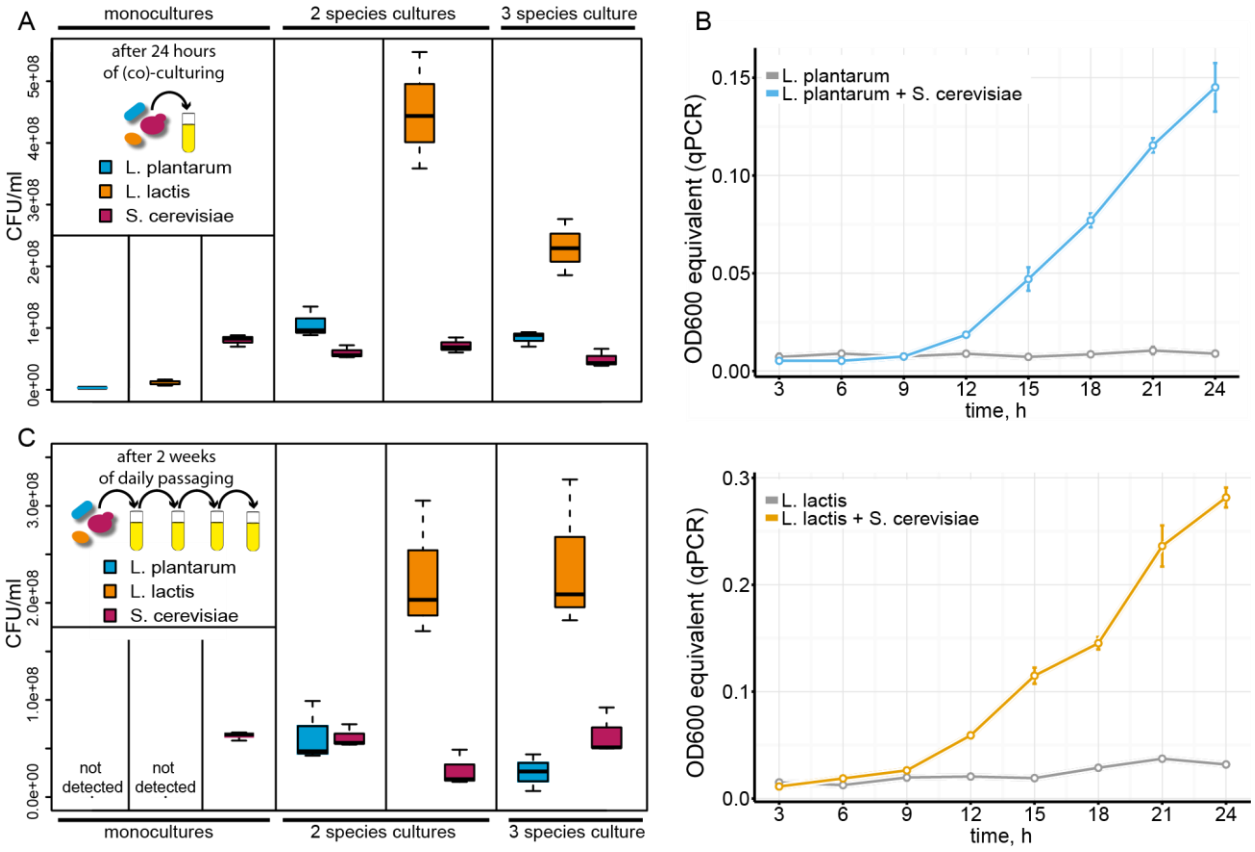

Figure 3.2: *S. cerevisiae* sustains growth of lactic acid bacteria. A and C. Co-culture of *S. cerevisiae*, *L. lactis* and *L. plantarum* in all combinations for 24 hours and 15 days with daily passaging. B. Dynamics of LAB growth in co-culture with *S. cerevisiae* (coloured line) and alone (grey line).

After of co-culturing of three species in all combinations for 24 hours, absolute amounts of living cells were quantified by CFU count. While yeast did not show any significant variation between co-cultures and monocultures, LAB could grow exclusively in presence of *S. cerevisiae* (Fig. 3.2A). Additionally, dynamics of LAB growth assessed by qPCR in monocultures and co-

cultures with yeast is shown on Fig. 3.2B – it clearly demonstrates positive effect of yeast culture on *L. lactis* and *L. plantarum*.

To check the stability of this positive interaction, co-culturing was extended to 15 days with daily passaging of microbial community into the fresh medium. At the end of the experiment in monocultures of lactic acid bacteria no living cells remained, but together with yeast LAB survived, forming stable communities, with especially high response of *L. lactis* (Fig. 3.2C). Proportions of species in mixed cultures after two weeks of propagation were similar to those established after 24 hours. In analogous two month long passaging experiment with binary combinations of yeast and LAB co-cultures remained stable. These findings reveal important property of designed synthetic community – stability over time, which indicates the possibility for this interaction to make a long lasting ecological and evolutionary impact on LAB survival. Robustness of microbial community is an indication of resistant interaction and, from biotechnology point of view, important pre-requisite for the development of mixed-culture applications.

3.2 Growth promoting effect of yeast on LAB is mediated by small metabolites

After capturing the dependency of lactic acid bacteria on co-culturing with *S. cerevisiae*, next step was to confirm or refute the hypothesis of yeast excreting metabolites that are used by bacteria as nutrients. To verify the role of extracellular metabolites, effect of yeast conditioned medium (cell-free filtrate of yeast culture) was tested on LAB (Methods). Conditioned medium was sufficient to reproduce co-culture effect and supported growth of *L. lactis* and *L. plantarum* (Fig. 3.3A). Results of this simple experiment can be summed up as follows:

(i) diffusible factor(s) are responsible for sustaining LAB growth,

(ii) direct physical contact between yeast and LAB is not required for interaction, and that

(iii) release of growth promoting factor(s) is a property of yeast monoculture and does not need to be induced by bacteria.

These findings allow assuming that indeed *S. cerevisiae* produces certain metabolite(s) that supplement(s) auxotrophy of lactic acid bacteria.

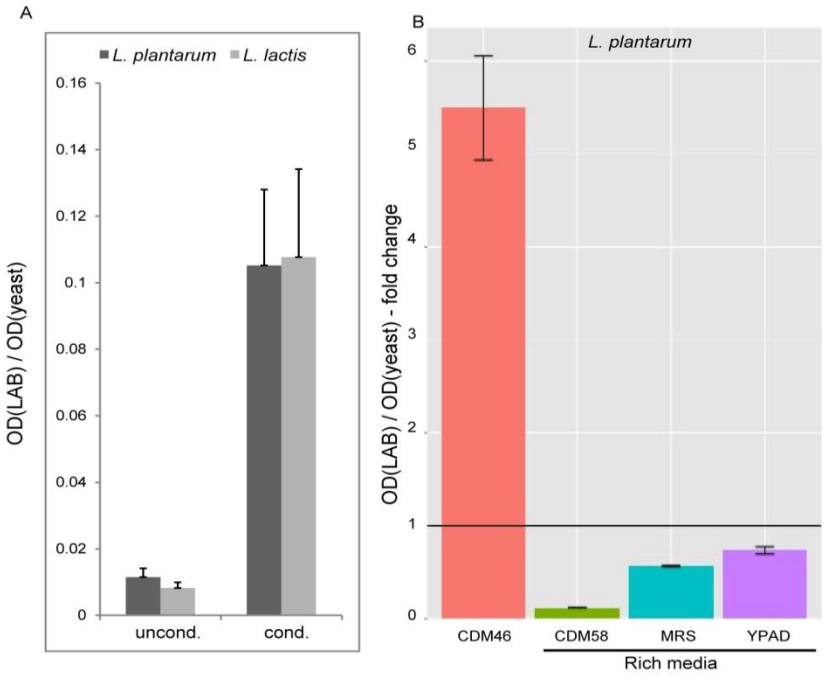

Figure 3.3: Effect of yeast conditioned medium on LAB. A. Effect of yeast conditioned (CDM47) medium on LAB, normalized by yeast cell density. B. Yeast conditioning of nutritionally challenging media improves growth of LAB, but in rich environments shows opposite effect, showing competition with bacteria for nutrients. CDM46 composition is same as that of CDM47 but w/o asparagine, CDM58 has 11 more amino acids then CDM47, MRS and YPAD are undefined rich media.

Composition of the medium used for conditioning experiment, again, matters a lot. Defined medium CDM47, unsuitable for mono-cultivation of *L. plantarum*, after conditioning with yeast can sustain growth of lactobacillus. At the same time conditioning of the rich medium, which alone can support growth of *L. plantarum*, reduces final yield of bacteria (Fig 3.3B). These differences between growth stimulating effects in rich and nutritionally limited media is quite intuitive. Conditioned medium is both supplemented with yeast fermentation products and depleted of primary nutrients. Thus, conditioning of incomplete minimal medium, even though reduces the total amount of nutrients, provides missing essential components and removes the narrowest nutritional bottleneck. On the contrary, conditioning of rich medium, which already contains all necessary nutrients for bacteria in excess, can only reduce the final yield by lowering the concentrations of carbon source, amino acids, vitamins and other nutrients.

It can be suggested, that metabolic exchanges are common in nature, but remain hidden when probed in plentiful environments. Medium-driven differences of microbial neighborship can serve as an illustration of balance between competition and cooperativity. These processes are

not mutually exclusive for two given species (Freilich et al., 2011), and are determined by the environmental properties, such as type and amount of nutrients.

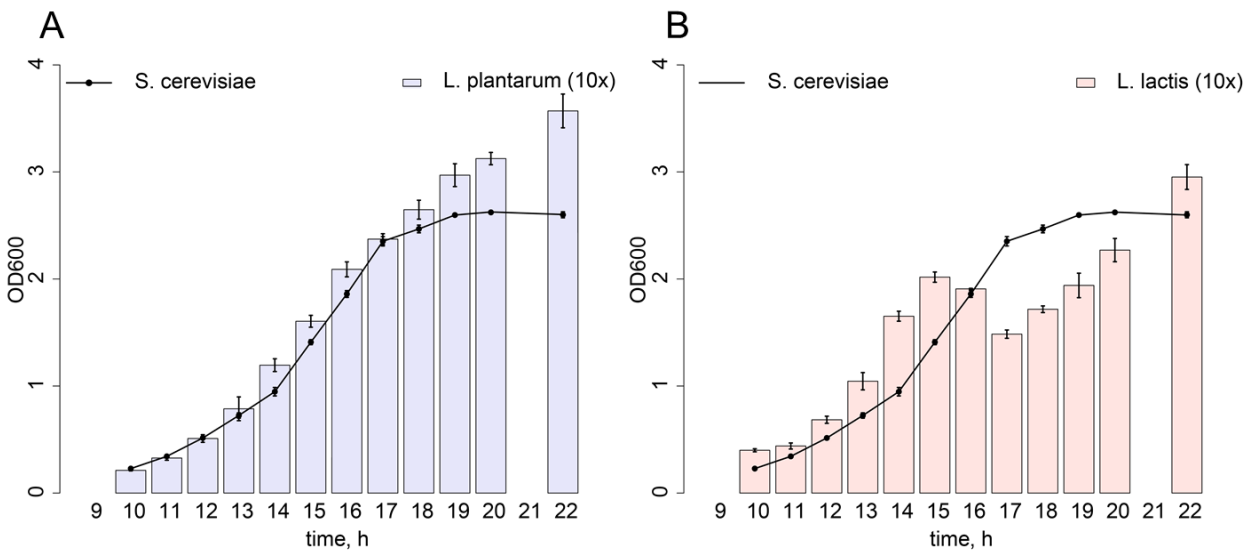

Figure 3.4: Effect of yeast growth phase on its interaction with LAB. **A.** Effect of conditioned medium on *L. plantarum*. **B.** Effect of conditioned medium on *L. lactis*.

To look at the dynamic changes of interaction in more detail, conditioned medium from different growth phases of yeast culture was characterized for their LAB growth-promoting effects (Fig. 3.4). Results demonstrated variation in effect on two lactic acid bacteria: *L. plantarum* grows proportionally to yeast culture density, but *L. lactis* grows less in conditioned medium from late-exponential/early-stationary phase. This hints at differences in LAB nutritional requirements – possibly two bacterial species live off different sets of metabolites produced by *S. cerevisiae*. Additionally, this experiment suggests a complex, dynamic and multicomponent nature of yeast exometabolome.

3.3 Yeast exometabolome analysis

Next step in characterizing bioactive molecules produced by *S. cerevisiae* was to identify their general chemical properties. Compounds responsible for LAB growth were resistant to autoclaving and protease treatment, remained in the flow through fraction following anione exchange chromatography and reverse phase solid phase extraction, passed through 3 kDa filter, could be precipitated with acetone, and could be partially extracted with acetonitrile but not with

low polarity organic solvents. These evidences pointed out that small hydrophilic metabolites were responsible for growth stimulation of the lactic acid bacteria.

3.3.1 Insights from untargeted metabolomics

To specifically identify metabolites secreted by *S. cerevisiae* and uptaken by LAB, microbial exometabolome dynamics was analyzed by using untargeted mass spectrometry (section 2.8). Samples of supernatant were taken during conditioning of the medium with yeast monoculture, and later during LAB cultivation in the yeast conditioned medium, revealing dynamic profiles for hundreds of ions in yeast-LAB exometabolome (data for *L. plantarum* is shown on Fig. 3.5). Metabolites that mediate yeast-LAB interaction accumulate with growth of *S. cerevisiae* and disappear from the medium with accumulation of bacterial biomass, creating bell shaped profiles (red cluster on Fig. 3.5).

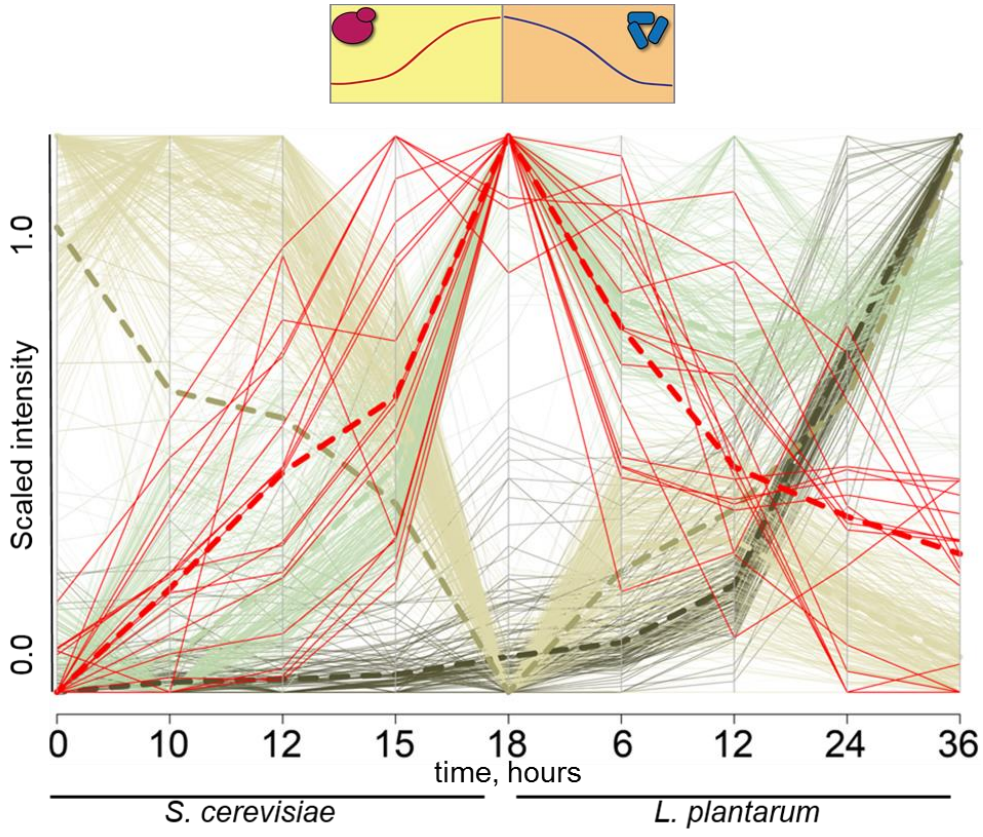

Figure 3.5: Exometabolome dynamics of *S. cerevisiae* and *L. plantarum* by untargeted metabolomics. Dotted lines show centroids for clusters of metabolites with similar dynamics. Group of candidate ions to be cross-fed from yeast to bacteria are highlighted in red.

This analysis revealed surprisingly large amount of metabolites flowing between microorganisms in the community. Top ranked exchange candidates (filtered by minimum 3x fold change and correlation with growth of microorganisms) match predominantly to the m/z of amino acids (labels in Fig. 3.6).

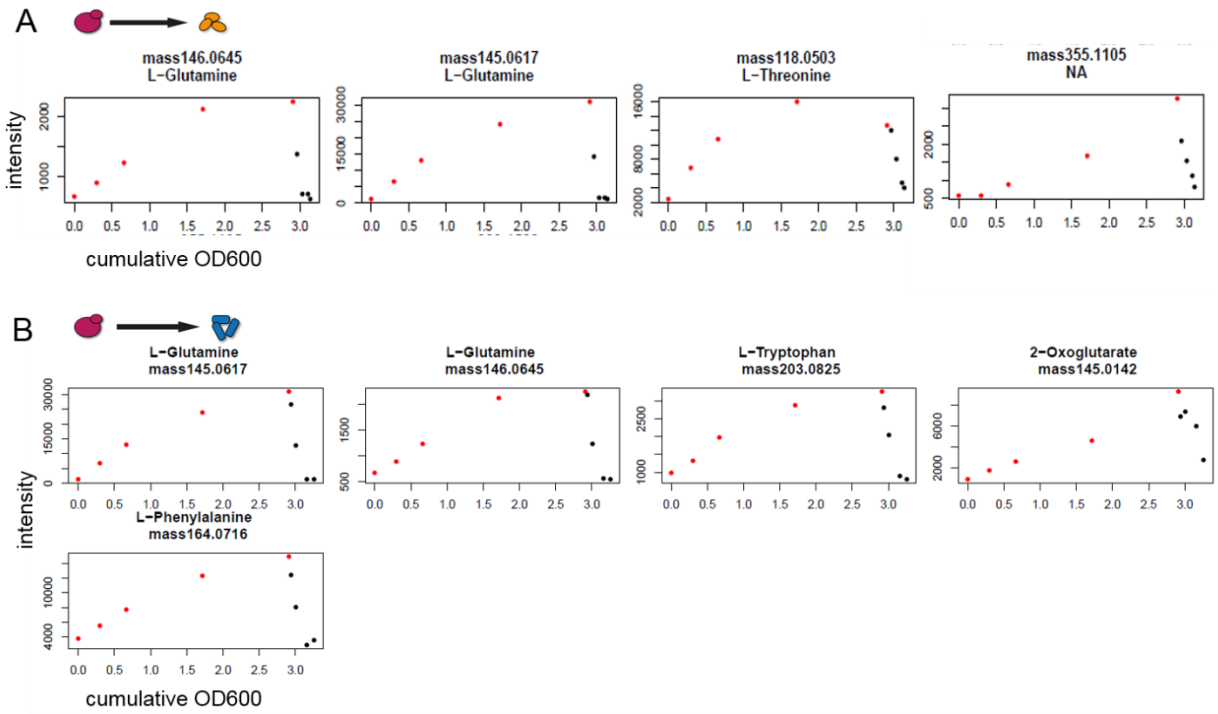

Figure 3.6: Ions produced by *S. cerevisiae* and consumed by lactic acid bacteria (at least 3x fold change). Ion intensity is plotted against cumulative OD, where red points show measurements during yeast grow and black points correspond to values during bacterial growth in yeast conditioned medium. **A.** Yeast – *L. lactis*. **B.** Yeast – *L. plantarum*.

Some of the ions have been mapped to known compounds, but identity of the others, including those well correlating with the growth of both microorganisms, could not be specified beyond particular m/z ratio. Described exometabolome diversity most likely shows a mere "tip of an iceberg" that can be captured by state of the art metabolomics, and further exploration is needed to expose the multitude of inter-species connections.

Detecting primary metabolites in culture medium has been for a long time considered an artefact of sample preparation or cell lysis effect. It took a sensitive instrument and extensive set of controls to prove otherwise and quantify curiously rich body of excreted compounds for yeast and some bacterial species (Paczia et al., 2012). Secretome of microorganisms is an emerging

topic that will keep gaining momentum as scientific community shifts from traditional monoculture to interactive multi-species experiments.

3.3.2 Quantification of secretome components with targeted metabolomics

To confirm identity and quantify amount of secreted amino acids yeast conditioned medium was analyzed for amino acids using a targeted LC-MS/MS setup. Among the quantified metabolites, most abundant amino acids were glutamine, threonine/homoserine (undistinguishable by the used MS method) and alanine, concentrated at 40-70 µM (Fig 3.7A). Targeted metabolomics results thus agree well with the untargeted experiment, as well as with the fact that *L. lactis* and *L. plantarum* are auxotrophic for some of these amino acids, originally missing from the medium.

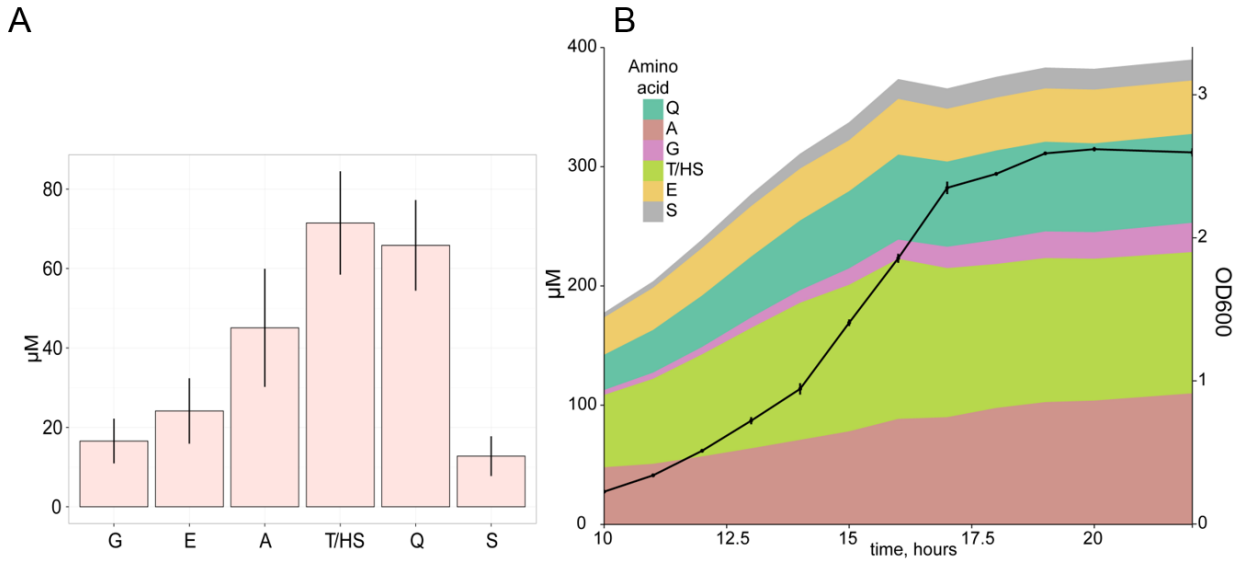

Figure 3.7: A. Amino acid concentration in yeast conditioned medium. Medium collected at log phase (OD600~1), measurements corrected for medium background. **B.** Dynamics of secreted amino acid concentrations in *S. cerevisiae* culture, black line showing yeast cell density.

Extracellular amino acid concentrations in yeast culture increase proportionally to the cell density and stabilize during stationary phase (Fig 3.7B). This result is important from two points of view. First, it serves as an additional argument against the possibility of nutrients release due to cell death and lysis. If the mentioned assumption were true, we should have observed an increase, rather than stabilization, in amino acid concentration during the stationary phase, when

cells run out of nutrients and thus are more likely to die. Second, overlaying this plot with the dependency of lactic acid bacteria response on yeast growth phase (Fig 3.4), it becomes evident that metabolic cross feeding between yeast and bacteria is not limited to measured amino acids, at least for *L. lactis*, whose growth yield has a prominent deep on the background of steadily growing concentration of amino acids.

In addition to amino acids, other components of yeast exometabolome were quantified, from expected fermentation by-products, such as succinate, to polyamines (Fig. 3.8). Known fermentation products of yeast metabolism were tested for ability to boost lactobacilli growth, but no positive effect was observed with succinic acid, citrate, isocitrate, 2-oxoglutarate or pyruvate.

A

Quantified compounds used for supplementation experiment
Pyruvate
Putrescine
Threonine
Alanine
2-oxoglutarate
Glycine
Glutamine
Glutamate
Serine
Aspartate

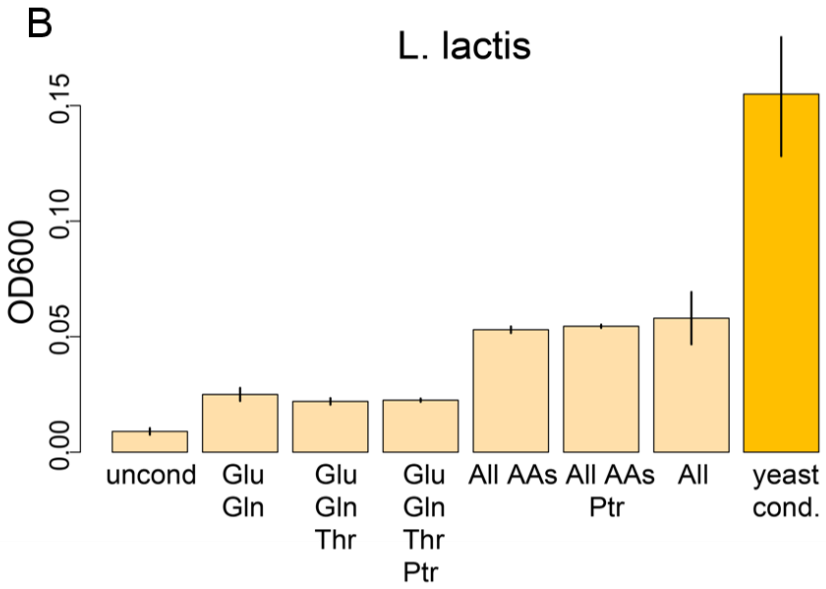

Figure 3.8: Conditioned medium effect can be partially recreated with metabolite mix. **A.** Compounds used for medium supplementation. **B.** Effect of different metabolite combinations on growth of *L. lactis* in comparison to effect of yeast conditioned medium (orange bar).

Supplementing naïve medium with the identified compounds (in corresponding concentrations) restored the growth of *L. lactis* to ~ 40 % [4] of that observed in yeast conditioned medium (Fig. 3.8B). This result agrees well with the prediction from genome scale modelling (see section 5.2) and the fact that glutamine is the only essential (for *L. lactis*) amino acid missing from the naïve

[4] This number is likely to increase significantly, as shown results are based on the old quantification data. Latest results (Fig. 4.7) were obtained using more sensitive method and show higher concentrations for all amino acids.

medium. Interestingly, glutamine was both necessary and sufficient to reproduce the majority of the effect, while other amino acids had stimulatory effect. Increasing concentration of either glutamine or non-essential amino acids improves growth of *L. lactis*. This suggests that the rest of the effect observed in yeast conditioned medium can be explained (among other options) by joined quantification errors for every quantified amino acid and by presence of other nitrogenous compounds that either were in concentrations below the limit of exact quantification (which was the case for phenylalanine) or could not be identified.

L. plantarum, however, showed very limited response to all quantified components, indicating that other essential and yet non-identified components are excreted by yeast cells. According to literature (Wegkamp et al., 2010), the here-used strain of *L. plantarum* requires at least four additional amino acids. Auxotrophies for threonine and glutamine are covered (according to quantitative metabolomics data), but phenylalanine and tryptophan that were identified in untargeted screen could not be quantified due to very low concentrations. Perplexingly, supplementing medium with even millimolar concentrations of glutamine, threonine, phenylalanine, and tryptophan did not restore growth of *L. plantarum*. In similar discord with literature evidence, putrescine did not stimulate grow of lactobacillus either. These discrepancies will have to be resolved by further supplementation testing. Missing pieces of the puzzle can possibly be found among aromatic catabolites or metabolism intermediates of tyrosine, which along with asparagine is being primarily uptaken by *S. cerevisiae* (data not shown).

4 GENETIC AND ENVIRONMENTAL DETERMINANTS OF YEAST-LAB SYMBIOSIS

4.1 Rapamycin increases growth-stimulating effect of yeast on LAB

Suspecting the involvement of nutrient sensing and signaling in yeast metabolite overproduction, we decided to test the effect of perturbing TOR (Target of Rapamycin) pathway, the major nutrient–sensing controller of eukaryotic cell. Treatment of yeast cells with rapamycin is specific in its point of action, but quite global in terms of its physiological effect (see Introduction). While affecting multiple processes in yeast, rapamycin treatment is reported to mimic starvation effect and activate nutrient metabolism and recycling pathways dormant in replete media.

Rapamycin expectedly reduced the yeast growth, and at the same time reinforced positive effect on bacterial growth in dose dependent manner. Drug treatment of yeast during medium conditioning caused *L. lactis* and *L. plantarum* to reach up to three folds higher yields (Fig. 4.1A). Dynamic exometabolome analysis (section 3.3.1) revealed that rapamycin increased secretion of multiple metabolites (Fig. 4.1B), especially of glutamine and aspartate. Interesting is appearance of pyridine metabolism intermediates (Fig. 4.2), however identity of these hits needs validation by another method. Many more metabolites, including those with no matches in the database, cross the threefold threshold in concentration change during production by yeast and consumption by lactic acid bacteria, proving the connection with increased growth of bacteria in rapamycin treated yeast conditioned media.

Surprisingly, yeast adapted to presence of rapamycin not only avoids growth reduction but even outperforms untreated culture in biomass yield (Fig 4.1C). The difference becomes apparent after 2-3 weeks of adaptive evolution. This effect needs further investigation to be explained, but in general suggests possible benefits behind an extended overflow metabolism exacerbated by rapamycin. Positive effects of rapamycin could be due to parallel use of multiple nitrogen sources, release of toxic/inhibitory components or reduction of end-product inhibition.

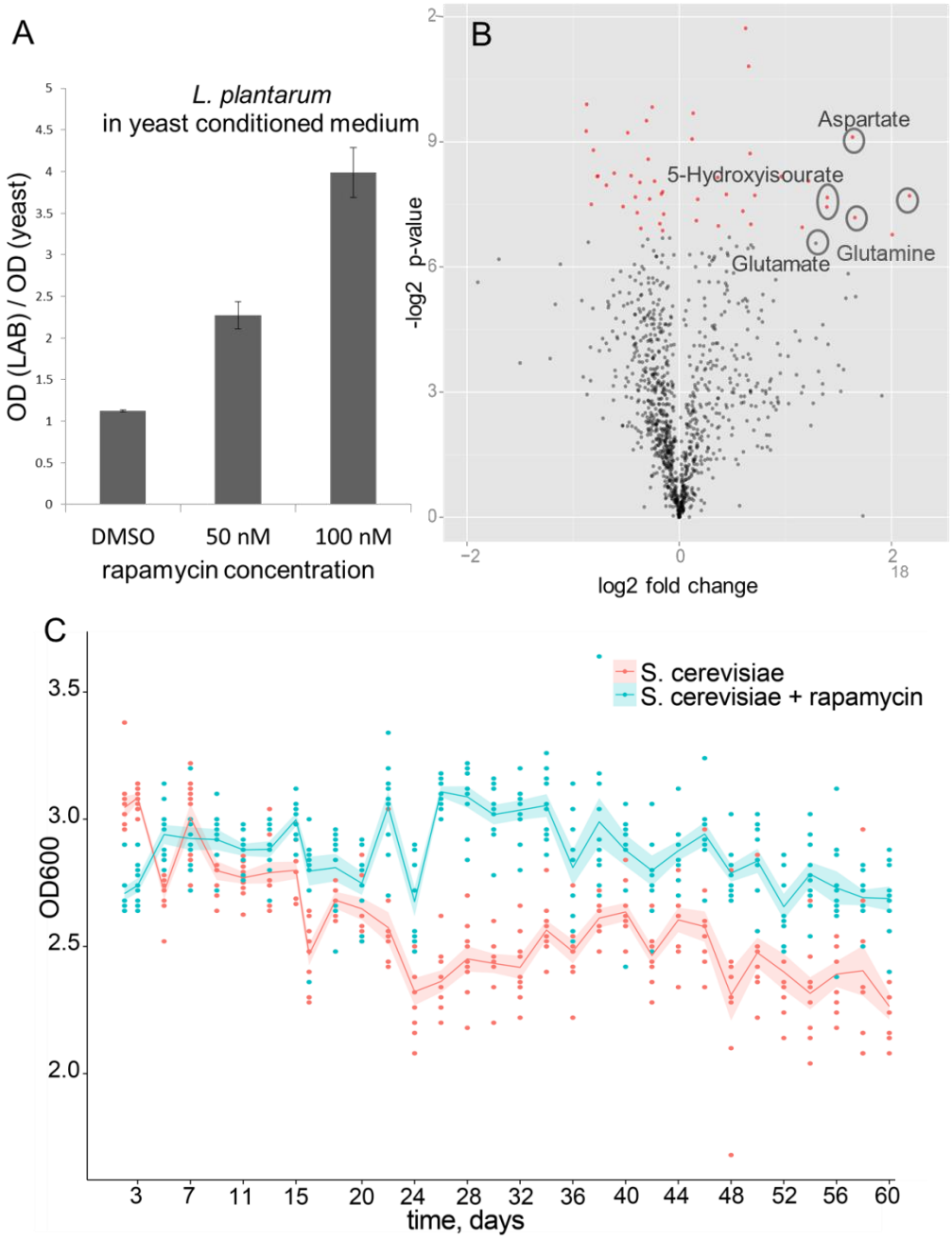

Figure 4.1: Effect of rapamycin on yeast secretome and fitness. **A.** Increased growth of *L. plantarum* in conditioned medium of rapamycin treated yeast. **B.** Secretome of rapamycin treated yeast versus untreated control (labelled are some metabolites most increased in presence of rapamycin). C. Long-term adaptation of *S. cerevisiae* to rapamycin demonstrates advantage of the drug treatment over naïve CDM47 in terms of cell yield. Data produced by intern student Laura R. Ripoll.

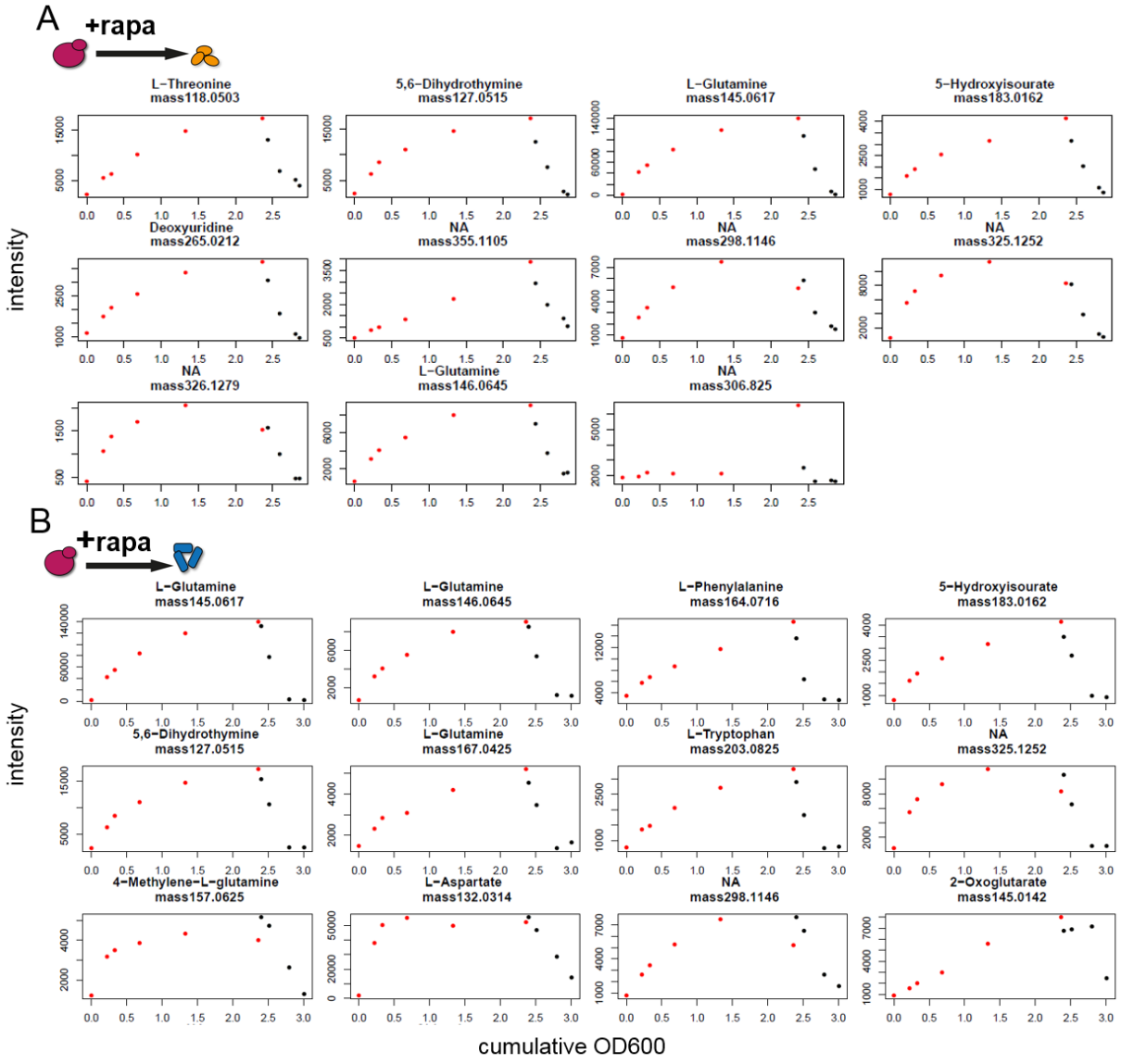

Figure 4.2: Ions produced by *S. cerevisiae* in presence of 20 nM rapamycin and consumed by lactic acid bacteria (at least 3x fold change). Ion intensity is plotted against cumulative OD, where red points show measurements during yeast grow and black points correspond to values during bacterial growth in yeast conditioned medium. A. Yeast – *L. lactis.* B. Yeast – *L. plantarum.*

4.2 Identification of genes that effect yeast-LAB interaction

To specify the involvement of TOR/rapamycin and to explore genetic underpinnings of yeast metabolite secretion, 88 yeast single gene knockout strains (Table 4.1) were selected for testing from a prototrophic deletion library (Mulleder et al., 2012). Since rapamycin specifically targets

TORC1 complex, deleted genes were chosen from upstream and downstream TORC1 route to clarify what specific parts of TOR-regulated processes are involved in interactions with *L. plantarum* and *L. lactis* (using conditioned medium assay). Most of the knockout strains affected bacteria similarly to the wild type, and a large fraction of tested mutants was not able to growth in CDM47 (Fig. 4.3A). Among genes that did modify the growth of LAB, 7 knockout mutants increased, and 2 reduced interaction strength in comparison to the wild type (Fig. 4.3A). To exclude the possibility of an artefact due to increased fragility of certain mutant strains (which could increase likelihood of cell lysis and thus inadvertently cause intracellular metabolite spill-out), fluorescent staining assay for cell damage/viability was done, showing no correlation between yeast mutants death / membrane permeability and their ability to promote bacterial growth (Fig. 4.3B).

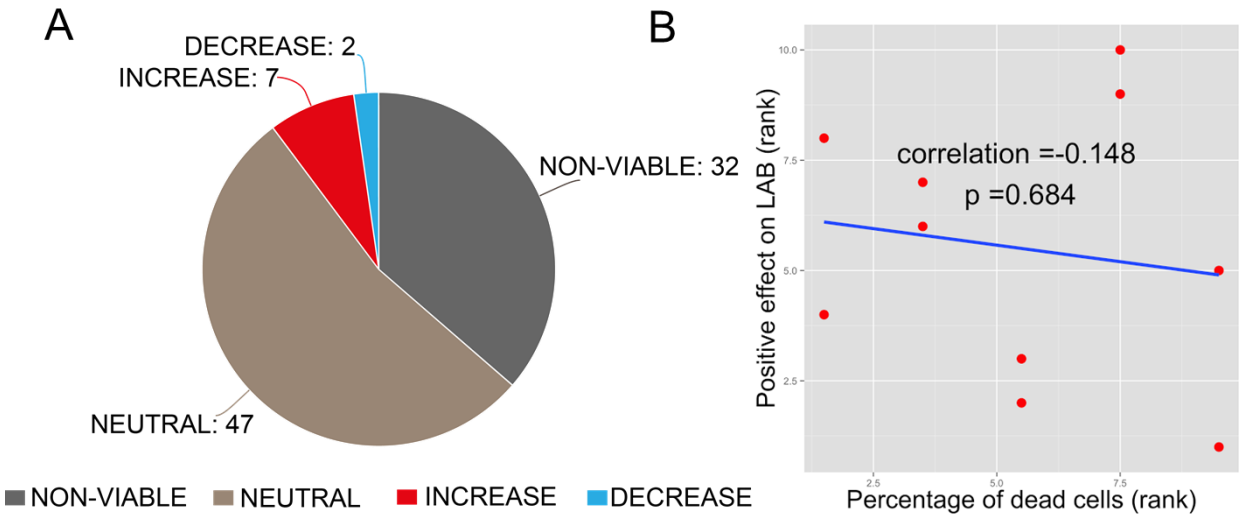

Figure 4.3: A. Effect of TORC1 pathway related single gene knockouts on LAB growth, relative to wild type, normalized by yeast culture cell density. B. No correlation observed between yeast mutants death / membrane permeability and their ability to promote bacterial growth.

Poor growth of the large fraction of mutants is not surprising since deleted genes coordinate nutrient response and metabolic regulation, and may affect growth in nutritionally replete medium. Additional instability may stem from plasmid genome used in the BY yeast library to compensate for the auxotrophies. Reproducible growth was later achieved for some strains by reproducing gene deletion in truly prototrophic *S. cerevisiae* of background S90 (shown in Figure 4.4 of this section).

Having a closer look at identified effector genes, seven knockout strains that increase interaction strength between yeast and LAB (*URE2, GTR1, PEP3, GCN1, ALT1, LST4,* and *NIR1)* showed effect on both *L. lactis* and *L. plantarum.* Deletion of either of two genes – *GLN3* or *DAL81* –

Table 4.1: Effect of yeast strains with deletions of TORC1 related genes on LAB growth in CDM47 conditioned medium (relative to wild type).

no difference		**not growing**	**increase**	**decrease**
DAL80	*UGA2*	*DAL82*	*URE2*	*GLN3*
TOR1	*UGA3*	*GZF3*	*GTR1*	*DAL81*
SIT4	*ARG4*	*RTG1*	*PEP3*	
DAL81	*GDH3*	*DUR3*	*GCN1*	
MSN2	*GCN2*	*NCR1*	*ALT1*	
MSN4	*LEU3*	*NCR2*	*LST4*	
GLT1	*MCH3*	*UGA4*	*NIR1*	
GAT1	*CAR1*	*SPE1*		
PTR2	*CAR2*	*SCH9*		
GNP1	*ARO9*	*RTG3*		
GAP1	*ARO10*	*TPO5*		
ASP1	*ARO80*	*GGC1*		
AVT6	*PUT1*	*GDH2*		
AGP1	*PUT2*	*URA2*		
AAT1	*VID30*	*KGD1*		
ZWF1		*ARG4*		
GCN4		*PPM1*		
GAP1		*TOD6*		
MEP3		*GLY1*		
GDH1		*PDC6*		
GNP1		*AVT4*		
ATG1		*NDE1*		
DAL1		*PDC5*		
DAL2		*AVT1*		
DAL3		*GCC1*		
DAL4		*GIS1*		
DAL5		*MKS1*		
DAL7		*PDR12*		
STP1		*CYS4*		
STP2		*HOM2*		
DUR1,2		*HOM3*		
UGA1		*PDC1*		

significantly reduces the growth promoting effect of *S. cerevisiae* on *L. lactis*, but absence of *GLN3* only reduces growth of *L. lactis*. This difference once again points out inequivalent means

by which two LAB species depend on yeast, and presents an opportunity to further investigate these differences.

Identified group of genes that alter yeast-LAB interaction are predominantly transcription factors, which can be caused by bias in knockouts selected for screening, but also could indicate that high level regulators can potentially provide more complex effect on metabolism than any single enzyme. Further in this work the focus will be on four knockout strains: Δ*gln3* and Δ*dal81* (with reduced effect on LAB growth) and Δ*ure2* with Δ*gtr1* (increasing LAB growth). Their quantitative effects comparing with the wild type are shown on Figure 4.4.

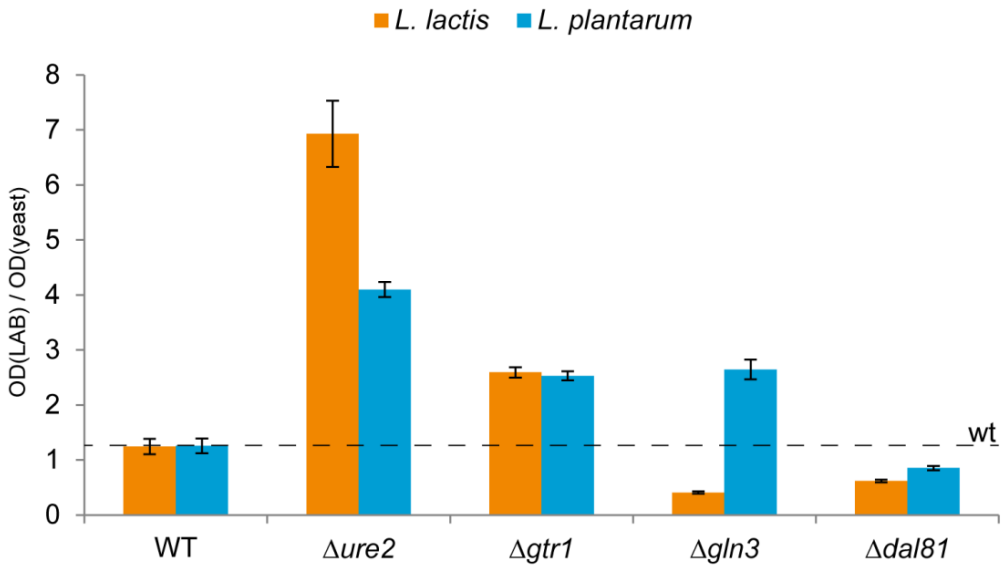

Figure 4.4: Gene knockouts that alter *S. cerevisiae* effect on LAB comparing with wild type.

Remarkably, the genes whose deletion up- or downregulates yeast metabolite secretion, belong to key regulators of nitrogen catabolite repression (NCR) - process responsible for selective utilisation of nitrogenous nutrients (Hofman-Bang, 1999). When grown on good nitrogen source (e.g. glutamine) NCR-sensitive genes are off, and cell is concentrated on an "easy" pathway for utilization of very limited number of nitrogen substrates. On the other hand, when preferred nitrogen is not available, NCR-regulators activate processing of alternative nutrients. Rapamycin treatment also regulates NCR (both trough and independent of TORC1 signalling) by lifting the repression and activating alternative nutrient salvaging pathways according to "starvation

protocol". Effect of rapamycin on yeast-LAB relationship phenotype agrees well with the picture obtained from knockouts testing, which can be traced to their common role in NCR gene regulation (Fig. 4.5). For description of interactions between NCR, TORC1, and other nitrogen regulators see also section 1.5.

Particularly practical for understanding of yeast phenotype-genotype relationship are mutations associated with loss of function, in our case reduction, through knockout of *GLN3* or *DAL81*. These are transcription factors that positively regulate uptake and degradation of less preferred nitrogen sources. Gln3 controls the majority of NCR-sensitive genes, including other key transcription factors, permeases, and catabolic enzymes (see section 1.5.2 for more details).

Dal81 has fewer known targets, but also is pleiotropic: it regulates pathways for degradation of such nitrogenous compounds as urea, allantoin, GABA (André et al., 1995; Bricmont et al., 1991; Talibi et al., 1995), mediates expression of amino acid permeases in response to signals from external amino acid sensing pathway (Abdel-Sater et al., 2004; Cardillo et al., 2010).

Ure2 is a negative regulator of Gln3 that by binding prevents Gln3 from migrating into nucleus and initiating transcription (Fig. 1.3). Ure2 binding to Gln3 is promoted by intracellular glutamine and/or active TORC1. Accordingly, Δ*gln3* and Δ*ure2* have antagonistic effect on interaction with LAB (Fig. 4.4). Gtr1 and Pep3 are components of upstream TORC1 activators, EGO complex and VPS complex respectively (Fig. 1.3, Fig. 4.5). Knockout of these factors would result in inhibition of TORC1 activity, and thus are confirmed by the fact that growth reduction in LAB is associated with Δ*gtr1* and Δ*pep3* deletion strains.

NCR does not rely entirely on Gln3 and Dal81. Downstream metabolic targets of NCR regulators tend to be cross-governed by several TFs, however, no effect was observed when testing knockouts of other NCR transcription factors: positive regulator GAT1, negative regulators Dal80 and Gzf3, together with a big set of downstream metabolic gene knockout (Table 3.1). Difference between targets of these TFs and those influencing metabolite overproduction might clarify specific metabolic players even further. Similarly, certain effect specificity seem to be responsible for interaction phenotype differences between Δ*gln3* and Δ*dal81* mutants (both decrease growth of *L. lactis* but only Δ*dal81* reduces growth of *L. plantarum*).

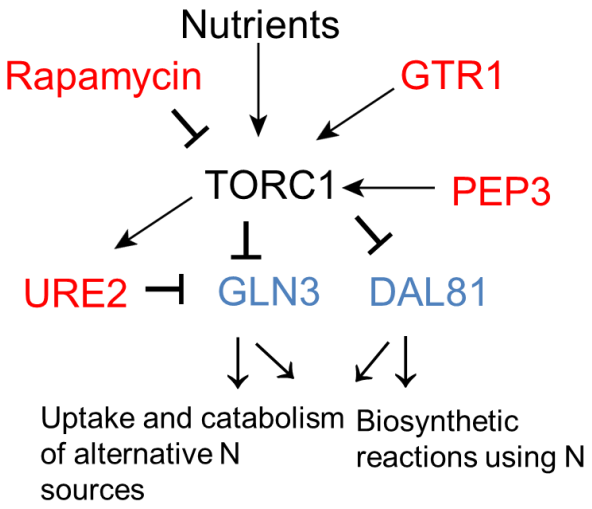

Figure 4.5: Interaction of NCR/TORC1 effectors agree with their effect on yeast-LAB interactions. Blue color highlites proteins which gene knockout downregulates effect of yeast on LAB, an red color shows effectors whose absence (addition in case of rapamycin) has a positive effect.

Effect of all abovementioned regulators and rapamycin converges on the processes of uptake and metabolism of alternative (non-preferred) nitrogen sources. These metabolic changes are collectively controlled by several nutrient-responsive pathways, as well as metabolites directly. In the next section will be explored some specific consequences that disruption of selected regulators brings to yeast exometabolome.

Surprisingly, deletion of TOR1 (specific Target Of Rapamycin) did not reproduce effect of rapamycin treated yeast on bacterial growth. Although Δ*tor1* and rapamycin treatment are considered to be similar, there are differences. For example, it was observed that some aspects of yeast response to rapamycin or nitrogen are TORC1 independent, such as sorting of general amino acid permease Gap1 (De Virgilio and Loewith, 2006a).

Several other sets of selected knockouts, besides TOR/NCR-related genes, were also tested: group of genes regulating autophagy and protein recycling (another process stimulated by rapamycin) and alcohol dehydrogenases and aldolases (these reactions are commonly involved in compound modification before release from yeast cell). However, results of these tests were negative (data not shown).

4.3 Exometabolome of yeast knockout strains

Since deletion of several identified transcription factors up- or downregulate the growth of lactic acid bacteria, changes in exometabolome of concerned yeast deletion strains were explored. Re-engineered deletion strains (in a more stable S90 background) were used to show significant differences in their amino acid profiles. Strains Δ*ure2* and Δ*gtr1* excrete more amino acids than the wild type, secreting more aspartate and proline respectively, and both overproducing glutamine and glutamate (Fig. 4.6). Especially dramatic are changes in Δ*ure2* strain, which produces aspartate and glutamine in millimolar concentrations.

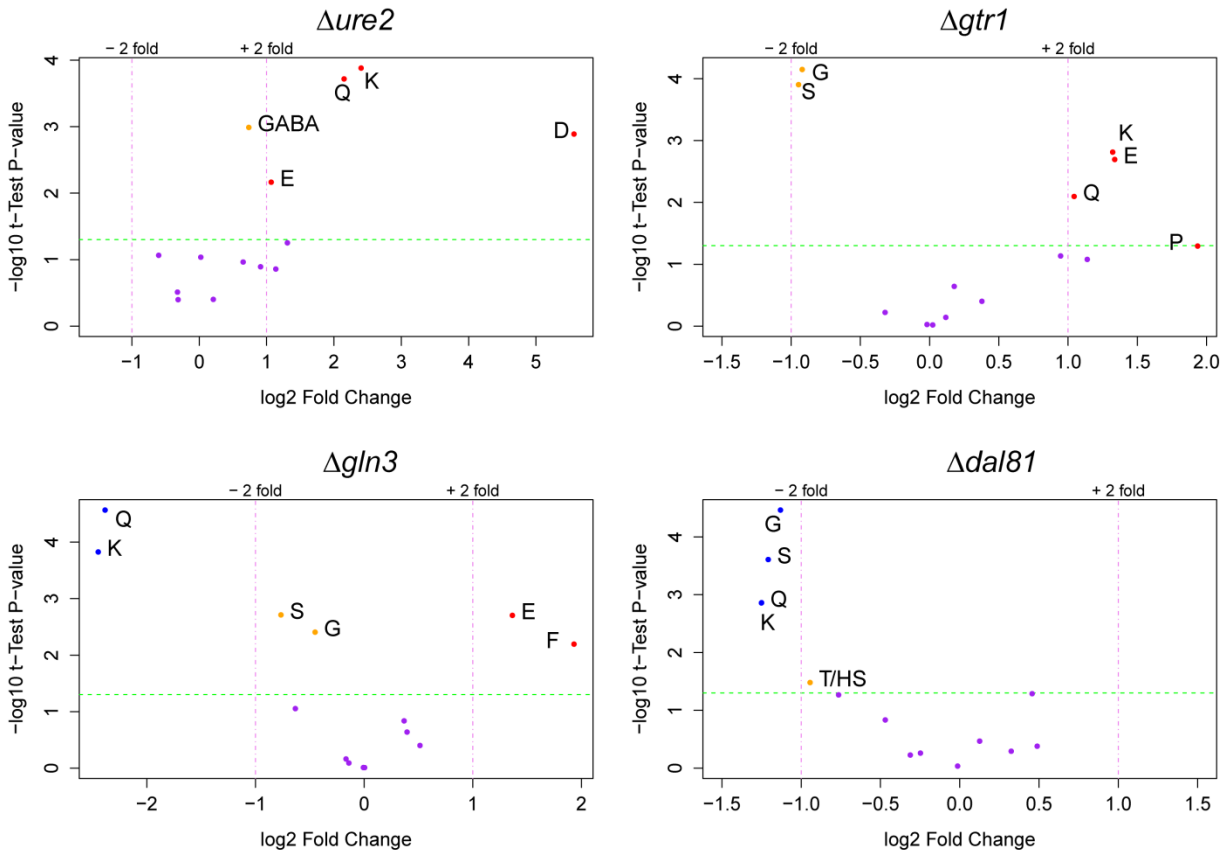

Figure 4.6: Concentrations of amino acids (OD normalized) in exometabolome of knockout yeast strains, fold changes to the wild type.

At the same time strains with reduced growth stimulating effect on LAB excrete in total fewer amino acids, cutting down on glutamine, serine and glycine. It is noteworthy, that Δ*gln3*, which reduces growth of *L. lactis*, but not of *L. plantarum*, together with reduction also shows elevation

of glutamate and phenylalanine – essential amino acids for *L. plantarum*, which potentially can explain the difference.

Altogether, total concentration of amino acids in conditioned medium of knockout yeast strains corresponds to growth promotion in LAB (Fig. 4.7).

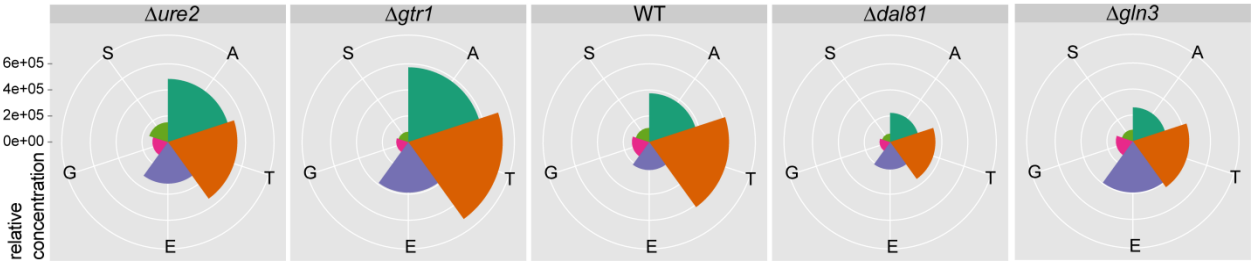

Figure 4.7: Amino acids (OD normalized) in exometabolome of yeast strains. Mutants with increased positive effect on LAB are on the left-hand side, and decreased are on the right-hand side from the wild type. Data for extremely overproduced in Δ*ure2* knockout amino acids aspartate and glutamine are omitted.

4.4 Effect of medium composition on excreted metabolites

Analysis of yeast knockout strains showed which of rapamycin-responsive processes are key to promoting growth of lactic acid bacteria. The branch of nitrogen catabolism genes, in particular those under NCR control, enable yeast for production of amino acids that are used as a nitrogen source by lactic acid bacteria. Since NCR is primarily regulated by quality of available nitrogen, next experiments involved testing the effect of different nitrogen sources on yeast-LAB interaction.

NCR genes are induced when good nitrogen sources are not available. Knowing this, we asked if culturing yeast on poor nitrogen source would additionally stimulate amino acid biosynthesis and enhance bacterial growth. Test conditions included standard CDM47 medium which contains eight amino acids and ammonium, CDM47 without arginine, asparagine and ammonium (three best nitrogen sources in CDM47), medium with proline as sole nitrogen source (least favorite amino acid), and CDM47 with single amino acid omissions (Fig. 4.9). On the contrary to expectations, poor nitrogen sources that de-repress NCR-sensitive genes important for amino acid overproduction not only did not increase the positive effect on growth of either lactic acid bacteria, but caused a significant reduction.

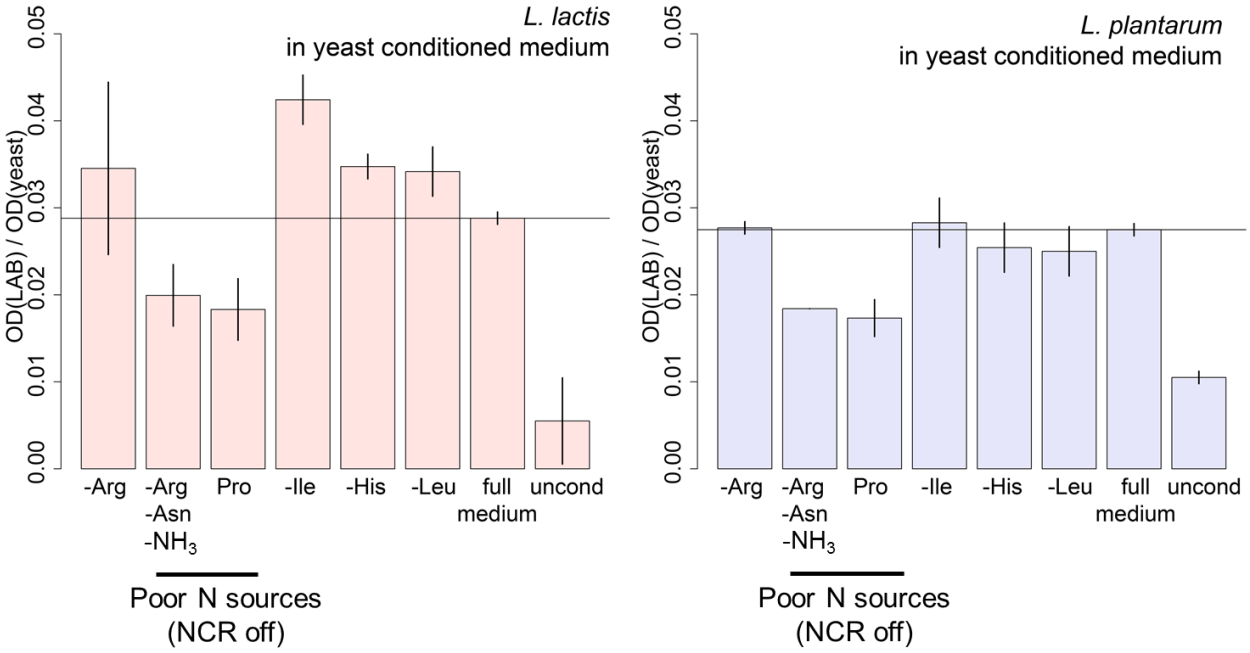

Figure 4.9: Effect of nitrogen source on yeast-LAB interaction. Poor nitrogen sources reduce growth-promoting effect.

Some of the media effects were also analyzed for differences in amino acid profiles. Test conditions included media with the following single nitrogen sources: glutamine (most favorite), proline (least favorite), ammonia (good), regular test medium CDM47 (containing ammonia and 8 different amino acids, including good nitrogen sources asparagine and arginine), and CDM47 with an exclusion of asparagine, arginine, and ammonia. In accordance with the observed good growth of LAB, conditioned CDM47 contained the highest concentrations for most amino acids (Fig. 4.10). Likewise, depleted media that sustain least interaction with bacteria - with only proline, only ammonium, or six less-preferred amino acids as nitrogen source – those contained much lower amounts of amino acids. In addition, when grown on the best possible sole nitrogen source, glutamine, yeast overproduced primarily glutamate. Two other preferred amino acids arginine and asparagine also seem to be critical for nitrogen overflow, but their individual roles still need to be delineated.

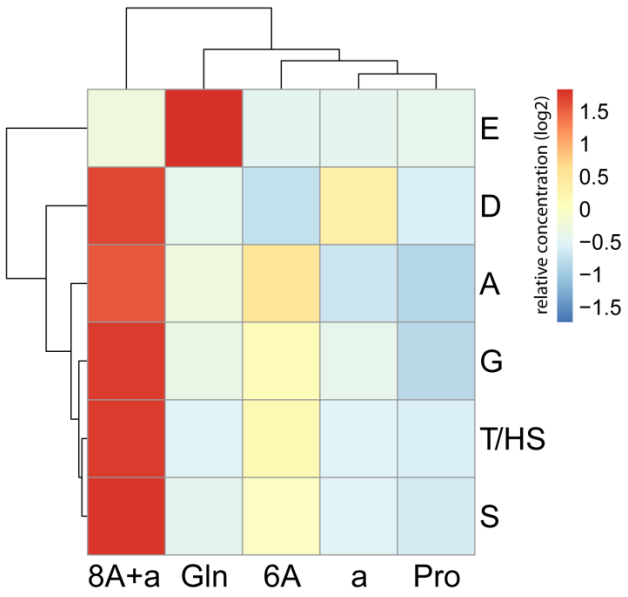

Figure 4.10: Effect of medium composition on amino acid composition of yeast exometabolome. Values are corrected for corresponding medium blanks.

Altogether, the results indicate that the observed connection between LAB and *S. cerevisiae* is linked to amino acid uptake and catabolism in yeast that are required /lead to the overflow metabolism. Uptake of amino acids does not result in their full utilization, but in interconversion and spill-out, rather similar to the production of ethanol and glycerol as carbon overflow during rapid growth on glucose. Although theories behind overflow in carbon assimilation are not yet reconciled, it is possible that some of them, such as regeneration of co-factors and restoration of redox balance apply to the situation we observe with nitrogen.

Amino acid profiles in different media correlate with the LAB response and depend on the number and quality of nitrogen source in the medium. Results suggest that presence of preferable amino acids is necessary for yeast to produce components helpful to LAB. Presence of good nitrogen source supposed to repress expression of NCR sensitive genes. Yet, counterintuitively, the activity of *GLN3* and *DAL81* (some of the main NCR players) was shown to be necessary for metabolite excretion as much as the presence of good nitrogen source. Perhaps NCR genes are important due to their involvement in amino acid permease sorting and can contribute at basal expression levels.

So far, obtained results suggest two components to be essential for amino acid overflow (Fig. 4.11). On one hand, presence of diverse amino acids, in particular those preferentially taken up

by yeast cell, gives material to convert into other amino acids (and other nitrogenous compounds) excreted into the culture medium. Preferred amino acids, such as asparagine and glutamine are more likely to "overflow", possibly because of unrestricted uptake and complete utilization of their degradation products. On the other hand, as it is evident from mutant analysis, genes responsible for incorporating secondary nitrogen sources into central metabolism are also important for the rich extracellular exometabolome. Both these factors are present when wild type yeast are grown in our regular test medium CDM47 – amino acid rich medium is complemented with basal level of expression of NCR genes (Fig. 4.11), thus yeast cells are in an overflow mode, extruding micromolar quantities of amino acids. However, if either of the driving forces gets diminished, either NCR (through knockout of essential transcription factor like *DAL81* or *GLN3*) or richness of the medium (by eliminating preferred nitrogen sources) - fewer nutrients are being released from the cell. In contrast, upregulating NCR with the deletion of alternative transcription factors (*URE2* or *GTR1*), while keeping the plentiful nitrogen sources, allows increasing the excretion and thus giving more benefit to LAB co-habitants.

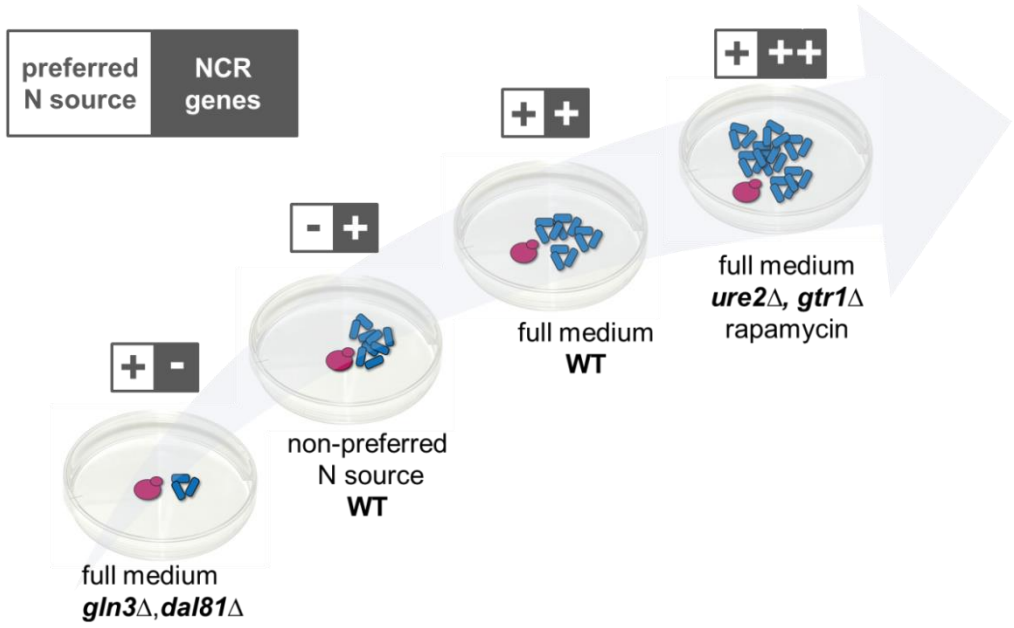

Figure 4.11: Driving forces of yeast-LAB interaction: metabolic genes under control of nitrogen catabolite repression and preferred (and diverse) nitrogen sources in the medium.

Described model (Figure 4.11) is a part of work in progress, requiring more of orthogonal confirmation from other experiments, since the picture metabolism regulation can be hard to deconvolute. A number of research publications suggest that yeast perceives amino acids in the medium not only as a substrate, but also as a signal to adjust regulatory state and metabolic activity. Presence of multiple amino acids in the test medium complicates the situation due to their interactions. Some amino acids can be easily interconverted to one another, in other case they can compete for the same transporter, thus the medium composition can be very different from what nutrients and in what amounts yeast cell can actually access. Although this thesis will reach conclusions in several more pages, work on this topic will go on.

4.5 Genome-wide analysis of effect of yeast gene knockouts on symbiotic LAB

Screening of genome-wide deletion strain collections is widely used in exploring genotype-phenotype relationship in many microorganisms. In case of yeast-LAB interaction, identifying yeast genetic modulators of metabolite production would prove extremely useful to pinpoint the genes involved in inter-species interaction. To this end, we used the ability of *L. plantarum*'s native beta-galactosidase to cleave X-gal molecule with production of colored metabolite. This color reaction enabled quantification of bacterial growth associated with each yeast knockout strain in co-cultures (section 2.3). Screening allowed to identify genes associated with lowest color development per yeast, i.e. whose knockout abolishes *L. plantarum* growth. (Table 4.2). Gene ontology annotation showed that this group of genes was significantly enriched for categories "structural constituent of ribosome" in molecular function (MF) ontology group, mitochondrial translation" in biological component (BP) group and "mitochondrial ribosome" in cellular component (CC) group (Fig. 4.12). Enrichment groups were identical irrespective of number of screen replicates tested. Correspondingly, KEGG pathway enrichment showed overrepresentation of "oxidative phosphorylation".

While results were significant and reproducible between replicates, they do not agree with the subsequent conditioned medium assays. Yeast knockouts that took top place in the library screen and developed co-cultures with no color showed support to LAB in conditioned medium assays.

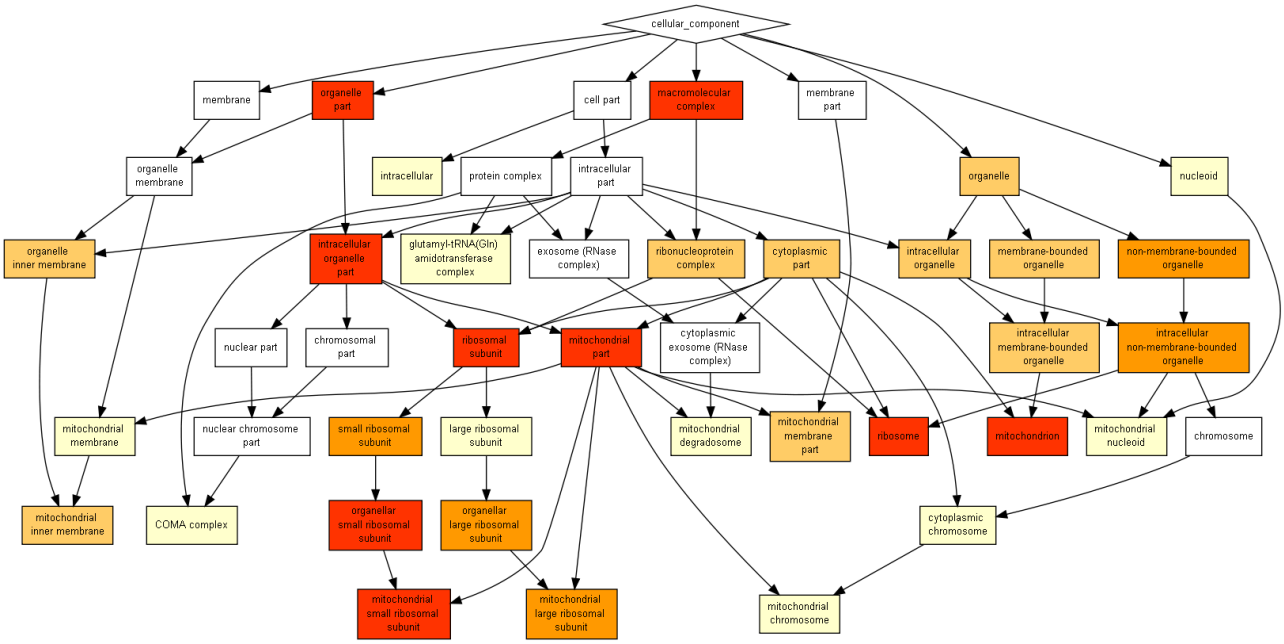

Figure 4.12: Gene onthology categories enriched among yeast deletion strains associated with reduction in *L. plantarum* growth. Colored boxes highlight (inverse-proportionally) groups with significant p-values (<0.05).

There are several possibilities that could have led to this discrepancy. First, high-throughput screening approach dictates conditions different from low throughput tube co-culture testing, and could have skewed the results. Specifically, small culture volume combined with repetitive shaking (required for optical density measurements) could have caused elevated levels of dissolved oxygen in the medium. Such effects are prune to be aggravated in BY (and S288c) background strain, as they tend to have lower cytochrome c accumulation and lower respiratory rates (Gaisne et al., 1999). In these conditions respiratory chain deficient mutants (hits) might not be able to use up the oxygen from the medium as well as other strains. This in turn could have caused impeded growth of microaerophilic *L. plantarum*. Second possibility includes interference of identified knockout strains not with the bacterial growth, but rather with the beta-galactosidase activity (color development). Third, screening was done in condition of true co-culture, thus in comparison with conditioned medium assay, other interaction effects could have been induced by presence of *L. plantarum* in co-culture. The latter being true, however, does not diminish the investigation of conditioned medium interaction, since this type of experiment provides an essential “baseline” interaction description.

It should also be noted, that the yeast library in background BY shows irreproducible growth pattern in our chemically defined medium even for the wild type. These problems can be partially explained by plasmid instability and secondary mutations pertaining to the single gene deletions (Teng et al.). Eventually drawbacks were circumvented by reproducing several key deletions in S90 yeast background, and doing the same for full library might be a necessary task for the future, as plasmid genome and multiple auxotrophies may play a role in an aberrant growth. Further experiments are required to contextualize obtained results.

Table 4.2: Yeast genes whose deletion is associated with reduction in *L. plantarum* growth

Gene	Name/Description	ORF	Hit replicated, #
ATG5	AuTophaGy related	YPL149W	3
ATP7	ATP synthase	YKL016C	2
COX11	Cytochrome c OXidase	YPL132W	2
CTF19	Chromosome Transmission Fidelity	YPL018W	2
EMI5	Succinate DeHydrogenase	YOL071W	3
GIN4	Growth Inhibitory	YDR507C	2
GLO3	GLyOxalase	YER122C	2
GSH1	glutathione (GSH)	YJL101C	2
HIT1	HIgh Temperature growth	YJR055W	2
MRP1	Mitochondrial Ribosomal Protein	YDR347W	3
MRP51	Mitochondrial Ribosomal Protein	YPL118W	2
MRPL37	Mitochondrial Ribosomal Protein Large subunit	YBR268W	3
MRPL9	Mitochondrial Ribosomal Protein Large subunit	YGR220C	2
PYC1	PYruvate Carboxylase	YGL062W	2
QCR2	QH2:cytochrome-C oxidoReductase	YPR191W	2
RPL21b	Ribosomal Protein of the Large subunit	YPL079W	2
RPS0b	Ribosomal Protein of the Small subunit	YLR048W	3
RRG8	Required for Respiratory Growth	YPR116W	2
RSA1	RiboSome Assembly	YPL193W	3
RSM27	Ribosomal Small subunit of Mitochondria	YGR215W	2
SHU2	Suppressor of HydroxyUrea sensitivity	YDR078C	3
TVP18	Tlg2-Vesicle Protein	YMR071C	2
YDL062w	Dubious	YDL062W	2
YKL169c	Dubious	YKL169C	2
YNL226w	Dubious	YNL226W	2
YPL080c	Dubious	YPL080C	2
YTA12	Yeast Tat-binding Analog	YMR089C	2

5 MODELLING OF METABOLIC INTERACTIONS IN MULTI-SPECIES COMMUNITIES

Mathematical modelling can be a powerful tool in predicting interspecies metabolic interactions. Simulation approach is also very resource efficient, requiring not more than genome sequences of individual community members (to be converted into genome scale metabolic models) and medium's chemical composition. It is the process of getting and tuning individual models that takes the lion's share of the effort. Automatic reconstruction of genome scale metabolic models is rapidly progressing, new optimized gap filling algorithms and annotation services appear (Dias et al., 2015; Hamilton and Reed, 2014), however, manually curated models are still considered to be more reliable. In both reconstruction cases though, one should be wary of the purpose models were designed to serve.

Majority of modelling applications involve tasks of estimating gene essentiality and growth yields, and all of these tasks are done in relation to biomass composition, making the biomass reaction a "heart" of the model. At the same time, since simulations are usually done under assumption of rich medium conditions (most commonly used in lab), much less attention is devoted to nutritional essentiality, in particular which metabolites can be transported in/out of the cell, what metabolites are essential for growth, and what interconversion can and cannot happen between metabolic species inside the cell. In order to use individual species genome scale metabolic models to predict metabolic exchanges in the community, the accuracy of the models regarding nutrient essentiality is decisive in prediction result. While quality control of this aspect is not yet feasible for large scale simulations, it is a reasonable step for 2-3 species communities.

5.1 Genome-scale multi-species modelling to explore metabolism of microbial communities[5]

Microbial communities are present virtually at every spot of the Earth, thus making the question of their assembly an important pursuit. Due to the scale of this problem, computational approach

[5] Work described in this subsection was done together with Aleksej Zelezniak and Sergej Andrejev and described in Zelezniak et al., 2015 (except for the kefir network, which is yet unpublished).

holds a lot of appeal. We investigated the role of inter-species metabolite cross-feeding in shaping microbial consortia by applying a new algorithm, SMETANA (species metabolic interaction analysis), to previously described dataset of microbiomes from 1.297 communities found in diverse habitats (Chaffron et al., 2010). SMETANA enumerates all possible metabolite exchanges in multi-species communities relying solely on genome sequences of identified species (Zelezniak et al., 2015). Unlike other methods, it works independent of growth rate optimization and is scalable to large communities.

We first tested SMETANA predictions on small microbial communities, with known metabolic cross-feeding relationships (Fig. 5.1). Correct predictions were obtained for ethanol, hydrogen (electron equivalents) and acetate exchange in three-species anaerobic community (Miller et al., 2010), as well as carbon dioxide and nitrogen source exchange between yeast and algae in synthetic assembly (Hom and Murray, 2014). In addition to experimentally reported metabolic dependencies, we have also identified new potential exchanged metabolites, which can possibly be relevant under different conditions (e. g. alternative medium composition, result of adaptive evolution etc.).

Next we compared SMETANA predictions on number of cross-fed metabolites in sample-level communities and in sub-communities of co-occurring species (species that live together with a chance higher than random across different sample communities). Results have shown that co-occurring microbial communities are more metabolically interdependent, and also enriched in cooperative (mutualistic) interactions (Zelezniak et al., 2015), thus suggesting nutrient exchange to be a driver of microbial co-habitation. Furthermore, metabolic cross-feeding outweighs the risk for competition: communities that live in similar environments tend to be metabolically more similar (habitat filtering), yet it does not preclude exploitation of their complementary biosynthetic abilities to support each other. These findings put the problem of nutrient exchange in a global perspective. They illustrate prevalence of metabolic interdependencies in natural systems and illuminate the role of community metabolism in microbial ecology. Next step on this road, in part addressed in this thesis, is to dwell on mechanisms of metabolite exchanges that make up the microbial world.

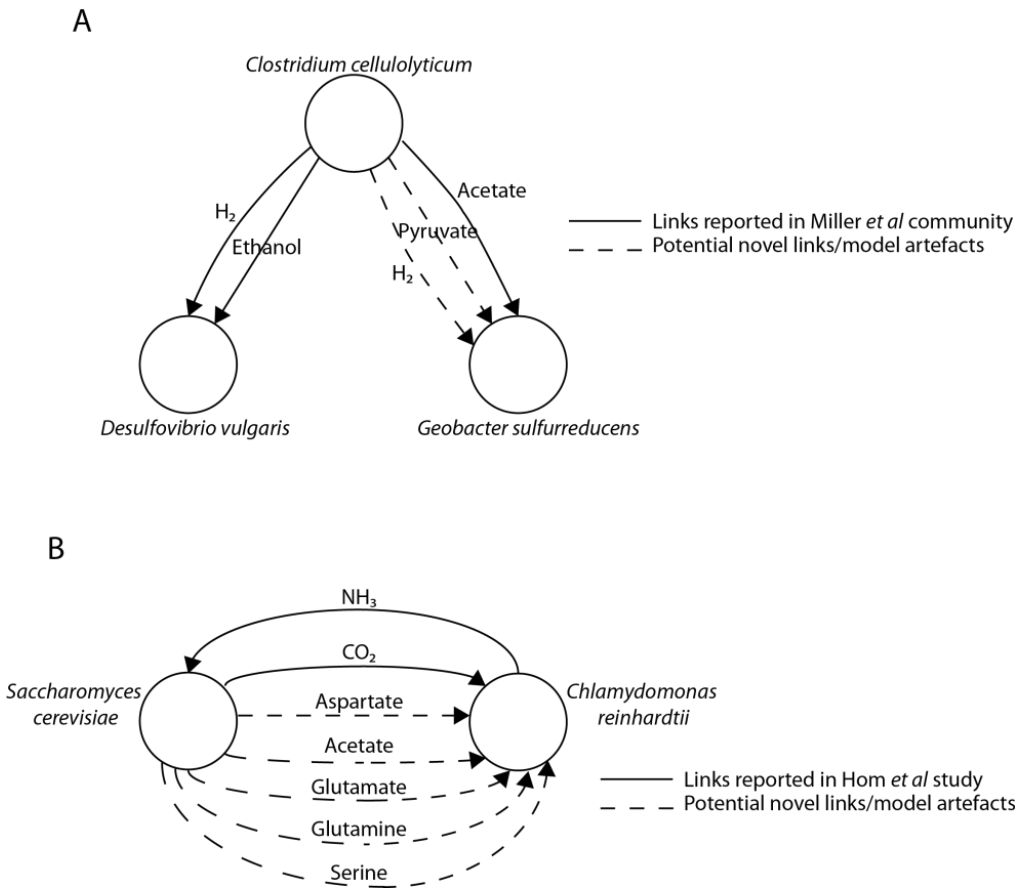

Figure 5.1: Metabolite cross-feeding in microbial communities as predicted by simulations (reconstructed as in Zelezniak et al., 2015). A and B. Interacting microbial communities with metabolic exchanges described in (Miller et al., 2010) and (Hom and Murray, 2014).

5.2 Simulating metabolic interactions in yeast-LAB community.

To simulate yeast-LAB community co-metabolism manually curated genome-scale metabolic models for *S. cerevisiae* (Zomorrodi et al., 2014), *L. lactis* (Oliveira et al., 2005), and *L. plantarum* (Teusink et al., 2005) were selected. Yeast metabolic model is the most developed model in the field, and did not raise concerns. *L. plantarum* model, in agreement with our experimental observations, did not show any growth in the synthetic CDM47 medium, but *L. lactis* was growing *in silico*, indicating an erroneous nutrient essentiality. In order to reproduce the observed *L. lactis* amino acid requirements, also available from the literature (van Niel et al., 1999), we have added to the original model or blocked total of 34 reactions (Appendix). These allowed accurately capturing the need for essential amino acids and restored nutrient dependent growth / no growth behavior observed experimentally.

Next, assembled community models were used to enumerate all possible flux-balanced interspecies metabolite exchanges (Methods 2.12) possible under CDM47 medium conditions. Results corroborate experimental findings, by showing nutrient flow from yeast to both lactic acid bacteria (Fig. 5.2). Glutamate / glutamine (interchangeable alternatives) are predicted to be secreted by yeast and consumed by *L. lactis* and *L. plantarum*, further cross-feeding of amino acids phenylalanine and proline (also detected in yeast exo-metabolome) from yeast to *L. plantarum* was observed (Fig. 3.20).

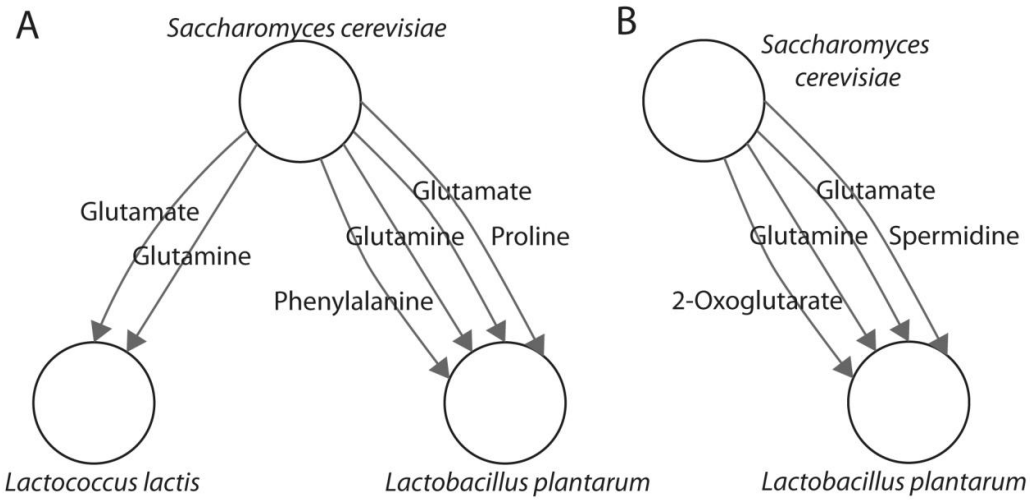

Figure 5.2: Metabolic cross-feeding between yeast and LAB as predicted by simulations. Yeast model taken from (Zomorrodi et al., 2014). **A.** Based on manual reconstruction of LAB models (*L. plantarum* (Teusink et al., 2005) and *L. lactis* (Oliveira et al., 2005), modified. **B.** Automatically reconstructed LAB models (Henry et al., 2010).

In addition, after comparison of simulation results obtained by using manually curated LAB models to the results obtained for automatically reconstructed models of lactic acid bacteria metabolism, similar results were obtained. Joint model predicted flow of glutamine / glutamate (as in manually created version), 2-oxoglutarate and spermidine from yeast to *L. plantarum*. In case of *L. lactis*, growth independent of yeast was observed, same as in case of manually created model before correction (described above). It is important to emphasize, that these simulations can capture only exchange of essential components that are absolutely necessary for growth, however in reality additional nutrients, with stimulatory effects could be involved (as shown in the experimental results section 3.3.2).

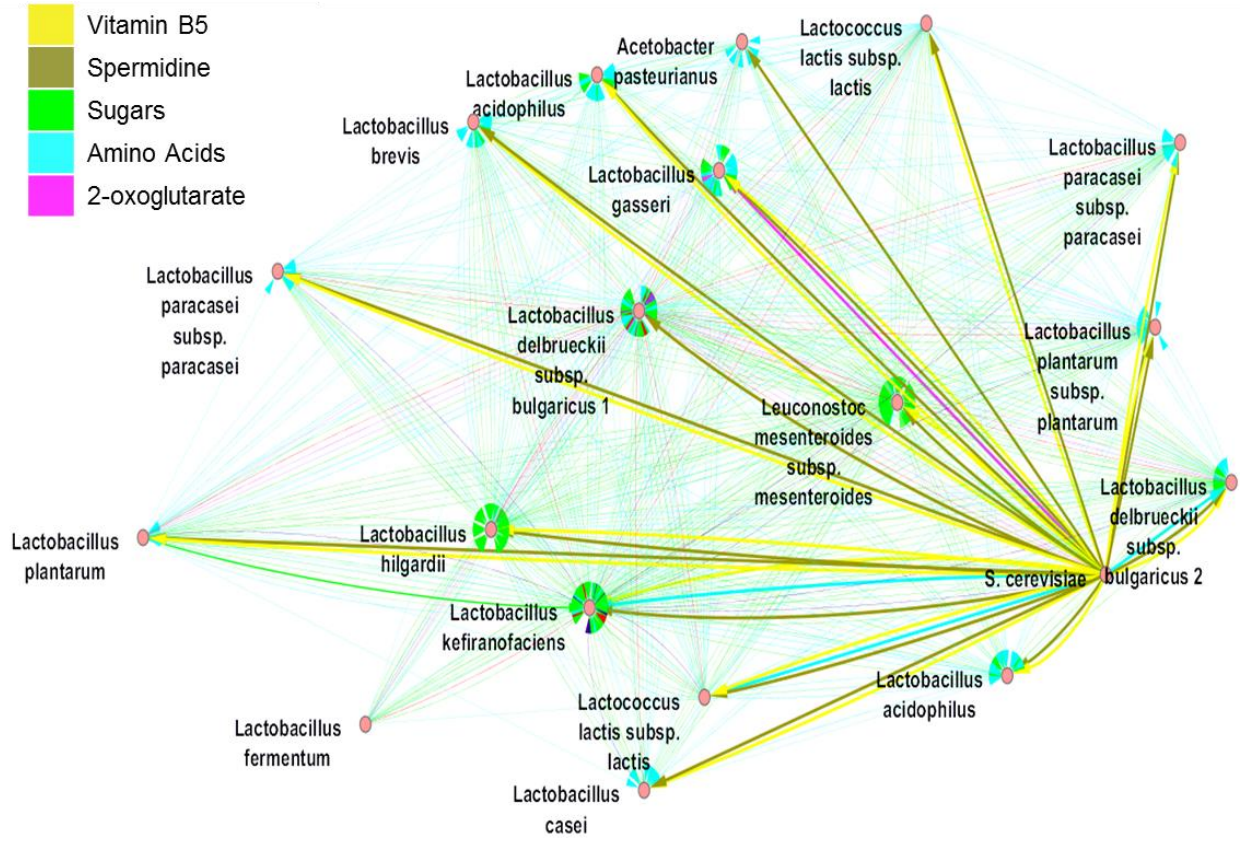

Figure 5.3: Enumeration of possible metabolic interactions in kefir community (Figure made by S. Andrejev).

Finally, to subject yeast-LAB interaction to a more natural environment, we ran exploratory simulations for the community of species that live together in natural communities of milk kefir grains and for which genome scale metabolic models were available (Henry et al., 2010). Resulting community of 19 species in a milk-like *in silico* medium demonstrated a number of potential inter-species metabolic exchanges (Figure 5.3). Notably, even in a rich milk-like medium *S. cerevisiae* forms a hub of the community by donating multiple metabolites to LAB.

6 CONCLUSIONS AND OUTLOOK

6.1 CONCLUSIONS

Our results show that *Saccharomyces cerevisiae* enables growth of *Lactobacillus plantarum* and *Lactococcus lactis* by secreting essential nutrients. Resulting three species community remains stable for at least two weeks and can be simply sustained by passaging. Yeast sustain otherwise impossible growth of lactic acid bacteria by providing them with essential nutrients. This metabolite overflow is independent of bacterial symbionts and happens by virtue of strategic regulatory response.

Untargeted mass spectrometry analysis provided a list of candidate exchange metabolites. Following targeted analysis confirmed that secretion of amino acids is responsible, at least partially, for growth of LAB. Metabolic modeling of three species community metabolism captured exchange of essential amino acids, demonstrating high predictive potential.

Additional analysis of knockout yeast strains demonstrates involvement of TORC1/NCR signalling in metabolite overproduction. Availability of preferred nitrogen source and activity of nitrogen metabolism, specifically genes sensitive to nitrogen catabolite repression, both are necessary for pronounced nitrogen overflow in yeast. The balance of these two components defines the strength of yeast-LAB interaction.

6.2 IMPLICATIONS AND OUTLOOK

Sharing nutrients is an important factor to consider in microbial physiology, ecology, biotechnology and evolution.

In physiology, fluxes through metabolic network depend on the input – medium composition. Although flexible, metabolic pathways operate under the set of physiological constrains, and thus lack or excess of metabolite in one branch has to be compensated with appropriate adjustments in the network. Such balancing mechanism was illustrated here, when nitrogen metabolism regulation in yeast resulted in overflow and excretion of still metabolizable compounds. As scientific community shift its attention from monocultures to complex communities, it becomes

apparent, that metabolic adjustments are not the matter of one cell anymore, and can considerably affect community life.

In ecology, metabolic exchanges guide development and survival of microbial communities. Metabolite excretion by yeast modifies the environment and thus constructs a niche for lactic acid bacteria. Beyond this specific example, interrogation of multiple natural bacterial communities illustrated implication of metabolic mutualism in bacterial co-occurrence. Investigating metabolic links would help to understand why certain species are where they are, with results reaching from quite general ecological "laws" to concrete applications, e.g. understanding development of polymicrobial infections.

In biotechnology, analysis of multiple factors that modulate exo-metabolome composition (genotype, nutrient availability, growth phase) provides the leverage point in regulating inter-species interactions and thus community composition and properties. For biotechnological purposes this knowledge can advance our understanding of microbiology of multispecies fermentation in food and beverage industry, where both yeast and LAB are commonly co-cultured. Moreover, grasping the reasons behind metabolite secretion/overflow will be instrumental in boosting efficiency of that or another biotechnologically relevant process/pathway.

In evolution, microbial fitness largely depends on their handling of nutrients. Efficient scavenging or, on the contrary, excretion of nutrients appear to be strategies whose purpose can be revealed by multi-angular scrutiny. This work represents one small attempt to address this problem. Un-engineered nature of studied cross-feeding organisms hints at possible modes of inter-species relationships in natural environments. Another important corollary of this work is that act of "feeding" unrelated microorganisms does not necessary evolve in response to some reciprocal benefit from that organism, but can be a mere consequence of internal metabolic regulation and/or metabolic strategy. Such a dependency can further evolve into a bona fide mutualism (when two organism benefit from each other equally), hence suggesting a possible route for the evolution of cooperation.

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