Coevolution of *Saccharomyces cerevisiae* and *Lactobacillus plantarum*: Engineering interspecies cooperation

by

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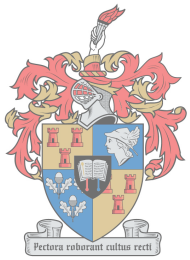

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Declaration

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Summary

Microbial interactions are ubiquitous in nature and play a vital role in economically important industrial processes like winemaking. *Saccharomyces cerevisiae* and *Lactobacillus plantarum* are important species responsible for the completion of alcoholic and malolactic fermentation (AF and MLF) respectively. Understanding how these species interact with each other and their environment is important to better manage successful completion of AF and MLF. However, the complexity of the wine matrix makes it nearly impossible to study these interactions in a natural environment and synthetic ecological systems can therefore be used to overcome these difficulties. This study was designed to establish a co-dependent, mutualistic relationship between *S. cerevisiae* and *Lb. plantarum* in order to gain insights into the cooperation between species, how pH, temperature, and inoculation dosages influences the interaction, and how the interaction evolves over time.

The interaction, centered on the reciprocal exchange of amino acids, was established between the lysine auxotrophic strain *S. cerevisiae* THI4 and the isoleucine, alanine, valine, and methionine auxotrophic strain *Lb. plantarum* B038. Different combinations of amino acids were omitted from the chemically defined synthetic grape juice-like media in order to find an amino acid treatment which promoted the best growth for both microorganisms. B038 showed excellent growth when co-cultured with THI4 for all the amino acid treatments, but THI4 struggled to grow under these conditions. The two treatments selected for further experiments were the Lys-Ile (lysine and isoleucine omitted) and Lys-Val (lysine and valine omitted) treatments since THI4 showed the best growth under these conditions.

Lower temperature and pH conditions had a negative effect on the growth and malic acid consumption of B038, but when co-cultured with THI4 the yeast appeared to stimulate the growth of the bacteria under both selective and control conditions. THI4 continued to show poor growth performance and sugar consumption under these conditions. However, when THI4 and B038 were inoculated at cell densities with similar biomass, the growth of THI4 improved significantly. It was expected that THI4 and B038 would show poor growth when grown in the absence of their respective auxotrophic amino acids and support of their respective partner. This proved true for all the amino acid treatments except when B038 was grown in the absence of lysine and valine. B038's ability to grow under these conditions was hypothesized to be linked to the uptake of glutamine and the production of γ-Aminobutyric acid (GABA), but further research is still required to investigate this.

Over continuous rounds of fermentation, THI4 adapted to the imposed selective conditions by increasing its consumption of glucose while cell density remained the same. Whether this is linked

to increased ethanol production still needs to be determined. No significant changes were observed in B038 after coevolving the strains. This study provides relevant insights into the industrially important interaction between *S. cerevisiae* and *Lb. plantarum* and also provides a basis for future work to create optimised yeast-bacteria pairings for both industrial applications in winemaking and to investigate the genetic changes involved in the establishment of cooperative interactions between species.

Opsomming

Mikrobiese interaksies is alomteenwoordig in die natuur en speel ‘n kern rol in ekonomies belangrike industriële prosesse soos wynproduksie. *Saccharomyces cerevisiae* en *Lactobacillus plantarum* is belangrike spesies onderskeidelik verantwoordelik vir die suksesvolle voltooiing van alkoholiese- en appelmelksuurgisting (AG en AMG). Hoe hierdie spesies teenoor mekaar asook hul omgewing reageer het ‘n invloed op fermentasie, dus is dit belangrik om hierdie interaksies te verstaan om sodoende AG en AMG beter te bestuur. Die kompleksiteit van die wynmatriks bemoeilik egter die studie van hierdie interaksies in ‘n natuurlike omgewing en sintetiese ekologiese sisteme verskaf ‘n alternatiewe wyse om hierdie interaksies te bestudeer. Hierdie studie het daarna gestreef om ‘n mede-afhanklike, mutualistiese verhouding te vestig tussen *S. cerevisiae* en *Lb. plantarum* om ten einde beter te verstaan hoe hierdie spesies saamwerk en hoe pH, temperatuur, en aanvanklike selkonsentrasies die interaksie beïnvloed, asook hoe die interaksie ontwikkel oor tyd.

Die interaksie, afhanklik op die wedersydse uitruil van aminosure, was gevestig tussen die lisien ouksotrofe ras *S. cerevisiae* THI4 en die isoleusien, alanien, valien, en metionien ouksotrofe ras *Lb. plantarum* B038. Verskillende kombinasies van aminosure was weggelaat uit sintetiese druiwemos om ten einde die aminosuurbehandeling te identifiseer wat die groei van beide organismes bevorder. B038 het goeie groei getoon in mede-kultuur met THI4 vir al die aminosuurbehandelinge, maar THI4 het gesukkel om te groei in hierdie kondisies. Die Lys-Ile (lisien en isoleusien weggelaat) en Lys-Val (lisien en valien weggelaat) aminosuurbehandelinge was geselekteer vir opvolgende eksperimente aangesien THI4 die beste groei getoon het onder hierdie kondisies.

‘n Afname in temperatuur asook pH het die groei en appelsuurmetabolisme van B038 vertraag, maar in die teenwoordigheid van THI4 het die groei van B038 verbeter. Inteendeel, onder hierdie toestande het THI4 nogsteeds vertraagde groei en suikermetabolisme getoon. Die groei van THI4 het egter aansienlik verbeter wanneer die gis en bakterium by ‘n soortgelyke biomassa geïnokuleer was in plaas van soortgelyke selkonsentrasies. Vertraagde groei was verwag vir THI4 en B038 in die afwesigheid van hul ouksotrofiese aminosure en die ondersteuning van hul vennoot. Dit was waargeneem vir al die aminosuurbehandelinge behalwe vir B038 in monokultuur in die afwesigheid van lisien en valien. Die bakterium se vermoë om in hierdie toestande te groei kan moontlik toegeskryf word aan die gesamentlike opname van glutamien en uitvoer van γ-aminobottersuur (GABA), maar verdere eksperimentele werk word vereis om die hipotese te bevestig.

Tydens aaneenlopende ko-fermentasie rondtes het THI4 se glukosemetabolisme aansienlik versnel, terwyl geen merkwaardige veranderinge waargeneem was vir B038 nie. Dit moet nog

bepaal word of etanol produksie moontlik toegeneem het weens die toename in glukosemetabolisme. Hierdie studie het interessante aspekte uitgelig in die industriële belangrike interaksie tussen *S. cerevisiae* en *Lb. plantarum.* Die resultate wat deur hierdie studie gegenereer is bied ‘n basis vir toekomstige werk om gis-bakterium pare te optimiseer vir industriële gebruik in wynfermentasies, asook om die genetiese veranderinge te bestudeer wat betrokke is in die vestiging van mutualistiese interaksies tussen spesies.

This thesis is dedicated to my father

Dr Pieter Christiaan du Toit, MD

“And when the night is cloudy
there is still a light that shines on me
shine until tomorrow
let it be”
-*The Beatles*

Biographical sketch

Sandra Christine du Toit was born on 7 February 1991 in the Free State. She matriculated from Paarl Girls' High School in 2009 and began studying at Stellenbosch University in 2012. Christine completed her BSc-degree (Molecular Biology and Biotechnology) in 2014. In 2015, she obtained her HonsBSc-degree (Wine Biotechnology) *cum laude* at the Institute for Wine Biotechnology where she continued studying towards her MSc in Wine Biotechnology in 2016.

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Preface

This thesis is presented as a compilation of 4 chapters. Each chapter is introduced separately and referencing is done according to the style of the International Journal of Food Microbiology.

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General introduction and project aims

Chapter 1 – General introduction and project aims

1.1 Introduction

Cooperative interactions between microorganisms are ubiquitous in nature and play a vital role in numerous environmental and industrial processes, including, but not limited to, environmental remediation, wastewater treatment, and food and beverage production (Brenner et al., 2008). Different species living in persistent physical contact with one another in symbiotic communities interact with each other in various ways (Thompson, 1999). Generally, species in microbial symbioses are physiologically coupled where chemical compounds, such as vitamins and amino acids, produced by one partner are used by another (Hoffmeister and Martin, 2003). Numerous symbiotic relationships between various species of bacteria, yeasts, and fungi have been described and studied, but how these associations form between previously free-living organisms is still poorly understood.

It is possible that novel symbioses may arise in nature through chance ecological encounters within a particular environment between species with complementary inherent traits (Hom and Murray, 2014). The interaction between these complementary species may provide the microbial partners with a competitive advantage which increases their chances of survival and allows them to grow within an environment not favorable to each species individually. Novel symbioses may also arise within a population through the coevolution of species (Thompson, 1999). Due to changes in population interactions, new adaptations are constantly evolving within species. These evolved species exert selection pressure on other species within the population which helps facilitate the evolution of reciprocal adaptations. Mutualistic symbioses are therefore central to the evolution of species and ecosystems. Consequently, research has been focused on both understanding and inducing these relationships between species (Shou et al., 2007; Brenner et al., 2008; Biliouris et al., 2012; Hom and Murray, 2014).

Interest in the cooperative interactions between species has led to the development of a new field of research aimed at creating synthetic microbial consortia programmed to behave in a desired manner, namely synthetic ecology (Brenner et al., 2008). Members of consortia are able to communicate with one another which enable them to perform complex functions through the division of labor. One way of engineering cooperation between organisms is through targeted genetic modifications (Shou et al., 2007). However, a relatively simpler approach was taken by Hom and Murray (2014) to establish an obligate mutualistic relationship between a yeast and alga. They selected organisms with inherent traits which complemented each other and established a cooperative interaction between them when grown under selective conditions. This study highlights the relative ease with which mutualistic symbioses can be established between species when they

are grown under conditions which force them to depend on one another. This could serve as the basis for establishing cooperative interactions between other species, such as *Saccharomyces cerevisiae* and *Lactobacillus plantarum* under oenological conditions.

S. cerevisiae is the principal yeast responsible for alcoholic fermentation (AF) during the vinification process, where sugars in the grape must are converted to ethanol and CO_2. Most red wines and some sparkling and full-bodied white wines undergo a secondary fermentation after the completion of AF, namely malolactic fermentation (MLF), where L-malic acid is converted to L-lactic acid by lactic acid bacteria (LAB) such as *Lb. plantarum* (Costello et al., 2003). MLF is important for deacidification, enhancing the microbial stability of wine, and has an effect on wine quality due to the development of flavour compounds.

Winemakers do however face uncertainty in obtaining successful and timely completion of MLF since LAB develop in physicochemically harsh conditions, such as low pH, high ethanol concentrations, the presence of sulfur dioxide, and low nutritional status, which may result in MLF failure (Alexandre et al., 2004). The success of MLF is also dependent on the yeast strain used, as certain strains have been observed to have a stimulatory or inhibitory effect on the growth of LAB and their malolactic activity (Liu et al., 2016). One feasible option to overcome these difficulties is through simultaneous inoculation of *S. cerevisiae* and *Lb. plantarum* as it is expected that the bacteria may adapt better to environmental conditions in grape must rather than wine (Guzzon et al., 2013). Studies have shown that simultaneous inoculation does not negatively affect the quality of wine or the successful completion of AF and also greatly reduces the overall fermentation duration (Jussier et al., 2006; Knoll et al., 2012; Guzzon et al., 2013; Tristezza et al., 2016). However, co-inoculation does not reduce the negative impact of inhibitory yeasts on the growth and malolactic activity of sensitive bacterial strains. Overcoming this problem could be achieved through various strategies, including, but not limited to, selection of compatible strains, mutagenesis, genetic engineering, and directed evolution.

Species have the capacity to acquire traits through evolutionary processes that increase their survival fitness if they are subjected to appropriate selective pressure (Harris et al., 2009). Various physical and chemical selective drivers can be used to direct the adaptation of a species to a particular environment, while biotic drivers may enhance a sensitive species' resistance to the inhibitory effects of another species in the environment. Coevolving complementary strains of *S. cerevisiae* and *Lb. plantarum* under oenological conditions may therefore increase the bacteria's resistance to the inhibitory mechanisms of the yeast and also establish a yeast-bacteria pairing capable of successfully completing AF and MLF in a cost and time efficient manner. It is still poorly understood how mutualistic associations form between organisms. Therefore, an increased understanding of the relationship between *S. cerevisiae* and *Lb. plantarum* will help in the

exploitation of these organisms for commercial applications and will also contribute to understanding mutualistic associations in general.

1.2 Project aims and objectives

The main aim of this project is to establish a co-dependent, mutualistic relationship between *S. cerevisiae* and *Lb. plantarum* centered on the reciprocal exchange of amino acids, in order to gain insights into the cooperation between the species. The study also aims to investigate how this relationship evolves over time under selective conditions. The main objectives are as follows:

1. Determine which selective conditions promote the best co-dependent growth of *S. cerevisiae* and *Lb. plantarum* strains with compatible auxotrophic traits;

2. Investigate the effect pH, temperature, and yeast-bacteria cell ratio has on the established relationship;

3. Coevolve *S. cerevisiae* and *Lb. plantarum* over continuous rounds of fermentation and;

4. Ascertain if any observed phenotypes of the evolved strains remain stable when the selective conditions are removed.

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Chapter 2

Literature review

Symbiotic microbial communities: Understanding interactions through natural and synthetic ecological systems with applications for winemaking

Chapter 2 – Symbiotic microbial communities: Understanding interactions through natural and synthetic ecological systems with applications for winemaking

2.1 Introduction

In nature, microorganisms can be found living within complex symbiotic communities where different species are constantly interacting with each other. Members of one species often have complex interactions with members from multiple other species within the community which can have both positive and negative implications for the partners involved in the interaction. Numerous examples of microbial symbioses have been researched and some interesting interactions between yeast and bacteria can be found in the production of a number of fermented food and beverage products, such as sourdough, Kombucha and wine.

Sourdough is a mixture of water and flour that is fermented by metabolically active yeast and lactic acid bacteria (LAB) strains originating from an inoculated starter culture or natural contaminants in the flour (De Vuyst and Neysens, 2005). Sourdough is an intermediate product used in the production of a number of bakery products, such as sourdough breads and sweet leavened baked goods, such as Panettone, Pandoro and Colomba cakes traditionally prepared in Italy. The LAB typically found in sourdough are Lactobacilli, most frequently *Lactobacillus sanfranciscensis, Lb. plantarum* and *Lb. brevis* while *Leuconostoc* spp. and *Enterococcus* spp. are also occasionally found (Gobbetti, 1998; De Vuyst and Neysens, 2005; Minervini et al., 2012; Venturi et al., 2012; Lattanzi et al., 2013; De Vuyst et al., 2014). Several yeast species occur within sourdough, especially species of the genera *Saccharomyces* and *Candida* such as *S. cerevisiae* and *C. humilis.* The microbial community in sourdough is responsive to different factors including, but not limited to, pH, leavening and storage temperature, back slopping, use of baker's yeast as inoculum, as well as the chemical and enzymatic composition of the flour used (Lattanzi et al., 2013; Gänzle, 2014). Additionally, the cooperative activity between the yeast and LAB communities, such as competition for carbohydrates and nitrogen compounds, production of carbon dioxide (CO_2) and other volatile compounds, as well as the production of antimicrobial products, play an important role in the organoleptic and nutritional properties of the sourdough bakery products (Gobbetti et al., 1994; Gobbetti, 1998). Traditionally, back slopping is used to keep the dominant microorganisms in the sourdough active (Lattanzi et al., 2013). During this process a fraction of the previous fermentation batch is used as a starter culture for the new fermentation batch. This practice is also predominantly used in the production of Kombucha.

Kombucha is a slightly sweet and acidic beverage that has been consumed in the East for thousands of years and is gaining popularity in the West due to the various health benefits claimed

to be linked to its consumption (Dufresne and Farnworth, 2000; Teoh et al., 2003; Kallel et al., 2012; Kozyrovska et al., 2012; Jayabalan et al., 2014). The carbonated beverage is produced by fermenting sweetened black or green tea with a symbiotic colony of bacteria and yeasts (SCOBY) which is also known as a tea fungus. The SCOBY is a cellulosic pellicle containing various yeast and bacteria species which floats on the tea broth. After completion of the fermentation process, the SCOBY is removed from the Kombucha beverage and reused for subsequent fermentations. This allows the SCOBY to thicken and grow over time due to the addition of a new cellulose layer during each round of fermentation. The exact microbial composition of Kombucha varies and is dependent on the source of the SCOBY (Jarrell et al., 2000). Bacterial species from the genera *Acetobacter* and *Gluconacetobacter* are commonly isolated from Kombucha, with *Acetobacter xylinum* reported to mainly be responsible for the production of bacterial cellulose while *Gluconacetobacter* spp. are responsible for the production of the compounds d-saccharic acid 1,4 lactone (DSL) and glucuronic acid which are claimed to contribute to the health benefits associated with Kombucha consumption and are therefore desired at high concentrations in the beverage (Yang et al., 2010; Jayabalan et al., 2014; Nguyen et al., 2015a, c). In addition to the acetic acid bacteria (AAB), *Lactobacillus* spp. are also frequently identified in Kombucha (Teoh et al., 2003; Marsh et al., 2014) with strains such as *Lb. casei* and *Lb. plantarum* stimulating glucuronic acid production in Kombucha (Yang et al., 2010; Nguyen et al., 2015a, b). Additionally, sucrose concentration, temperature, pH, and duration of fermentation were also shown to have a significant effect on glucuronic acid production (Nguyen et al., 2015a). The yeast population in Kombucha is generally diverse and species of the genera *Brettanomyces/Dekkera, Candida, Hanseniaspora, Mycotorula, Mycoderma, Pichia, Saccharomyces, Saccharomycodes, Schizosaccharomyces, Torulaspora,* and *Zygosaccharomyces* have been reported (Teoh et al., 2003; Jayabalan et al., 2014; Marsh et al., 2014; Chakravorty et al., 2016). Yeast species present in Kombucha are responsible for the conversion of sugars to organic acids, CO_2, and ethanol. AAB subsequently use ethanol to produce acetaldehyde and acetic acid and they also produce cellulose and gluconic acid from yeast-derived glucose (Marsh et al., 2014). The microbial community structure and dynamics in Kombucha play an important role in the chemical composition of the final product (Chakravorty et al., 2016) which is also true for sourdough products (Gobbetti et al., 1994; Gobbetti, 1998) and wine (Pozo-Bayón et al., 2005; Jussier et al., 2006; Knoll et al., 2012; Medina et al., 2013; Tristezza at al., 2016; Cappello et al., 2017).

Wine is a commercially important alcoholic beverage produced through the fermentation of grape must by various *Saccharomyces* and non-*Saccharomyces* yeast species. These indigenous yeasts present in grape must at the start of fermentation are predominantly derived from grape berries and the composition of the yeast community varies greatly between different grape musts (Bisson and Joseph, 2009; Bagheri et al., 2015; Liu et al., 2017). Species belonging to the genera *Hanseniaspora, Metschnikowia* and *Candida* are commonly present in grape must at significant

cell numbers while *S. cerevisiae* strains are initially present at very low levels but tend to outcompete the other yeast species to dominate the later stages of spontaneous fermentations. *S. cerevisiae* has also been shown to suppress the growth of certain non-*Saccharomyces* species such as *Wickerhamomyces anomalus* while positively influencing the growth of other species such as *H. vineae* (Bagheri et al., 2017). Commercial *S. cerevisiae* starter cultures are generally used in winemaking for the successful completion of alcoholic fermentation (AF), where sugars are converted to ethanol. Most red wines and some sparkling and full-bodied white wines also undergo a secondary fermentation, namely malolactic fermentation (MLF), which is performed by inoculated commercial LAB strains or LAB species naturally present in the grape must (Costello et al., 2003). Yeast-bacteria interactions play an important role in the successful completion of both AF and MLF during winemaking and will be discussed in more detail later in this review.

The microbial communities present in and responsible for the production of sourdough, Kombucha and wine highlight the complexity of natural symbiotic communities, how community structure and dynamics play an important role in shaping the final product, as well as how the structure and dynamics change due to varying biological, chemical, and physical factors. Understanding yeast-bacteria interactions is therefore important to better manage the production of these food and beverage products. This review will broadly discuss a few key topics important for understanding microbial interactions as well as the use of synthetic ecological systems to better investigate these interactions. Lastly, yeast-bacteria interactions in wine will be discussed along with strategies that can potentially be used to optimize these interactions.

2.2 Community structure and classification of microbial interactions

Understanding the composition and abundance or richness of individual community members, namely the structure of the community, is important to subsequently understand the function of the community. The functional properties of the microbial community are related to the behaviour of the community, such as how substrates are metabolised and how the community interacts with and responds to changes in its environment (Little et al., 2008). Factors driving the interactions between microorganisms also play a role in shaping the functional properties of the whole microbial community. Therefore, understanding individual microbial interactions within the community is important to ultimately understand the functional properties of the community. Microorganisms can have beneficial (+), detrimental (-), or neutral (0) effects on each other and symbiotic relationships are divided into six different categories (Table 2.1) based on the effects of these pairwise interactions (Song et al., 2014).

Table 2.1 Classification of symbiotic interactions (adapted from Song et al., 2014)

Symbiotic interaction	Nature of pairwise interaction
Mutualism	[+/+]
Commensalism	[+/0]
Parasitism/ Predation	[+/-]
Competition	[-/-]
Amensalism	[0/-]
Neutralism	[0/0]

[+] Positive: beneficial; [-] Negative: detrimental; [0] Null: neutral/ no effect

Mutualisms are interactions where both partners benefit from the relationship and can be further divided into obligate associations, where partners depend on each other for survival and reproduction, and facultative associations, where one or both partners can survive without the other (Little et al., 2008). Many facultative mutualisms involve the exchange of nutritional resources between partners and species can cooperate metabolically if one species is capable of using by-products generated by another or if species have metabolic capabilities which complement each other. Additionally, mutualisms can also be classified as syntrophic interactions. This involves the coupling of metabolic processes between two partners, usually through the transfer of electrons between them, which allows organisms to engage in metabolic activities that would otherwise not be thermodynamically favourable. However, for partners to participate in these cooperative beneficial interactions they have to be in close proximity to each other which can be achieved by the formation of biofilms or aggregates comprised of multiple species. This in turn makes the population vulnerable to invasion by non-co-operator species who are able to exploit and benefit from cooperative interactions between species, but without contributing to the interaction (Xavier, 2011). For example, sucrose hydrolysis by *S. cerevisiae* requires the production of the enzyme invertase which converts sucrose to glucose and fructose that can be used by cells (Gore et al., 2009). This conversion occurs in the periplasmic space of cells and converted sugars are subsequently lost to neighbouring cells. Species incapable of invertase production can therefore benefit from sugars released in the environment without contributing to the conversion. Cooperation between species therefore comes with the risk that non-co-operator species can invade and dominate the population.

2.3 Mechanisms for microbial interactions

Microorganisms interact or communicate with each other through the exchange of molecular and genetic information (Braga et al., 2016). The molecules involved in these interactions, such as metabolites or signalling molecules, are produced in response to biotic or abiotic stimuli in the environment which triggers gene expressions in the individual species present in the symbiotic community. These interactions can be contact-based or contact-independent where physical cell-

cell contact is respectively necessary and unnecessary (Song et al., 2014). Quorum sensing is an example of a contact-independent interaction where microorganisms in a community use signalling molecules to coordinate group behaviours such as virulence and biofilm formation. In contact-based interactions, molecules like DNA and proteins can be transferred from donor to recipient cells through the formation of a conjugation between cells such as in the case of the bacterial type IV secretion systems (Alvarez-Martinez and Christie, 2009).

2.4 Benefits of microbial communities

Microbial communities consisting of multiple species have a more diverse range of genes and metabolic capabilities than single species systems. Two important characteristics of microbial communities are robustness and division of labour (Little et al., 2008; Hays et al., 2015). Robustness refers to the microbial community's ability to survive fluctuations in the environment. Lichens, communities of bacterial, fungal, and algal species, and biofilms, microbial communities that attach to surfaces, are examples of symbiotic communities that exhibit higher resilience to environmental perturbations than the single species found in these communities (Kranner et al., 2005; Zhang et al., 2011; Lee et al., 2014; Perez et al., 2014). Additionally, the large diversity of species present in microbial communities and the ability of species to metabolise by-products produced by partner species increases the functional properties of the community and allows for metabolic labour to be divided across species (Brenner et al., 2008; Mee and Wang, 2012; Steenackers et al., 2016).

2.5 Evolution of microbial interactions

Numerous symbiotic relationships between various species of bacteria, yeasts, and fungi have been described and studied, but how these associations form between previously free-living organisms and the genetic changes involved in their establishment are still poorly understood. Novel symbioses may arise within a particular environment through chance ecological encounters between species with complementary inherent traits (Hom and Murray, 2014). The interaction between these complementary species may provide the microbial partners with a competitive advantage which increases their chances of survival and allows them to grow within an environment not favourable to each species individually (Figure 2.1A). Novel symbioses may also arise through the coevolution of species (Thompson, 1999). Due to changes in population interactions, new adaptations are constantly evolving within species. These evolved species exert selection pressure on other species within the population which helps facilitate the evolution of reciprocal adaptations (Figure 2.1B).

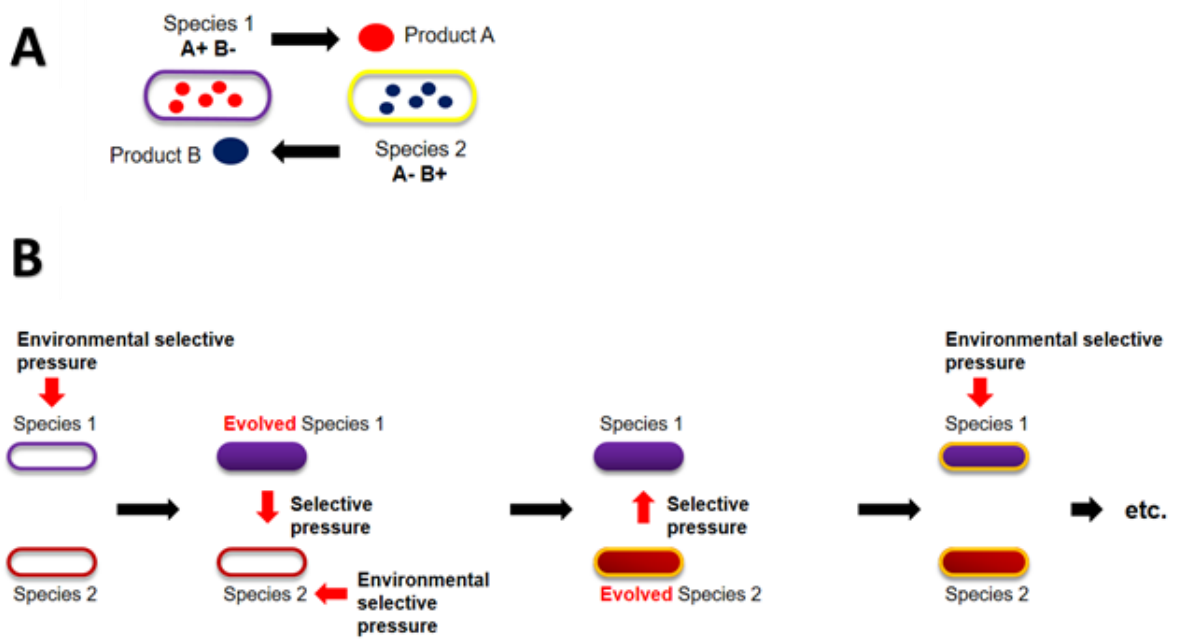

Figure 2.1 Establishment of novel symbiotic interactions through chance ecological encounters between species with complementary inherent traits (A) and through the coevolution of species where biotic and abiotic selective pressures causes genetically based adaptations in one species which then invokes reciprocal genetic changes in another species (B). Image A adapted from Hays et al. (2015).

Understanding why species cooperate with other species in their environment has been extensively researched. In 1964, Hamilton formulated a model, known as Hamilton's rule, based on three factors vital for cooperative interactions between species, namely the fitness cost to the actor, the fitness benefit to the recipient, and the relatedness between the actor and recipient (Hamilton, 1964a, b). Since then, Hamilton's rule has been extensively debated and reviewed (Xavier, 2011; van Veelen et al., 2017) and cooperative behaviors are now believed to evolve if they directly benefit the actor along with the recipients or if they benefit individuals related to the actor (Smukalla et al., 2008). The main driver for evolutionary change is still being debated and different models have been proposed that hypothesize the dominant driving factors, abiotic or biotic, for the evolution of species (Benton, 2009, 2010; Nowak et al., 2010; Morris et al., 2012, 2014; Mas et al., 2016). Additionally, Lehman and Miikkulainen (2015) used computational models to show the importance of extinction events for increasing the evolvability of species. This is due to fierce competition between surviving species to repopulate niches within the environment following the extinction of the majority of the population. One interesting hypothesis that was recently formulated is the Black Queen hypothesis which proposes that dependencies between microorganisms evolves through adaptive gene loss. According to this model, free-living organisms in an environment will lose a certain function if other organisms in the environment publicly and continuously provide for this function (Morris et al., 2012, 2014; Mas et al., 2016). The Black Queen hypothesis was proposed only recently and therefore still needs to be thoroughly tested. Although various hypotheses have been formulated and tested to explain the evolution of social

interactions between species, the genetic changes involved in their establishment still remains elusive. Studying evolutionary changes in natural microbial systems is difficult and model systems are therefore needed that can be rigorously controlled.

2.6 Complexity of microbial communities

Natural microbial communities are generally comprised of multiple species, as previously highlighted. These communities also have high species richness and unevenness and most microorganisms found in nature are unable to be cultured under laboratory conditions (Curtis et al., 2002, 2004). Understanding the structure and function of natural microbial communities is therefore difficult. Culture-dependent methods for species identification and enumeration are only able to describe community members that can be cultured and these species usually represent the minority in microbial communities. Culture-independent techniques, like molecular methods, are able to describe community structure more thoroughly and sequence analysis of the 16S rRNA gene is the most popular method for identifying microorganisms (Curtis et al., 2006). Only small samples of whole microbial communities are analysed and estimates of species richness in communities are then made. This brings with it different challenges due to different species not always being evenly distributed in communities (Curtis et al., 2006). Natural microbial communities are also constantly exposed to changing biological, chemical, and physical factors which has an influence on community structure and function. Additionally, detecting metabolic interactions between species is difficult due to the ubiquitous nature of metabolic exchanges within communities (Ponomarova and Patil, 2015). This degree of complexity makes it difficult to study the ecological and evolutionary patterns of natural microbial communities. Synthetic ecological systems, maintained under controlled conditions, can therefore be used to study microbial community structure and function in order to understand the forces that govern them and make predictions for practical applications in natural communities.

2.7 Understanding microbial interactions through synthetic ecology

Synthetic microbial ecology, as defined by Dolinšek et al. (2016), is focused on designing, building, and analysing the dynamic behaviour of ecological circuits, consisting of a set of interacting microbial genotypes, to understand how community-level properties emerge as a consequence of those interactions. Although synthetic ecology is considered a relatively new field of research, the numerous advances made in the field have already been extensively reviewed (Brenner et al., 2008; Mee and Wang, 2012; Großkopf and Soyer, 2015; Song et al., 2014; Zomorrodi and Segrè, 2015; Dolinšek et al., 2016).

2.7.1 Building microbial communities

There are many factors that need to be considered when building a synthetic interactive system, some of which were highlighted in this review, such as the structure and function of the system, the type of interactions, and the mode of interaction or communication between organisms, but a more thorough review was done by Dolinšek et al. (2016). Two broad approaches can be used to design such a system, namely a bottom-up approach where different organisms with known properties are used to achieve a desired set of interactions or a top-down approach can be used where organisms are selected and randomly paired to obtain a system with certain compositional aspects, for example systems with varying degrees of functional or taxonomic diversity. Additionally, it is important in systems based on cooperative behaviour that the interaction is robust to environmental variations and invasion by exploitative organisms (Wintermute and Silver, 2010). Principles of synthetic biology can also be used to create genetically modified organisms for applications in synthetic ecology systems.

2.7.2 Synthetic microbial systems

Various synthetic microbial interactive systems have been successfully engineered, such as the complex predator-prey system where two genetically engineered *Escherichia coli* populations were able to control the survival and death of their partners through different quorum sensing signals (Balagaddé et al., 2008). Simpler systems have also been engineered based on the reciprocal exchange of metabolic compounds between yeast and cyanobacteria species (Li et al., 2017), yeast and LAB species (Ponomarova et al., 2017), and between auxotrophic *E. coli* mutants that were able to complement the growth of their partners by providing them with essential metabolites (Wintermute and Silver, 2010). Wintermute and Silver (2010) also found that the metabolites most readily exchanged between mutants were the metabolites with the lowest production cost to the producing strain. However, Harcombe (2009) was able to evolve a *Salmonella* strain to excrete a costly amino acid in order to improve the growth of an *E. coli* mutant unable to synthesize the amino acid. This cooperative interaction was only maintained when *Salmonella* was able to consume the metabolic waste from *E. coli* in lactose media.

Shou et al. (2007) were able to engineer two auxotrophic *S. cerevisiae* strains to be dependent upon one another for survival. One strain overproduced lysine, but was unable to synthesize adenine and the other strain overproduced adenine, but was unable to synthesize lysine (Figure 2.2A). Amino acid synthesis was delayed until near death when strains were grown in monoculture, but the mutualism between the strains was successful when they were grown in co-culture. The engineered strains adapted to co-culture conditions over time and showed resilience to reductions in population density when cells were diluted and regrown in new media.

Hom and Murray (2014) were also able to establish an obligate mutualistic relationship between the yeast *S. cerevisiae* and the alga *Chlamydomonas reinhardtii*, but without any genetic modifications to either organism. The yeast and alga both had inherent traits which complemented each other and established a cooperative interaction between them. *S. cerevisiae* provided the alga with a carbon source, in the form of carbon dioxide (CO_2) obtained through glucose metabolism, while *Ch. reinhardtii* provided the yeast with a nitrogen source, in the form of ammonia (NH_3) obtained through nitrite reduction (Figure 2.2B). This study highlights the relative ease with which mutualistic symbioses can be established between species when grown under conditions which force them to depend on one another.

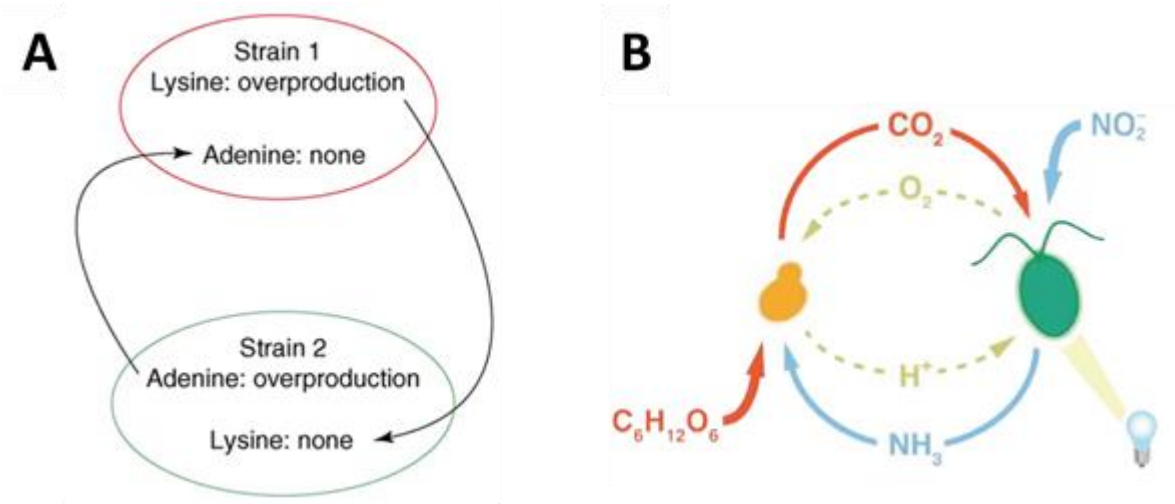

Figure 2.2 Synthetic mutualistic relationships maintained through the reciprocal exchange of amino acids (A), as established by Shou et al. (2007), and through the exchange of carbon dioxide and ammonia (B), as established by Hom and Murray (2014). Image A obtained from Brenner et al. (2008).

Klitgord and Segrè (2010) used computational models which indicated that environmental fluctuations may be able to induce symbiotic interactions between microorganisms more successfully than genetic modifications to the microbes. In a study by Hu et al. (2010), two *E. coli* populations were genetically engineered to use quorum sensing signals to increase antibiotic resistance gene expression in their partner when co-cultured in the presence of ampicillin and kanamycin. This study showed that changing environmental factors, like different antibiotic concentrations and initial cell densities, influenced the interactive dynamics between the populations, resulting in extinction, obligatory and facultative mutualisms, and commensalism. This highlights the importance of community robustness in maintaining cooperative interactions.

2.8 Yeast-bacteria interactions in wine and strategies for their improvement

S. cerevisiae is arguably the most important yeast species involved in the winemaking process due to its strong fermentative capabilities and role in completing AF. Malolactic fermentation is a

secondary fermentation performed by LAB, usually after the completion of AF, during the production of most red wines and some sparkling and full-bodied white wines (Costello et al., 2003). Traditionally, *Oenococcus oeni* strains are preferred for MLF since *Lactobacillus* and *Pediococcus* species have been reported to negatively affect the sensory characteristics of wine (Cappello et al., 2017). However, *Lb. plantarum* strains are being commercialised as MLF starter cultures due to their potential use in co-inoculation with *S. cerevisiae* and their potential to positively affect the organoleptic properties of wine through the production of a number of enzymes (du Toit et al., 2011; Bartowsky et al., 2015; Cappello et al., 2017). The species is also frequently isolated from grapes and in wine, where it is involved in spontaneous MLF. As mentioned above, MLF is traditionally performed after the completion of AF by inoculated commercial LAB strains or species naturally present in the grape must (spontaneous MLF). This practice is associated with the possibility of obtaining sluggish or stuck MLF since LAB develop in physicochemically harsh conditions, such as low pH, high ethanol concentrations, the presence of sulphur dioxide (SO_2), and low nutritional status (Alexandre et al. 2004). Simultaneous inoculation of *S. cerevisiae* and LAB can be used to overcome these difficulties since LAB are expected to adapt better to environmental conditions in grape must rather than wine (Guzzon et al., 2013). Studies have shown that simultaneous inoculation does not negatively affect the quality of wine or the successful completion of AF and also greatly reduces the overall fermentation duration (Jussier et al., 2006; Knoll et al., 2012; Guzzon et al., 2013; Tristezza et al., 2016).

However, the success of MLF is also dependent on the yeast strain used, as certain strains have been observed to have a stimulatory or inhibitory effect on the growth of LAB and their malolactic activity (Alexandre et al., 2004; du Toit et al., 2011; Liu et al., 2016, 2017). Yeast strains can inhibit the growth of LAB through the production of ethanol, SO_2, medium-chain fatty acids (MCFAs), and antimicrobial proteins. While yeast strains can stimulate LAB growth and MLF through the release of nutrients, such as nitrogenous compounds, during yeast autolysis as well as the production of mannoproteins, which are able to absorb MCFAs and enhance the nutritional content of wine when hydrolysed by LAB. It has also been reported that *Lactobacillus* species can inhibit yeast growth and AF through the production of acetic acid and other short-chain carboxylic acids. Certain LAB species are also capable of producing extracellular β-1,3-glucanase enzymes and bacteriocin-like compounds which can potentially inhibit yeast growth (Liu et al., 2017). Furthermore, nutrient competition is another factor which could inhibit the growth of both yeast and LAB species.

In addition to selecting compatible *S. cerevisiae* and LAB strains for successful AF and MLF, recombinant and non-recombinant methods can be used to improve and optimise strains for application under winemaking conditions (Sumby et al., 2014; Betteridge et al., 2015). Recombinant methods are generally focused on the addition or deletion of specific genes and require prior knowledge regarding the genome of the targeted organism. Multiple genes in *O. oeni*

are involved in stress resistance during MLF which makes targeted genetic manipulation difficult and risky since the modification of a single gene could have a negative effect on the overall ability of the species to survive other stresses. Non-recombinant methods such as directed evolution and coevolution of strains offer a potential alternative to genetic manipulation for strain improvement, as these methods are relatively simple and no prior genetic information is required. Other non-recombinant methods such as mutagenesis and genome shuffling can also be used (Betteridge et al., 2015), but will not be focused on in this review.

2.8.1 Strain selection

Commercial yeast strains can be classified as MLF positive (*MLF*+) or MLF negative (*MLF*-) depending on the respective stimulatory or inhibitory effects they have on MLF (Liu et al., 2017). Costello et al. (2003) developed a standardised methodology to test the intrinsic effects of wine yeast strains on LAB and their subsequent MLF activity. In order to determine if the yeast strain tested had a positive or negative effect on the tested LAB strain, the growth of the bacterial strain was compared in synthetic wine and synthetic reference wine with similar compositions. The synthetic wine was prepared by fermenting synthetic grape juice with the yeast strain being tested for LAB compatibility while the synthetic reference wine was chemically prepared and not fermented by any yeast strains. Although this methodology enables identification of compatible yeast and bacteria pairings, it does not provide any information on MLF biomarkers responsible for the observed phenotypes. Liu et al. (2016) used a non-targeted metabolomics approach to compare the extracellular metabolic profiles of *MLF*+ and *MLF*- yeast strains in order to identify these stimulatory and inhibitory MLF biomarkers. D-gluconic acid, trehalose, and citric acid were identified as compounds produced by *MLF*+ yeast strains while sulphur-containing peptides were found to be associated with *MLF*- yeast strains. These compounds can potentially be used in future work to screen yeast and LAB strain compatibility.

Although *S. cerevisiae* is still the predominant yeast species used for wine inoculations, interest is growing in the potential use of commercial non-*Saccharomyces* species like *Torulaspora delbrueckii* and *Metschnikowia pulcherrima* in sequential inoculations with *S. cerevisiae* in order to enhance the positive organoleptic properties of wine (de Koker, 2015). Inhibition of MLF activity by non-*Saccharomyces* species was shown to be strain specific when used in sequential inoculation with a commercial *O. oeni* strain (du Plessis et al., 2017). Nutrient depletion played a role in this inhibition for some non-*Saccharomyces* strains, while some strains likely produced other compounds that inhibited MLF. A more complex yeast consortium comprised of different wine related species was also investigated that could potentially be used in future for wine production (Bagheri et al., 2017). The synthetic yeast consortium provided valuable insights regarding the

dynamics of yeast ecosystems. It would be interesting to still investigate the effect this yeast consortium has on stimulating or inhibiting the growth and MLF activity of LAB.

2.8.2 Directed evolution and coevolution

Directed evolution (DE) or adaptive evolution is based on the ability of an organism to adapt to stress conditions through genome-wide mutations selected for by a gradual increase in the specific stress condition (Dragosits and Mattanovich, 2013; Sumby et al., 2014). Different *Lactobacillus* species (Teusink et al., 2009; Zhang et al., 2012; Wu et al., 2014) and a *Lactococcus lactis* strain (Bachmann et al., 2012) have previously been successfully modified through DE and the ethanol tolerance and MLF activity of an *O. oeni* culture has also been shown to significantly improve after the bacterial population was exposed to increasing ethanol concentrations over numerous generations (Betteridge et al, 2017). Additionally, DE has also been successfully used to improve stress tolerance in wine yeast species (McBryde et al., 2006; Tilloy et al., 2014). These studies highlight the value of using DE as a method to improve and optimize different yeast and bacterial strains. Although abiotic factors were previously used to drive the adaptation of the species, biotic factors can also be used. DE can therefore be used to increase the resistance of wine related yeast and bacteria to the negative effects of antagonistic strains as well as the harsh conditions associated with winemaking. Zhou et al. (2017) were able to direct the evolution of *Lachancea kluyveri* by using different bacterial strains with increasing tolerance to ethanol concentrations in competitive co-culture with the yeast. Over approximately 960 generations, *L. kluyveri* adapted by rapidly consuming glucose and converting it to ethanol in a likely attempt to outcompete the bacterial strains by increasing the ethanol in the environment which is toxic to the bacteria. Interactions between species and the strength of the selective pressures exerted on species therefore play vital roles in driving the coevolution of reciprocal adaptations within populations. However, in order for two species to truly coevolve there needs to be a degree of dependency between the species in order to ensure species are not driven to extinction by their evolutionary partners.

2.9 Conclusions

Symbiotic microbial communities present in natural environments consists of a complex, interactive network of various species. Deciphering this network is challenging and synthetic ecology provides a means of achieving this task. Understanding the forces that govern microbial community structure and function is important in order to subsequently make predictions for practical applications in natural communities. The back slopping techniques used in the production of sourdough and Kombucha allow the different species in the respective microbial communities to coevolve, leading to the adaptation and specialization of these communities for sourdough and Kombucha production respectively. The principles of back slopping and synthetic ecology can be

used to improve and optimise the interaction between *S. cerevisiae* and LAB wine strains to ensure successful and timely completion of alcoholic and malolactic fermentation. Selecting compatible yeast and bacteria strains is an important winemaking practice and directed evolution experiments have already proven successful in improving both yeast and bacterial strains. However, using a combination of these strategies could potentially provide the most effective method for optimizing wine strains. Selecting yeast and bacteria strains with specific characteristics, either compatible or antagonistic, and then allowing them to coevolve under oenological conditions could potentially establish desirable yeast-bacteria pairings. These pairings could be suitable as commercial starter cultures for simultaneous alcoholic and malolactic fermentation. Coevolving yeast and bacteria strains under oenological conditions can also improve the strains' resistance to biotic inhibitory mechanisms.

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Chapter 3

Research results

Induced cooperation between *Saccharomyces cerevisiae* and *Lactobacillus plantarum*: Investigating the impact of environmental parameters and the evolvability of the interaction

Chapter 3 – Induced cooperation between *Saccharomyces cerevisiae* and *Lactobacillus plantarum*: Investigating the impact of environmental parameters and the evolvability of the interaction

3.1 Introduction

Grape juice contains a complex, interactive microbial community of various yeast and bacteria responsible for its biochemical conversion to wine. Initially, various non-*Saccharomyces* yeast species are present, some of which may contribute to the organoleptic properties of the wine, but *Saccharomyces cerevisiae* outcompetes these species to dominate the later stages of a spontaneous fermentation (Bisson and Joseph, 2009; Bagheri, 2014; Liu et al., 2017). *S. cerevisiae* is therefore the primary yeast species responsible for the completion of alcoholic fermentation (AF), where sugars such as glucose and fructose present in the grape must are converted to ethanol. During AF, the ethanol concentration increases and many nutrients are depleted by the yeast. This creates a harsh environment for lactic acid bacteria (LAB) to subsequently grow in and complete a secondary fermentation, where malic acid is converted to lactic acid, known as malolactic fermentation (MLF) (Alexandre et al., 2004). *Oenococcus oeni* is the primary species used in commercial starter cultures to initiate MLF, but research has shifted to other LAB species like *Lactobacillus plantarum* as possible alternatives (du Toit et al., 2011).

Simultaneous inoculation of yeast and LAB can possibly overcome some of these difficulties since LAB are expected to adapt better to environmental conditions in grape must rather than wine (Guzzon et al., 2013). However, this practice results in the yeast and bacteria forming biomass and being metabolically active while in direct contact with each other. Nutrient competition is therefore a factor which could inhibit the growth of these organisms. LAB have limited biosynthetic capabilities and are auxotrophic for various amino acids and vitamins (Terrade and Mira de Orduña, 2009; du Toit et al., 2011). In co-culture, MLF may be inhibited if the yeast rapidly depletes the nutrients or AF may be sluggish if the LAB use up trace elements and other survival factors (Bayrock and Ingledew, 2004; Liu et al., 2017). Competition for biochemical compounds between yeast and LAB is however not yet fully understood (Liu et al., 2017).

Numerous factors affect the growth of *S. cerevisiae* and LAB during fermentation, including pH, temperature, and nitrogen availability. Additionally, some interactions between the species such as physical contact between cells and release of extracellular compounds have also been reported (Alexandre et al., 2004; Liu et al., 2017). Such interactions can be stimulatory or inhibitory to the growth of the organisms and the successful completion of AF and MLF respectively and are in part dependent on specific strains and not always applicable at species level (Nehme et al., 2008; Liu

et al., 2016). Yeast species can inhibit bacterial growth and MLF through the release of medium-chain fatty acids (MCFAs) into the extracellular environment (Alexandre et al., 2004). MCFAs deprotonate within cells, resulting in intracellular acidification, loss of the transmembrane gradient, and subsequent inhibition of ATPase, an important enzyme for MLF (Tourdot-Maréchal et al., 1999). This inhibition is increased under low pH conditions and in the presence of ethanol and is also dependent on the concentration of individual MCFAs (Capucho and Romão, 1994). *Lactobacillus* species have been reported to inhibit yeast growth and AF through various mechanisms, such as the production of acetic acid and other short-chain carboxylic acids which causes acidification of the yeast intracellular environment and subsequent cell death (Bayrock and Ingledew, 2004). LAB also have extracellular β-1,3-glucanase activity which means that they could potentially degrade yeast cell walls, and have been reported to produce bacteriocin-like compounds capable of potentially inhibiting the growth of other LAB (Guilloux-Benatier et al., 2000; Yurdugül and Bozoglu, 2002; Liu et al., 2017).

Selecting compatible *S. cerevisiae* and LAB strains is therefore important for successful AF and MLF (Costello et al., 2003). To further improve such compatibility, a directed evolution approach has been suggested to improve and optimise strains for application under winemaking conditions by increasing their resistance to the inhibitory effects of antagonistic strains and the harsh winemaking conditions (Sumby et al., 2014; Betteridge et al., 2017).

Here we propose a synthetic ecological approach to better control and optimise the interaction between *S. cerevisiae* and *Lb. plantarum*. *S. cerevisiae* and *Lb. plantarum* strains, auxotrophic for specific amino acids, were grown in a chemically defined synthetic grape juice-like medium under mono- and co-culture conditions. Selected amino acids were omitted from the medium in an attempt to establish a co-dependent, mutualistic relationship between the species. The relationship was investigated under different pH, temperature, and yeast-bacteria cell ratio conditions in order to understand their biochemical interaction. The relationship was also evolved over a number of generations to investigate how the continuous selective pressure might affect the species. A broader understanding of the relationship between *S. cerevisiae* and *Lb. plantarum* could help to improve AF and MLF. Furthermore, this study served as a proof of concept to establish a basis for the coevolution of the species to understand the genetic changes involved in the process.

3.2 Materials and methods

3.2.1 Microbial species and culture conditions

Saccharomyces cerevisiae strain THI4 and *Lb. plantarum* strain B038, obtained from the culture collection of the Institute for Wine Biotechnology (IWBT) at Stellenbosch University, were used for all experiments. THI4 is a deletion mutant (EUROSCARF reference: BY4742) and is unable to

produce lysine and B038 was isolated from South African wine at the IWBT. Previous amino acid auxotrophic screening assays performed on various LAB strains in the IWBT culture collection (unpublished data) indicated that B038 is auxotrophic for isoleucine, alanine, valine, and methionine and prototrophic for all other amino acids listed in Table 3.1, including lysine and glutamine. B038 showed particularly good growth in the absence of lysine. Auxotrophic screening assays for the relevant amino acids were performed for THI4 and B038 as a control measure before experiments were started and PCR amplification of the *recA* gene was done on B038 to confirm the identity of the species.

Strain THI4 was grown at 30ºC in yeast peptone dextrose (YPD) broth and on YPD agar plates (Biolab diagnostics, Wadenville, South Africa) supplemented with 0.025 mg/L chloramphenicol (Roche, Sandton, South Africa) for the inhibition of bacterial growth when appropriate. Strain B038 was grown anaerobically at 30ºC in MRSA broth (MRS broth (Biolab diagnostics) with 20% preservative free apple juice (Ceres fruit juices (Pty) Limited, Paarl, South Africa) and on MRST agar plates (MRS broth with 20 g/L Bacteriological agar (Biolab diagnostics) and 10% preservative free tomato juice (Tiger Food Brands Limited, Sandton, South Africa) supplemented with 0.05 mg/L Delvocid® Instant (DSM Food Specialities, Delft, The Netherlands) which contains 50% natamycin for the inhibition of yeast growth when appropriate. The pH of the MRSA broth and MRST agar plates were adjusted to pH 5.2 using 6 M HCl and the MRSA broth was filter sterilised through a 0.22-µm syringe filter before use. B038 cultures grown on MRST agar plates were anaerobically incubated using anaerobic containers with Anaerocult A (Merck, Darmstadt, Germany) as per the manufacturer's instructions.

Wet stock freeze cultures were prepared for strains THI4 and B038 from cultures grown in YPD and MRSA broth respectively. A single yeast or bacterial colony was used to inoculate 5 ml of broth which was then incubated for 14 h. After the incubation period 1 ml of culture was centrifuged at 6000 rpm for 5 min, the supernatant was discarded, the cells were re-suspended in fresh broth, and 1 ml 80% glycerol was added. The wet stock freeze cultures were stored at -80ºC in order to pre-culture all strains from the same culture stock solution.

3.2.1.1 Amino acid auxotrophic screening assay

Synthetic grape juice-like (SGJ) agar plates, containing 2% agar, were prepared in order to evaluate the growth of strains THI4 and B038 in the absence of lysine, isoleucine, alanine, valine, methionine, and glutamine. Seven different amino acid stock solutions were prepared. One stock solution contained the 19 amino acids as listed in Table 3.1 (positive control) and the remaining six stock solutions contained only 18 of the listed amino acids with one of the following amino acids omitted: lysine, isoleucine, alanine, valine, methionine, or glutamine.

SGJ base medium was prepared as described by Bely et al. (1990) and Henschke and Jiranek (1993). The carbon sources, acids, salts, and ammonium sulphate (Table 3.1) were added to distilled water and the pH of the medium was adjusted to pH 3.5 with KOH before being autoclaved. The amino acids, trace elements, and vitamins were filter sterilised using a 0.22-µm syringe filter and added to the SGJ base medium along with the anaerobic factors and 0.02 mg/L uracil. An 8% agar solution was prepared in distilled water with Bacteriological agar (Biolab diagnostics). After the agar solution was autoclaved, the SGJ medium was added to the hot agar and the solution was poured into petri dishes to solidify. The seven amino acid stock solutions were used to prepare seven different batches of SGJ agar plates. The negative control SGJ agar plates contained no amino acids.

Single colonies of strains THI4 and B038, from YPD and MRST agar plates respectively, were streaked out on positive and negative control SGJ agar plates and on the six single amino acid omission SGJ plates. After incubation at 30ºC for 96 h, the growth of strains THI4 and B038 on the single amino acid omission SGJ plates were compared to their respective positive and negative control SGJ plates. All plates were streaked out in triplicate.

Table 3.1 Chemical composition of synthetic grape juice-like medium (SGJ) as formerly described by Henschke and Jiranek (1993) with adjusted nitrogen sources as previously described by Bely et al. (1990). The amount of each component is given as the final concentration per 1 L of SGJ.

		Amount per litre
Carbon sources	Glucose	100 g
	Fructose	100 g
Acids	Potassium L-Tartrate Monobasic	2.5 g
	L-Malic acid	4 g
	Citric acid	0.2 g
Salts	Potassium phosphate dibasic (K_2HPO_4)	1.14 g
	Magnesium sulphate heptahydrate ($MgSO_4.7H_2O$)	1.23 g
	Calcium chloride dihydrate ($CaCl_2.2H_2O$)	0.44 g
Nitrogen sources	Ammonium sulphate	0.3 g
	Amino acids (prepared as 100X stock solution in 20 g/L $NaHCO_3$ buffer solution)	
	- Tyrosine	0.014 g
	- Tryptophane	0.137 g
	- Isoleucine	0.025 g
	- Aspartic acid	0.034 g
	- Glutamic acid	0.092 g
	- Arginine	0.286 g
	- Leucine	0.037 g
	- Threonine	0.058 g
	- Glycine	0.014 g
	- Glutamine	0.386 g
	- Alanine	0.111 g
	- Valine	0.034 g
	- Methionine	0.024 g
	- Phenylalanine	0.029 g
	- Serine	0.060 g
	- Histidine	0.025 g
	- Lysine	0.013 g
	- Cysteine	0.010 g
	- Proline	0.468 g
Trace elements (prepared as 100X stock solution)	Manganese(II) chloride tetrahydrate ($MnCl_2.4H_2O$)	200 µg
	Zinc(II) chloride (ZnCl)	135 µg
	Iron(II) chloride ($FeCl_2$)	30 µg
	Copper(II) chloride ($CuCl_2$)	15 µg
	Boric acid (H_3BO_3)	5 µg
	Cobalt(II) nitrate hexahydrate ($Co(NO_3)_2.6H_2O$)	30 µg
	Sodium molybdate dihydrate ($NaMoO_4.2H_2O$)	25 µg
	Potassium iodate (KIO_3)	10 µg
Vitamins (prepared as 100X stock solution)	Myo-inositol	100 mg
	Pyridoxine hydrochloride	2 mg
	Nicotinic acid	2 mg
	Calcium pantothenate	1 mg
	Thiamin hydrochloride	0.5 mg
	PABA.K	0.2 mg
	Riboflavin	0.2 mg
	Biotin	0.125 mg
	Folic acid	0.2 mg
Anaerobic factors (prepared as 10X stock solution in hot 96% EtOH)	Ergosterol	10 mg
	Tween 80	0.5 ml

3.2.1.2 PCR amplification

The PCR reaction mixture (48 µL) contained 200 µM dNTP's, 0.5 µM planF and 0.5 µM PRev primers (Table 3.2), 1.5 mM magnesium chloride, 1.25 U of Taq DNA polymerase (Promega), Taq buffer, and a single bacterial colony. The thermal cycling conditions were as follows: initial denaturation at 94ºC (5 min); 30 cycles of denaturation at 94ºC (30 s), annealing at 56ºC (10 s), and elongation at 72ºC (30 s); and final extension at 72ºC (5 min). The PCR products were separated on a 2% agarose gel stained with Gelred Nucleic Acid Gel stain® (Biotium, Hayward, CA, USA).

Table 3.2 Species specific primers used in this study to identify *Lb. plantarum.*

Primer name	Gene	Primer sequence (5'3')	Amplicon size (bp)	Reference
planF (fwd)	*recA*	CCGTTTATGCGGAACACCTAA	318	Torriani et al. (2001)
PRev (rev)		TCGGGATTACCAAACATCAC		

3.2.1.3 Determination of growth phases for strains

Growth curves monitored over 48 h in YPD and MRSA broth, for THI4 and B038 respectively, were used to differentiate the growth phases for the strains. Single colonies of strains THI4 and B038, from YPD and MRST agar plates, were used to inoculate 5 ml of YPD and MRSA broth respectively which were then incubated at 30ºC for 16 h. After incubation, the cultures were used to inoculate 50 ml of YPD and MRSA broth at OD_{600} 0.1 (THI4) and 0.01 (B038) respectively. The cultures were sampled after 0, 4, 8, 12, 16, 20, 24, 28, 36, and 48 h. OD_{600} measurements were taken and serial dilutions of the samples were prepared in 0.9% saline solution. Two dilutions for each sample was plated on agar plates to determine CFUs/ml. Biomass was also determined by centrifuging the cells at 6000 rpm for 5 min, discarding the supernatant, and washing the cells twice with sterile water. The pellets were then dried at 60ºC until the weight of the pellets remained constant. All growth curves were performed in triplicate.

3.2.2 Pre-culture and fermentation conditions

Monoculture and co-culture fermentations were all performed in triplicate. The yeast and bacteria strains were pre-cultured by first inoculating a single colony of THI4 and B038 in 5 ml YPD and MRSA broth respectively. The cultures were incubated at 30ºC for 14 h. Thereafter, the cultures were used to inoculate 30 ml of YPD and MRSA broth at OD_{600} 0.1 (THI4) and 0.01 (B038) respectively. These cultures were incubated at 30ºC for 14 h in order to generate cells for inoculation that were in mid-exponential growth phase. Before inoculation, the cells were washed two times and re-suspended in sterile water. THI4 and B038 were not subjected to a starvation

period before inoculation as it was assumed that any amino acids stored within the cells during pre-culturing would be essential for initial growth to establish cooperation between the strains.

All fermentations were performed in glass flasks with fermentation caps containing 80 ml SGJ medium (Table 3.1). The SGJ medium was prepared as previously described except the anaerobic factors were added to each fermentation flask after the medium had been dispensed. The strains were inoculated at OD_{600} 0.1 (± 2E+06 CFUs/ml, THI4) and 0.01 (± 4E+06 CFUs/ml, B038) in both monoculture and co-culture fermentations, the initial pH of the medium was pH 3.5, and all fermentations were incubated at 20ºC, except where stated otherwise. Weight loss was measured every 24 h and was used to calculate CO_2 release. Sampling was done at 0, 48, 120, and 192 h, except where stated otherwise, to measure CFUs/ml as well as glucose, fructose, and malic acid concentrations (section 3.2.3).

3.2.2.1 Amino acid selective conditions

Five amino acid treatments were initially used in order to select two treatments for further experiments. Four of the treatments contained 17 of the 19 listed amino acids (Table 3.1) with lysine and one of the following amino acids omitted: isoleucine, alanine, valine, or methionine. The remaining treatment contained 14 amino acids with the following amino acids omitted: lysine, isoleucine, alanine, valine, and methionine (Table 3.3). The positive and un-inoculated controls contained all 19 amino acids. Fermentations were carried out for 336 h and sampling was done at 0, 48, 120, 192, 240, and 336 h. High performance liquid chromatography (HPLC) was used for the quantification of amino acids for treatment Lys-Ile and Lys-Val as well as the positive control (section 3.2.4). Treatment Lys-Ile and Lys-Val were selected for all other experiments.

Table 3.3 Amino acid treatments

Treatment name	Amino acids omitted	YAN concentration (mg/L)
Control	None	322.48
Lys-Ile	Lysine Isoleucine	317.32
Lys-Ala	Lysine Alanine	302.54
Lys-Val	Lysine Valine	315.93
Lys-Met	Lysine Methionine	317.74
Lys-Ile-Ala-Val-Met	Lysine Isoleucine Alanine Valine Methionine	293.55
Lys-Val-Gln	Lysine Valine Glutamine	241.93

3.2.2.2 Fermentation perturbations

The influence of pH, temperature, and cell ratio was investigated. The effect of pH was investigated at pH 3 and pH 4, temperature at 15 and 25ºC, and cell ratio at an inoculation OD_{600} for B038 at 0.05, 0.1, and 10 and an inoculation OD_{600} for THI4 at 0.007. The initial pH, temperature, and inoculation dosages were the same for these experiments as previously described, except for the single parameter being investigated which was changed as indicated. The effect of lysine addition, to a final concentration of 0.013 g/L, after 48 h of fermentation was also investigated. Lastly, the effect of glutamine omission from the Lys-Val treatment (Table 3.3) was also investigated.

3.2.2.3 Coevolution of yeast and bacteria

THI4 and B038 were grown in co-culture batch fermentations using the treatments Lys-Ile and Lys-Val, in order to coevolve the species. All fermentations were performed in triplicate. Fermentations were carried out for 192 h and sampled at t = 0 h and t = 192 h to monitor cell counts. After 192 h, 20 ml fermentation medium was centrifuged at 6000 rpm for 5 min in order to harvest the yeast and bacteria cells which were then re-inoculated into new SGJ media. The change in cell count was used to determine the average number of generations obtained during one fermentation round. A fermentation round ended after 192 h and a new round started when the cells were re-inoculated, with the first fermentation starting at round 0 (R0).

During round 11 (R11), fermentations were carried out for 336 h and sampling was performed at 0, 48, 120, 192, 240, and 336 h to measure cell counts and glucose, fructose, and malic acid concentrations. Weight loss was also monitored every 24 h and the fermentations were performed in mono- and co-culture conditions using treatments Lys-Ile and Lys-Val, as well as the positive control. YPD and MRST agar plates containing THI4 and B038 colonies from R10 (t = 192 h) were used to pre-culture yeast and bacteria cells which were then inoculated into SGJ media at OD_{600} 0.1 and 0.01 respectively for round 11. THI4 and B038 were pre-cultured by randomly selecting 10 yeast and 10 bacterial colonies which were then inoculated into 10 ml YPD and MRSA broth respectively. This was done separately for each biological repeat and amino acid treatment.

3.2.3 Glucose, fructose, and malic acid measurement

An automated analyser (Konelab Arena 20XT, Thermo Electron Corporation, Finland) was used to enzymatically measure the concentrations of glucose (Enzytec™ Fluid D-glucose Id-No: E5140, Roche, R-Biopharm), fructose (Enzytec™ Fluid D-fructose Id-No: E5120, Roche, R-Biopharm), and L-malate (Enzytec™ Fluid L-malate Id-No: E5280, Roche, R-Biopharm). Malolactic fermentation was considered to be completed if the concentration of L-malate was equal to or less than 0.3 g/L and alcoholic fermentation was considered to go to dryness if the residual sugar concentration was less than 4 g/L.

3.2.4 Quantification of amino acids by HPLC

Amino acids were quantified by high performance liquid chromatography (HPLC), Agilent 1100 (Agilent Technologies, Waldbronn, Germany) by pre-column derivatisation and fluorescence detection based upon a method previously described by Henderson and Brooks (2010). The following modifications were made to the derivatisation and injection: a Zorbax Eclipse plus C18 Rapid Resolution column (4.6 x 150 mm, 3.5 µm particle size; Agilent Technologies) was used following derivatisation of the amino acids. Three different reagents were used to perform derivatisation: iodoacetic acid (Sigma Aldrich) for cysteine, o-phthaldialdehyde (OPA, Sigma Aldrich) for primary amino acids, and fluorenylmethyloxycarbonyl chloride (Sigma Aldrich) for secondary amino acids. Internal standards, norvaline (Sigma Aldrich) and sarcosine (Sigma Aldrich) were spiked to each sample before derivatisation. One millilitre of each filtered sample was used for time point 0 h and 192 h.

3.2.5 Calculation of generations obtained during fermentation

The number of generations obtained during the fermentation period, for all experiments, was calculated according to the following formula:

$$\frac{\ln\left(\frac{End\ point\ CFUs/ml}{Starting\ point\ CFUs/ml}\right)}{\ln 2}$$

Generations obtained was used to compare treatments to each other under both mono- and co-culture conditions in order to determine if the treatment or culture conditions had a significant effect on the growth kinetics of the strains. Statistical analyses were only performed on this data if there were no significant statistical differences between the initial cell counts of the treatments and conditions being compared.

3.2.6 Statistical analysis

Data were analysed by performing a one-way analysis of variance (ANOVA) followed by a Tukey's HSD test using XLSTAT (version 2016.05.33324, Addinsoft). Differences between treatments and culture conditions were considered as significant when the p-values were below 0.05.

3.3 Results

3.3.1 Characterization of strains

S. cerevisiae strain THI4 was confirmed to be auxotrophic for lysine and prototrophic for isoleucine, alanine, valine, methionine, and glutamine. *Lb. plantarum* strain B038 was confirmed to be auxotrophic for isoleucine, alanine, valine and methionine, and prototrophic for lysine and glutamine. PCR amplification of the *recA* gene also confirmed the species identity of this strain (data not shown). THI4 and B038 had relatively similar growth phases when grown in YPD and MRSA broth respectively, but B038 had higher cell numbers and OD values compared to THI4. B038 had a slightly shorter lag phase than THI4, but both strains were in mid-exponential growth phase after approximately 14 h (data not shown). The strains were therefore pre-cultured for 14 h to generate cells for inoculation.

3.3.2 Initial screening for amino acid selective conditions

THI4 and B038 were initially grown under mono- and co-culture conditions for five amino acid treatments as well as a control treatment in order to select two amino acid treatments for further experiments. Amino acids were omitted from the treatments based on the auxotrophic profiles of the strains. Since THI4 is only auxotrophic for one amino acid and B038 is auxotrophic for four, four different combination ratios of amino acids omitted could be tested, namely 1:1, 1:2, 1:3, and 1:4. All possible 1:1 ratios were tested as well as the 1:4 ratio (Table 3.3). The total YAN concentrations of all the amino acid treatments tested ranged between 241.93 and 322.48 mg/L YAN (Table 3.3) which is adequate for *S. cerevisiae* to complete AF (Bely et al., 1990).

Growth kinetics

THI4 showed relatively poor growth under monoculture conditions for all five amino acid treatments when inoculated at approximately 6.30 log CFUs/ml (Figure 3.1), which was expected to occur in the absence of lysine and B038. However, there were some significant differences between the number of generations obtained over the 14-day incubation period for the different treatments, which ranged between 0.72 – 1.47 generations (Table 3.4). For the control, containing the full complement of amino acids, THI4 obtained 2.68 generations by the end of the incubation period. As expected, this was significantly higher than the number of generations obtained for the different amino acid omission treatments (Table 3.4).

THI4 showed relatively poor growth under co-culture conditions as well, for all five amino acid treatments (Figure 3.1). The number of generations obtained by the end of the incubation period ranged between 0.46 – 1.78 for the different treatments (Table 3.4). The highest number of generations obtained under co-culture conditions were for the Lys-Ile and Lys-Val treatments at 1.78 and 1.70 generations respectively. For the control, THI4 obtained 2.78 generations which was not significantly different to the number of generations obtained under monoculture conditions. The number of generations obtained over the 14-day incubation period were significantly different between mono- and co-culture conditions for the Lys-Ile, Lys-Ala, and Lys-Val treatments (Table 3.4). For the Lys-Met and Lys-Ile-Ala-Val-Met treatments, the number of generations obtained between mono- and co-culture conditions were not significantly different.

B038, inoculated at approximately 6.60 log CFUs/ml, showed relatively poor growth under monoculture conditions for the Lys-Ile, Lys-Ala, and Lys-Ile-Ala-Val-Met treatments (Figure 3.1). During the 14 day incubation period, the log CFUs/ml decreased significantly for these three treatments while there was a slight increase in the log CFUs/ml for the Lys-Met treatment and a significant increase for the Lys-Val treatment (Figure 3.1). However, the increase in log CFUs/ml for the Lys-Val treatment was significantly lower than the increase observed under monoculture conditions for the control containing all amino acids.

B038 showed good growth for all five amino acid treatments when co-cultured with THI4 (Figure 3.1). The log CFUs/ml increased significantly within the first two days of the incubation period for all treatments. The number of generations obtained for the Lys-Val, Lys-Ile-Ala-Val-Met, and control treatments were 5.99, 5.41, and 5.60 respectively and were not significantly different from each other (Table 3.4). For the Lys-Ile, Lys-Ala, and Lys-Met treatments, B038 obtained 6.47, 6.37, and 6.27 generations respectively over the incubation period which were significantly higher than the number of generations obtained for the control treatment. The number of generations obtained over the 14-day incubation period were significantly different between mono- and co-culture conditions for all treatments, including the control (Table 3.4).

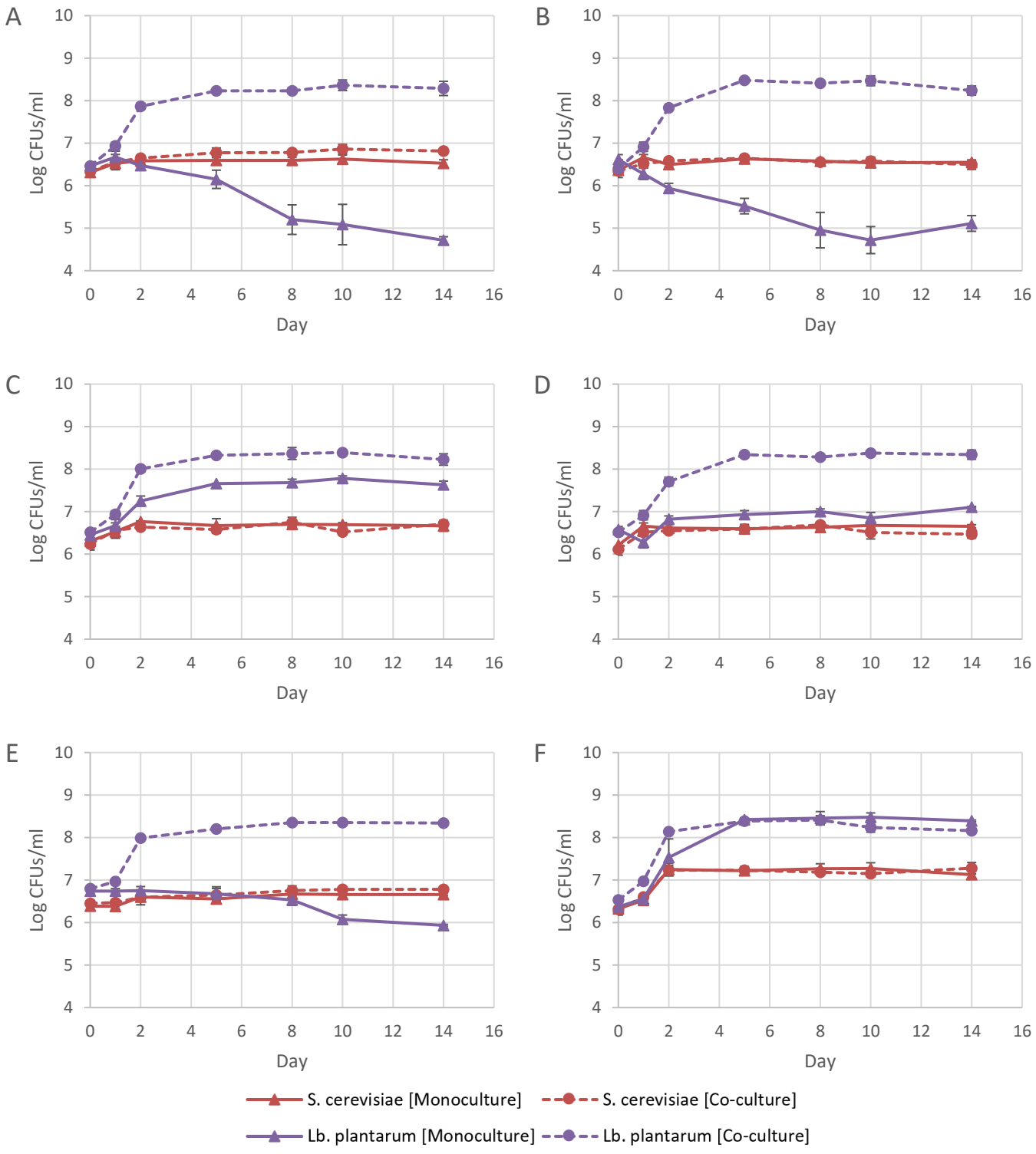

Figure 3.1 Log CFUs/ml for *S. cerevisiae* strain THI4 and *Lb. plantarum* strain B038 over the 14 day incubation period for the Lys-Ile (A), Lys-Ala (B), Lys-Val (C), Lys-Met (D), Lys-Ile-Ala-Val-Met (E), and Control (F) treatments. Data shown are means of triplicates with error bars representing standard deviation.

Table 3.4 Number of generations obtained by day 14 for *S. cerevisiae* strain THI4 and *Lb. plantarum* strain B038 under mono- and co-culture conditions for different amino acid treatments. Data shown are means of triplicates with standard deviation. Means within rows without common uppercase letter are significantly different ($p < 0.05$) and means within columns without common lowercase letter are significantly different for *S. cerevisiae* and *Lb. plantarum* respectively.

	Amino acid treatment					
	Lys-Ile	**Lys-Ala**	**Lys-Val**	**Lys-Met**	**Lys-Ile-Ala-Val-Met**	**Control**
***S. cerevisiae* THI4**						
Monoculture	0.92^{aA}	0.72^{aA}	1.39^{aBC}	1.47^{aC}	1.02^{aAB}	2.68^{aD}
	± 0.12	± 0.07	± 0.06	± 0.18	± 0.15	± 0.20
Co-culture	1.78^{bC}	0.46^{bA}	1.70^{bC}	1.35^{aB}	1.20^{aB}	2.78^{aD}
	± 0.02	± 0.04	± 0.14	± 0.16	± 0.19	± 0.11
***Lb. plantarum* B038**						
Monoculture	-5.56^{aA}	-4.68^{aB}	3.88^{aC}	1.56^{aD}	-2.68^{aE}	6.72^{aF}
	± 0.20	± 0.24	± 0.08	± 0.09	± 0.19	± 0.58
Co-culture	6.47^{bC}	6.37^{bC}	5.99^{bBC}	6.27^{bC}	5.41^{bA}	5.60^{bAB}
	± 0.13	± 0.02	± 0.14	± 0.16	± 0.35	± 0.22

Glucose, fructose and malic acid consumption

THI4 consumed less than 1 g/L of glucose and fructose under monoculture conditions for each of the five amino acid treatments over the 14 day incubation period (Figure 3.2). For the control, THI4 consumed 30.19 g/L glucose and 9.86 g/L fructose. No malic acid was consumed by THI4 in monoculture.

In co-culture, THI4 showed an overall preference for glucose instead of fructose and the amount of sugar consumed for the five amino acid treatments by day 14 ranged from 4.62 – 12.28 g/L for glucose and 1.81 – 6.56 g/L for fructose. THI4 consumed the highest amount of sugar for the Lys-Ile (12.28 g/L glucose, 6.56 g/L fructose) and Lys-Val (11.50 g/L glucose, 4.03 g/L fructose) treatments. For the control, THI4 consumed 39.19 g/L glucose and 12.33 g/L fructose which were slightly higher than the amount of sugar consumed in monoculture.

B038 was unable to complete malolactic fermentation (MLF) by day 14 for the Lys-Ile, Lys-Ala, and Lys-Ile-Ala-Val-Met treatments under monoculture conditions, while MLF was completed by day 8, day 14 and day 5 for the Lys-Val, Lys-Met, and control treatments respectively (Figure 3.3). No glucose or fructose was consumed by B038 in monoculture. Under co-culture conditions, B038 was able to consume all the malic acid present in the medium by day 5 for all five amino acid treatments as well as the control treatment (Figure 3.3).

B038 showed good growth for all five amino acid omission treatments when co-cultured with THI4, while THI4 struggled to grow in these conditions. The Lys-Ile and Lys-Val treatments were

therefore selected for subsequent experiments and as the conditions for the coevolution of the strains, since THI4 showed the best growth for these two treatments. Since THI4 consumed significantly less sugar by day 14 for the 1:4 ratio (Lys-Ile-Ala-Val-Met treatment) compared to the 1:1 ratios (Lys-Ile, Lys-Ala, Lys-Val, and Lys-Met treatments), the assumption was made that a 1:1 ratio of amino acids omitted would promote the best growth for THI4 (Figure 3.2). Therefore, the possible 1:2 and 1:3 ratios were not tested.

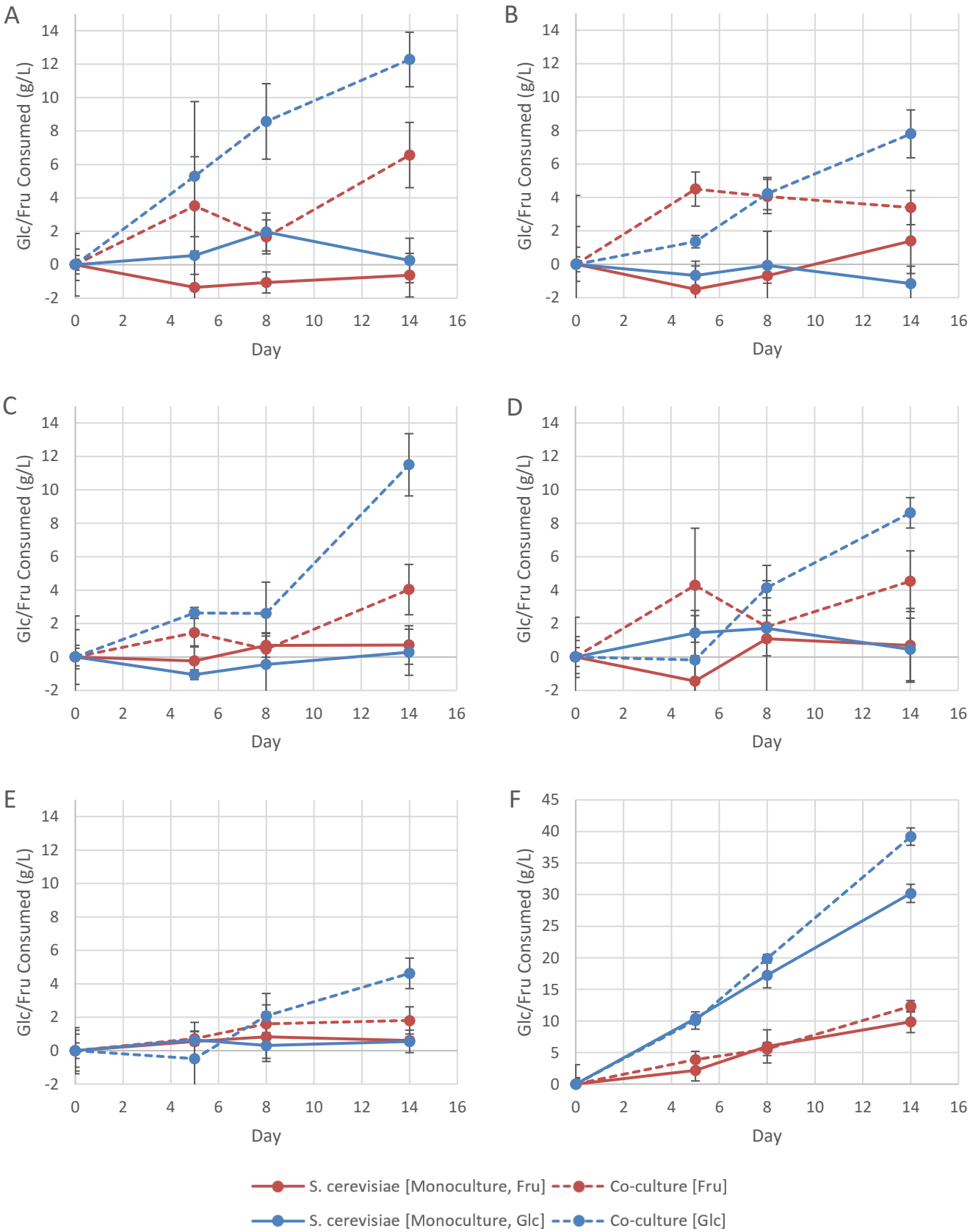

Figure 3.2 Total glucose (Glc) and fructose (Fru) consumed over the 14 day incubation period by *S. cerevisiae* mono- and co-cultures for the Lys-Ile (A), Lys-Ala (B), Lys-Val (C), Lys-Met (D), Lys-Ile-Ala-Val-Met (E), and Control (F) treatments. Data shown are means of triplicates with error bars representing standard deviation.

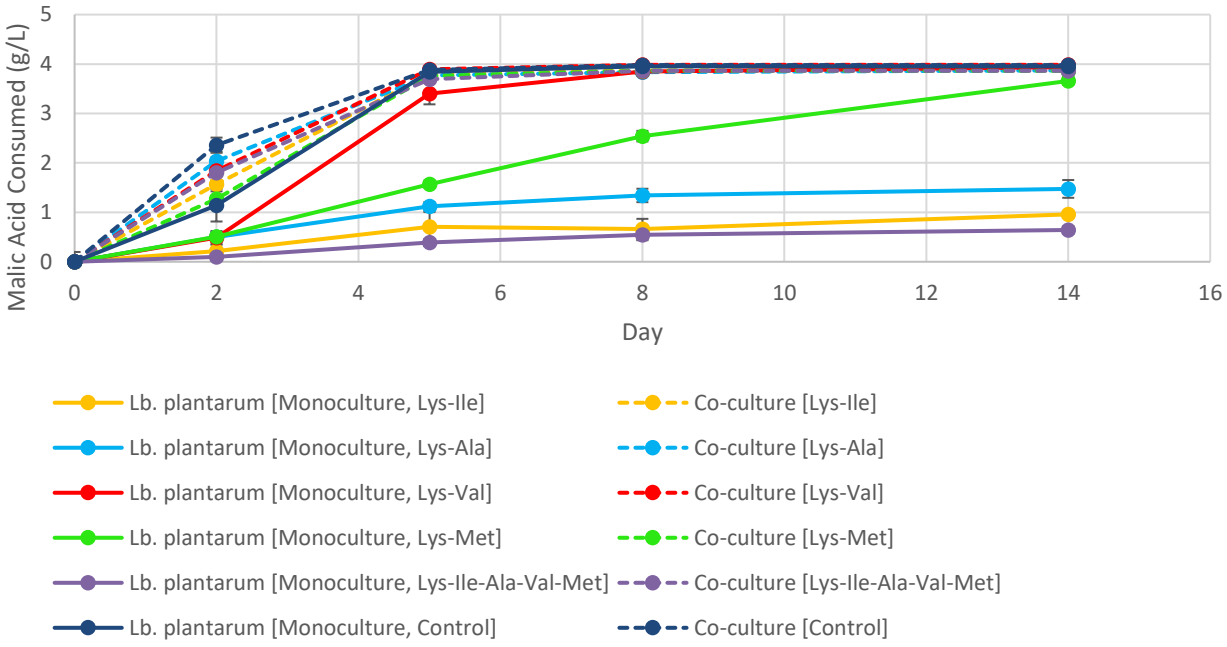

Figure 3.3 Total malic acid consumed over the 14 day incubation period by *Lb. plantarum* mono- and co-cultures for the different amino acid and control treatments. Data shown are means of triplicates with error bars representing standard deviation.

3.3.3 Amino acid uptake

HPLC analysis was used to detect any extracellular lysine and isoleucine in the Lys-Ile treatment, as well as any lysine and valine in the Lys-Val treatment on day 0 (before inoculation) and day 8. None of these amino acids were detected in their respective treatments on day 0, as expected, since they were not added to the treatments. This was done to establish a cooperative interaction between THI4 and B038 based on the reciprocal exchange of amino acids. These amino acids were also not detected in their respective treatments on day 8 (Figure 3.4).

S. cerevisiae THI4 monoculture

When THI4 was grown under monoculture conditions the percentage glutamine (Gln), glycine (Gly), arginine (Arg), alanine (Ala), tryptophan (Trp), and phenylalanine (Phe) taken up differed significantly between the Lys-Ile, Lys-Val, and control treatments (Figure 3.4A). However, for the Lys-Ile and Lys-Val treatments, only tryptophan and phenylalanine had an uptake of more than 20%. For the Lys-Val and control treatments, the uptake of methionine (Met) was similar at 97.88% and 100.00% respectively. For the Lys-Ile treatment, methionine (Met, 55.14%), tryptophan (Trp, 49.82%), and phenylalanine (Phe, 27.23%) had the highest uptake and there was a slight increase in the amount of tyrosine (Tyr), proline (Pro), and valine (Val). For the Lys-Val treatment, methionine (Met, 97.88%), tryptophan (Trp, 60.32%), and phenylalanine (Phe, 21.70%) also had the highest uptake and there was a slight increase in the amount of glutamine (Gln), glycine (Gly),

alanine (Ala), tyrosine (Tyr), and proline (Pro). For the control treatment, methionine (Met, 100.00%), tryptophan (Trp, 93.69%), and lysine (Lys, 80.50%) had the highest uptake.

Lb. plantarum B038 monoculture

When B038 was grown under monoculture conditions the percentage aspartic acid (Asp), glutamic acid (Glu), threonine (Thr), arginine (Arg), tyrosine (Tyr), and phenylalanine (Phe) taken up differed significantly between the Lys-Ile, Lys-Val, and control treatments (Figure 3.4B). However, for the Lys-Val treatment, only tyrosine and phenylalanine had an uptake of more than 20%. All the previously mentioned amino acids had an uptake of less than 20% for the Lys-Ile treatment. For the Lys-Val and control treatments, glutamine (Gln) and methionine (Met) had an uptake of 100.00%. For the Lys-Ile treatment, glutamine (Gln, 25.74%), methionine (Met, 55.77%), and tryptophan (Trp, 36.21%) had the highest uptake. For the Lys-Val treatment, glutamine (Gln, 100.00%), methionine (Met, 100.00%), and tryptophan (Trp, 35.37%) also had the highest uptake and there was a slight increase in the amount of glutamic acid (Glu). For the control treatment, glutamine (Gln, 100.00%), methionine (Met, 100.00%), and tryptophan (Trp, 80.97%) also had the highest uptake.

S. cerevisiae and Lb. plantarum co-culture

When THI4 and B038 were grown under co-culture conditions the percentage aspartic acid (Asp), glutamic acid (Glu), arginine (Arg), alanine (Ala), tyrosine (Tyr), and phenylalanine (Phe) taken up differed significantly between the Lys-Ile, Lys-Val, and control treatments (Figure 3.4C). However, for the Lys-Ile and Lys-Val treatments, only tyrosine and phenylalanine had an uptake of more than 20%. For the Lys-Ile treatment, 27.25% aspartic acid was taken up while 33.36% (4.35 ± 0.17 mg/L) aspartic acid was produced in the Lys-Val treatment. For the Lys-Ile, Lys-Val, and control treatments, glutamine (Gln) and methionine (Met) had an uptake of 100.00%. Glycine (Gly) had a significantly higher uptake in the Lys-Ile and Lys-Val treatments, compared to the control, and isoleucine had a similar uptake in the Lys-Val and control treatments, 93.69% and 93.11% respectively.

For the Lys-Ile treatment, glutamine (Gln, 100.00%), methionine (Met, 100.00%), and tryptophan (Trp, 74.40%) had the highest uptake. For the Lys-Val treatment, glutamine (Gln, 100.00%), methionine (Met, 100.00%), and isoleucine (Ile, 93.69%) had the highest uptake and there was a significant increase in the amount of aspartic acid, as mentioned previously. For the control treatment, glutamine (Gln, 100.00%), methionine (Met, 100.00%), and tryptophan (Trp, 97.09%) had the highest uptake.

Asparagine production

Asparagine (Asn) was not added to the medium and was not detected on day 0 in any of the amino acid treatments. However, on day 8, asparagine was detected at varying quantities in the different treatments. When THI4 was grown in monoculture, 0.24 ± 0.01 mg/L, 0.36 ± 0.01 mg/L, and 1.54 ± 0.03 mg/L asparagine was released into the extracellular medium in the Lys-Ile, Lys-Val, and control treatments respectively. These amounts differed significantly between the treatments. When B038 was grown in monoculture, asparagine was only released in the Lys-Val treatment (0.24 ± 0.03 mg/L). In co-culture, 1.07 ± 0.09 mg/L, 1.03 ± 0.08 mg/L, and 1.15 ± 0.08 mg/L asparagine was released in the Lys-Ile, Lys-Val, and control treatments respectively. These amounts were not significantly different between the treatments (data not shown).

γ-Aminobutyric acid (GABA) production

When THI4 was grown in monoculture, 1.99 ± 0.05 mg/L, 2.73 ± 0.59 mg/L, and 4.55 ± 0.06 mg/L GABA was released in the Lys-Ile, Lys-Val, and control treatments respectively. When B038 was grown in monoculture, 1.86 ± 0.04 mg/L, 50.65 ± 3.00 mg/L, and 3.28 ± 1.10 mg/L GABA was released in the Lys-Ile, Lys-Val, and control treatments respectively. In co-culture, 3.40 ± 0.05 mg/L, 2.84 ± 0.01 mg/L, and 4.82 ± 0.29 mg/L GABA was released in the Lys-Ile, Lys-Val, and control treatments respectively (data not shown).

3.3.4 Influence of environmental factors

The Lys-Ile, Lys-Val, and control treatments were initially performed at pH 3.5, incubated at 20ºC, and the inoculation dosages for THI4 and B038 were OD_{600} 0.1 and 0.01 respectively. These factors were changed in order to investigate their effect on the yeast and bacteria when grown in mono- and co-culture.

3.3.4.1 Influence of temperature

Growth Kinetics

THI4 showed relatively similar and poor growth performance in mono- and co-culture for the Lys-Ile and Lys-Val treatments at 15°C, 20°C, and 25°C. For the Lys-Ile and Lys-Val treatments the population remained relatively constant over the 8 day incubation period. For the control treatment THI4 grew exponentially within the first 2 days, after which the population remained relatively constant at approximately 7.20 log CFUs/ml for the rest of the incubation period. Temperature did not have a significant effect on the number of generations obtained, which ranged from 2.25 – 2.82 generations under monoculture conditions and from 2.38 - 2.78 generations under co-culture conditions for the control treatment (data not shown).

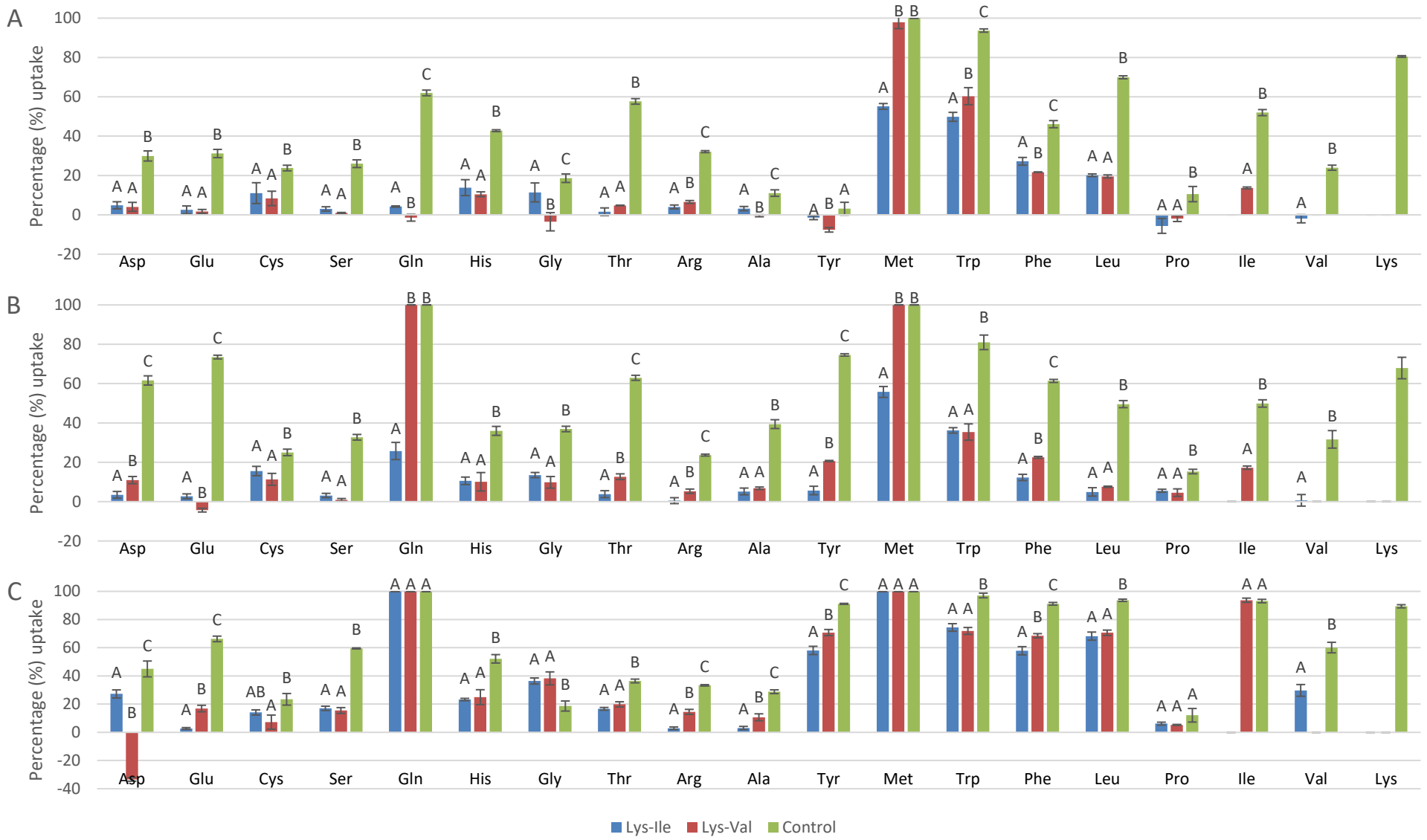

Figure 3.4 Total percentage uptake for individual amino acids by day 8 for Lys-Ile, Lys-Val, and Control treatments under *S. cerevisiae* monoculture (A), *Lb. plantarum* monoculture (B), and co-culture (C) conditions. Data shown are means of triplicates with error bars representing standard deviation. Means for different treatments, for each amino acid, without common uppercase letter are significantly different ($p < 0.05$) under *S. cerevisiae* monoculture, *Lb. plantarum* monoculture, and co-culture conditions respectively.

Temperature had a significant effect on the growth of B038 in mono- and co-culture for all the amino acid treatments. For the Lys-Ile treatment, the log CFUs/ml decreased over the 8 day incubation period under monoculture conditions at 15°C, 20°C, and 25°C. Under co-culture conditions, the number of generations obtained differed significantly between temperatures and were 3.95, 6.04, and 5.31 at 15°C, 20°C, and 25°C respectively. For the Lys-Val treatment, the log CFUs/ml decreased under monoculture conditions at 15°C and 25°C, but increased significantly at 20°C (as previously discussed). Under co-culture conditions, the number of generations obtained differed significantly between temperatures and were 4.39, 6.53, and 5.89 at 15°C, 20°C, and 25°C respectively. For the control treatment, the log CFUs/ml decreased at 15°C and increased at 20°C (7.27 generations) and 25°C (5.27 generations) in monoculture. Under co-culture conditions, the number of generations obtained differed significantly between temperatures and were 3.95, 6.25, and 5.10 at 15°C, 20°C, and 25°C respectively (data not shown).

Glucose, fructose and malic acid consumption

THI4 consumed relatively low amounts of glucose and fructose in monoculture for the Lys-Ile and Lys-Val treatments at 15°C, 20°C, and 25°C (Table 3.5). For the control treatment, THI4 showed an overall preference for glucose instead of fructose and consumed a total of 8.55 g/L, 17.23 g/L, and 22.85 g/L glucose and 2.15 g/L, 5.97 g/L, and 7.98 g/L fructose at 15°C, 20°C, and 25°C respectively. No malic acid was consumed by THI4 under monoculture conditions. In co-culture, THI4 showed an overall preference for glucose instead of fructose. For the Lys-Ile treatment, THI4 consumed relatively similar amounts of glucose at 15°C (10.64 g/L), 20°C (8.57 g/L), and 25°C (13.17 g/L). For the Lys-Val treatment, THI4 consumed the highest amount of glucose at 25ºC (9.11 g/L). THI4 consumed a total of 7.51 g/L, 19.87 g/L, and 25.89 g/L glucose and 8.01 g/L, 5.55 g/L, and 11.15 g/L fructose at 15°C, 20°C, and 25°C respectively for the control treatment.

Temperature had a significant effect on malic acid consumption. For the Lys-Ile treatment, B038 was able to consume all the malic acid present in the medium by day 5 at 20ºC and 25ºC and by day 8 at 15ºC in co-culture (Figure 3.5). In monoculture, B038 was unable to complete malolactic fermentation (MLF) by day 8 at the different temperatures. However, more malic acid was consumed as the temperature increased. For the Lys-Val treatment, B038 was able to consume all the malic acid present in the medium by day 5 at 20ºC and 25ºC and by day 8 at 15ºC in co-culture. At 15ºC and 25ºC, B038 was unable to complete MLF by day 8 in monoculture but was able to do so at 20ºC. For the control treatment, all the malic acid was consumed by day 5 at 20ºC and 25ºC in mono- and co-culture and by day 8 at 15ºC in co-culture. MLF was not completed by day 8 at 15ºC when B038 was grown in monoculture. Malic acid was consumed faster at higher temperatures and also when co-cultured with THI4. No glucose or fructose was consumed by B038 in monoculture.

Table 3.5 Total amount of glucose and fructose consumed by day 8 for Lys-Ile, Lys-Val and control treatments at different incubation temperatures and pH values. For treatments at 15ºC, 20ºC, and 25ºC the pH of the medium was at 3.5. For treatments at pH 3, 3.5, and 4 the incubation temperature was at 20ºC. Data shown are means of triplicates with standard deviation.

	Lys-Ile			**Lys-Val**			**Control**		
	15°C	**20°C**	**25°C**	**15°C**	**20°C**	**25°C**	**15°C**	**20°C**	**25°C**
				Total glucose (g/L) consumed by day 8					
THI4 Monoculture	2.56 ± 2.06	1.96 ± 1.13	1.90 ± 1.93	0.53 ± 0.57	-0.45 ± 1.90	2.24 ± 2.15	8.55 ± 0.58	17.23 ± 1.99	22.85 ± 2.60
Co-culture	10.64 ± 2.67	8.57 ± 2.26	13.17 ± 1.78	2.44 ± 0.366	2.61 ± 1.87	9.11 ± 1.24	7.51 ± 0.17	19.87 ± 0.67	25.89 ± 1.17
				Total fructose (g/L) consumed by day 8					
THI4 Monoculture	-0.19 ± 1.97	-1.06 ± 0.63	0.17 ± 1.70	-1.07 ± 1.85	0.70 ± 0.71	0.66 ± 1.53	2.15 ± 1.64	5.97 ± 2.63	7.98 ± 0.38
Co-culture	5.71 ± 1.85	1.67 ± 1.02	3.67 ± 1.87	4.39 ± 3.37	0.47 ± 0.78	-1.20 ± 2.12	8.01 ± 0.33	5.55 ± 1.03	11.15 ± 1.52
	Lys-Ile			**Lys-Val**			**Control**		
	pH 3	**pH 3.5**	**pH 4**	**pH 3**	**pH 3.5**	**pH 4**	**pH 3**	**pH 3.5**	**pH4**
				Total glucose (g/L) consumed by day 8					
THI4 Monoculture	0.94 ± 1.71	1.96 ± 1.13	0.83 ± 0.30	1.97 ± 1.36	-0.45 ± 1.90	0.84 ± 1.89	20.74 ± 1.60	17.23 ± 1.99	22.68 ± 0.51
Co-culture	2.63 ± 1.50	8.57 ± 2.26	8.43 ± 1.40	5.05 ± 0.44	2.61 ± 1.87	13.69 ± 1.28	24.20 ± 0.12	19.87 ± 0.67	24.05 ± 1.77
				Total fructose (g/L) consumed by day 8					
THI4 Monoculture	0.32 ± 0.26	-1.06 ± 0.63	-0.79 ± 0.83	0.86 ± 1.19	0.69 ± 0.71	-0.02 ± 1.60	3.23 ± 0.20	5.97 ± 2.63	5.81 ± 0.14
Co-culture	-0.20 ± 1.67	1.67 ± 1.02	1.19 ± 1.11	0.22 ± 1.29	0.47 ± 0.78	3.31 ± 1.19	7.15 ± 0.02	5.55 ± 1.03	8.21 ± 0.14

3.3.4.2 Influence of pH

Growth Kinetics

THI4 showed relatively similar and poor growth performance in mono- and co-culture for the Lys-Ile and Lys-Val treatments at pH 3, 3.5, and 4. For the Lys-Ile and Lys-Val treatments the population remained relatively constant over the 8 day incubation period. There was a slight decrease in the log CFUs/ml at pH 3 for these two treatments in mono- and co-culture and also at pH 4, but only in monoculture for both treatments. At pH 4, THI4 obtained 1.19 and 1.39 generations for the Lys-Ile and Lys-Val treatments respectively in co-culture. At pH 3.5, 1.08 and 1.56 generations were obtained for the Lys-Ile treatment in mono- and co-culture respectively and 1.37 and 1.53 for the Lys-Val treatment. For the control treatment THI4 grew exponentially within the first 2 days, after which the population remained relatively constant at approximately 7.20 log CFUs/ml for the rest of the incubation period. The pH did not have a significant effect on the number of generations obtained, which ranged from 2.59 – 2.94 generations in monoculture and from 2.19 – 2.86 generations in co-culture (data not shown).

The pH had a significant effect on the growth of B038 in mono- and co-culture for all the amino acid treatments. For the Lys-Ile treatment, the log CFUs/ml decreased over the 8 day incubation period under monoculture conditions at pH 3, 3.5, and 4. In co-culture, the number of generations obtained differed significantly for the different pH values and were 5.46, 6.04, and 5.69 at pH 3, 3.5, and 4 respectively. For the Lys-Val treatment, the number of generations obtained differed significantly and were 4.68, 3.90, and 0.31 in monoculture and 5.62, 6.53, and 5.94 in co-culture at pH 3, 3.5, and 4 respectively. For the control treatment, the number of generations obtained differed significantly and were 4.96, 7.27, and 5.86 in monoculture and 3.31, 6.25, and 5.25 in co-culture at pH 3, 3.5, and 4 respectively (data not shown).

Glucose, fructose and malic acid consumption

THI4 consumed relatively low amounts of glucose and fructose in monoculture for the Lys-Ile and Lys-Val treatments at pH 3, 3.5, and 4 (Table 3.5). For the control treatment, THI4 showed an overall preference for glucose instead of fructose and consumed a total of 20.74 g/L, 17.23 g/L, and 22.68 g/L glucose and 3.23 g/L, 5.97 g/L, and 5.81 g/L fructose at pH 3, 3.5, and 4 respectively in monoculture. Under co-culture conditions, THI4 showed an overall preference for glucose instead of fructose. For the Lys-Ile treatment, THI4 consumed 2.63 g/L, 8.57 g/L, and 8.43 g/L glucose at pH 3, 3.5, and 4 respectively. For the Lys-Val treatment, THI4 consumed 5.05 g/L, 2.61 g/L, and 13.69 g/L glucose at pH 3, 3.5, and 4 respectively. THI4 consumed a total of 24.20 g/L, 19.87 g/L, and 24.05 g/L glucose and 7.15 g/L, 5.55 g/L, and 8.21 g/L fructose at pH 3, 3.5, and 4 respectively for the control treatment. No malic acid was consumed by THI4 in monoculture.

The pH had a significant effect on malic acid consumption. For the Lys-Ile treatment, B038 was able to consume all the malic acid present in the medium by day 5 at pH 3, 3.5, and 4 in co-culture, but was not able to complete MLF by day 8 in monoculture (Figure 3.6). Malic acid was consumed slightly faster at pH 4 in co-culture. For the Lys-Val treatment, B038 was able to consume all the malic acid present in the medium by day 5 at pH 3, 3.5, and 4 in co-culture, with malic acid degradation occurring slightly faster at pH 4. At pH 3 and 3.5, B038 was able to complete MLF by day 8 in monoculture, with malic acid degradation occurring slower at pH 4. For the control treatment, all the malic acid was consumed by day 5 at pH 3.5 and 4 under mono- and co-culture conditions, with MLF progressing faster in co-culture. At pH 3, MLF was completed by day 5 in co-culture and by day 8 in monoculture. Malic acid was consumed faster at higher pH values and also when co-cultured with THI4. No glucose or fructose was consumed by B038 in monoculture.

3.3.4.3 Glutamine omission from Lys-Val treatment

Glutamine was suspected of enabling B038 to grow in the monoculture fermentations in the absence of lysine and valine. The amino acid was therefore omitted from the treatment in order to test this hypothesis.

Growth kinetics

No significant change was observed in the growth performance of THI4 for the Lys-Val-Gln treatment for mono- and co-culture conditions, compared to the Lys-Val treatment. The treatment did however have a significant effect on the growth of B038 in monoculture. For the Lys-Val treatment, the log CFUs/ml increased significantly within the first two days of the incubation period while the log CFUs/ml remained constant for the Lys-Val-Gln treatment during this time. The log CFUs/ml was also higher for the Lys-Val treatment (7.68 log CFUs/ml) by day 8 than for the Lys-Val-Gln treatment (7.22 log CFUs/ml).

Glucose, fructose and malic acid consumption

THI4 consumed less than 1 g/L of glucose and fructose for the Lys-Val and Lys-Val-Gln treatments in monoculture. In co-culture, 0.47 g/L fructose and 2.61 g/L glucose was consumed for the Lys-Val treatment and 4.04 g/L fructose and 8.25 g/L glucose for the Lys-Val-Gln treatment by day 8. No malic acid was consumed by THI4 in monoculture.

In co-culture, malic acid was completely degraded by day 5 for the Lys-Val and Lys-Val-Gln treatments (Figure 3.7). For the Lys-Val treatment, B038 completed MLF by day 8 in monoculture but was unable to complete MLF by day 8 for the Lys-Val-Gln treatment and consumed significantly less malic acid over the incubation period under monoculture conditions. No glucose or fructose was consumed by B038 in monoculture.

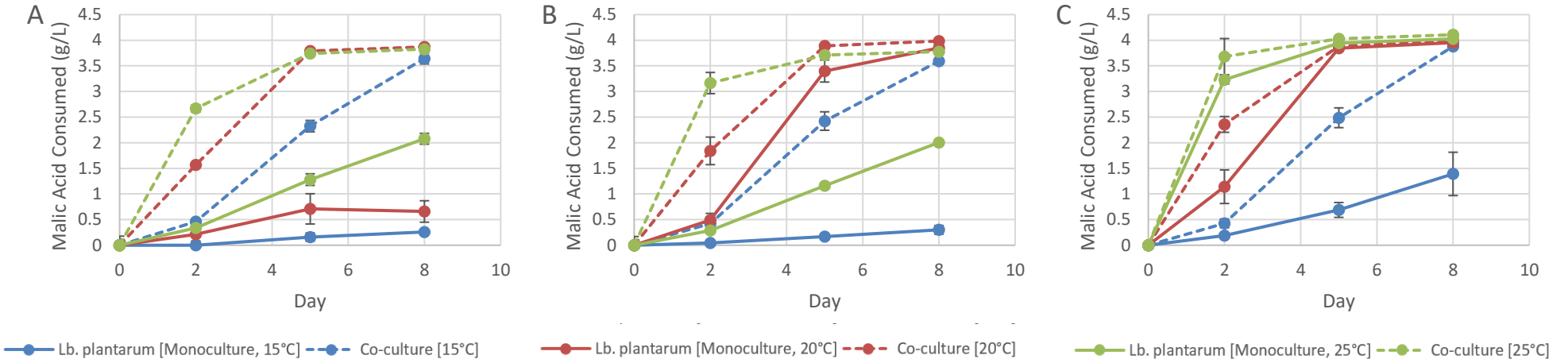

Figure 3.5 Influence of temperature on malic acid consumption for *Lb. plantarum* in mono- and co-culture for Lys-Ile (A), Lys-Val (B), and Control (C) treatments. Data shown are means of triplicates with error bars representing standard deviation.

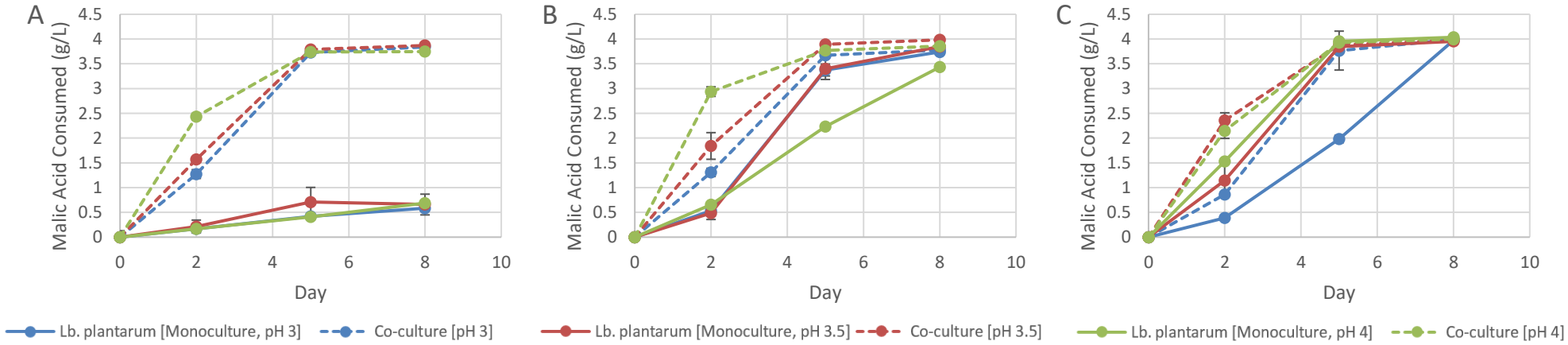

Figure 3.6 Influence of pH on malic acid consumption for *Lb. plantarum* in mono- and co-culture for Lys-Ile (A), Lys-Val (B), and Control (C) treatments. Data shown are means of triplicates with error bars representing standard deviation.

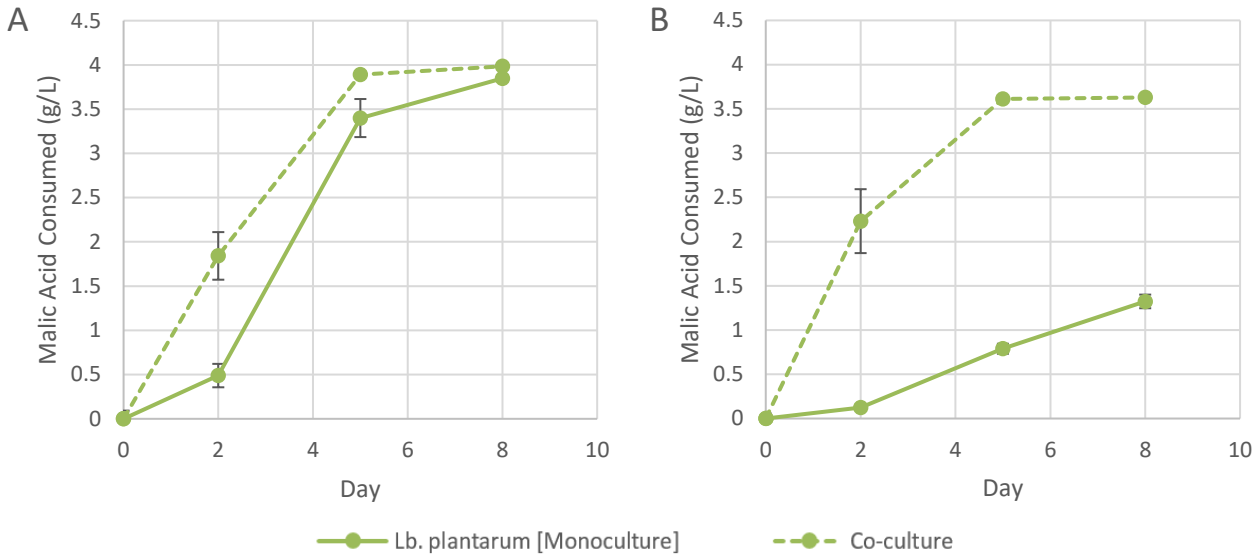

Figure 3.7 Malic acid consumption for *Lb. plantarum* B038 in mono- and co-culture for Lys-Val (A) and Lys-Val-Gln (B) treatments. Data shown are means of triplicates with error bars representing standard deviation.

3.3.4.4 Addition of lysine to fermentations

It was initially suspected that B038 was outcompeting THI4 for an essential vitamin or trace element, which resulted in the poor growth performance of the yeast when co-cultured with the bacteria. To test this hypothesis, lysine was added to treatments Lys-Ile and Lys-Val to a final concentration of 0.013 g/L (amount present in control treatment at start of fermentation) after 2 days of incubation at 20ºC. The lysine addition had no significant effect on the growth of B038 in co-culture. In monoculture, the lysine addition had no effect on the growth of B038 for the Lys-Val treatment but the log CFUs/ml remained relatively constant for the Lys-Ile treatment after the addition instead of declining as observed to occur without lysine addition.

Lysine addition had a significant effect on the growth of THI4 under mono- and co-culture conditions. After the addition, the log CFUs/ml increased significantly and were similar to the values observed at day 5 and day 8 for the control treatment when all amino acids were present (data not shown).

3.3.4.5 Influence of inoculation dosage

The addition of lysine to the fermentations and the subsequent improvement in the growth of THI4 confirmed that the current bacterial population density was not able to provide enough lysine to the yeast population. At the initial inoculation cell densities the yeast biomass was approximately 12.5 fold higher than the biomass for the bacteria. Initially THI4 was inoculated at OD_{600} 0.1 and B038 at OD_{600} 0.01. In order to evaluate the effect the inoculation dosage has on the yeast and bacteria, B038 was inoculated at OD_{600} 0.05, 0.1, and 10, while the yeast inoculation dosage remained the

same. However, as the inoculation dosage of the bacteria increased the number of generations obtained for B038 decreased and the population always stabilised at around 8.30 log CFUs/ml. When B038 was inoculated at OD_{600} 10, there was no increase in log CFUs/ml and the population remained at approximately 9.60 log CFUs/ml for the duration of the 8 day incubation period. The increased inoculation dosage of B038 had no effect on the growth of THI4. Instead the yeast was inoculated at OD_{600} 0.007 while B038 was inoculated at OD_{600} 0.01, which yielded a similar amount of biomass (wet weight) for THI4 and B038.

Growth kinetics

THI4 showed good growth for the Lys-Ile and Lys-Val treatments in co-culture when inoculated at a lower dosage (Figure 3.8). In monoculture, there was a slight decrease in log CFUs/ml for the Lys-Ile treatment and THI4 obtained 0.61 generations for the Lys-Val treatment. In co-culture, THI4 obtained 4.52 and 4.06 generations for the Lys-Ile and Lys-Val treatments respectively. For the control treatment, THI4 obtained significantly more generations in co-culture (6.23) compared to monoculture (5.86).

B038 showed good growth under co-culture conditions for all the treatments and obtained 5.50, 5.06, and 6.25 generations for the Lys-Ile, Lys-Val, and control treatments respectively when THI4 was inoculated at OD_{600} 0.007 (Figure 3.8). However, when THI4 was inoculated at OD_{600} 0.1 the generations obtained for the Lys-Ile (6.04) and Lys-Val (6.53) treatments where significantly higher while the generations obtained for the control (6.25) treatment were the same. In monoculture, there was a slight decrease in log CFUs/ml for the Lys-Ile treatment and B038 obtained 3.87 and 6.35 generations for the Lys-Val and control treatments respectively.

Glucose, fructose and malic acid consumption

THI4 consumed less than 1 g/L of glucose and fructose for the Lys-Ile and Lys-Val treatments in monoculture. In co-culture, THI4 consumed 2.60 g/L fructose and 3.50 g/L glucose for the Lys-Ile treatment and 1.48 g/L fructose and 5.98 g/L glucose for the Lys-Val treatment. For the control, THI4 consumed 3.65 g/L fructose and 10.19 g/L glucose in monoculture and 4.68 g/L fructose and 12.70 g/L glucose in co-culture. No malic acid was consumed by THI4 in monoculture.

For the Lys-Ile treatment, B038 degraded all the malic acid by day 5 in co-culture and was not able to complete MLF by day 8 in monoculture (Figure 3.8). Malic acid was degraded at approximately the same rate in co-culture when THI4 was inoculated at different dosages. For the Lys-Val treatment, B038 degraded malic acid significantly slower under co-culture conditions when THI4 was inoculated at OD_{600} 0.007 compared to malic acid degradation under co-culture conditions when THI4 was inoculated at a higher dosage. In monoculture, malic acid degradation by B038 was also faster compared to co-culture conditions at the lower yeast inoculation dosage. For the

control treatment, malic acid degradation was similar in mono- and co-culture conditions. However, MLF was faster in co-culture when THI4 was inoculated at a higher OD. No glucose or fructose was consumed by B038 in monoculture.

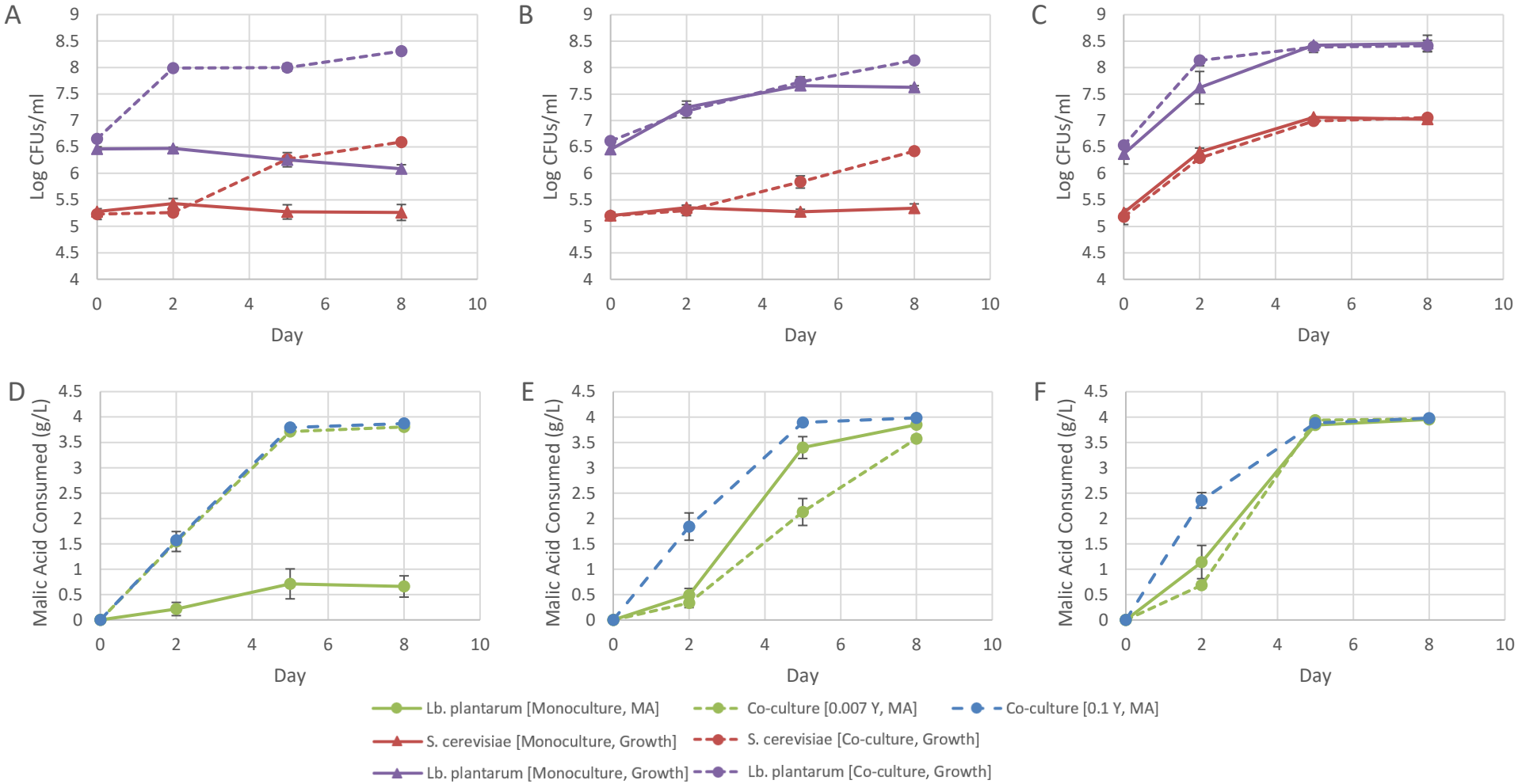

Figure 3.8 Influence of inoculation dosage on log CFUs/ml (Growth) for *S. cerevisiae* THI4 and *Lb. plantarum* B038 under mono- and co-culture conditions (A,B,C) and malic acid consumption (MA) for *Lb. plantarum* B038 under mono-and co-culture conditions (D,E,F) for Lys-Ile (A,D), Lys-Val (B,E), and Control (C,F) treatments. In the legend, "Co-culture [0.007 Y, MA]" is malic acid consumption when THI4 was inoculated at OD_{600} 0.007 and B038 at OD_{600} 0.01 and "Co-culture [0.1 Y, MA]" is malic acid consumption when THI4 was inoculated at OD_{600} 0.1 and B038 at OD_{600} 0.01. The latter was included on the graphs to indicate the difference in malic acid consumption when THI4 was inoculated at a higher cell density. For the Lys-Val (E) and Control (F) treatments the higher THI4 inoculation dosage resulted in faster malic acid consumption. Data shown are means of triplicates with error bars representing standard deviation.

3.3.5 Initial design for coevolution of *S. cerevisiae* THI4 and *Lb. plantarum* B038

The pH of grape musts generally range from 3 to 4 and AF is typically performed at 15ºC for white wine and 25ºC for red wine while MLF is performed between 18 - 20ºC. It was therefore decided to adjust the pH of the SGJ medium to 3.5 and to incubate the fermentations at 20ºC, as these values are at the centre of the pH and temperature ranges respectively. *S. cerevisiae* THI4 and *Lb. plantarum* B038 were grown under co-culture conditions over 11 fermentation rounds. In that time, THI4 and B038 obtained approximately 30 and 55 generations respectively. For simplicity, the original THI4 and B038 strains will be referred to as the parental strains and the THI4 and B038 strains that underwent 11 rounds of fermentation will be referred to as the evolved populations. However, it is not implied that these strains underwent any significant evolutionary changes since relatively few generations were obtained due to time constraints. The evolved isolates obtained from the fermentations were pooled together (THI4 and B038 populations kept separate) to test the population response and not the response of individual isolates.

Growth kinetics

The only significant difference between the growth kinetics of the parental strains and evolved populations was for B038 in monoculture for the Lys-Ile treatment (Figure 3.9A). The log CFUs/ml started to decrease significantly after day 2 for the parental strain, but the log CFUs/ml remained relatively constant for the evolved population. The slight differences observed on day 2 between the B038 parental strain and evolved B038 population were due to marginal differences in the starting log CFUs/ml (Figure 3.9A, B and C).

Glucose, fructose and malic acid consumption

For the Lys-Ile treatment, there was a significant difference between the total amount of glucose consumed by day 14 for the parental and evolved co-cultures (Figure 3.10A). The parental co-culture consumed a total of 12.23 g/L glucose while the evolved co-culture consumed nearly twofold the amount at 25.55 g/L. The amount of fructose consumed under co-culture conditions were similar between the parental strain and evolved population, as was the amount of glucose and fructose consumed under THI4 monoculture conditions. For the Lys-Val treatment, similar trends were observed as for the Lys-Ile treatment (Figure 3.10B). However, the difference between the total amount of glucose consumed by day 14 for the parental and evolved co-cultures was not as large. The parental co-culture consumed a total of 11.50 g/L glucose while the evolved co-culture consumed 19.23 g/L. For the control treatment, there were slight differences between the total amount of fructose consumed by day 14 under THI4 mono- and co-culture conditions for the parental strains and the population evolved under the Lys-Ile treatment (Figure 3.10C). The total amount of glucose consumed by day 14 differed significantly between the THI4 parental strain and

evolved population under monoculture conditions (30.19 g/L and 37.67 g/L respectively), but not under co-culture conditions (39.12 g/L and 43.84 g/L respectively). For the other control treatment, the total amount of glucose consumed by day 14 differed significantly between the parental strain and population evolved under the Lys-Val treatment (Figure 3.10D). Under monoculture conditions the THI4 parental strain and evolved population consumed 30.19 g/L and 42.47 g/L glucose respectively while these amounts were 39.12 g/L and 51.55 g/L under co-culture conditions respectively. The total amount of fructose consumed by day 14 differed significantly between the parental strain and evolved population under co-culture conditions (12.33 g/L and 19.44 g/L respectively), but not under monoculture conditions (9.86 g/L and 14.59 g/L respectively).

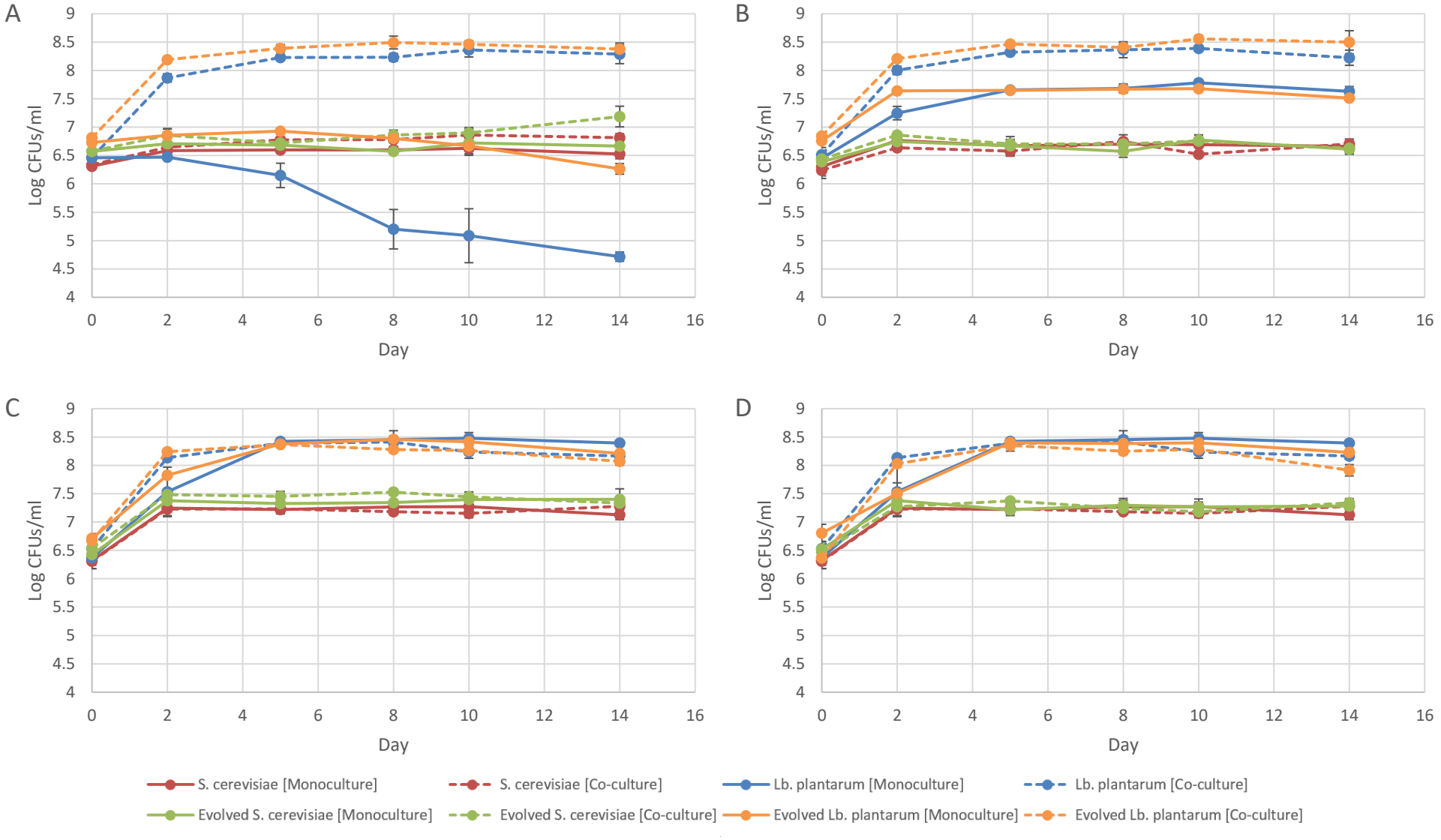

Figure 3.9 Log CFUs/ml for parental and evolved *S. cerevisiae* THI4 and *Lb. plantarum* B038 over the 14 day incubation period for the Lys-Ile (A), Lys-Val (B), and Control (CD) treatments. Graph C compares the THI4 and B038 parental strains to the THI4 and B038 populations evolved under the Lys-Ile treatment and graph D compares the parental strains to the populations evolved under the Lys-Val treatment. Data shown are means of triplicates with error bars representing standard deviation.

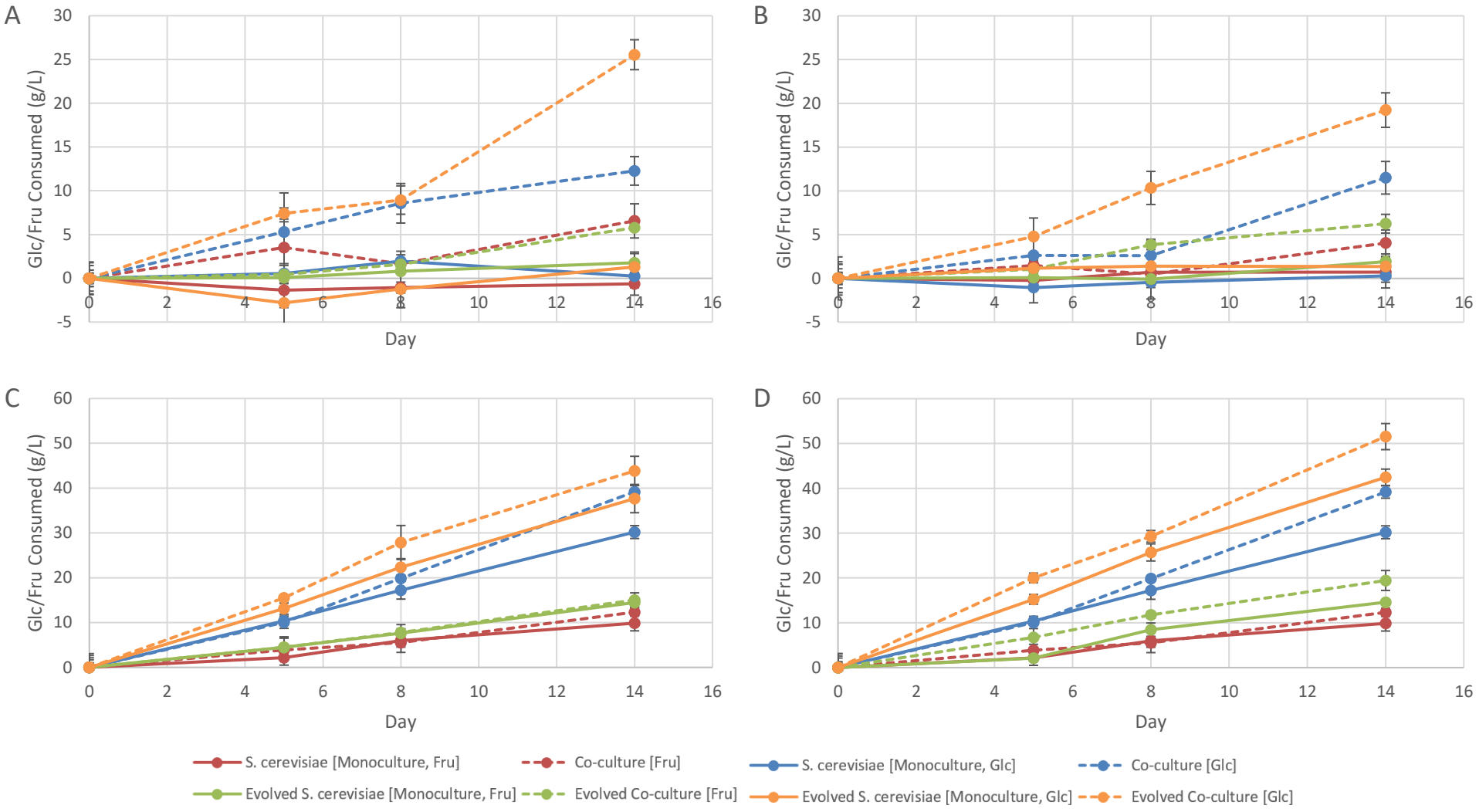

Figure 3.10 Total glucose (Glc) and fructose (Fru) consumed over the 14 day incubation period by *S. cerevisiae* THI4 in mono- and co-culture for the Lys-Ile (A), Lys-Val (B), and Control (CD) treatments. Graph C compares the parental strain to the population evolved under the Lys-Ile treatment and graph D compares the parental strain to the population evolved under the Lys-Val treatment. Data shown are means of triplicates with error bars representing standard deviation.

For the Lys-Ile treatment, there was a significant difference between the total amount of malic acid consumed by day 14 for the B038 parental and evolved monocultures (Figure 3.11A). Neither one was able to complete MLF by day 14, but the evolved B038 population consumed 1.90 g/L malic acid by day 14 while the parental strain only consumed 0.96 g/L. In co-culture, MLF was completed by day 5 for both the parental strain and evolved population with no significant difference between their performances. For the Lys-Val treatment, under co-culture conditions, similar trends were observed as for the Lys-Ile treatment (Figure 3.11B). In monoculture, the evolved B038 population degraded malic acid slower than the parental strain. For the control treatments, containing all amino acids, there were no differences between the parental strain and evolved population (Figure 3.11C and D). No malic acid was consumed by the THI4 parental strain or evolved population in monoculture and no glucose or fructose were consumed by the B038 parental strain or evolved population in monoculture.

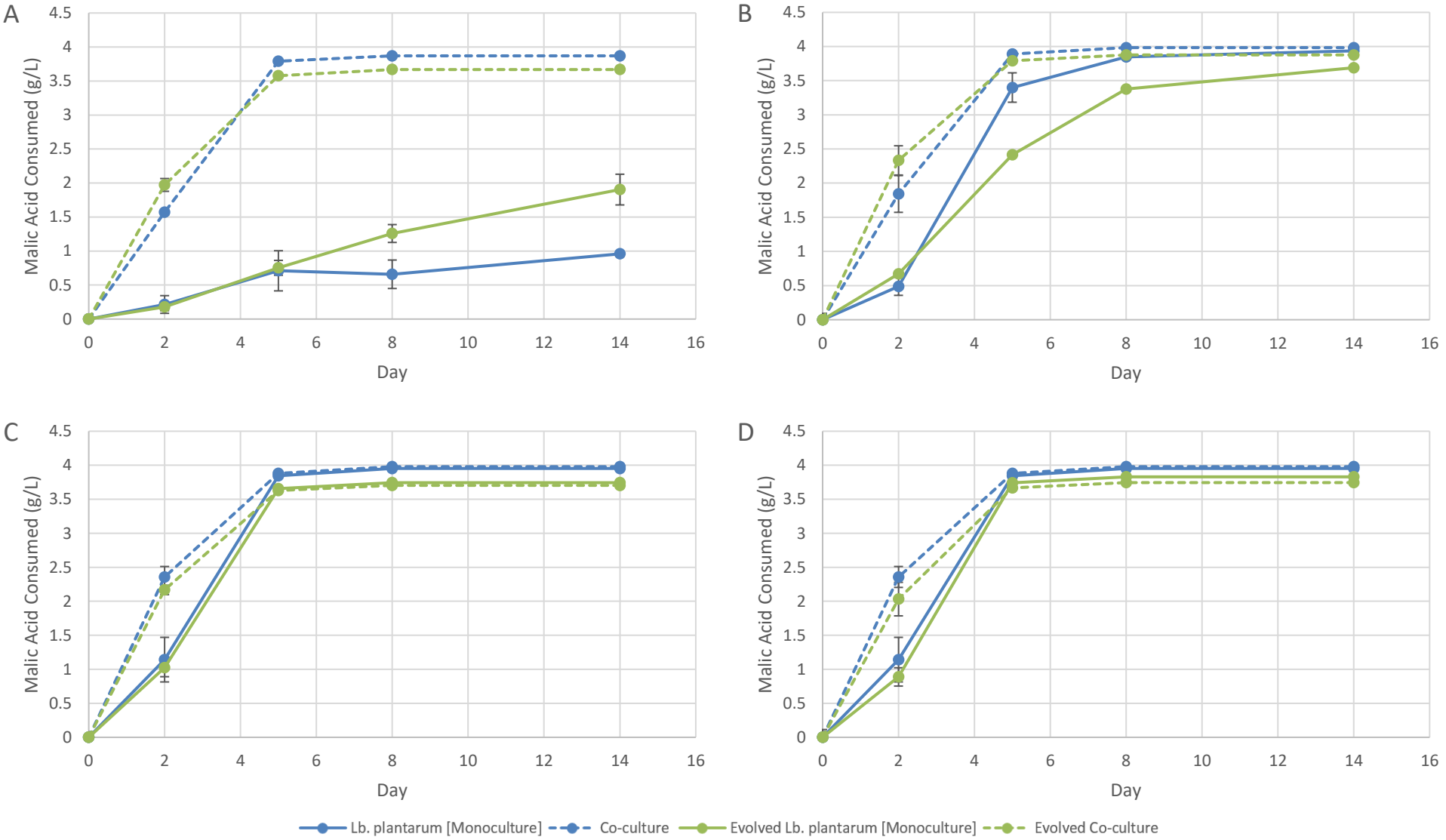

Figure 3.11 Total malic acid consumed over the 14 day incubation period under *Lb. plantarum* strain B038 mono- and co-culture conditions for the Lys-Ile (A), Lys-Val (B), and Control (CD) treatments. Graph C compares the parental strain to the population evolved under the Lys-Ile treatment and graph D compares the parental strain to the population evolved under the Lys-Val treatment. Data shown are means of triplicates with error bars representing standard deviation.

3.4 Discussion

3.4.1 Selection of amino acid auxotrophic conditions

S. cerevisiae THI4 is auxotrophic for lysine and the amino acid was therefore omitted from all five amino acid treatments. Since *Lb. plantarum* B038 is prototrophic for lysine, and showed good growth in the absence of the amino acid during initial auxotrophic screenings, it was hypothesised that THI4 would be able to grow in the absence of lysine when co-cultured with B038 as the bacteria would provide the yeast with the lysine required for its growth. In return, the yeast would provide the bacterium with the amino acids isoleucine, alanine, valine, and/or methionine which B038 is unable to synthesise. It was also hypothesised that THI4 and B038 would show poor growth performance in monoculture when grown within an environment lacking their respective auxotrophic amino acids and the support of their respective partner. The overall results shown provide support for both these hypotheses, except in the case where B038 was grown in monoculture conditions for the Lys-Val treatment which will be discussed in section 3.4.4.

THI4 is a lab strain, poorly adapted to synthetic grape juice conditions, and it was not expected that the yeast would perform as well as commercial wine strains. The growth of B038 was strongly supported in this synthetic relationship, while THI4 showed relatively limited growth performance and sugar consumption under these conditions. However, THI4's growth and sugar consumption was improved in co-culture with B038 compared to monoculture. It was therefore decided to select the Lys-Ile and Lys-Val treatments for further investigation, since THI4 obtained the highest amount of generations under these conditions, which is important for the coevolution of the strains.

3.4.2 Uptake of amino acids by THI4 and B038

Cellular regulation of nitrogen transport and metabolism in *S. cerevisiae* is complex and under the control of the nitrogen catabolite repression (NCR) and Ssy1p-Ptr3p-Ssy5p (SPS) regulatory systems. These systems, as well as *S. cerevisiae*'s preference for certain nitrogen sources, have been extensively reviewed and studied and will not be detailed in this discussion (McKinnon, 2013; Smit, 2013; de Koker, 2015). Nitrogen preferences of *Lb. plantarum* have not yet been extensively studied under oenological conditions and research is still needed in this regard. However, it was not within the scope of this study to investigate the preferential uptake of amino acids by *S. cerevisiae* or *Lb. plantarum.*

It is difficult to formulate any conclusions from the uptake of amino acids by THI4 and B038 in mono- and co-culture. Higher amino acid uptake was expected for the control treatment since yeast and bacteria growth is significantly better under these conditions. Higher amino acid uptake

was also expected under co-culture conditions since THI4 and B038 improve the growth of their respective partner (for the Lys-Ile and Lys-Val treatments) and they also compete for nutrients which might result in a higher uptake of certain amino acids based on preference and availability. Therefore, the combined percentage uptake of a specific amino acid by THI4 and B038 under monoculture conditions will not be equal to the percentage uptake of the same amino acid under co-culture conditions. Also, it is not possible to discern which amino acids and how much of each were taken up by THI4 and B038 individually when grown under co-culture conditions. HPLC analyses did provide some noteworthy results regarding the production of asparagine (Asn) and γ-Aminobutyric acid (GABA) as well as the uptake of glutamine (Gln) by B038 under monoculture conditions for the Lys-Val treatment (section 3.3.3 and Figure 3.4) which will be discussed further in section 3.4.4. The production of aspartic acid (Asp) under co-culture conditions for the Lys-Val treatment can also be investigated further since THI4 and B038 both assimilated Asp when grown under monoculture conditions.

3.4.3 Establishment of a mutually beneficial relationship between THI4 and B038

The established THI4-B038 relationship strongly supported the growth of B038, but growth of THI4 was more limited. This is likely due to the release of amino acids by THI4, as Ponomarova et al. (2017) have previously shown that *S. cerevisiae* secretes amino acids into the environment, to relieve excess nitrogen present in cells, which has a stimulatory effect on LAB. The presence of B038 had a significant stimulatory effect on sugar consumption by THI4, suggesting that the LAB either provided some required resources to the yeast or triggered an adaptive response. This relationship was maintained at the different temperature and pH conditions tested, which is important for application under different winemaking conditions. Temperatures between 20ºC and 25ºC is optimal for LAB growth in wine, with temperatures at 15ºC and lower inhibiting LAB growth, especially when ethanol is present (du Toit et al., 2011). The same trends were observed in this study. A decrease in temperature had a negative effect on the growth and MLF activity of B038. A decrease in pH also negatively affected B038, which was expected as *Lactobacillus* species generally grow better in wines with pH levels above 3.5 (du Toit et al., 2011). The presence of THI4 under co-culture conditions did however significantly improve the growth of B038 under these conditions. The improved growth and MLF activity of B038 when co-cultured with THI4 could be due to a stimulatory effect that the yeast has on the bacteria (Nehme et al., 2008; Liu et al., 2016) or it could be due to competition where the strains are stimulated to grow by the presence of another organism in the environment in order to outcompete their competitor (Alexandre et al., 2004).

The poor growth performance of THI4 was initially suspected of being either the result of B038 outcompeting the yeast for essential vitamins and/or trace elements, as an auxotrophy for

riboflavin has been identified in wine-related *Lactobacillus* strains (Terrade and Mira de Orduña, 2009), or due to B038 not providing THI4 with enough lysine. In order to investigate this, lysine was added to the Lys-Ile and Lys-Val treatments on day 2, after the exponential growth phase of B038 (section 3.3.4.4). After the addition of lysine, the growth of THI4 improved significantly which indicates the poor growth performance of THI4 is due to a shortage of lysine and not an essential vitamin or trace element. It was therefore suspected that the B038 population did not release enough lysine to support the THI4 population at the current THI4:B038 biomass ratio of approximately 12.5:1. Initially, the inoculation dosage of B038 was increased which did not have an effect on THI4 as the bacterial population always stabilised around 8.30 log CFUs/ml (section 3.3.4.5). This was suspected of being the population threshold for B038, which was confirmed by inoculating B038 into 10 ml SGJ medium containing all the amino acids at OD_{600} 10 in order to see if there was an increase in the population, which did not occur. Therefore, the inoculation dosage of THI4 was decreased to OD_{600} 0.007, which yielded a similar amount of biomass as B038 at OD_{600} 0.01 and a THI4:B038 cell ratio of approximately 1:25. At this cell ratio, there was a significant improvement in the growth of THI4 under co-culture conditions for the Lys-Ile and Lys-Val treatments compared to monoculture conditions (Figure 3.8A and B). However, when THI4 was inoculated at a lower dosage the growth of B038 was negatively affected under co-culture conditions for the Lys-Ile and Lys-Val treatments and the MLF activity of B038 was negatively affected for the Lys-Val and control treatments (Figure 3.8E and F). This further supports the findings that THI4 stimulates the growth and MLF activity of B038 under co-culture conditions and also indicates that this stimulatory effect is dependent on the size of the THI4 population. It is worth investigating further how lower THI4 inoculation dosages will affect the growth of B038 and how higher B038:THI4 cell ratios will affect the growth of THI4. Hom and Murray (2014) showed that the cell ratio of alga:yeast stabilised at a ratio of 1:2 in their system, even when the organisms were inoculated at a number of different cell ratios. Therefore, the ideal THI4:B038 cell ratio under these selective conditions is still yet to be determined.

3.4.4 Growth of B038 in monoculture in the absence of lysine and valine

B038 was expected to show poor growth performance under monoculture conditions for the Lys-Val treatment (at pH 3.5 and 20ºC), but instead the bacterium grew relatively well and was able to complete MLF. HPLC analysis of the amino acid uptake by B038 under monoculture conditions revealed only 25.74% of the initial glutamine (Gln) present was taken up by day 8 for the Lys-Ile treatment, while all the Gln was taken up for the Lys-Val and control treatments. HPLC analyses also revealed that asparagine (Asn) was produced and released by B038 under monoculture conditions only for the Lys-Val treatment and B038 also released significantly more GABA (50.65 mg/L) under these conditions compared to the Lys-Ile (1.86 mg/L) and control treatments (3.28 mg/L). The ability of B038 to grow in monoculture in the absence of lysine and valine only occurs

within a narrow temperature range around 20ºC, is improved under low pH conditions, and is possibly linked to the uptake of Gln and the production of Asn and GABA.

A study by Dai et al. (2013) investigated the effect of Gln on the utilization of amino acids by mixed bacteria cultures isolated from the small intestines of pigs. The total utilization of certain amino acids, including valine, decreased significantly when Gln was present at increasing concentrations i.e. the more Gln added to the medium the less valine was utilized by the cells. *Lb. plantarum* was not one of the pure culture bacterial species used in their study, but it is possible that similar results could be observed for *Lb. plantarum* as for the mixed intestinal bacterial cultures, since *Lactobacillus* species are known to be part of the normal gut microbiota of pigs (Suo et al., 2012). This could explain why B038 was able to grow relatively well in the absence of valine and THI4, even though B038 is auxotrophic for valine. The uptake of Gln, production of GABA, and B038's increased growth performance at low pH conditions could be linked to an acid resistance system that relies on the uptake of Gln, which was characterized in *Escherichia coli* (Lu et al., 2013). In this system GadC, an amino acid antiporter with specificity for Gln, glutamate (Glu), and GABA, exchanges intracellular Glu or GABA for extracellular Gln (Figure 3.12). Once inside the cell, Gln is converted to Glu by YbaS, a glutaminase activated and functional under low pH conditions. This conversion releases ammonia which can be used to increase the intracellular or environmental pH by neutralising a proton. The Glu that is formed can either be exchanged for extracellular Gln or converted to GABA by GadA/GadB which is then used by the GadC antiporter to import Gln into the cell. The activity of YbaS increases significantly as the pH decreases and is only properly functional at pH 6 or lower. The glutamate decarboxylase genes *gadA* and *gadB* as well as the glutamate:GABA antiporter gene *gadC* have previously been identified in *Lactobacillus brevis* (Li et al., 2013). It is therefore possible that a similar acid resistance system is present in *Lb. plantarum* which could explain the high uptake of Gln, the production of GABA, and why the growth of B038 improved when the pH of the medium was decreased.

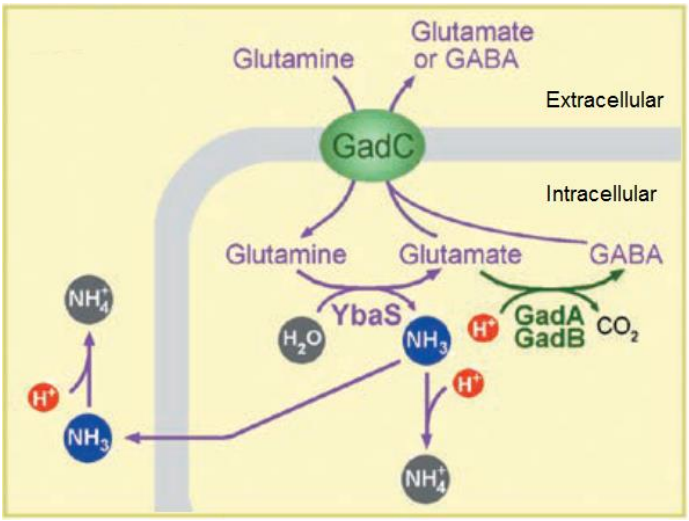

Figure 3.12 GadC-YbaS acid resistance system characterized in *Escherichia coli* (Adapted from Lu et al., 2013). GadC is an antiporter that exchanges extracellular glutamine (Gln) for intracellular glutamate (Glu) or γ-Aminobutyric acid (GABA). Gln inside the cell is converted to Glu by the acid-activated glutaminase Ybas which is coupled with the release of ammonia. The free ammonia can then be used to neutralise an acid (proton) inside or outside the cell in order to increase the intra- or extracellular pH respectively. Glu can also be converted to GABA by the two glutamate decarboxylases GadA and GadB. This reaction results in the fixation of a proton and subsequent increase in the intracellular pH as well as the release of CO_2 which can be used to neutralize a base (hydroxide ion).

In order to test this hypothesis, Gln was omitted from the Lys-Val treatment (Figure 3.7). When Gln was no longer present in the medium, along with lysine and valine, B038 was unable to complete MLF by day 8 and the growth performance of the bacteria was also negatively affected. B038 is prototrophic for Gln so the bacteria is able to synthesise the amino acid. These results provide further support for the hypothesis that the uptake of Gln by B038 is central to its ability to grow under monoculture conditions in the absence of lysine and valine. However, this was only a preliminary experiment and further investigation is still required.

The connection between B038's ability to grow in the absence of lysine and valine under monoculture conditions and the production of Asn was not investigated further in this study. The production of Asn could be linked to citrate metabolism, as previous studies have found that LAB capable of metabolising citrate are able to synthesise amino acids like leucine, valine, aspartate, and asparagine from precursors produced during citrate metabolism (Marty-Teysset et al., 1996; Goupil-Feuillerat et al., 1997; Magni et al., 1999; Pudlik and Lolkema, 2012). Citrate concentrations were not measured in this study, but it is worth investigating in future work in order to determine if the production of Asn is linked to citrate metabolism. It is also worth investigating if the production of aspartic acid (Asp) seen under co-culture conditions for the Lys-Val treatment (Figure 3.4C) may be linked to citrate metabolism as well.

3.4.5 Coevolution of *S. cerevisiae* THI4 and *Lb. plantarum* B038

THI4 and B038 were continuously grown under co-culture conditions in the absence of lysine and isoleucine (Lys-Ile treatment, referred to as LI population) as well as in the absence of lysine and valine (Lys-Val treatment, referred to as LV population). The THI4-B038 population was diluted and inoculated into new SGJ medium after every 8 days, which was considered one round of fermentation. Only 11 rounds of fermentation were performed and during that time THI4 and B038 obtained approximately 30 and 55 generations respectively, with no significant difference between the generations obtained for the Lys-Ile and Lys-Val treatments. This is a relatively short evolutionary time period and it was not expected that any major evolutionary changes would occur within so few generations. However, there was a significant increase in the amount of glucose consumed by the LI- and LV-THI4 evolved populations (Figure 3.10). The increased glucose consumption of the evolved populations was maintained when they were grown in the absence of B038 and in the presence of all the amino acids (control treatment). Similar results, related to increased glucose consumption, were seen in a study by Zhou et al. (2017) where *Lachancea kluyveri* was coevolved with different bacterial strains. The yeast adapted by rapidly consuming glucose and converting it to ethanol in a likely attempt to outcompete the bacterial strains by increasing the ethanol in the environment which is toxic to the bacteria. The results seen by Zhou et al. (2017) were obtained after coevolving *L. kluyveri* for approximately 960 generations, which is significantly longer than the 30 generations obtained in this study. However, it is possible that the stronger selective pressures in this study (competition with B038 and absence of select amino acids) accelerated the selection of the yeasts with advantageous mutations. Ethanol concentrations were not measured in this study, but are worth investigating in future work. It is also important to investigate *Lb. plantarum* B038's tolerance to increasing ethanol concentrations and to determine if there are any changes between the parental strain and evolved populations. If the assumption is made that the LI- and LV-THI4 populations produce higher ethanol concentrations than the parental strain, it is possible that the LI- and LV-B038 populations are more adept at tolerating these higher concentrations since the bacterial log CFUs/ml remained constant and did not decrease after the initial exponential growth period (Figure 3.9). Lastly, it is also important to evaluate individual evolved strains when a greater number of generations has been reached, as the results obtained for this experiment are for the evolved populations as a whole.

3.5 Conclusion

This study confirmed the relative ease with which cooperative interactions can be engineered between species when grown under conditions which force them to depend on one another, such as the reciprocal exchange of essential nutrients absent from their external environment. The study also showed that the relationship between *S. cerevisiae* and *Lb. plantarum* is maintained for temperature and pH conditions typically found under winemaking conditions. Initially, the

relationship was unbalanced with *Lb. plantarum* being favoured while *S. cerevisiae* struggled to grow. However, this was rectified by the adjustment of the yeast-bacteria cell ratio as more *Lb. plantarum* cells are needed to support the growth of a single *S. cerevisiae* cell. Furthermore, the results of this study also allowed for the generation of an interesting hypothesis regarding *Lb. plantarum* B038's ability to grow in monoculture in the absence of lysine and valine, which can be further investigated. Lastly, the coevolution of the strains provided insights regarding how these species adapt to specific selective pressures. However, time was a limiting factor in this study and relatively few generations were obtained. Despite the limited evolutionary time period, the results obtained regarding the increased glucose consumption of the evolved yeast population correlates with the results obtained by Zhou et al. (2017). Future work should investigate if this is inked to an increase in ethanol production and the established relationship between *S. cerevisiae* and *Lb. plantarum* should be allowed to evolve further over numerous generations to fully investigate how the dynamic between these two species changes.

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Chapter 4

General discussion and conclusions

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4.1 General discussion and conclusion

Microorganisms are ubiquitous in nature and play a vital role in numerous economically important industrial processes, such as food and beverage production where communities of yeast and bacteria are responsible for the production of products like sourdough, Kombucha and wine. Understanding how these organisms interact with each other, the factors influencing these interactions and the mechanisms that drive these organisms to form cooperative interactions is important to better manage the production of these products (Brenner et al., 2008). The complexity of natural symbiotic communities makes it nearly impossible to study these interactions in their natural environment. Therefore, synthetic model systems that can be controlled are necessary to understand the molecular mechanisms that drive and shape these interactions.

Grape must contains a complex, interactive community of yeast and bacteria species and understanding how these different species interact with each other is important for the successful completion of alcoholic and malolactic fermentations (AF and MLF), and to improve the positive organoleptic properties of wine. Studying the interaction between *Saccharomyces cerevisiae* and lactic acid bacteria, such as *Lactobacillus plantarum,* is difficult in grape must due to the large number of other yeast and bacteria species present (Bagheri, 2014; Liu et al., 2017). By using the principles of synthetic ecology (Shou et al., 2007; Brenner et al., 2008; Biliouris et al., 2012; Hom and Murray, 2014), an artificial wine matrix can be created in order to study these interactions more effectively since the chemical composition of wine has been well studied and can be synthetically reproduced to some degree (Bely et al., 1990; Henschke and Jiranek, 1993). A complex synthetic environment can therefore be created to investigate these organisms and drive their evolution, which would partially reflect the processes occurring in a natural wine environment. *S. cerevisiae* and *Lb. plantarum* are both industrially important organisms and there are vast genetic resources available for *S. cerevisiae* and also for *Lb. plantarum,* but to a lesser extent. Investigating the interaction between these two species will therefore provide important insights that could potentially be relevant for industrial applications.

In this study, a co-dependent, mutualistic relationship, centered on the reciprocal exchange of amino acids, was established between the lysine auxotrophic strain *S. cerevisiae* THI4 and the isoleucine, alanine, valine, and methionine auxotrophic strain *Lb. plantarum* B038. Different combinations of amino acids were omitted from the chemically defined synthetic grape juice-like (SGJ) media in order to find an amino acid treatment which promoted the best growth for both microorganisms. B038 showed excellent growth when co-cultured with THI4 for all the amino acid treatments, but THI4 struggled to grow under these conditions. The two treatments selected for

further experiments were the Lys-Ile (lysine and isoleucine omitted) and Lys-Val (lysine and valine omitted) treatments since THI4 showed the best growth under these conditions.

The established interaction was also investigated under different pH and temperature conditions. Lower temperature and pH conditions had a negative effect on the growth of B038, which is consistent with literature (du Toit et al., 2011), but when co-cultured with THI4 the yeast appeared to stimulate the growth of the bacteria under both selective and control conditions. This can either be due to the release of growth promoting metabolites by the yeast or due to improved growth performance resulting from competition for nutrients and space (Alexandre et al., 2004, Nehme et al., 2008; Liu et al., 2016). Regardless, it is an important finding demonstrating the applicability of the relationship under various winemaking conditions. THI4 continued to show poor growth under these conditions and it was initially suspected that B038 was outcompeting the yeast for a vital vitamin or trace element, as wine-related *Lactobacillus* species have been found to be auxotrophic for riboflavin (Terrade and Mira de Orduña, 2009). Lysine was added to the media after the exponential growth phase of B038 and the subsequent improvement in the growth of THI4 confirmed that the bacterial population was not providing the yeast population with enough lysine to support its growth. The initial THI4:B038 biomass ratio of approximately 12.5:1 was therefore adjusted to approximately 1:1. This significantly improved the growth of THI4 under co-culture conditions, but the lower yeast cell numbers had a negative effect on B038. However, B038 still grew exceptionally well under these conditions. This shows that population size is an important factor for interactive communities of microorganisms, a finding supported by Hom and Murray (2014). In their system the cell ratio of alga:yeast stabilised at a ratio of 1:2, even when the organisms were inoculated at different cell ratios.

It was expected that THI4 and B038 would show poor growth when grown in the absence of their respective auxotrophic amino acids and support of their respective partner. This proved true for all the amino acid treatments except when B038 was grown in the absence of lysine and valine. HPLC analysis revealed that this ability of B038 to grow relatively well under these conditions could be related to the uptake of glutamine and the production of γ-Aminobutyric acid (GABA) and asparagine. It was hypothesised that B038 uses a pH dependent antiporter (Lu et al., 2013) to import glutamine into the cell by exporting GABA. As glutamine has previously been shown to decrease the need for valine in the gut bacteria of pigs (Dai et al., 2013), it is possible that the uptake of glutamine and subsequent effect it has on the utilization of valine could be why B038 was able to grow under these conditions. Glutamine was therefore omitted form the Lys-Val treatment to test this hypothesis, which resulted in B038's inability to grow despite the bacteria's ability to synthesise glutamine. B038 still grew exceptionally well under these conditions when co-cultured with THI4. Only preliminary experiments were done in this study to test this hypothesis and more

research is still required. However, these findings do provide some insight into *Lb. plantarum*'s utilization of amino acids under oenological conditions, which has not yet been extensively studied.

Lastly, by coevolving THI4 and B038 over continuous rounds of fermentation, this study showed that THI4 adapted to the imposed selective conditions by increasing its consumption of glucose while cell density remained the same. This may be linked to increased ethanol production in a competitive strategy to inhibit the growth of B038, as proposed by Zhou et al. (2017). However, further research is still required to confirm this since THI4 is dependent on B038 for growth and inhibiting the growth of the bacteria is expected to negatively affect the growth of the yeast. This study also showed that the observed phenotype of the evolved THI4 population was not dependent on the presence of B038 or absence of select amino acids, which indicates the observed changes may be linked to genetic or epigenetic changes in the yeast genome. However, this still needs to be further investigated.

The system used in this study is based on the reciprocal exchange of essential amino acids between the yeast and LAB within the synthetic environment created for them. Nutrient exchange between different species often occurs in natural systems, such as in the case of lichens, which makes it an ideal means of interaction to recreate under synthetic conditions (Li et al. 2017). The natural environments where yeast and LAB can be found in symbiotic communities, such as sourdough and grape must, is rich in nutrients and contains various amino acids, peptides, and proteins. Therefore, auxotrophic yeast and LAB may not necessarily depend on one another in natural systems for amino acids, since they can obtain them from the environment. The observed interaction between THI4 and B038 would therefore not be applicable in grape must. However, through coevolution it is possible that the yeast and bacteria may develop other dependencies, perhaps through adaptive gene loss (Morris et al., 2012, 2014; Mas et al., 2016), which would make their interaction obligatory in the absence of the current selective conditions. The current system therefore has potential to further investigate the interaction between *S. cerevisiae* and *Lb. plantarum.*

In conclusion, this study used the principles of synthetic ecology to establish a co-dependent relationship between *S. cerevisiae* and *Lb. plantarum.* The co-dependence was then used to drive the coevolution of the strains. This study showed that cooperative interactions can be induced with relative ease when species are forced to depend on one another for survival. Furthermore, this study also showed that strong selective pressure had a significant effect on the adaptation of *S. cerevisiae* within a relatively short time period. These results correlate with the results obtained by Zhou et al. (2017) which contribute to understanding how interactions between species drive their adaptation. Lastly, this study also served as a proof of concept for subsequent work focused on understanding the genetic changes involved in the coevolution of species. These findings provide

relevant insights into the industrially important interaction between *S. cerevisiae* and *Lb. plantarum* and also established a foundation for inducing cooperative interactions between these two organisms. This can be built upon to ultimately create yeast-bacteria pairings capable of successfully completing AF and MLF in a time and cost efficient manner. Further work, aimed at identifying the genetic targets of the coevolution of the yeast and bacteria, will ultimately contribute to a better understanding of the evolution of cooperation between different species.

4.2 Limitations of the study

There were certain limitations to this study which will briefly be discussed. Firstly, the limited amount of sampling points during fermentation made it impossible to determine the growth and substrate consumption rates for the strains. Calculation of these rates would have allowed for a more thorough comparison of the strains under the different conditions and treatments tested. In future studies, it is therefore important to increase the amount of sampling points, especially during the first two days of fermentation. Furthermore, pH was not measured throughout fermentation. This could potentially have provided interesting data, since pH was shown to have a significant effect on the growth of the strains, especially on B038 under monoculture conditions in the absence of lysine and valine. In addition, the strains were not subjected to a starvation period before inoculation into SGJ media. The strains were pre-cultured in rich media and it's likely that amino acids were stored within the cells when they were inoculated into the fermentation media which could have provided them with a growth advantage. This was deliberately done as it was expected that the strains would initially struggle to grow under the selective conditions and subjecting them to a starvation period would only increase their struggle. However, it was not determined to what degree this improved their growth and should therefore still be investigated. Lastly, as mentioned, time was limited for this study and the evolutionary potential of the established relationship between *S. cerevisiae* and *Lb. plantarum* could not be fully investigated within the timeframe of the study.

4.3 Potential future studies

Future research should focus on continuing to evolve *S. cerevisiae* THI4 and *Lb. plantarum* B038 in order to fully investigate how these strains adapt to the imposed selective pressures. It is also important to investigate the genetic changes linked to the phenotypes observed for the evolved isolates in order to better understand how cooperative interactions form between species. This can be achieved through whole-genome sequencing. Furthermore, the fermentation and growth kinetics of the evolved isolates from this study need to be fully characterized. It is also important to investigate the ethanol production by the parental THI4 strain and the evolved isolates, as

mentioned previously. Lastly, the ability of B038 to grow in the absence of lysine and valine and how this is dependent on the uptake of glutamine should be further investigated.

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