Evaluating the vitamin requirements of wine-related yeasts and the resultant impact on population dynamics and fermentation kinetics

by

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Dedication

My parents, whom never stopped believing in me and always supported my dreams and ideals in life. Your prayers were the wings to my spirit. You never gave up on me and made me always hopeful that things will work how they are supposed to, if I just keep good faith and strive towards excellence in everything that I do. You have asked, and God answered.

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Summary

Within the vineyard environment, grape berries serve as a habitat to various microorganisms including bacteria, filamentous fungi and yeasts, of which some play distinct roles in winemaking. Studies on yeast species other than *Saccharomyces cerevisiae*, commonly referred to as non-*Saccharomyces* (NS) yeasts in oenology, have evaluated the ability of these yeast to modulate the sensory profile of wine. In the early stages of spontaneous fermentation when the ethanol concentrations are low, the NS yeast population increases, but is progressively replaced by *S. cerevisiae*, which is better adapted to the environmental conditions associated with fermenting grape juice. The overall sensory profile of wine is in part a result of the metabolite production of yeasts, and the extent of the contribution of each species will depend on the total metabolic activity of each species. Metabolic activity is directly related to the availability of nutrients such as carbon, nitrogen, vitamins and trace elements. These nutrients are indeed converted to biomass and other metabolites, many of which are aroma and flavour active by-products. Only limited information regarding the nutrient requirements of wine-related yeasts other than *S. cerevisiae* has been published. Several studies have explored the carbon and nitrogen requirements of some NS species, but the vitamin requirements of many biotechnologically relevant species remains to be determined. Vitamins are organic compounds, mostly of a complex chemical nature, and serve as cofactors in metabolic reactions. Vitamins occur in small quantities in grapes and grape juice, but some data suggest that they may in some cases be limiting for yeast growth in this environment, affecting metabolism and ultimately impact the final wine. This knowledge gap motivates the current study, which focuses on the growth and fermentation kinetics of different NS yeasts when presented with varying concentrations of the relevant vitamins: biotin, pantothenate, inositol, thiamine and pyridoxine. In a first section, a high-throughput microtiter plate assay was optimised to allow for the rapid screening of the vitamin requirements of NS yeasts. The results of this assay showed differences in the vitamin requirements amongst the different yeasts. The statistically most significant vitamin-dependent yeast phenotypes from the screen were selected for further investigation. These included the dependence of Viniflora® *P. kluyveri* Frootzen ™ on biotin and thiamine and of Viniflora ® *L. thermotolerans* Concerto ™ on inositol. The data obtained from this study provide a better understanding of the vitamin requirements of NS yeasts and how these requirements can potentially enhance the growth performance of NS yeasts. The data suggest that targeted nutrient additions may lead to a better modulation of the overall sensory profile of wine.

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Opsomming

In ‘n wingerdomgewing dien druiwebessies as 'n habitat vir verskeie mikroörganismes, insluitend bakterieë, filamentiese swamme en giste, wat almal verskillende rolle speel in wynmaak. Studies oor gis spesies behalwe *Saccharomyces cerevisiae*, wat algemeen bekend staan as nie-*Saccharomyces* (NS) giste in wynkunde, het die vermoë van hierdie giste geëvalueer om die sensoriese profiel van wyn te moduleer. In die vroeë stadiums van spontane fermentasie wanneer die etanolkonsentrasies laag is, is daar ‘n toename in die NS-gispopulasie wat geleidelik vervang deur *S. cerevisiae*, wat beter aangepas is by die omgewingstoestande wat geassosieer word met die fermentasie van druiwesap. Aangesien die algemene sensoriese profiel van wyn gedeeltelik afhanklik is van die metabolietproduksie deur giste, sal die omvang van die bydrae van elke betrokke spesie afhang van die totale metaboliese aktiwiteit van elke spesie. Die metaboliese aktiwiteit is gedeeltelik afhanglik van die beskikbaarheid van voedingstowwe soos koolstof, stikstof, vitamiene en spoorelemente. Hierdie voedingstowwe word omskep in biomassa en neweprodukte deur middel van metaboliese aktiwiteit. Beperkte inligting aangaande die voedingsvereistes van NS giste is gepubliseer. Terwyl verskeie studies die koolstof- en stikstofvereistes van sommige NS spesies ondersoek het, is die vitamien-behoeftes van hierdie giste onbepaald. Vitamiene is organiese verbindings wat van 'n komplekse chemiese aard is en hoofsaaklik dien as kofaktore in metaboliese reaksies. Vitamiene kom in klein hoeveelhede in druiwe voor en kan moontlik vir gisgroei beperk word, wat metabolisme beïnvloed en uiteindelik die finale wyn beïnvloed. Dus, het hierdie studie fundamenteel gefokus op die groei- en fermentasie kinetika van verskillende NS-giste wanneer dit aangebied word met wisselende konsentrasies van die mees relevante vitamiene vir giste: biotien, pantotenaat, inositol, tiamien en piridoksien. In 'n eerste afdeling is 'n hoë-deurlaat mikrotiter plaat toets geoptimaliseer om voorsiening te maak vir die vinnige bepaling van die vitamienbehoeftes van NS-giste. Die resultate van hierdie toets het verskille getoon in die vitamienbehoeftes onder die verskillende giste. Sommige van die statisties mees betekenisvolle vitamien-afhanklike gis fenotipes van die toets was gekies vir verdere ondersoek: die afhanklikheid van *P. kluyveri* Frootzen van biotien en tiamien sowel as dié van *L. thermotolerans* Concerto aangaande inositol. Die data verkry uit hierdie studie het 'n beter begrip aangaande die vitamienbehoeftes van NS-giste aangebied en hoe hierdie vereistes die groeiprestasie van NS-gis kan verbeter. Die data dui daarop dat geteikende voedingtoevoegings kan lei tot 'n beter modulasie van die algehele sensoriese profiel van wyn

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Biographical sketch

Jerobiam Marvin Julies was born in Malmesbury in the Western Cape on 2 November 1993. He attended Vooruitsig Primary and matriculated from Schoonspruit Secondary in 2011. In 2012, he enrolled at the Stellenbosch University and completed a BSc in Molecular Biology and Biotechnology in 2015. In 2016 he obtained a HonsBSc in Microbiology from Stellenbosch University. Since the beginning of 2017 he has been working towards obtaining his MSc in Wine Biotechnology at the Institute for Wine Biotechnology at Stellenbosch University.

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Preface

This thesis is comprised out of four chapters, with each chapter introduced separately.

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General introduction; problem statement; aim and objectives

Chapter 2 Literature review

Vitamins as growth factors for yeasts

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Chapter 1

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1.1 General introduction

The indigenous community of yeasts that are potentially found on grape berries includes a large population of wine-related yeasts that are colloquially termed as non-*Saccharomyces* (NS) yeasts by wine microbiologists (Jolly *et al*., 2014). These NS yeasts are usually divided into three groups: (1) strict aerobic yeasts such as *Cryptococcus* and *Rhodoturala* spp.; (2) apiculate yeasts with poor fermentative power such as *Hanseniaspora* spp., and (3) yeasts that have fermentative metabolism, for example, *Lachancea* spp. and *Torulaspora delbrueckii* (Fleet *et al*., 1984; Querol *et al*., 1990; Bisson & Kunkee, 1991; Longo *et al*., 1991; Lonvaud-Funel, 1996; Lorenzini, 1999; Torija *et al*., 2001; Combina *et al*., 2005). The NS yeasts are carried over to the must when the grape berries are crushed and are consequently introduced to fermentation (Jolly *et al*., 2014). In the early stages of fermentation when the ethanol concentrations are low, the populations of some NS yeast species increases but these species tend to be progressively replaced by *S. cerevisiae*, which is generally the primary yeast to drive alcoholic fermentation (Albergaria & Arneborg, 2016). Although *S. cerevisiae* is commonly found in limited numbers on grapes, it can ascertain dominance over NS yeasts as fermentation progresses. This is due to its high fermentative power, rapid fermentation rates and adaptation to cope with increasingly harsh environmental conditions such as low pH, limited oxygen availability and increasing levels of ethanol and organic acids (Albergaria & Arneborg, 2016).

The NS yeasts have received attention due to their contribution towards the overall sensory profile of wine including complexity, quality, and aroma when used in co-cultures with *S. cerevisiae* (Rojas *et al*., 2001; Jolly *et al*., 2003; Swiegers *et al*., 2005; Jolly *et al*., 2006; Domizio *et al*., 2007; Renouf *et al*., 2007; Anfang *et al*., 2009). However, some of the NS yeasts have properties that can have a negative impact on wine fermentations: low fermentative power, slow fermentation rates and poor adaptations to cope with harsh environmental conditions. Furthermore, the use of these yeasts may increase the risk of spoilage (Albergaria & Arneborg, 2016), since previous studies have reported high volatile acidity levels and other adverse compounds that may be produced by these yeasts (Castor, 1954; Amerine & Cruess, 1960; Van Zyl *et al*., 1963; Amerine *et al*., 1967, 1972)

To date, wine research involving the use of NS yeasts has primarily focused on topics such as:

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1. The origin of these yeasts and their interaction with grapes, along with the production of ethanol, acetate and glycerol (Jolly *et al*., 2006; Contreras *et al*., 2014).
2. The secretome of these yeasts during early fermentation (Mostert & Divol, 2014).
3. The production of secondary metabolites such as esters and higher alcohols (Charoenchai *et al*., 1997; Ciani & Maccarelli, 1998; Manzanares *et al*., 2000; Andorrá *et al*., 2012).
4. Interactions between these yeasts and *S. cerevisiae* (Fernández *et al*., 2000; Holm Hansen *et al*., 2001; Fleet, 2003; Nissen *et al*., 2004; Clemente-Jimenez, 2005; Ciani *et al*., 2006; Pérez-Navado *et al*., 2006; Mendoza *et al*., 2007; Bely *et al*., 2008; Anfang *et al*., 2009; Comitini *et al*., 2011; Domizio *et al*., 2011; Viana *et al*., 2011; Gobbi *et al*., 2013; Sun *et al*., 2014; Wang *et al*., 2015, 2016; Rossouw *et al*., 2015)
5. Nutrient requirements (carbon and nitrogen) (Ugliano *et al*., 2009; Schnierda *et al*., 2014; Taillandier *et al*., 2014).

A limited amount of research has evaluated the nutrient requirements for carbon and nitrogen of NS yeasts, and how deficiencies in these nutrients can affect fermentation and the resulting wine (Bely *et al*., 1990; Bataillon, 1996; Bisson, 1999; Wang *et al*., 2003; Bohlsheid *et al*., 2007). However, little is known about other nutrients such as vitamins which may also serve as growth factors for yeasts. Vitamins are organic compounds that allow for yeasts to maintain cell proliferation and viability, as well as the survival of yeast cells in unfavourable conditions (Julien *et al.,* 2017). A deficiency in a vitamin may influence the fermentation kinetics, especially when yeast is incapable of producing a vitamin *de novo* (Julien *et al.,* 2017). Most vitamins act as enzyme cofactors and may also aid in reactions involving energy transfer (Julien *et al.,* 2017). Of all vitamins, biotin and thiamine are considered the most important since their availability can affect fermentations considerably (Julien *et al.,* 2017). The impact of biotin and thiamine on fermentations is likely linked to the fact that most yeasts are not able to produce these vitamins (Pirner & Stolz, 2006), and to the important roles these vitamins play in metabolism. Biotin is indeed required for the synthesis of DNA, amino acids, fatty acids and degradation of carbohydrates, whereas thiamine plays a role in amino acid and carbohydrate catabolism. (Streit & Entcheva, 2003; Li *et al*., 2010).

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Regarding the impact on wine fermentations, with specific focus on *S. cerevisiae*, biotin may enhance yeast cell viability and increase the production of esters, but a deficiency in biotin can affect cell growth significantly (Bohlsheid *et al*., 2007). Thiamine affects the fermentation rate and biomass production, and the lack of this vitamin may ultimately lead to stuck fermentations (Julien *et al*., 2017). Other vitamins such as pantothenic acid or inositol can also impact fermentations. Pantothenic acid may prevent H_2S production and volatile acidity, whereas inositol impacts on membrane integrity (Julian *et al*., 2017).

1.2 Problem statement

Many of the specific mechanism of interaction between NS yeasts and *S. cerevisiae* remain unclear. In particular, it would be of value to further investigate the nutrient requirements of these yeasts since nutrient availability significantly impacts on biomass production and fermentation (Julien *et al.*, 2017). All yeast take up exogenous nutrients from the external environment, and general nutrient addition has become a common practice in wineries. Some reports have also shown that vitamin addition can lead to improved fermentation kinetics (Ough & Kunkee, 1967; Ough *et al*., 1989; Landolfo *et al*., 2010; Redón *et al*., 2009; Varela *et al*., 2012). It remains to be determined to what degree the NS yeasts incorporate exogenous vitamins and how vitamins impact on the fermentative power of these yeasts in the presence of *S. cerevisiae*.

1.3 Aim and objectives

The primary research aim of this project was to evaluate the impact of varying concentrations of vitamins on the growth performance of selected NS yeasts, in pure culture, but also in the context of a mixed culture with *S. cerevisiae*. The specific research objectives of the project were as follows:

1. Assessing the impact of vitamins, provided at different concentrations, on the growth of a few NS yeasts using a high-throughput microtiter plate experimental design.
2. Assessing some of the growth kinetics at a larger scale in mixed culture with *S. cerevisiae* to confirm the results obtained in the first objective and assess the potential competition for vitamins

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Chapter 2

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Literature review: vitamins as growth factors for yeasts

2.1 Introduction

In general, nutrient requirements for yeasts include nitrogenous compounds, carbohydrates, lipids, vitamins and various minerals (Walker, 1998). These nutrient requirements vary between species and even strains within a given species. Certain environmental factors, for example, temperature and the presence of certain compounds, may impact the need for specific nutrients and yeasts' ability to adapt to the composition of a matrix. Yeasts' requirement for certain nutritional elements, especially nitrogen and carbon, are widely reported on in literature, with relatively limited information on other nutrients such as vitamins and trace elements. Vitamins are known to impact the metabolism of yeasts and could impact fermentation as a result (Julien *et al*., 2017). However, very little is known about the vitamin requirements of yeasts, and studies that have reported on those only partially reported on vitamins as growth factors by yeasts as well as the interactions with other nutrients such as nitrogen (Bohlscheid, 2007). Vitamins are organic compounds, often complex in chemical nature, and act as essential enzyme cofactors in metabolic reactions, aiding in energy transfer, cell survival, cell proliferation as well as the production of precursors that may affect the overall sensory profile of wine (Ough *et al*., 1989; Bohlscheid *et al*., 2007; Julien *et al*., 2017). A deficiency in a vitamin may influence the fermentation kinetics, especially when yeast is incapable of producing the vitamin *de novo* (Olson & Johnson, 1949; Julien *et al.*, 2017). Yeasts have the necessary enzymatic machinery to synthesise most vitamins *de novo*, but it has been reported that most strains of *S. cerevisiae* are unable to synthesise biotin (Pirner & Stolz, 2006).

This review will provide an overview on our current understanding of the role of vitamins and will primarily focus on some of the vitamins that will be further investigated in this study, in particular, biotin, thiamine, pyridoxine, pantothenate and inositol. The synthesis and function of these vitamins in yeasts will be discussed together with their impact on fermentation. Moreover, this review also briefly introduces the yeasts species that are the focus of this study.

2.2 Vitamins as growth factors for yeasts and their role in yeast metabolism

The current knowledge of vitamins as growth factors stems from several studies which started in the 19th century on the nutritional factors that are required by microorganisms such as yeasts. Following the observations made by Von Liebig (1870), who suggested that a synthetic culture

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medium needs to contain stimulatory components to allow for the growth of yeasts, Wildier (1901) identified an unknown substance that served as a stimulus in a well-defined chemical medium. The unknown substance was then termed “bios”.

Bios is now regarded as a mixture of vitamins that are strain-specific. Five vitamins, in particular, have been described as essential vitamins required by yeasts: biotin, inositol, pantothenic acid, pyridoxine and thiamine (Burkholder, 1943). These vitamins are complex in chemical nature. Humans cannot synthesise vitamins *de novo* and will have to acquire them from their diet. Yeasts can synthesise most of these vitamins, but this is species and strain-dependent. Also, yeasts are not able to produce vitamins under all growth conditions but can incorporate vitamins that become limiting from the environment (Bataillon *et al*., 1996). The correlation between bios and growth of yeast set a platform of great interest and allowed for parallel research to be made in several research fields including microbiology, biochemistry and animal nutrition. These research fields usually associated vitamins as growth factors required by microorganisms with those of animals, allowing for fascinating discoveries and progress of biological significance to be made (Burkholder, 1943).

2.2.1 Biotin

Biotin is a cofactor involved in the central metabolic pathways of microorganisms that are essential for yeasts. Of all the vitamins mentioned above, biotin is regarded as a vital vitamin for yeasts (Walker, 1998). The high priority of biotin by yeasts is due to biotin being involved in the synthesis of DNA, amino acids, fatty acids and degradation of carbohydrates (Lafon-Lafourcade & Guimberteau, 1962; Walker, 1998).

2.2.1.1 Biotin-synthesis in yeasts

The biosynthesis pathways of biotin in microorganisms have been explored in detail since 1967 (Streit & Entcheva, 2003). The genes that encode the relevant enzymes have been identified in bacteria, archaea and eukarya (the latter being the domain to which yeasts belong). In eukaryotes, knowledge regarding the genes involved in biotin biosynthesis has been obtained from studies on plants and yeasts as model organisms, primarily in *S. cerevisiae*. In *S. cerevisiae*, the genes are contained in a cluster which can be found on chromosome 14 except for one gene (Streit & Entcheva, 2003). This cluster comprises the following genes, commonly referred to as *BIO*-genes, which encode for proteins that play pivotal roles in biotin biosynthesis: *BIO2* (biotin synthase which is located on chromosome 7); *BIO4* (dethiobiotin

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synthetase) and *BIO5* (7, 8-diaminopelargonic acid aminotransferase). Of these genes, *BIO5* encodes for Bio5p (7, 8-diaminopelargonic acid aminotransferase), an important protein involved in translocation of precursor molecules such as 7,8-diamino-pelargonic acid (DAPA) and 7-keto-8-amino-pelargonic acid (KAPA) (Zhang *et al*., 1994; Weaver *et al*., 1996; Phalip *et al*., 1999). It has been reported that most strains of *S. cerevisiae* are not able to synthesise biotin *de novo* due to the lack of specific genes that form part of the biosynthesis pathway of biotin (Pirner & Stolz, 2006). However, these strains can perform the last three steps in biotin synthesis and is presented in **Figure 2.1** (Pirner & Stolz, 2006).

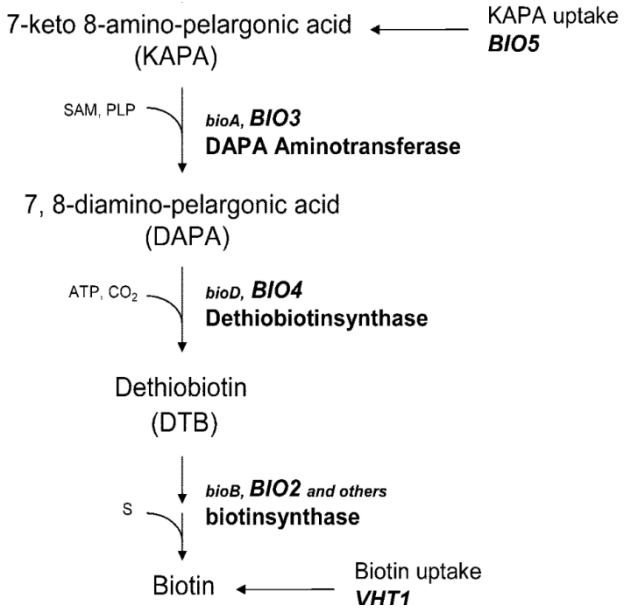

Figure 2.1: The last three steps in biotin synthesis that yeasts can perform (Adapted from Wu *et al*., 2005)

2.2.1.2 Role of biotin in yeast metabolism

Six carboxykinase reactions have been identified as being dependent on biotin (Romero-Navarro *et al*., 1999):

1) Acetyl-coenzyme A (CoA) carboxylase: catalyses the binding of bicarbonate to acetyl-CoA, resulting in the formation of malonyl-CoA. This step is crucial in fatty acid synthesis (Streit & Entcheva, 2003). Since fatty acids play a role in maintaining membrane integrity in yeast cells, this function may account for the biotin requirement by most yeasts, including non-*Saccharomyces* yeasts, to survive osmotic and ethanol stress during fermentation in the presence of more robust yeasts such as *S. cerevisiae*.
2) Pyruvate carboxylase: catalyses the formation of oxaloacetate from HCO_3^- and pyruvate (Streit & Entcheva, 2003).

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3) Methyl crotonyl-CoA carboxylase: catalyses the condensation of 3-methylcrotonyl-CoA and $HCO3^-$ to form methyl-glutaconyl-CoA. This step is required to degrade leucine to use it alternatively as a carbon rather than a nitrogen source (Streit & Entcheva, 2003).
4) Propionyl-CoA carboxylase: catalyses important steps in various metabolic reactions, for example, amino acids, cholesterol, as well as odd-chain fatty acids.
5) Geranyl-CoA carboxylase: catalyses isoprenoid catabolism, though limited information exists regarding the organisms it entails.
6) Urea carboxylase (part of urea amidolyase): catalyses the carboxylation of urea to form urea allophanate. Allophanate can undergo hydrolysis by allophanate lyase, an enzyme part of urea amidolyase, which may produce bicarbonate and ammonium ions (Whitney & Cooper, 1972).

Overall, biotin plays a significant role in amino acid metabolism, fatty acid synthesis, as well as gluconeogenesis- all of which highlights its importance in the central metabolic network of microorganisms. It has been reported by Dixon and Rose (1964) that in the case of extreme biotin deficiency in yeasts, the cell wall thickens more than usual due to the production of lipids that results from the overproduction of short chain fatty acids. Also, biotin deficiency may lead to increased levels of acetyl-CoA as well as mitochondrial hyperacetylation, which in turn may introduce alterations in the cellular respiration in yeasts as well as increased reactive oxygen species (Madsen *et al*., 2015).

2.2.1.3 Impact of biotin on wine fermentations

Compared to other fruits, grapes have been reported to contain the lowest amounts of biotin (Radler, 1957), although its concentration differs between grape cultivars (Ough & Kunkee, 1967). Ough and Kunkee (1967) further reported that, overall, red grapes contain more biotin than white grapes. Varying concentrations of biotin may have significant effects on the resulting fermentation, especially considering that the growth of certain strains depends on biotin availability in the medium, as reported above. To investigate the impact of biotin on fermentation rates, Ough and Kunkee (1967) added double the amount of biotin to what was found initially in several white grape juices, and observed no increase in fermentation rate, suggesting that, although low, the biotin concentration in white grape juice is generally sufficient to support the growth of the yeasts.

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In a more comprehensive study performed by Ough *et al*. (1989), the effects of several vitamins on growth, fermentation rates and cell viability were investigated. These authors reported a decrease in fermentation rate and growth when biotin was omitted from the synthetic grape juice used. This observation was found for all the strains they investigated. The findings of Ough *et al*. (1989) suggest that biotin is essential and that yeasts rely on extracellular biotin for their metabolism.

More recently, the interaction between nitrogen and biotin in alcoholic fermentation was investigated (Bohlsheid *et al*., 2007). The study reported on biotin affecting yeast growth, with the support of at least 1 $\mu g.L^{-1}$ in the fermentation medium allowing for maximum population growth to be observed. As for the impact of nitrogen alone, the study found that higher amounts of yeast assimilable nitrogen (YAN) led to increased fermentation rates, yet stable yeast growth among the various levels of YAN used in the experimental design. The effect of the interaction between biotin and YAN on fermentation times provided noteworthy results. The authors reported that in conditions of high YAN the fermentation time decreased with the increase of biotin concentrations ranging from 1 $\mu g.L^{-1}$ to 10 $\mu g.L^{-1}$. It is possible that with high YAN, yeasts are more metabolically active and would accordingly require more pyruvate carboxylase, urea carboxylase and acetyl-CoA. The activity of these enzymes is dependent on the presence of biotin. These requirements will, therefore, increase the demand for biotin. Since a decrease in fermentation time rather than fermentation rate was observed with increasing concentrations of biotin, the availability of biotin might play a crucial role in the late phases of growth during fermentation.

2.2.2 Thiamine

Thiamine is an essential cofactor for enzymes and functions in the carboxylation of reactions including carbohydrate catabolism, as well as the decarboxylation of α-keto acids during amino acid metabolism (Li *et al*., 2010). Yeasts can synthesise thiamine *de novo* as well as assimilate it from the environment (Hohmann & Meacock, 1998).

2.2.2.1 Thiamine-synthesis in yeasts

In its ability to reverse the adverse effects associated with thiamine deficiency, termed beriberi in animals, thiamine was extensively studied as the first water-soluble B-vitamin (Eijkman, 1990; Kowalska & Kozik, 2008; Li *et al*., 2010). A schematic representation of *de novo*, as well as external uptake of thiamine, is represented in **Figure 2.2**.

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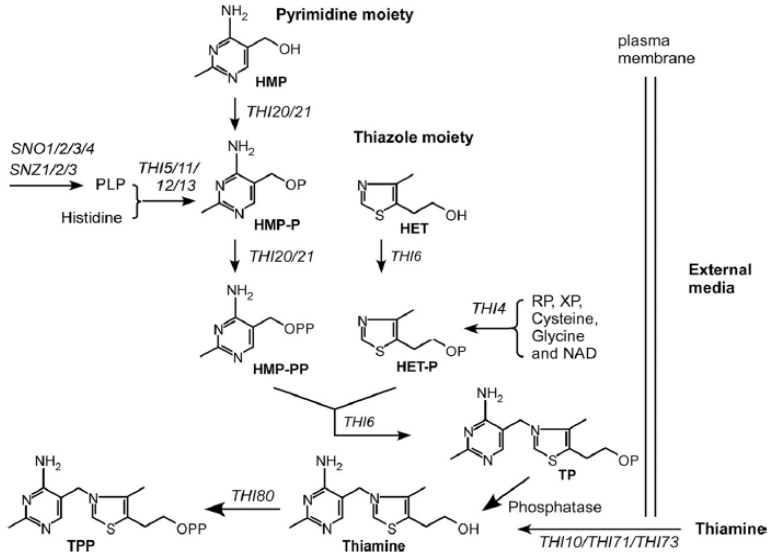

Figure 2.2: Schematic illustration of the synthesis and genes involved of thiamine both *de novo* and uptake from environment in yeasts. HET-P, 5- (2-hydroxyethyl)-4-ethylthiazole phosphate; RP, ribulose-5-phosphate; XP, xylose-5-phosphate; NAD, nicotinamide adenine dinucleotide; HMP-PP, 4-amino-5-hydroxymethyl-2- methylpyridimidine diphosphate; PLP, pyridoxal-5-phosphate; TPP, thiamine pyrophosphate; TP, thiamine monophosphate Genes involved are in italics (From: Li *et al*., 2010).

In two separate reactions, the yeasts start by synthesising two precursor molecules, including 5- (2-hydroxyethyl)-4-ethylthiazole phosphate (HET-P) and 4-amino-5-hydroxymethyl-2-methylpyridimidine diphosphate (HMP-PP). These two precursor molecules are then condensed to form thiamine monophosphate. In yeast cells, HMP-PP is synthesised from histidine as well as pyridoxal-5-phosphate (PLP, a component of pyridoxine), providing thus a linkage between the thiamine and pyridoxine. Due to this interlink, the requirement of pyridoxine by yeast may be dependent on the presence and concentration of thiamine. Substrates including cysteine, D-pentulose-5-phosphate and glycine are required for the synthesis of HET-P (Kowaliska & Kozik, 2008). However, it was found that the moiety of HET-P might also be synthesised via an advanced intermediate, the Thi4 protein, which in turn consumes nicotinamide adenine dinucleotide (NAD^+) to yield nicotinamide (NAM) as a by-product (Chatterjee *et al*., 2007). NAM and nicotinic acid (NA) are both alternative forms of niacin, also commonly referred to as vitamin B_3. Genes involved in thiamine synthesis is only expressed when thiamine is required (Hohmann & Meacock, 1998). For *S. cerevisiae*, the uptake of thiamine is an energy-dependent process (K_m = 3 µg.L^{-1}) and is mediated by a single transport system (Bataillon *et al*., 1996). As for other yeast species, such as *Kloeckera apiculata*, the energy-dependance of thiamine transport are still unclear (Versavaud *et al*., 1995; Schütz & Gafner, 1993).

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2.2.2.2 Impact of thiamine on wine fermentations

The thiamine concentration in grape musts has been reported to be between 150 and 750 $\mu g.L^{-1}$ (Peynaud & Lafourcade, 1977). Thiamine is accumulated in very high amounts by yeasts, as reported in a study focusing on *K. apiculata*, being able to accumulate amounts of thiamine that are equivalent to one-tenth of dry cell weight (Ough *et al*., 1989). In a different study performed by Bataillon *et al.* (1996), it was reported that fermentation kinetics depends on the initial concentration of thiamine in a pre-culturing medium and at what time cells are harvested for consequent fermentation. In addition, the assimilation of thiamine or any vitamin by wild yeasts in the early stages of alcoholic fermentation might influence the availability of these vitamins for later assimilation by *S. cerevisiae*. This deficiency could lead to stuck and sluggish fermentations. The study by Bataillon *et al*. (1996) focused on the use of *K. apiculata* and *S. cerevisiae*, thus interactions between other wild yeasts and *S. cerevisiae*, for example, *Hanseniaspora*, *Pichia*, *Metschnikowia*, *Torulaspora* and *Lachancea*, still need to be defined.

In a more recent study, low levels of thiamine have been reported to affect the metabolism rate of yeasts at a lower temperature especially in the lag-phase (Ferreira *et al*., 2017). The authors explained this to be due to low metabolic rates at a lower temperature, resulting in reduced thiamine uptake which may ultimately lead to longer lag-phases. The report of Ferreira *et al.* (2017) concurs with previous literature, suggesting that thiamine supports resistance to osmotic, thermal and oxidative stresses, especially in the early stages of growth (Wolak *et al.*, 2014). However, responses to stress conditions are strain -specific (Ferreira *et al*., 2017).

2.2.3 Pantothenate

Pantothenate serves as a precursor molecule for CoA which in turn serves as a crucial cofactor in a wide range of metabolic reactions (White *et al*., 2001). Pantothenate is also involved in lipid metabolism (Duc *et al*., 2017). Previous work which focused on the nutritional requirements of yeasts, specifically *S. cerevisiae*, reported on β-alanine serving as an alternative to pantothenate (Leonian & Lilly, 1945). This finding implied that *S. cerevisiae* possesses the necessary enzymes to allow for pantothenic acid biosynthesis except for aspartate-1-decarboxylase (Williamson & Brown, 1979; Cronan, 1980). It is known that a structural homolog of aspartate-1-decarboxylase is absent in yeast (Hodges *et al*., 1999), but that structural homolog of all the other enzymes (*S. cerevisiae* as reference) in the pantothenic acid biosynthesis pathway are found in yeast. Thus, *S. cerevisiae* can either incorporate

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pantothenate directly from the exogenous environment or use β-alanine as a precursor to synthesise pantothenate *de novo*.

2.2.3.1 Pantothenate synthesis in yeasts

A schematic illustration of pantothenate-synthesis in yeasts is presented in **Figure 2.3**. The formation of β-alanine results from the formation of spermine through amine oxidase, encoded by *FMS1*. For the processes to be successful, the β-alanine moiety of pantothenate results from the conversion of methionine through the *S*-adenosylmethionine and polyamine pathway.

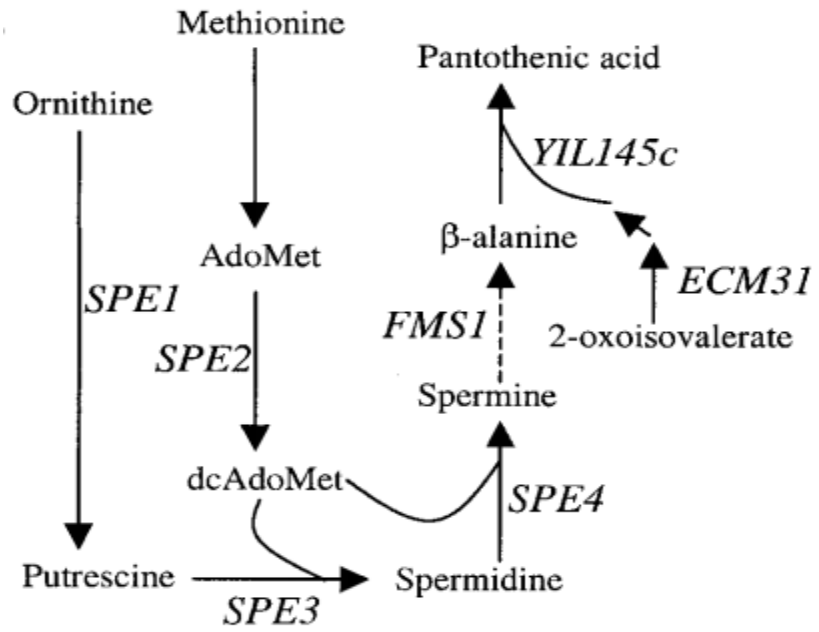

Figure 2.3: Schematic illustration of the synthesis and genes involved in pantothenic acid biosynthesis (From:White *et al*., 2001).

2.2.3.2 Impact of pantothenate on wine fermentations

Research has focused on the impact of pantothenate alone as well as on its impact with other growth factors. The amount of pantothenate in grapes have been reported to be approximately 6.8 $mg.L^{-1}$ and 8.5 $mg.L^{-1}$ in white and red grapes, respectively. Furthermore, the concentration of pantothenate in grapes can vary, suggesting that limitations can occur, depending on the extent of the variation (Hagen *et al.* 2008).

In a study carried out by Ough *et al*. (1989), different strains of *S. cerevisiae* were investigated concerning the impact of pantothenate on fermentation rate and cell viability. The authors found that elimination of pantothenate from the growth medium resulted in a decrease of

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fermentation rate and cell viability amongst all the strains investigated. In a different study (Hosono *et al*., 1972), the authors focused on the impact of pantothenate deficiency on an *S. cerevisiae* strain regarding respiration rate and hydrogen sulphide (H_2S). Cells sufficiently provided with pantothenate had an oxygen uptake rate approximately fifteen-fold higher than those deficient in pantothenate. Furthermore, the fermentation rate in early exponential growth phase was also higher than the late exponential growth phase when pantothenate was sufficient to support growth. An unfavourable compound in wine fermentations, H_2S, significantly accumulated when cells were deficient of pantothenate, approximately ten-fold more than cells presented with sufficient amount of pantothenate.

A different study evaluated the impact of pantothenate on wine fermentations (Wang *et al*., 2003). This study concurred with that by Hosono *et al*. (1972) who also reported on the production of H_2S in conditions deficient of pantothenate. Interestingly, the authors also reported on pantothenate deficiency leading to a decrease in higher alcohols as well as affecting lipid metabolism in *S. cerevisiae*. The following rationale can explain the impact on higher alcohols. The production of higher alcohols is partially due to initial transamination reactions between an amino acid and an α-keto acid followed by decarboxylation and reduction (Lambrechts & Pretorius, 2000). Isobutyl alcohol and isoamyl alcohol have an α-keto acid precursor in common, α-ketoisovalerate, as reported by Slaughter and McKernan (1988). Since the synthesis of isobutyl alcohol is dependent on acetyl-CoA, the dependency on pantothenate to produce this higher alcohol is emphasised, since pantothenate serves as a precursor in the synthesis of acetyl-CoA (Rucker & Bauerly, 2007).

Apart from its impact on fermentation kinetics and higher alcohols in wine aroma, pantothenate also may have an impact on lipid metabolism of yeasts. The production of medium-chain fatty acids to serve as intermediates for longer-chain fatty acids and ethyl esters, are of significance concerning the presence of pantothenate. Fatty acid-synthesis is mainly dependent on acetyl-CoA carboxylase as well as fatty acid synthetase (Lynen, 1980). Since pantothenate is a precursor in the synthesis of acetyl-CoA, a decrease in pantothenate could result in a decrease in acid-synthesis which may have an impact on a yeast's cell membrane integrity and at utmost cell survival, since the cell membranes of yeasts mostly consist of phospholipids, protecting against harsh conditions.

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The impact of pantothenic acid addition on H_2S production by two *Saccharomyces* yeasts have been investigated (Edwards & Bohlsheid, 2007). It was reported that H_2S production varied depending on the *S. cerevisiae* strain and on the time when the vitamin was added to the growth medium (from the onset; 48 hours after onset; 96 hours after the onset). Findings reported no distinct differences in the population size or fermentation rate of the two yeast. However, differences were observed in the cumulative H_2S production over time. Findings displayed an increase in H_2S production, regardless of the yeast used, when no pantothenate was added to the growth medium, followed by (in decreasing order of contribution towards H_2S production): addition of pantothenate 96 hours after, 48 hours after and from the onset. These findings may be crucial for winemakers, as H_2S is an undesired compound in wine (odour suggestive of rotten eggs). Thus, the concentration of pantothenate before the start of fermentation is vital to support beneficial auxotrophic yeasts, as well as to prevent off-flavour in the form of H_2S production.

In a more recent study (Duc *et al*., 2017), the impact of pantothenate limitations on yeast cell death in a nitrogen-dependent manner has been investigated. It was found that in an excess amount of nitrogen within a synthetic fermentation medium, a commercial strain of *S. cerevisiae* (EC1118) died during fermentation when pantothenate was omitted. However, the same phenomenon was not observed when thiamine, biotin and inositol were absent yet slow fermentations were observed. These findings suggest that the loss in cell viability during fermentation of *S. cerevisiae* in response to the limitation of pantothenate may be modulated by the amount of assimilable nitrogen that is present in the growth medium. This phenomenon is being compensated for in the wine industry, by adding nitrogen, usually in the form of NH_4^+ (Duc *et al*., 2017). The results of Duc *et al*. (2017) concur with other studies previously performed (Wang *et al*., 2003; Hagen *et al*., 2008).

2.2.4 Pyridoxine

Pyridoxine is part of a complex of other B_6-vitamins, consisting of pyridoxine, pyridoxal and pyridoxamine (Castor, 1953). Specificity for one or two of the three components results from some microorganisms either not being able to convert unphosphorylated forms to active enzyme forms, i.e. pyridoxal-P-phosphate and pyridoxamine-P-phosphate, or difficulty regarding transport (Snell & Rannefeld, 1945; McNutt & Snell, 1950; Rabinowitz & Snell, 1953; McCormick *et al*., 1961). Pyridoxine, in its biologically active components, including pyridoxal-5'-phosphate (PLP) and pyridoxamine-5'-phosphate, is a versatile cofactor.

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Pyridoxine plays significant roles in the metabolism of amino acids and is also commonly found in various metabolic pathways, for example, interconversion pathways converting amino acids into antibiotic compounds in humans (Schneider *et al*., 2000).

2.2.4.1 Pyridoxine-synthesis in yeasts

A schematic illustration of pyridoxine-synthesis in yeasts is presented in **Figure 2.4**. Synthesis of pyridoxine starts with ribulose-5-phosphate which makes up the 2'-4'portion of the pyridoxine molecule initially. The ribose can be made available by means of ribose-5-phosphate ketol-isomerase, a gene product from *RKI1*, which helps in mediating the interconversion of ribose-5-phosphate and ribulose-5-phosphate in the pentose phosphate pathway (Dong *et al*., 2004). Other than ribose as a pentulose, glycine and an S-source also serves as substrates for pyridoxine synthesis (Zeidler *et al*., 2003). Sno1p and Snz1 serve as a glutaminase, providing ammonia for the nitrogen ring of pyridoxine. The process of incorporating Snz1p is unclear (as indicated by '?'), however, is assumed to be due to condensation, with the additional aid of the encircled P (phosphoryl group).

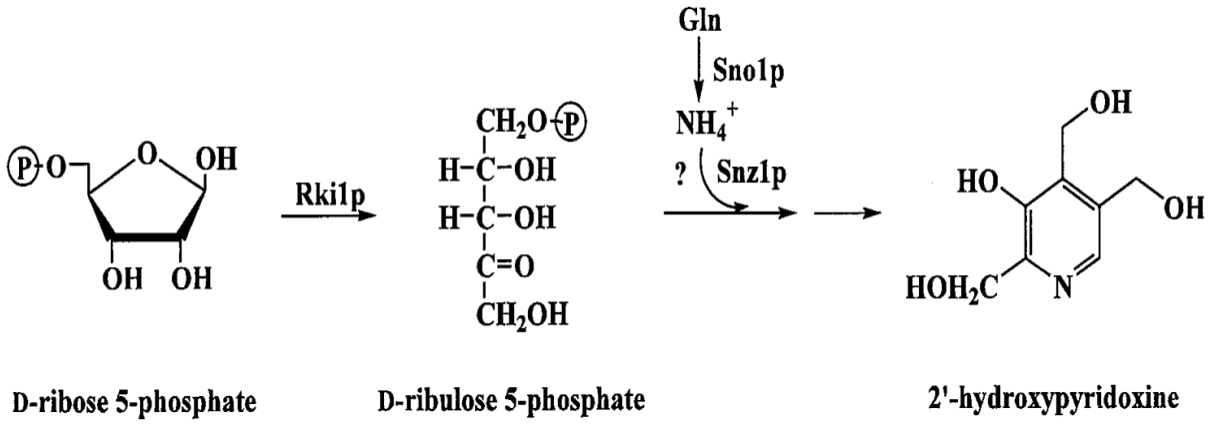

Figure 2.4: Synthesis of pyridoxine in yeasts (From: Kondo *et al*., 2014)

2.2.4.2 Impact of pyridoxine on wine fermentations

The amount of vitamin B_6 in grapes has been reported (Hall *et al*., 1956) and differs between white and red grapes, with an average content in white grapes of approximately 8.8 g. L^{-1}, whereas with red grapes the average vitamin B_6 content is approximately 12.5 g. L^{-1}. Very few studies have focused on the potential impact of a deficiency in pyridoxine on wine fermentations. In a study by Ough *et al*. (1989), the elimination of pyridoxine from the growth medium had different impacts on the fermentation rates of three different strains of

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S. cerevisiae. However, no change in cell viability was observed. When both thiamine and pyridoxine were removed from the growth medium, a similar response was exhibited by all the strains than that when only thiamine was eliminated. These observations raised the question whether a specific interaction between thiamine and pyridoxine exists, especially with the knowledge that some studies reported on certain microorganisms being only able to synthesise or metabolise pyridoxine when exogenous thiamine is present (Moses & Joselyn, 1953; Zeidler *et al*., 2003). The interrelationship between thiamine and pyridoxine have received ongoing investigation with the one of the earliest publications suggesting that: 1) thiamine or pyridoxine may serve as a precursor for an intermediate that may allow for the synthesis of the other or otherwise allow for the catalysis of the other (reversible interconversion); (2) one of the two vitamins might serve as a replacement for the other in the synthesis of an important intermediate produced by the influence of the other (Moses &Joselyn, 1953). Later on, more research shed light on the relations between thiamine and pyridoxine (Zeidler *et al*., 2003). It was reported that pyridoxine serves as an intermediate in the synthesis of thiamine in *S. cerevisiae* cells, of which the pyrimidine unit of thiamine is synthesised from histidine and pyridoxine.

A more recent study has updated on previous reviews regarding the interrelationship that exists between thiamine and pyridoxine, as well as evaluated the combined effect of these two vitamins on alcoholic fermentations (Xing, 2007). For the addition of only pyridoxine, similar results in comparison to the addition of only thiamine were found for yeast growth and fermentation rate. The addition of pyridoxine resulted in increasing fermentation rates and yeast growth, irrespective of the levels of YAN. These findings indicated that only pyridoxine affected the growth and fermentation kinetics of the fermentation. In addition, it was reported that pyridoxine could have also influenced specific reactions in yeast metabolism, for example in biochemically active form, pyridoxine exists as PLP which can undergo various reactions with amino acids. Xing (2007) further discussed the effect of nitrogen in combination with pyridoxine, by concluding that since nitrogen is usually found in larger quantities than pyridoxine and other vitamins in general, a masking effect may occur from the dominant nutrient that becomes limiting. This study also highlighted the impact of pyridoxine on H_2S production. The findings suggest that low levels of pyridoxine may result in lower levels of H_2S production. This is possible through PLP being required for certain condensations steps in sulphur metabolism pathway (Wiebers & Garner, 1967; Botsford & Parks 1969). Xing (2007) reported on the combined impact of thiamine and pyridoxine. Concurring with the findings by

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Zeidler *et al*. (2003), it was reported that an increase in thiamine resulted in decreased yeast growth and proved to be inhibitory (Xing, 2007). Since pyridoxine is an intermediate in thiamine synthesis, once thiamine is supplemented, the concentration of pyridoxine also needs to be increased to prevent inhibition.

2.2.5 Inositol

The synthesis and metabolism of inositol play a significant role in contributing to the membrane integrity of yeasts (Majerus *et al*., 1986). In yeasts, inositol and its role in phospholipid metabolism, are highly regulated at the cytoplasmic enzyme, MI-1-P-synthase (Majerus *et al*., 1986).

2.2.5.1 Inositol-synthesis in yeasts

A specific pathway for the synthesis of inositol has been reported for *S. cerevisiae* and is presented in **Figure 2.5** (Henry *et al*., 2014). The synthesis of inositol starts with the MI-1-P synthase, encoded by the *INO1* gene, catalysing the synthesis of inositol-3-phosphate *de novo* through the ring formation of glucose-6-phosphate (Eisenberg & Bolden, 1962; Loewus & Kelly, 1962; Eisenberg *et al*., 1964). The process involves the requirement of NAD and no net gain in NADH, suggesting that the overall reaction comprises of highly coupled oxidation and reduction reactions (Kiely & Sherman, 1975).

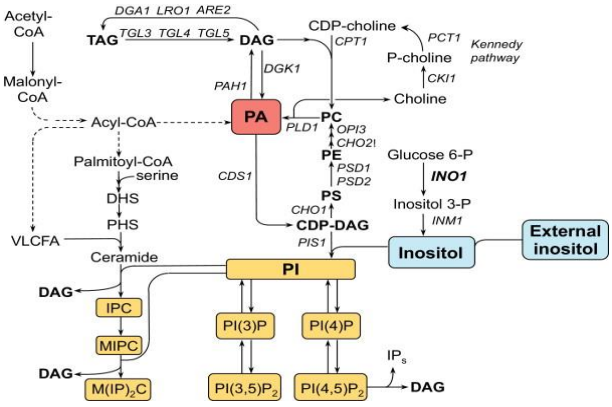

Figure 2.5: Schematic illustration of inositol synthesis *de novo* in *S. cerevisiae* and other important phospholipids, sphingolipids, phosphoinositides and triacylglycerols. DAG, diacylglycerol; CDP-DAG, cytidine diphosphate diacylglycerol; CDP-choline, cytidine diphosphate choline; PA, phosphatidic acid; PI, phosphatidylinositol; PI(4)P, phosphatidylinositol 4-phosphate; $PI(4,5)P_2$, phosphatidylinositol 4,5-*bis*phosphate; PI(3)P, phosphatidylinositol 3-phosphate; $PI(3,5)P_2$, phosphatidylinositol 3,5-*bis*phosphate; IPC, inositol-phosphorylceramide; MIPC, mannosyl-inositol-phosphorylceramide; $M(IP)_2C$, mannosyl-diinositol-phosphorylceramide; PE, phosphatidylethanolamine; PS, phosphatidylserine; PC, phosphatidylcholine; TAG, triacylglycerols; FS, free sterols; FFA, free fatty acids; SE, steryl esters; PL, phospholipids; VLCFA, very-long-chain fatty acids; DHS, dihydrosphingosine; PHS, phytosphingosine. IP_s refers to the inositol soluble phosphates. Solid arrows: direct conversion, dashed arrows requires more than one conversion step (Adapted from: Henry *et al*., 2014).

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2.2.5.2 Impact of inositol on wine fermentations

The amount of inositol in grapes is unclear; however, it can be expected to be quite high as a review by Robinson (1951) indicated high amounts of inositol to be found in plant tissue, cereal and fruit. In addition, inositol is usually found in the form of phosphoric esters in nature. As with pyridoxine, little to no studies have focused on the impact inositol might have on wine fermentations. A study by Ough *et al.* (1989) found that the elimination of inositol from the growth medium resulted in a decrease in both fermentation rate and cell viability amongst all the strains of *S. cerevisiae*. The limitation of their study was the specific focus on only *S. cerevisiae*. Therefore, it might be more valuable to investigate the impact of inositol on wine fermentations, especially with the use of NS yeasts.

In a recent study, the importance of inositol is emphasised when the impact of low temperature on yeast cell survival was investigated. Low temperature is considered to improve sensorial qualities of wine (mainly white and rosé wines), but could have certain drawbacks, for example, long lag phases, slow growth rate and stuck or sluggish fermentations (López-Malo *et al*. 2015). When the temperature is low, the molecular order of the membrane lipids is increased through rigidification (Russell, 1990). In response to rigidification, yeasts adapt their membrane lipid composition to allow for cell survival (Patton-Vogt & Henry, 1998; Fisher *et al*., 2005). In limited inositol conditions, the strain investigated was not able to reshape its membrane lipid composition to adapt to low temperature as a harsh condition which could potentially affect cell survival. In addition, the membrane phospholipid content was two-fold less in inositol-deficient conditions in comparison to when inositol was sufficient in the growth medium (López-Malo *et al*., 2015). These findings contribute to highlight the importance of inositol in yeast survival during fermentation, which if deficient, could potentially affect the final wine.

2.3 The linkage between vitamins and the aroma profile of a wine

As mentioned above, vitamins could play a potential role in the overall sensory profile of wine, by being actively involved in the synthesis or metabolism of precursor molecules that could ultimately affect the final wine. In **Figure 2.6**, certain parts of importance in the pathways are highlighted where certain vitamins might play a significant role, for example, both thiamine and pantothenate facilitate metabolic reactions that produce precursors for higher alcohols.

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2.4 Yeasts of interest and their contribution to wine aroma: a brief overview

The vitamin requirements of yeasts are strain-specific, and for the yeasts of focus, these requirements are summarised in **Table 2.1**.

Table 2.1: The vitamin requirements of selected yeast species (adapted from Barnett *et al*., 2000), evaluated based on the growth response when the particular vitamin was present or not.

Species	Biotin	Thiamine	Inositol	Pyridoxine	Pantothenate
S. cerevisiae	V	V	V	-	V
L. thermotolerans	-	V	+	V	-
T. delbrueckii	V	-	V	-	-
P. kluyveri	V	-	-	+	-
M. pulcherrima	-	V	+	V	-
H. vineae	V	V	+	+	+

+: required; -: not required; V: variable (might be strain-specific)

From **Table 2.1**, it is clear that the vitamin requirements are diverse and different amongst yeasts. Variability amongst these requirements could potentially be ascribed to the respective yeasts inherent capabilities to synthesise a vitamin *de novo*. A high amount of variability regarding biotin and thiamine as vitamins is apparent amongst the yeasts. Inositol appears to be a essential vitamin by most of the yeasts other than *S. cerevisiae*. In contrast, pantothenate appears to be not as required by most of the yeasts.

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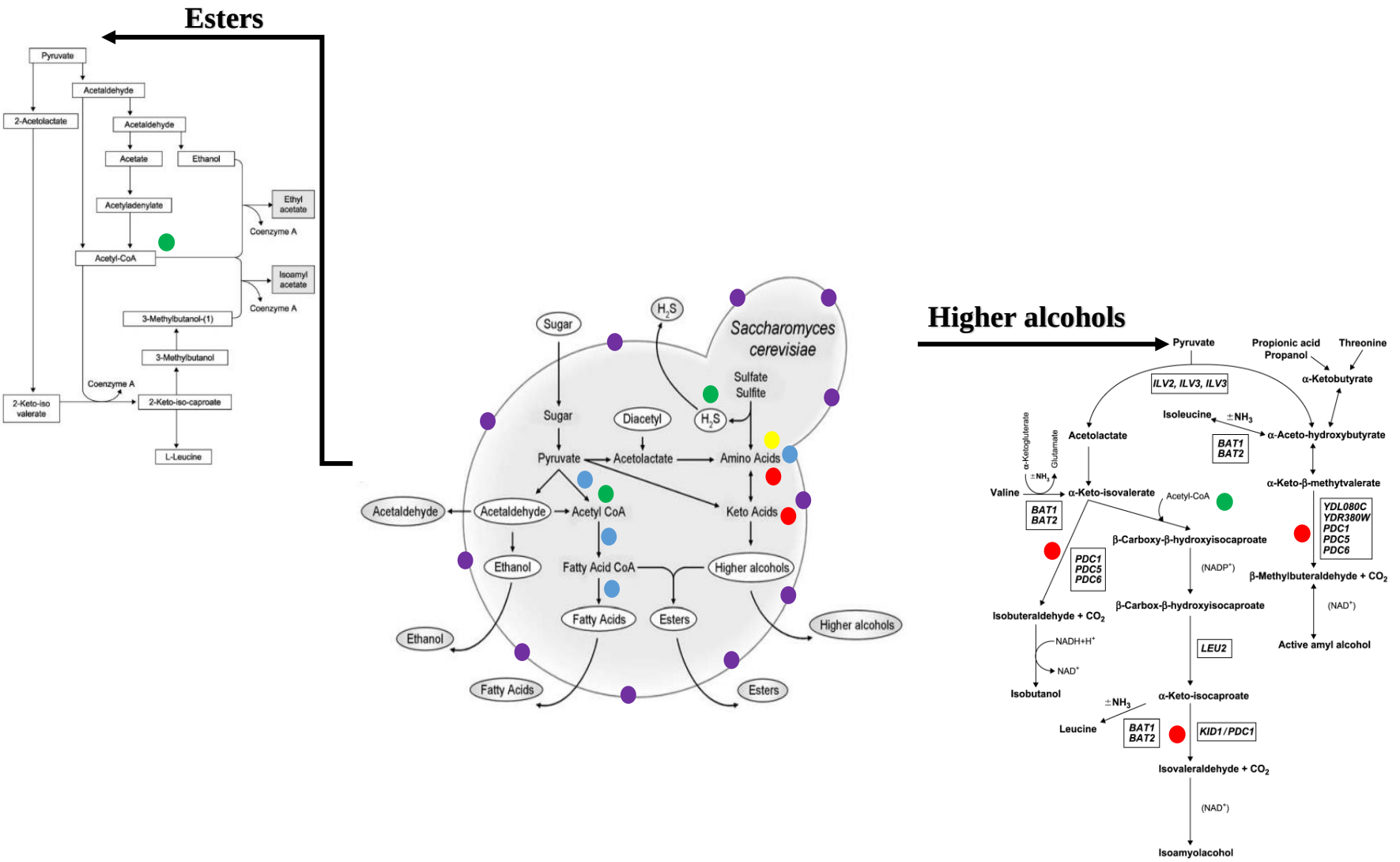

Figure 2.6: Schematic illustration of the possible points of impact by vitamins on wine aroma (Adapted from: Swiegers & Pretorius, 2005). Key: ●, biotin; ●, pantothenate; ●, thiamine; ●, pyridoxine; ●, inositol.

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2.4.1 *Saccharomyces cerevisiae*

Apart from its central involvement in alcoholic fermentation, *S. cerevisiae* also contributes to the formation of important secondary metabolites in wine (Fleet, 1993) as well as the formation of precursors necessary to allow for the development of various aromas in wine (Darriet *et al*., 1995; Tominaga *et al*., 1996; Ribéreau-Gayon *et al*., 2000; Howell *et al*., 2004). With extensive research done on *S. cerevisiae* and its involvement in winemaking, researchers have tried to apply the same techniques towards the possible use of NS yeasts in winemaking (**Table 2.2**).

Table 2.2: The impact of non-*Saccharomyces* yeasts on the aroma and quality of wine when fermenting in pure culture as compared to a mixed culture with *S. cerevisiae* (adapted from Ciani & Comitini, 2011)

Non-*Saccharomyces* yeasts	Impact	Effect of mixed fermentation with *S. cerevisiae*
L. thermotolerans	Produces lactic acid Low producer of acetaldehyde	Increase in acidity Reduction in formation of acetaldehyde
T. delbrueckii	Low producer of acetic acid	Reduction in the production of acetic acid
P. kluyveri	High 3-mercaptohexyl acetate producer	Increase in the concentration of thiols
M. pulcherrima	High producer of ethyl octanoate	Increase in overall quality (grape and strain specific)
H. vineae	High producer of acetate esters	Increase in flavour density

2.4.2 *Torulaspora delbrueckii*

With its anamorph as *Candida colliculosa*, *T. delbrueckii* which was formerly known as *Saccharomyces rosei* is one of the first commercially sold NS yeasts. This yeast has been previously suggested for the fermentation of grape musts low in sugar and acidity in the production of red and rosé wines in Italy (Castelli, 1955). In comparison to *S. cerevisiae*, reports were made on the observation of lower levels of acidity being produced by pure cultures of *T. delbrueckii* and allowed for the latter to be used in the fermentation of botrytized grapes which are high in sugar (Moreno *et al.*, 1991; Bely *et al*., 2008; Renault *et al*., 2009). Furthermore, *T. delbrueckii* can produce succinic acid (Ciani & Maccarelli, 1998) and linalool (derived from monoterpene alcohols) that adds flavour

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complexity to, especially Muscat type wines (King & Dickenson, 2000). The production of succinic acid and linalool is reported to be strain-specific (King & Dickenson, 2000). In addition, *T. delbrueckii* is remarkable in not only adding to the varietal aroma but also modulating and affecting wine aroma (Jolly *et al*., 2014).

Jolly *et al*. (2017) performed fermentation on Sauvignon Blanc and Chenin Blanc with the use of the co-inoculation of *T. delbrueckii* and *S. cerevisiae* in comparison to their *S. cerevisiae* reference wine, five and 18 years after their production. They found improved aroma profiles from co-inoculation. Furthermore, Azzolini *et al*. (2012) performed a sequential inoculation fermentation with *T. delbrueckii* and *S. cerevisiae* on Amarone wines and observed an increase in certain attributes of aroma intensity, such as sweetness and 'ripe red fruit' aroma. Also, a decrease in vegetal attributes was reported. Although first sold as a blend of active commercial dried yeast in 2013, with other manufacturers, including Lallemand (2012) and Laffort oenology (2013), different strains of this yeast are being sold. This may be indicative that some winemakers prefer some selected and tested strains of *T. delbrueckii* and possibly other NS yeasts.

2.4.3 *Lachancea thermotolerans*

Formerly known as *Kluyveromyces thermotolerans* (Lachance & Kurtzman, 2011), *L. thermotolerans* has been reported to allow for high production of glycerol, lactic acid and 2-phenylethanol during the mixed fermentation of grape musts (Kapsopolou *et al*., 2007; Comitini *et al*., 2011; Gobbi *et al*., 2013). Gobbi *et al*. (2013) reported on distinct 'spicy', and 'acidity' attributes being ascribed to the aroma profile of 10 000 L Sangiovese grape fermented with *L. thermotolerans* in co-culture with *S. cerevisiae*. In comparison, the wine aroma that results from the chemical composition of wine is known to be affected by the time of inoculation of *L. thermotolerans* with *S. cerevisiae* (Kapsopolou *et al*., 2007; Gobbi *et al*., 2013). These authors further stated that the later *L. thermotolerans* are inoculated with *S. cerevisiae*, the higher the amount of lactic acid in glycerol in the final fermented must.

In 2004 (Anonymous, 2004b), a commercial blend of dried yeast pertaining *L. thermotolerans* (marketed as *K. thermotolerans* at the time) was made available for commercial use as Viniflora® Symphony (Chr. Hansen). This commercial yeast was believed to allow the improved development of aroma and flavour of white and red wine varieties, explicitly contributing floral and tropical fruity notes to a wine. However, contrasting results were obtained when tests with this yeast led to a decrease in the aroma of red wines and no specific "spicy" notes as believed (Takush & Osborne,

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2012). Furthermore, when compared to the commercial strain *S. cerevisiae* EC1118, the commercial yeast produced wines that had less intensity of general, 'dark fruit' and 'jammy/ cooked' aroma. These findings suggest that the choice of *S. cerevisiae* as co-fermenting strain may also have an influence on the final aroma profile of wine in mixed fermentations. With these findings, Viniflora® Symphony was released as single active dry yeast in 2012.

2.4.4 *Pichia kluyveri*

The co-fermentation of *P. kluyveri* with *S. cerevisiae* allows for an increase in thiol production, especially 3-mercaptohexyl acetate (Anfang *et al*., 2009). Interestingly, it has been reported that *P. kluyveri* can produce zymocins, which are killer toxins that may inhibit the growth of *S. cerevisiae* (Middelbeek *et al*., 1980). This phenomenon may allow for interesting results to be observed due to the possible up-regulation of genes involved in fermentative upon the supplementation of vitamins, allowing for competition to be observed between *P. kluyveri* and *S. cerevisiae* when grown in co-culture. On a commercial scale, a strain of *P. kluyveri* is available and has the unique characteristic in comparison to other yeasts to extract flavour precursor metabolites at a much higher level. This yeast strain is recommended for the fermentation of Riesling, Chardonnay and Sauvignon Blanc grape musts by the manufacturer. Different from other commercially sold yeasts, *P. kluyveri* as a commercial product is not an active dry yeast, but rather stored and delivered frozen (-45°C) and can be directly added to a fermentation growth medium without rehydration.

2.4.5 *Metschnikowia pulcherrima*

With its anamorph as *Candida pulcherrima*, *M. pulcherrima* is also a commercially available NS yeast. This commercial yeast secretes α-arabinofuranosidase that affects terpenes and volatile thiol levels (Lallemand, 2012). In addition, this yeast occurs frequently in grape musts (Schütz & Gafner, 1993; Pretorius *et al*., 2003) and produces large amounts of esters (Bisson & Kunkee, 1991; Rodríguez *et al*., 2010), especially those that are associated with the flavour of pears, for example ethyl octanoate (Lambrechts & Pretorius, 2000; Clemente-Jimenez *et al*., 2004). When tested on Sauvignon Blanc, Muscat d'Alexandrie and Chenin Blanc grape musts, the sequential inoculation of *M. pulcherrima* demonstrated a higher quality score than control wines fermented with *S. cerevisiae* (Joly *et al*., 2003b; Rodríguez *et al*., 2010).Similarly, Parapouli *et al*. (2010) found an increase in the intensity of aroma and flavour of Debina wines with the use of an indigenous strain of *M. pulcherrima* in sequential inoculation with *S. cerevisiae*. In contrast, the inferior attributes of Chardonnay wine were observed, suggesting that the impact of *M. pulcherrima* on the overall sensory profile of wine may be dependent on the grape must as well as strain.

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2.4.6 *Hanseniaspora vineae*

Other than the above-mentioned non-*Saccharomyces* yeasts which have obtained commercial status, other NS yeasts have been investigated. These include members of *Hanseniaspora* spp., especially *H. uvarum* and *H. vineae*. The latter has been shown to provide an intense flowery, fruity and honey flavour to a wine (Medina *et al.*, 2013). Research has ascribed the contribution of this yeast to aroma due to its high production of acetate esters, especially benzenoids and phenylpropanoid esters (Martin *et al*., 2016). Although *H. vineae* has been associated with good aroma properties, not much has been reported by literature regarding its fermentation dynamics in grape musts, as well as its interaction with other yeasts found in grape musts (Lleixà *et al*., 2016). Nonetheless, some strains of *H. vineae* have a four times greater cell lysis rate compared to *S. cerevisiae*, which is a critical characteristic in wine ageing (Martin *et al*., 2016).

2.5 Conclusion

This chapter reported on the vitamin requirements of yeasts and how these requirements could potentially impact winemaking, especially with the knowledge that vitamins can impact yeast growth by serving as cofactors in metabolic reactions. These metabolic reactions result in the production of secondary metabolites, which in turn serve as precursors for compounds that can contribute to wine aroma. Therefore, the concentration of the secondary metabolites and as a result, the precursors contributing to wine aroma, may be affected by the availability of vitamins amongst other growth factors, for example, nitrogen, carbon and trace minerals. This chapter also aimed to report on the vitamin requirements of some wine-related yeasts other than *S. cerevisiae*, colloquially known by wine microbiologists as non-*Saccharomyces* (NS) yeasts, due to their contribution in modulating wine aroma in unique ways. However, more clarity is required regarding the impact of vitamins on winemaking especially regarding population dynamics and fermentation kinetics. Since not all the vitamin requirements of NS yeasts and the resultant impact on wine fermentations have been explored, it could be of value to investigate more into this field to further innovation in the winemaking industry.

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Chapter 3

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Research results and discussion: Evaluating the vitamin requirements of yeasts and the impact on population dynamics and fermentation kinetics

3.1 Introduction

In grape musts, a broad diversity of yeast genera and species occur. Amongst them, *S. cerevisiae* is the primary yeast driving alcoholic fermentations despite generally being present at very low cell densities initially. In a spontaneous fermentation, the other yeast species (collectively referred to as "non-*Saccharomyces*" (NS) yeasts in oenology), begin the fermentation process but are eventually outcompeted by *S. cerevisiae*, although some of them can persist until the late stages of fermentation (Ciani *et al*., 2010; Fleet, 2008). Their decline is typically attributed to many factors including primarily the gradually increasing concentration of ethanol and the rapid decrease of dissolved oxygen, but other factors probably also play a role. Despite their decline during fermentation, indigenous NS yeasts have been shown to contribute to the aroma profile by producing various aroma compounds, such as esters, higher alcohols and volatile acids or by releasing varietal aromas from their non-odorous precursors, for example, monoterpenes and volatile thiols (Swiegers *et al*., 2005). The diversification of aroma profiles with the involvement of NS yeasts in spontaneous fermentations is, therefore, a possible way to meet the ever-changing consumer expectations. The extent of the contribution of NS yeasts depends significantly on their persistence during fermentation. A targeted strategy to enhance persistence can only be achieved through in-depth knowledge of the reasons behind their early decline, which in part may be linked to unmet nutrient requirements. Recently, the nitrogen requirements of several important species have started to be investigated (Ugliano *et al*., 2009; Schnierda *et al*., 2014; Taillandier *et al*., 2014). However, the vitamin requirements of these yeasts and the possible impact on wine fermentation are still poorly understood.

The five essential vitamins required by yeasts are biotin, thiamine, pantothenate, pyridoxine and inositol - all fulfilling specific metabolic functions (Burkholder, 1943). Biotin is involved in the synthesis of DNA, amino acids, fatty acids and carbohydrates (Lafon-Lafourcade & Guimberteau, 1962; Walker, 1998). Thiamine is an essential cofactor for several enzymes, especially decarboxylases involved in carbohydrate and amino acid catabolism (Li *et al*., 2010). Pantothenate serves as a precursor molecule for coenzyme A, which is a crucial cofactor in a wide range of metabolic reactions (White *et al*., 2001). Pyridoxine is part of a complex of other B_6-vitamins and in its biologically active components, including pyridoxal-5'-phosphate and pyridoxamine-5'-phosphate, play significant roles in the metabolism of amino acids and are also commonly found in various metabolic pathways, for example, pathways converting amino acids into

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antibiotic compounds (Schneider *et al*., 2000). The synthesis and metabolism of inositol plays a significant role in contributing to the membrane integrity of yeasts (Majerus *et al*., 1986). In yeasts, inositol and its role in phospholipid metabolism, are highly regulated at the cytoplasmic enzyme, MI-1-P-synthase (Majerus *et al*., 1986). Yeasts contain the necessary enzymatic machinery to synthesise most of the vitamins mentioned above *de novo*, except for biotin (Streit & Entcheva, 2003).

Numerous studies have focused on the impact of vitamins on the growth and fermentation kinetics (Castor, 1953; Hall *et al*., 1956; Rogers & Lichstein, 1969; Peynaud & Lafourcade, 1977; Lynen, 1980; Slaughter & McKernan, 1988; Ough *et al*., 1989; Bataillon *et al*., 1996; Lambrechts & Pretorius, 2000; Wang *et al*., 2003; Bohlsheid *et al*., 2007). Also, the impact of vitamins on the production of compounds relating to the aroma profile of wine has been investigated. However, not all yeasts have been evaluated for their vitamin requirements, neither has the impact of all essential vitamins on fermentation been investigated (Suomalainen & Keranen, 1963; Rankine, 1967; Hosono *et al*., 1972; Lynen, 1980; Rapp & Mandery, 1986; Jordan & Slaughter, 1986; Thomas & Surdin-Kerjan; 1997; Lambrechts & Pretorius, 2000; Sohn & Kuriyama, 2001; Bohlscheid *et al*., 2007).

In this study, we investigated the impact of different concentrations of these vitamins on the growth of several commercially relevant NS yeasts using a high-throughput microtiter plate experimental design. After that, the most significant results were confirmed at a larger scale and in mixed culture with *S. cerevisiae* in order to assess the potential competition between yeasts for vitamins. All of these experiments were performed in synthetic grape juice under fermentative conditions.

3.2 Materials and methods

3.2.1 Yeasts strains used

Six yeast strains either commercialised or previously isolated from South African grape juice were investigated in this study (**Table 3.1**).The yeasts strains were grown on Yeast Peptone Dextrose (YPD) agar (20 $g.L^{-1}$ glucose, 20 $g.L^{-1}$ peptone, 10 $g.L^{-1}$ yeast extract, and 20 $g.L^{-1}$ agar) and maintained at 4°C. Where appropriate, Wallenstein (WL) Nutrient Agar was used to allow for differentiation between yeast species (*S. cerevisiae*: big white colonies; *L. thermotolerans*: dark green; *T. delbrueckii*: mint-green; *P. kluyveri*: white with irregular circumference pattern of the colony; *M. pulcherrima*: cream with a hint of red and *H. vineae*: bright green).

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Table 3.1: Yeast species used in this study.

Yeast	Strain	Region of isolation/ institution	Manufacturer	Location of manufacturer's headquarters
S. cerevisiae	Lalvin® EC1118	Champagne, France	Lallemand	Canada
L. thermotolerans	Viniflora® Concerto	Greece	Chr. Hansen	Denmark
T. delbrueckii	Biodiva™ TD291	North America	Lallemand	Canada
P. kluyveri	Viniflora® Frootzen™	Auckland University, New Zealand	Chr. Hansen	Denmark
M. pulcherrima	Flavia™ MP346	Universidad de Santiago de Chile	Lallemand	Canada
H. vineae	IWBT Y980	Institute for Wine Biotechnology, Stellenbosch University	n/a	n/a

3.2.2 Pre-culture conditions

Pre-cultures of the yeast strains were performed as described by Prior (2017), except for the use of Yeast Nitrogen Base with amino acids and 2% glucose as a minimal medium. All chemicals were obtained from Difco (Detroit, U.S.A) or Sigma-Aldrich (St. Louis, U.S.A) and were of highest-grade purity.

3.2.3 Fermentation conditions

3.2.3.1 Microtiter plate assay

A high-throughput method as proposed by Toussaint and Conconi (2006) was used to allow for screening of the vitamin requirements of the different yeast strains used. The fermentation medium used was synthetic grape juice prepared as described by Henschke and Jiranek (1993), with specific amendments shown in **Table 3.2**. The pH of the fermentation medium was adjusted to 3.5 with potassium hydroxide. Nitrogen sources at the concentrations described by Bely *et al*. (1990) were provided. Fermentations for each yeast were performed in a sterile 96-well microtiter plate (Sterilin,

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Sterilin Limited, London, U.K) in triplicate and randomly placed with a final volume of 200 µL per well (**Figure 3.1**). The fermentation design (in general) included a control (all vitamins kept at concentrations representative of grapes proposed by Henschke and Jiranek (1993)); double the concentration of specific vitamin and the absence of a specific vitamin while keeping the other vitamins at control concentrations, respectively. Fermentations took place for 48 h at 25°C, with automated absorbance readings at 600 nm every 2 h by the Powerwave$_x$ Microplate Spectrophotometer (BioTek Instruments Inc.). Also, sample readings were accompanied by automated agitation at medium speed, to avoid spillage, before every reading. The microtiter plates were covered with parafilm to prevent cross-contamination and minimise evaporation. This protocol involved the addition of different compounds at different concentrations resulting in increased exposure to contamination either through the user or air. Considering the aforementioned, samples were plated out on a differential medium such as WL-agar to verify if cross-contamination took place.

Fermentations with selected yeast and media combinations were repeated in 50 ml of synthetic grape juice at the same conditions as in the microplate plates in order to validate the results obtained from the microplate reader. The conditions of the microtiter plate assay were mimicked, that is fermentation at 25°C and no agitation, except before taking a sample. Fermentations were performed in triplicate in suitable Erlenmeyer flasks fully equipped with fermentation caps and stoppers.

3.2.3.2 Selected fermentations in larger volume

From the microtiter plate assay, some of the findings were investigated in fermentations at a larger volume. For fermentations at a large volume, yeasts cells following pre-culturing were inoculated at an OD_{600}nm: 0.1 in 200 ml synthetic grape juice. Fermentations were performed in triplicate in suitable Erlenmeyer-flasks fully equipped with fermentation caps and stoppers. Fermentations were conducted at 25°C with agitation at 100 rpm. Yeast population dynamics was monitored daily by aseptically taking 1ml of sample and plating on WL-agar. WL-agar was used to differentiate between yeasts species where applicable and to verify the absence of contaminants. To measure fermentation kinetics, accumulated weight loss was monitored throughout fermentation until weight loss was less than 0.1g for three consecutive days.

3.2.4 Statistical analyses

The variability in the experiments was determined by calculating the standard deviations of the biological repeats. All data obtained were analysed using repeated measurements analysis of variances at a 5% significance level. The software used to compute these statistical results was Statistica Version 13.3.

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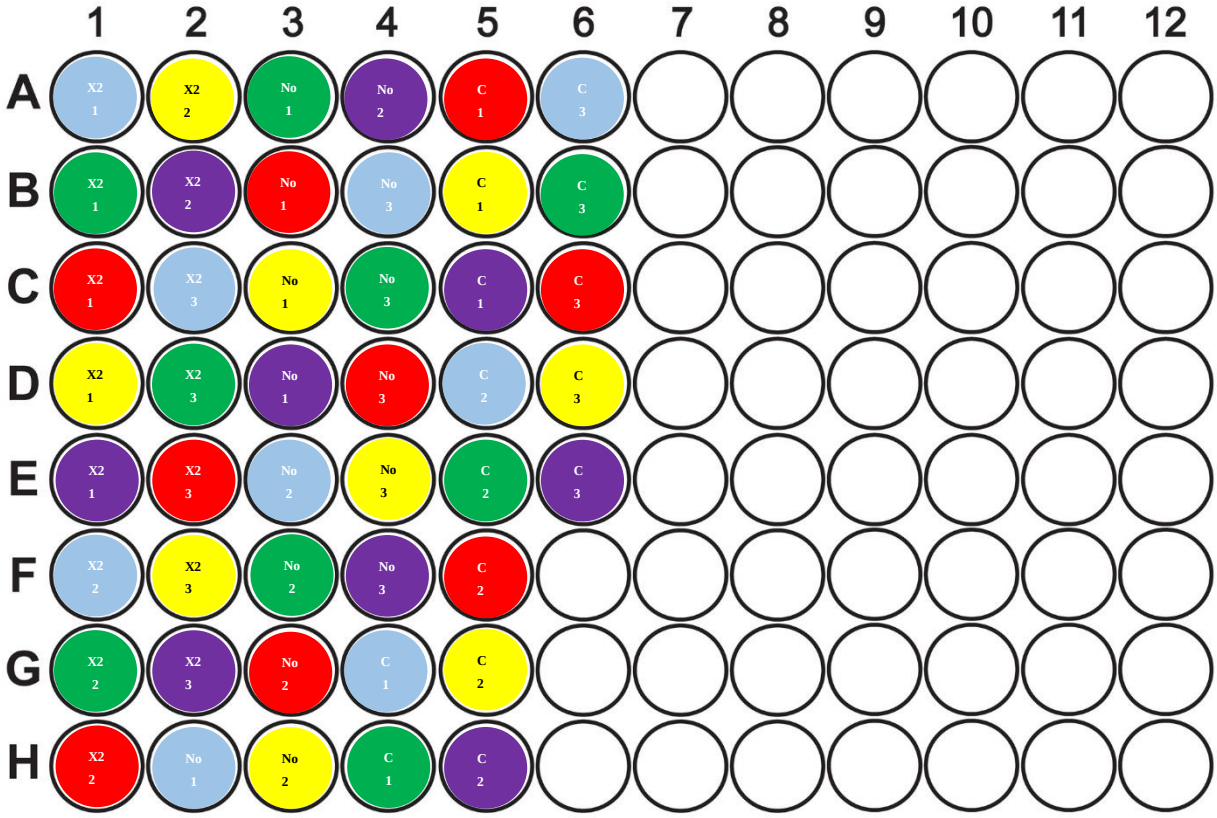

Figure 3.1: Example of randomisation with a 96-well microtiter plate for fermentations. Key: , biotin; , pantothenate; , thiamine; , pyridoxine; , inositol; X2: double the specific vitamin whilst keeping the other vitamins at controlled amounts; No: the elimination of the specific vitamin whilst keeping the other vitamins at controlled amounts; c: control. The key indicators are accompanied by the replicate number. Image provided by Edita Aksamitiene 2009©.

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Table 3.2: Synthetic grape juice medium as proposed by Henschke and Jiranek (1993). The pH of the medium was set to 3.5 with KOH.

Source	Components	Quantity
Carbon (g.L^{-1})	Glucose	115*
	Fructose	115*
Nitrogen sources	Bely *et al.* (1990)	Bely *et al.* (1990)
Acids (g.L^{-1})	Potassium tartrate	2.5
	L-malic acid	3
	Citric acid	0.2
Salts (g.L^{-1})	Potassium phosphate	1.14
	Magnesium sulphate	1.23
	Calcium chloride	0.44
Trace elements (µg.L^{-1})	Manganese chloride	200
	Zink chloride	135
	Iron chloride	30
	Copper chloride	15
	Boric acid	5
	Cobalt (II) nitrate	30
	Sodium molybdenum oxide	25
	Potassium iodate	10
Vitamins (mg.L^{-1})	Myo-inositol	100
	Pyridoxine hydrochloride	2
	Nicotinic acid	2
	Calcium pantothenate	1
	Thiamine hydrochloride	0.5
	Potassium para-amino benzoic acid	0.2
	Riboflavin	0.2
	Biotin	0.125
	Folic acid	0.2
Anaerobic factors (mg.L^{-1})	Ergosterol	10
	Tween 80	0.5

*Amendments from original medium described by Henschke and Jiranek (1993)

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3.3 Results

3.3.1 Microtiter plate assay

The primary research aim of this study was to evaluate the impact of varying concentrations of vitamins on the growth performance of selected, commercially relevant NS yeast strains, in pure culture and in the context of a mixed culture with *S. cerevisiae*. In order to screen a large number of conditions, a microtiter plate assay was adapted for this specific purpose (Toussaint and Conconi; 2006). The experimental lay-out included three variable conditions: synthetic grape juice, referred to as the control in this study, as used by many research teams around the world (Henschke & Jiranek, 1993), a condition where the concentration of individual vitamins was doubled, and a condition where individual vitamins were omitted while all other vitamins were kept at the standard concentrations.

The evaluation of the vitamin requirements of yeasts with a microtiter plate assay presented many challenges. These challenges were linked to the sensitivity of the assay and required careful optimisation of pipetting techniques during inoculation to reach a sufficient level of precision and accuracy to obtain reproducible results. The microtiter plate assay proved to be capable of detecting specific growth responses by yeasts towards variations in the concentration of the investigated vitamins. However, in some cases, high variability was found between biological repeats. The high variability may have allowed for other possible statistically significant differences in yeast growth responses to be understated. Therefore, the assay should be rather considered as a rapid-screening protocol and followed-up by additional experiments to verify the significance of the results obtained.

3.3.1.1 Synthetic grape juice does not meet the vitamin requirements of some non-*Saccharomyces* strains

The concentrations of individual vitamins were doubled from their recommended concentration in synthetic grape juice (Henschke & Jiranek, 1993). The performance of *P. kluyveri* Frootzen improved for every individual vitamin (**Figure 3.2**). This finding suggests that the concentrations of the particular vitamins investigated is limiting for the growth of *P. kluyveri* Frootzen and that modification of synthetic grape juice might be necessary. Yeasts including *S. cerevisiae* EC1118 (**Figure 3.3**) did not benefit from increased concentrations of the individual vitamins. This result may be due to the synthetic grape juice being designed initially based on the vitamin and other nutrient requirements of *S. cerevisiae* (Henschke & Jiranek, 1993). The yeast *T. delbrueckii* Biodiva did also not benefit from the increase in the concentration of any of the vitamins. On the contrary, increased concentrations of thiamine and pyridoxine, respectively, was shown to have an inhibitory impact on the growth of *T. delbrueckii* Biodiva (**Figure 3.4**). The growth of *M. pulcherrima* Flavia appeared to have also been inhibited by the increase in the concentration of all individual vitamins, except

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pantothenate. Nonetheless, only the growth responses of *M. pulcherrima* Flavia to increasing concentrations of inositol was computed as statistically significant due to high variability presented in response to increasing concentrations of the other individual vitamins (**Figure 3.5**).

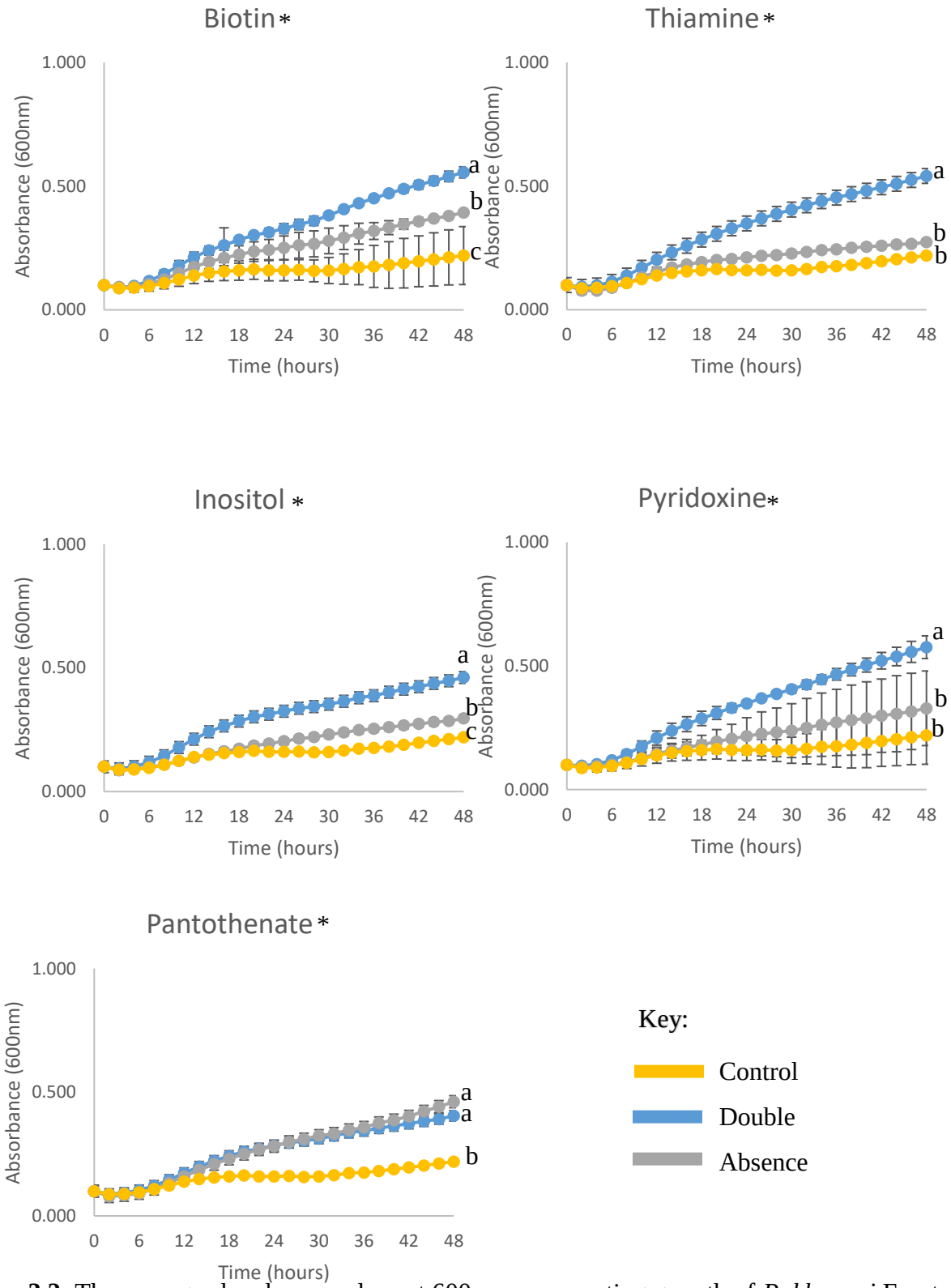

Figure 3.2: The average absorbance values at 600 nm representing growth of *P. kluyveri* Frootzen in response to varying concentrations of particular vitamins in 200 µL synthetic grape juice medium. Outputs with statistically significant differences are indicated with * ($p < 0.05$) and different letters infer that the variable means are different. Error bars represent standard deviation (n=3 biological repeats).

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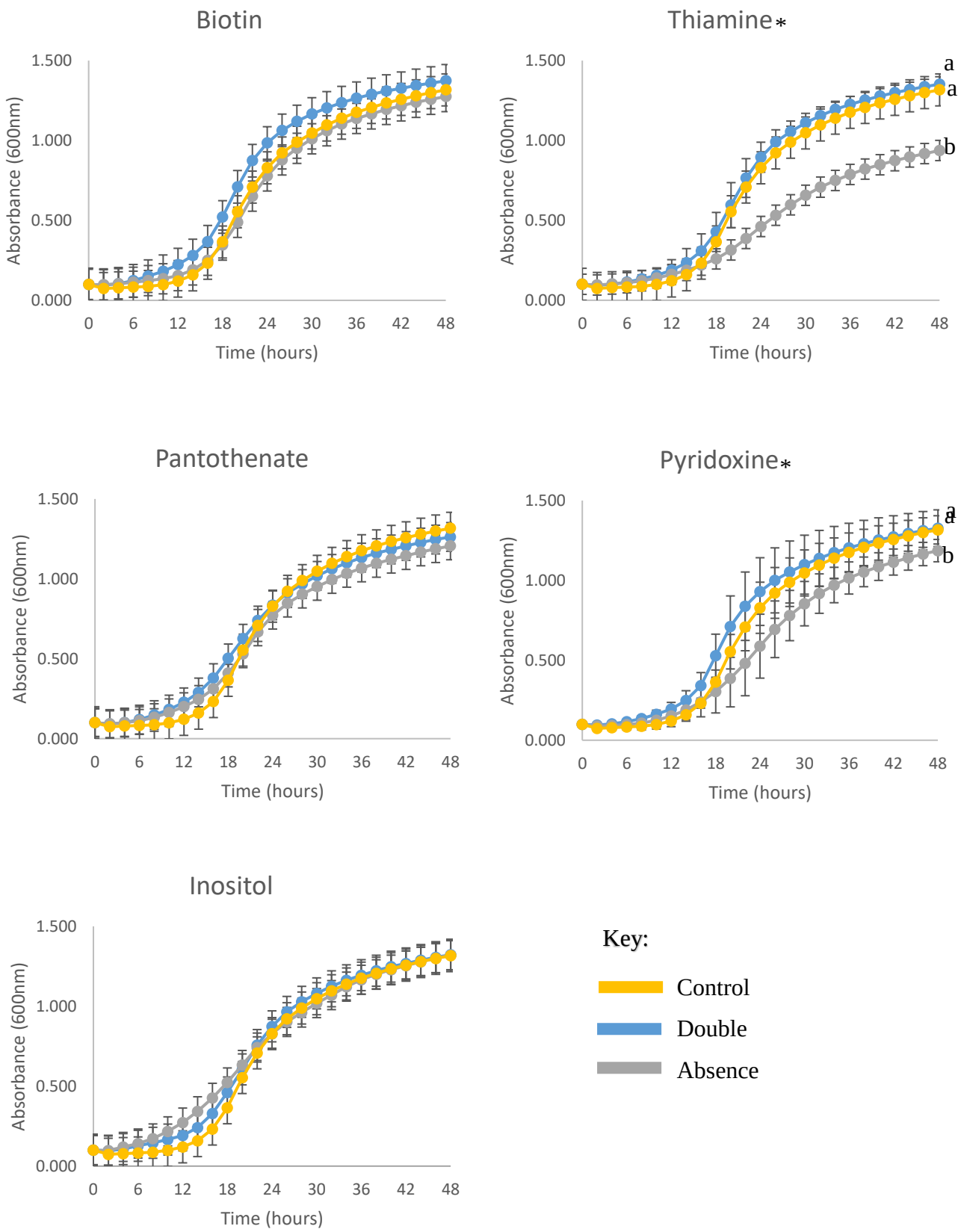

Figure 3.3: The average absorbance values at 600 nm representing *S. cerevisiae* EC1118 in response to varying concentrations of particular vitamins in 200 µL synthetic grape juice medium. Outputs with statistically significant differences are indicated with * ($p < 0.05$) and different letters infer that the variable means are different. Error bars represent standard deviation (n=3 biological repeats).

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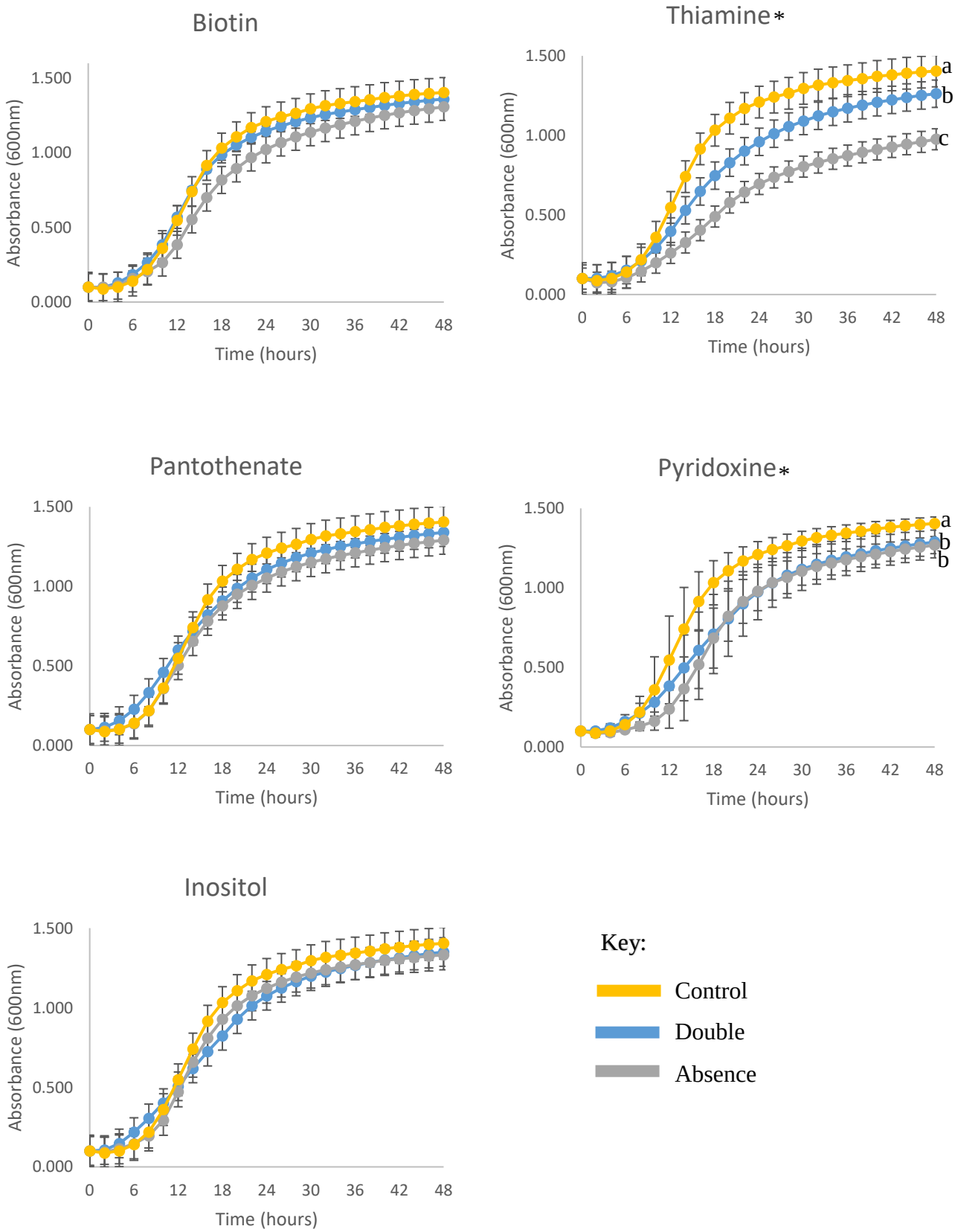

Figure 3.4: The average absorbance values at 600 nm representing *T. delbrueckii* Biodiva in response to varying concentrations of particular vitamins in 200 µL synthetic grape juice medium. Outputs with statistically significant differences are indicated with * ($p < 0.05$) and different letters infer that the variable means are different. Error bars represent standard deviation (n=3 biological repeats).

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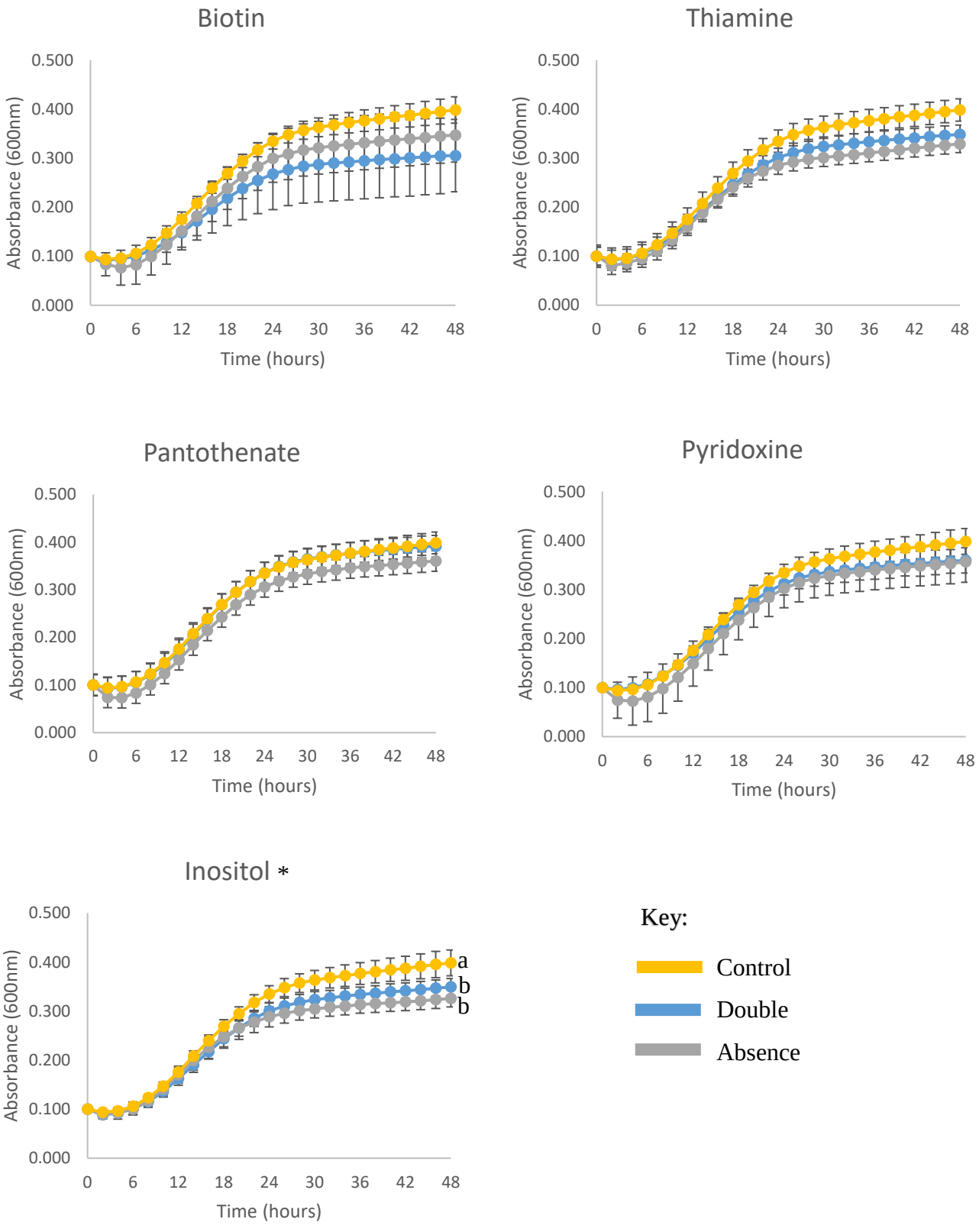

Figure 3.5: The average absorbance values at 600 nm representing *M. pulcherrima* Flavia in response to varying concentrations of particular vitamins in 200 µL synthetic grape juice medium. Outputs with statistically significant differences are indicated with * ($p < 0.05$) and different letters infer that the variable means are different. Error bars represent standard deviation (n=3 biological repeats).

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The omission of particular vitamins found in synthetic grape juice had adverse effects on the growth of several yeast species. The omission of any of the particular vitamins investigated, respectively, resulted in the weakest growth of *M. pulcherrima* Flavia (**Figures 3.5**), however, as mentioned above, the high variability amongst biological repeats did not allow for any conclusions to be made. The omission of thiamine resulted in the suboptimal levels of growth for *S. cerevisiae* EC1118 (**Figure 3.3**), *T. delbrueckii* Biodiva (**Figure 3.4**) as well as *L. thermotolerans* Concerto (**Figure 3.6**), resulting in slower growth rates and lower final levels of biomass. These yeasts, except for *L. thermotolerans* Concerto, also displayed poor growth in response to pyridoxine (**Figures 3.3-3.4**). The absence of inositol was unfavourable for the growth of most of the NS yeasts, including *L. thermotolerans* Concerto, *H. vineae* IWBT Y980 and *M. pulcherrima* Flavia, with a more prominent impact on the growth of *L. thermotolerans* Concerto (**Figure 3.7**). In the absence of inositol, *L. thermotolerans* Concerto reached a maximum level of growth that was approximately three-fold less in comparison to recommended concentrations of inositol in synthetic grape juice.

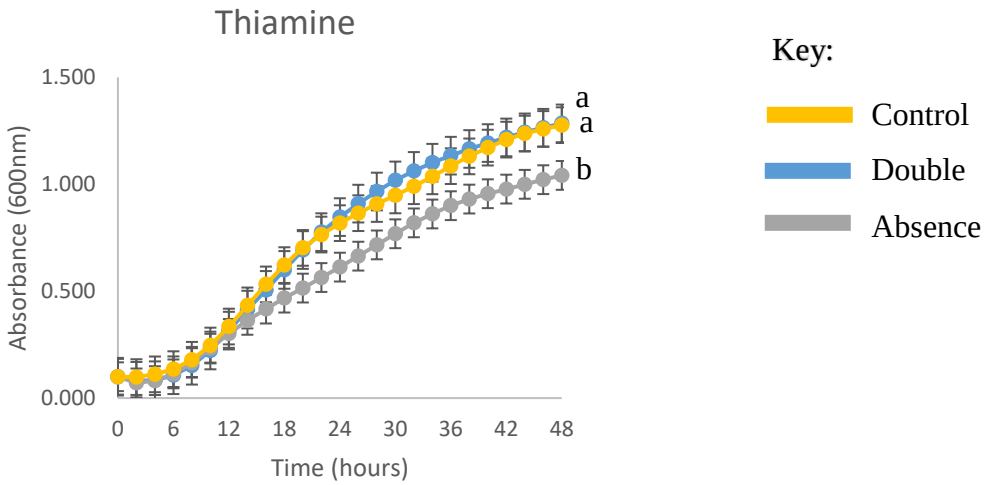

Figure 3.6: The average absorbance values at 600 nm representing *L. thermotolerans* Concerto in response to varying concentrations of thiamine in 200 µL synthetic grape juice medium. Different letters infer that the variable means are different ($p < 0.05$). Error bars represent standard deviation (n=3 biological repeats).

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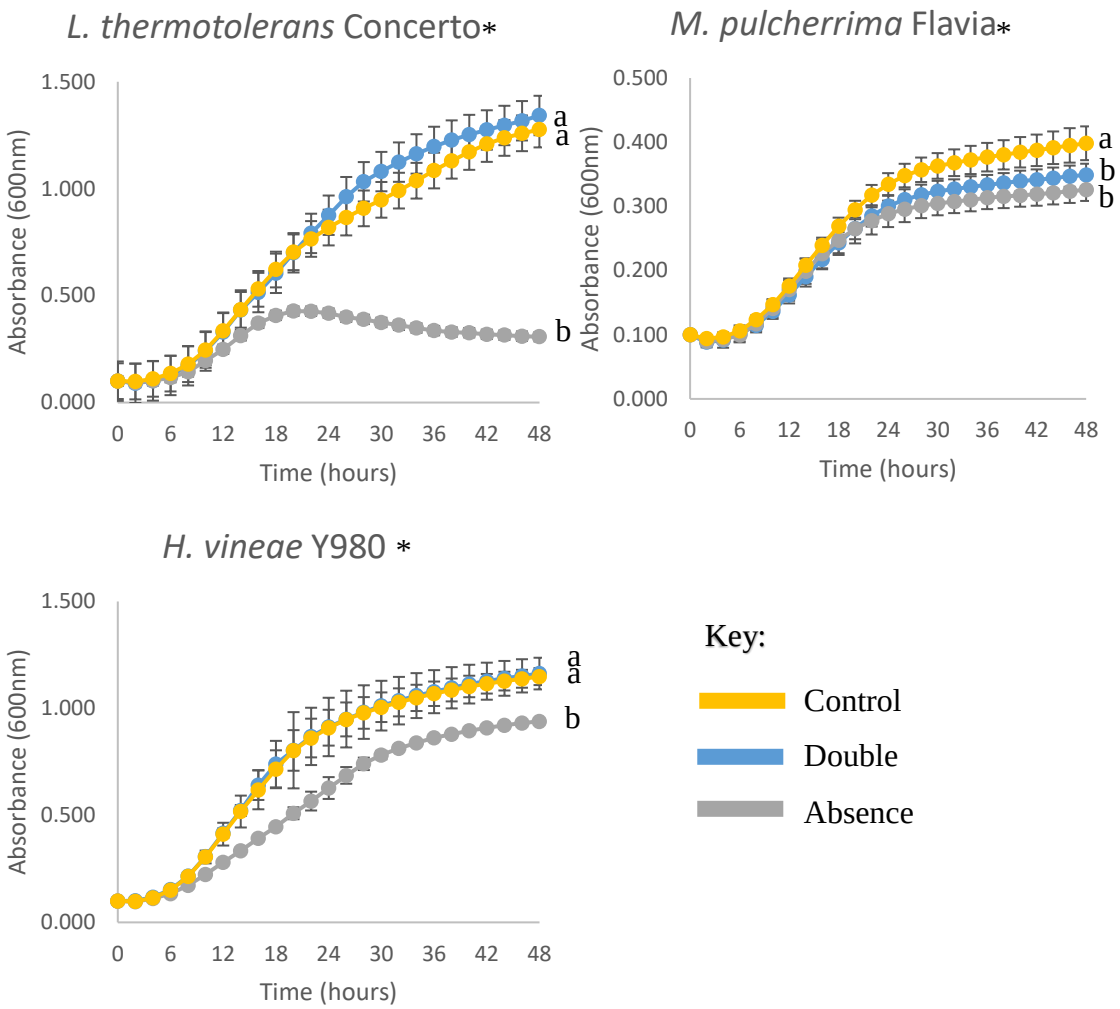

Figure 3.7: The average absorbance values at 600 nm representing yeast growth in response to varying concentrations of inositol in 200 µL synthetic grape juice medium. Outputs with statistically significant differences are indicated with * ($p < 0.05$) and different letters infer that the variable means are different. Error bars represent standard deviation (n=3 biological repeats).

3.3.1.4 Biotin

In response to varying biotin concentrations, only *P. kluyveri* Frootzen showed significant differences in its growth responses (**Figure 3.2**). These differences were observed from the start of the exponential growth phase. The yeast *P. kluyveri* Frootzen benefited from increased concentrations of biotin by displaying faster growth rates and by reaching higher levels of biomass.

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3.3.1.5 Thiamine

Four of the yeasts investigated which included *S. cerevisiae* EC1118 (**Figure 3.3**), *L. thermotolerans* Concerto (**Figure 3.6**), *T. delbrueckii* Biodiva (**Figure 3.4**) and *P. kluyveri* Frootzen (**Figure 3.2**), showed significant differences in their growth responses towards varying thiamine concentrations. These differences were illustrated from the onset of exponential growth of the respective yeasts. When compared to the control, which represents all vitamins and other growth factors at concentrations of real grape juice as reported by Henschke & Jiranek (1993), double the concentration of thiamine had no impact on the growth of *S. cerevisiae* EC1118 and *L. thermotolerans* Concerto, respectively. However, an increase in the concentration of thiamine had an inhibitory effect on the growth of *T. delbrueckii* Biodiva. This inhibitory effect was sustained until early stationary phase. Unlike *S. cerevisiae* EC1118, *L. thermotolerans* Concerto and *T. delbrueckii* Biodiva, double the concentration of thiamine allowed for the improved growth performance of *P. kluyveri* Frootzen. Although the absence of thiamine when compared to the control resulted in the significantly poorer growth of *S. cerevisiae* EC1118, *L. thermotolerans* Concerto and *T. delbrueckii*, no impact on the growth of *P. kluyveri* Frootzen was observed.

3.3.1.6 Pantothenate

Similar in response to biotin, only *P. kluyveri* Frootzen showed significant differences in its growth responses response to varying pantothenate concentrations (**Figure 3.2**). These differences were observed from the start of the exponential growth phase. The increase in pantothenate resulted in faster growth rates of *P. kluyveri* Frootzen, as well as higher biomass.

3.3.1.7 Pyridoxine

The same yeasts that illustrated significant differences in their growth responses towards thiamine, except *L. thermotolerans* Concerto, displayed significant differences in their growth responses towards varying concentrations of pyridoxine (**Figures 3.2-3.4**). These significant differences were also observed at the onset of the exponential growth phase. Different from what was observed in its response to varying concentrations of thiamine, the growth of *T. delbrueckii* Biodiva was inhibited to the same extent towards the increase in the concentration of pyridoxine as to when pyridoxine was absent (**Figure 3.4**).

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3.3.1.8 Inositol

Significant differences in growth responses towards inositol were found for most of the NS yeast investigated. These significant differences were found in early stages of fermentation for *P. kluyveri* Frootzen from 12 h (**Figure 3.2**), *H. vineae* Y980 (**Figure 3.7**) and *M. pulcherrima* Flavia (**Figure 3.7**) both from 8 h, whereas for *L. thermotolerans* Concerto (**Figure 3.7**) these differences were displayed from 16 h onward. When compared to the control, double the concentration of inositol showed to be inhibitory for the growth of *M. pulcherrima* Flavia. However, the increase in the concentration of inositol had no impact on the growth of *H. vineae* Y980 and *L. thermotolerans* Concerto. As for *P. kluyveri* Frootzen, double the concentration of inositol improved growth. Moreover, *L. thermotolerans* Concerto was unable to grow in the absence of inositol from 18 h onward, while the other yeasts still managed to grow, but at a slower rate.

3.3.1.9 Verification of microtiter plate assay results

To verify whether similar results are obtained in a larger volume, fermentations in 50 ml of synthetic grape juice were repeated to allow for comparisons. An example of the results obtained from fermentations performed in a larger volume is presented in **Figure 3.8**. Although more deviations amongst the sample readings were computed, the results obtained from fermentations performed in a larger volume were similar to what was observed from the microtiter plate assay.

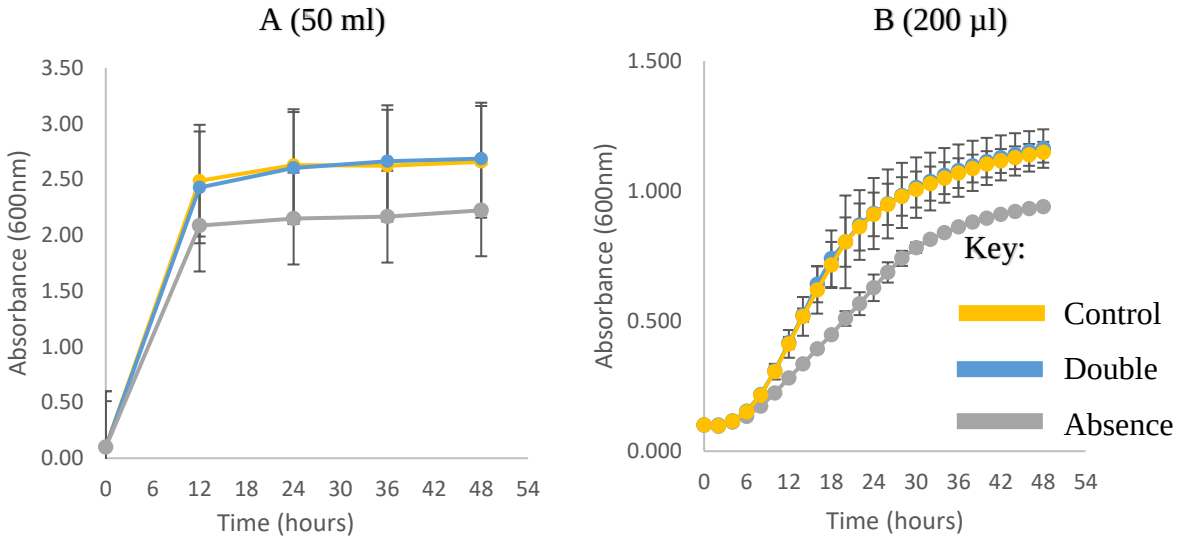

Figure 3.8: Validation of the fermentations, involving *H. vineae* Y980 IWBT in response to inositol, performed in a larger volume (**A**) in comparison to microplate reader results (**B**). Error bars represent standard deviation (n=3 biological repeats).

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From the microtiter plate assay, some significant findings were further assessed at a larger scale in mixed culture with *S. cerevisiae* to confirm the results obtained in the first objective and to assess the potential competition for vitamins. The yeasts *L. thermotolerans* Concerto in response to inositol, and *P. kluyveri* Frootzen in response to biotin and thiamine, respectively, was assessed in mixed cultures with *S. cerevisiae* EC11118. Fermentations were performed according to what has been described in section **3.2.3.2**, and yeast population dynamics, as well as fermentation kinetics, were monitored.

3.3.2 Selected fermentations in larger volume

3.3.2.1 Population dynamics of *L. thermotolerans* Concerto and *S. cerevisiae* EC1118 in response to varying concentrations of inositol

Similar trends in the growth patterns of *L. thermotolerans* Concerto and *S. cerevisiae* EC1118 in response to inositol, when grown in monoculture in a larger volume (**Figure 3.9 A** and **B** respectively), was observed compared to those from the microtiter plate assay (**Figures 3.7** and **3.3**). However, fermentation in a larger volume led to specific differences which were not observed in the results from the microtiter plate assay. For example, (i) *L. thermotolerans* Concerto did not grow at all in the absence of inositol, whereas in the microtiter plate assay, growth was observed until approximately 16 hours before *L. thermotolerans* Concerto decreased in population size and (ii) the absence of inositol had a significant impact on the growth of *S. cerevisiae* EC1118 in the early and late stages of fermentation.

When grown simultaneously (**Figure 3.9 D**), the respective population sizes of *L. thermotolerans* Concerto and *S. cerevisiae* EC1118 were lower than their respective population sizes in monocultures (**Figure 3.9 A** and **B**). Furthermore, double the concentration of inositol appeared to better support the optimum growth of *L. thermotolerans* Concerto in the early stages of fermentation when grown in co-culture with *S. cerevisiae* EC1118 (**Figure 3.9 C** and **D**). These findings were in contrast to when *L. thermotolerans* Concerto was grown in monoculture, where the control rather than double the concentration of inositol appeared to support optimal growth in the early stages of fermentation. Overall, the results indicated a form of competition for inositol between *L. thermotolerans* Concerto and *S. cerevisiae* EC1118.

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3.3.2.2 Fermentation kinetics of *L. thermotolerans* Concerto and *S. cerevisiae* EC1118 in response to varying concentrations of inositol

No difference in the growth rate of *L. thermotolerans* Concerto in monocultures was observed when the concentration was maintained at control levels or doubled (**Figure 3.10 A**). The variation in the concentration of inositol compared to the control resulted in slower exponential growth rates of *S. cerevisiae* EC1118 when grown in monocultures, but the final amounts of CO_2 produced were similar toward the endpoint. When *S. cerevisiae* EC1118 was grown in sequential cultures with *L. thermotolerans* Concerto (**Figure 3.10 C**), an extended lag duration was observed when inositol was absent from the growth medium. Soon after, the fermentation rate rapidly increased and appeared to be faster in comparison to that of the control and double the concentrations of inositol, resulting in a higher final CO_2-production. In contrast to the findings of the sequential cultures, no lag phase was observed in the simultaneous when inositol was absent, yet a similar final amount of CO_2 production was observed.

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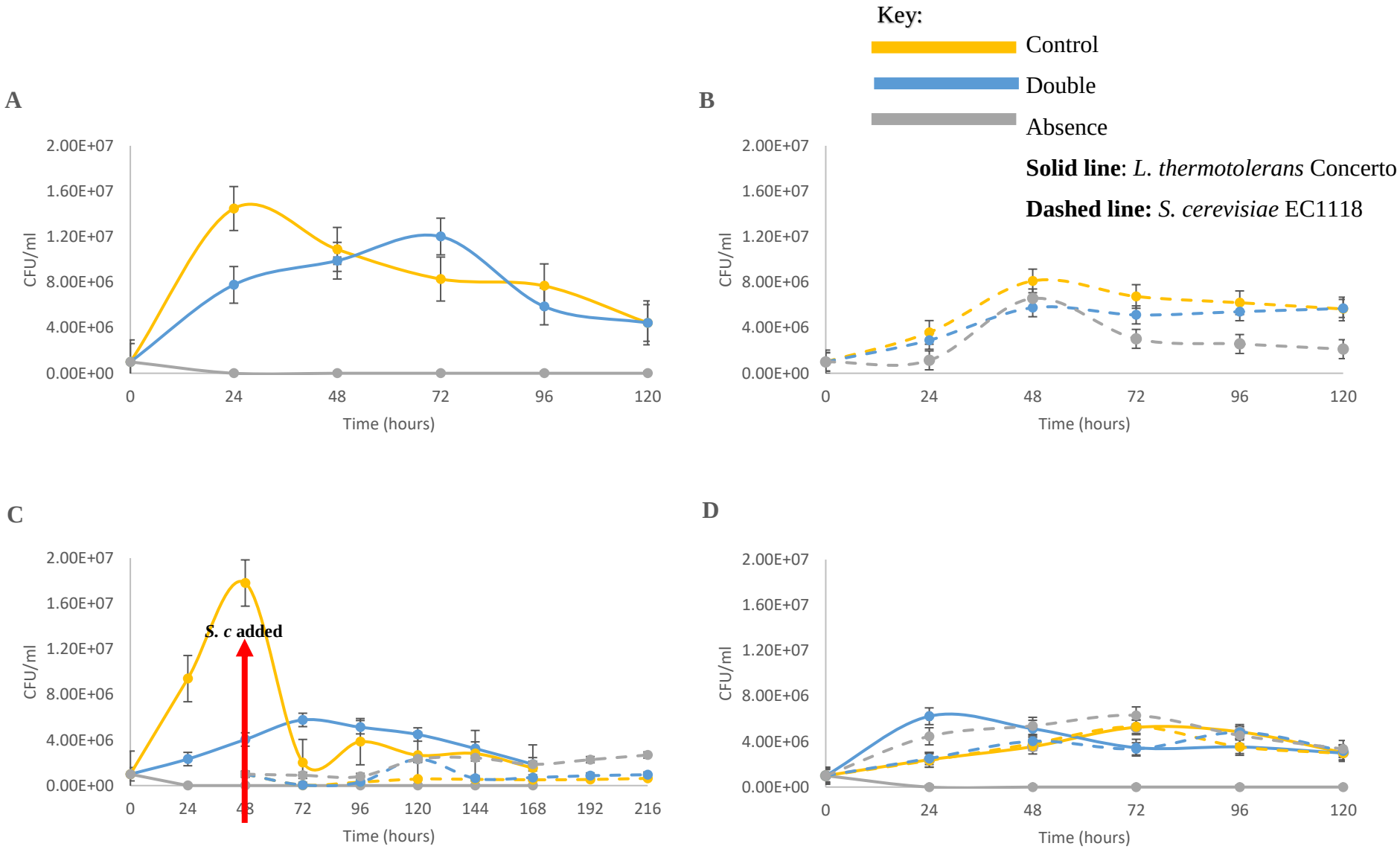

Figure 3.9: Population dynamics expressed as average growth in colony forming units (CFU)/ml of *L. thermotolerans* Concerto (**A**) and *S. cerevisiae* EC1118 (**B**) in monocultures respectively, as well in co-cultures (**C**: sequential; **D**: simultaneous). Error bars represent standard deviation (n=3 biological repeats).

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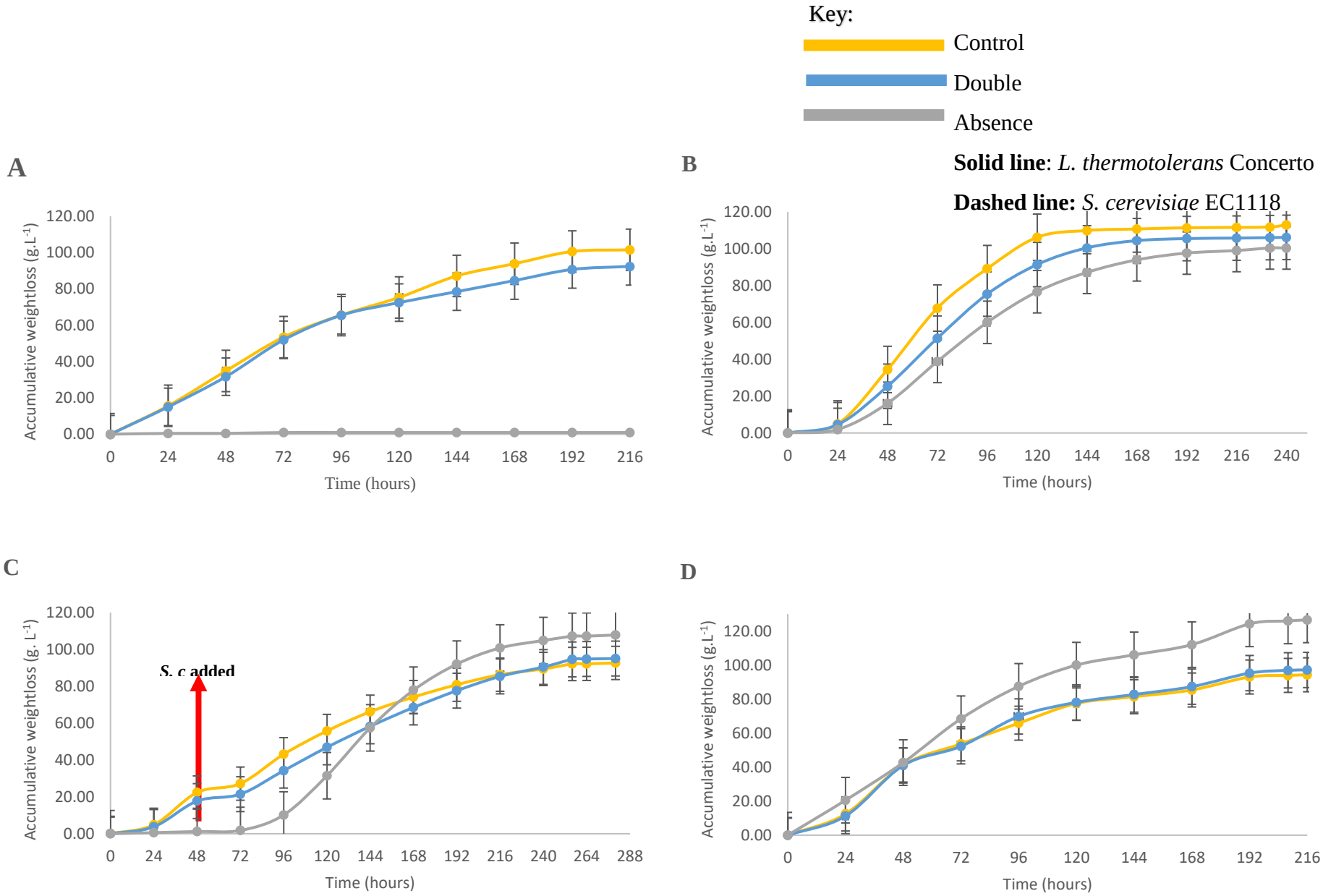

Figure 3.10: Fermentation kinetics expressed as accumulated weight loss in flasks due to CO_2-production by *L. thermotolerans* Concerto (**A**) and *S. cerevisiae* EC1118 (**B**) in monocultures respectively, as well in co-cultures (**C**: sequential; **D**: simultaneous). Error bars represent standard deviation (n=3 biological repeats).

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3.3.2.3 Population dynamics of *P. kluyveri* Frootzen and *S. cerevisiae* EC1118 in response to varying concentrations of biotin

The population dynamics of *P. kluyveri* Frootzen in larger volume (**Figure 3.11**), displayed marked differences in comparison to that of the microtiter plate assay (**Figure 3.2**). Changes in the concentration of biotin had no impact on the respective monocultures of *P. kluyveri* Frootzen and *S. cerevisiae* EC1118 (**Figure 3.11 A** and **B** respectively). More specifically, statistical analyses showed that double the concentration of biotin allowed for a significant improvement in the growth of *S. cerevisiae* EC1118 in the exponential growth stages of fermentation, but a faster decrease in the population size in the later stages of fermentation when compared to control or absence of biotin.

The increase in the population size of *S. cerevisiae* EC1118 was reduced when grown sequentially after *P. kluyveri* Frootzen, as displayed in **Figure 3.11 C**. When grown in a simultaneous culturing modality rather than sequentially, illustrated by **Figure 3.11 D**, control rather than double the concentrations of biotin appeared to favour the growth of *S. cerevisiae* EC1118 in the early stages of fermentation. However, the opposite was found when *S. cerevisiae* EC1118 was grown in monocultures. Furthermore, *S. cerevisiae* EC1118 displayed dominance in population size over that of *P. kluyveri* Frootzen when grown simultaneously, with *P. kluyveri* Frootzen, which in turn, decreased in population size as soon as from 24 hours.

3.3.2.4 Fermentation kinetics of *P. kluyveri* Frootzen and *S. cerevisiae* EC1118 in response to varying concentrations of biotin

Variations in the concentration of biotin had no impact on the fermentation rates of *P. kluyveri* Frootzen and *S. cerevisiae* EC1118 when grown in monocultures (**Figure 3.12 A** and **Figure 3.12 B** respectively). Although the fermentation rates were also similar amongst the different concentrations of biotin investigated in the co-culture modalities (**Figure 3.12 C** and **D**), lower final CO_2-production was observed when these two yeasts were grown sequentially (**Figure 3.12 C**), compared to when grown simultaneously (**Figure 3.12 D**).

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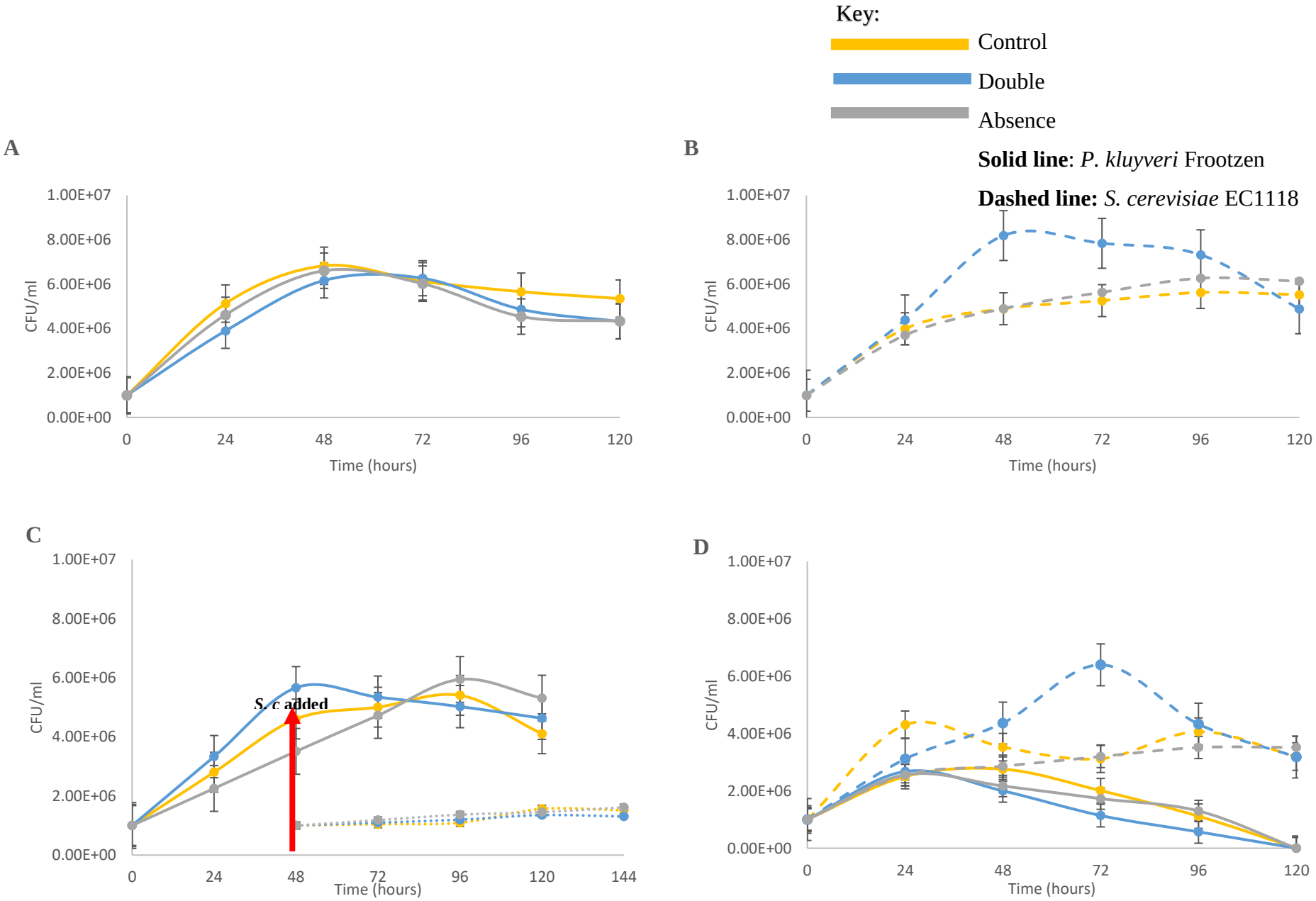

Figure 3.11: Population dynamics expressed as average growth in colony forming units (CFU)/ml of *P. kluyveri* Frootzen (**A**) and *S. cerevisiae* EC1118 (**B**) in monocultures respectively, as well in co-cultures (**C**: sequential; **D**: simultaneous). Solid line: *P. kluyveri* Frootzen; dashed line: *S. cerevisiae* EC1118. Error bars represent standard deviation (n=3 biological repeats).

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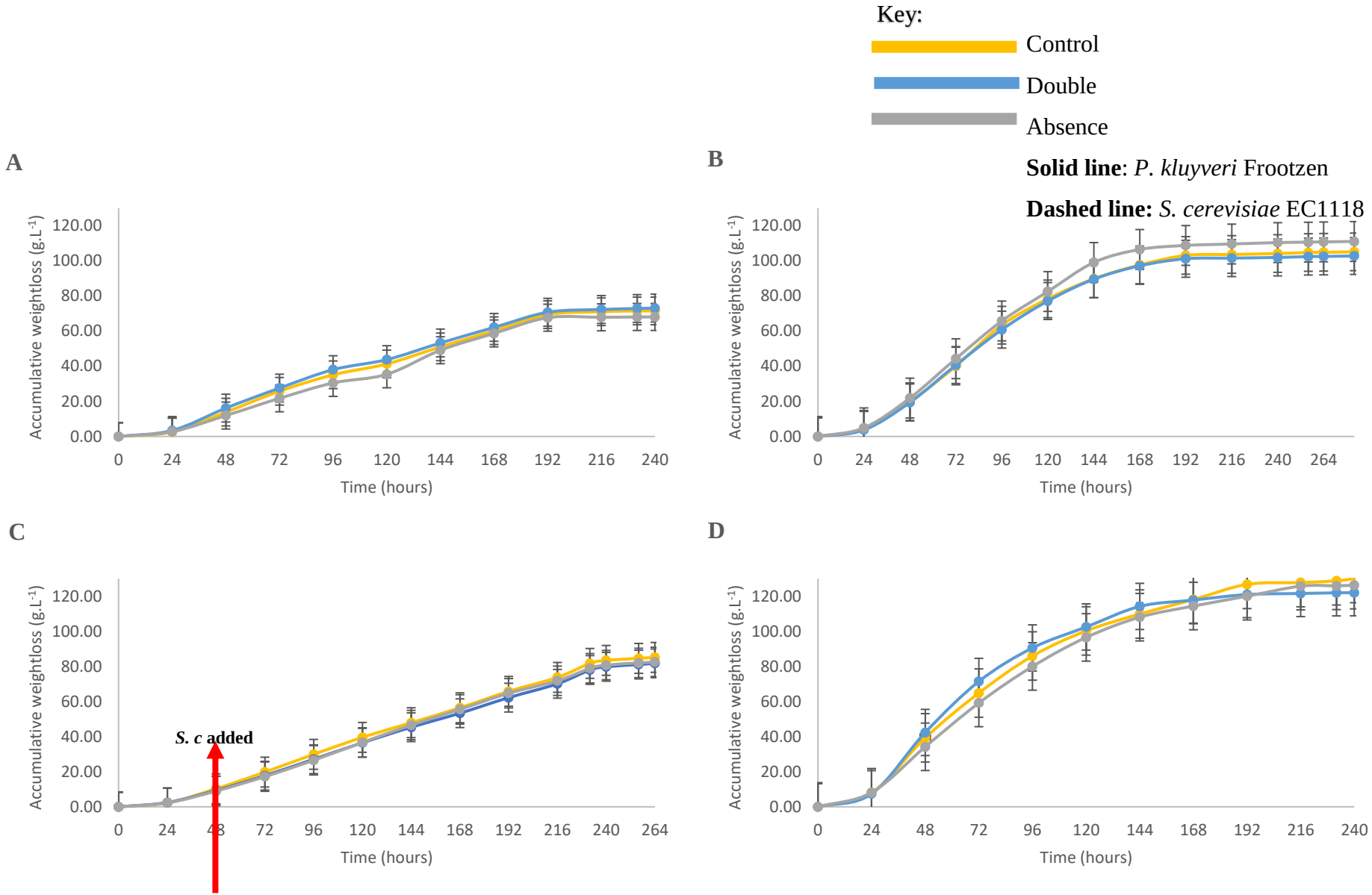

Figure 3.12: Fermentation kinetics expressed as accumulated weight loss in flasks due to CO_2-production by *P. kluyveri* Frootzen (**A**) and *S. cerevisiae* EC1118 (**B**) in monocultures respectively, as well in co-cultures (**C**: sequential; **D**: simultaneous). Error bars represent standard deviation (n=3 biological repeats).

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3.3.2.5 Population dynamics of *P. kluyveri* Frootzen and *S. cerevisiae* EC1118 in response to varying concentrations of thiamine

The population dynamics of *P. kluyveri* Frootzen grown as monocultures in a larger volume (**Figure 3. 13 A**) showed marked differences in comparison to the results obtained from the microtiter plate assay (**Figure 3.2**). Compared to the control, the absence of thiamine resulted in the significantly poorer growth of *P. kluyveri* Frootzen throughout fermentation when grown in monocultures. As for when *S. cerevisiae* EC1118 was grown in monocultures (**Figure 3.13 B**), the absence of thiamine only had a significant impact on the growth response during the early to mid-stages of fermentation, after which similar growth was observed in response to the different concentrations of thiamine. In sequential cultures (**Figure 3.13 C**), a decrease in the population size of *P. kluyveri* Frootzen was observed after the addition of *S. cerevisiae* EC1118. This was mainly in conditions of control and double the concentration of thiamine, with a notable decrease when the concentration of thiamine was double that of the control. In addition, the significant increase in the population size of *S. cerevisiae* EC1118 was more observed when double the concentration of thiamine was present in comparison to the control and absence of thiamine. These findings indicated a form of competition for thiamine between the two yeasts. When grown simultaneously, the population size of *P. kluyveri* Frootzen was dominant over that of *S. cerevisiae* EC1118 (**Figure 3.13 D**), a different finding to what was found the simultaneous culturing of these two yeasts were presented with varying concentrations of biotin (see section **3.3.2.3**).

3.3.2.6 Fermentation kinetics of *P. kluyveri* Frootzen and *S. cerevisiae* EC1118 in response to varying concentrations of thiamine

The absence of thiamine resulted in significantly slower fermentation rates of both *P. kluyveri* Frootzen and *S. cerevisiae* EC1118 when grown in monocultures as well as co-cultures (**Figure 3.14**). In addition, lower levels of CO_2-production in the absence of thiamine were observed for all the modalities mentioned above. However, when *S. cerevisiae* EC1118 was grown in monocultures, no significant impact on the maximum level of CO_2-production was observed in the absence of thiamine, compared to that of the control and double the concentration of thiamine, at the late stages of fermentation.

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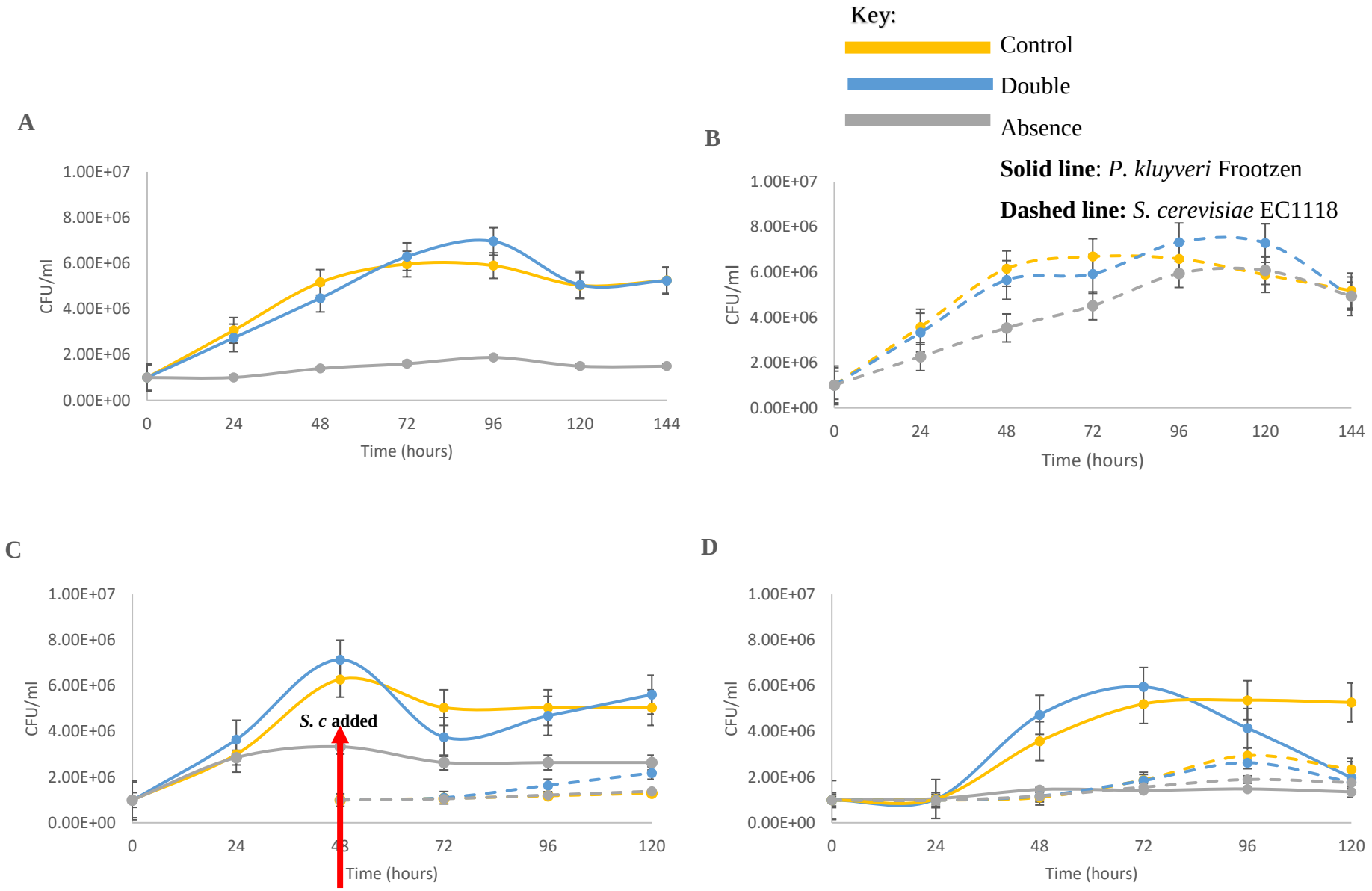

Figure 3.13: Population dynamics expressed as average growth in colony forming units (CFU)/ml of *P. kluyveri* Frootzen (**A**) and *S. cerevisiae* EC1118 (**B**) in monocultures respectively, as well in co-cultures (**C**: sequential; **D**: simultaneous). Error bars represent standard deviation (n=3 biological repeats).

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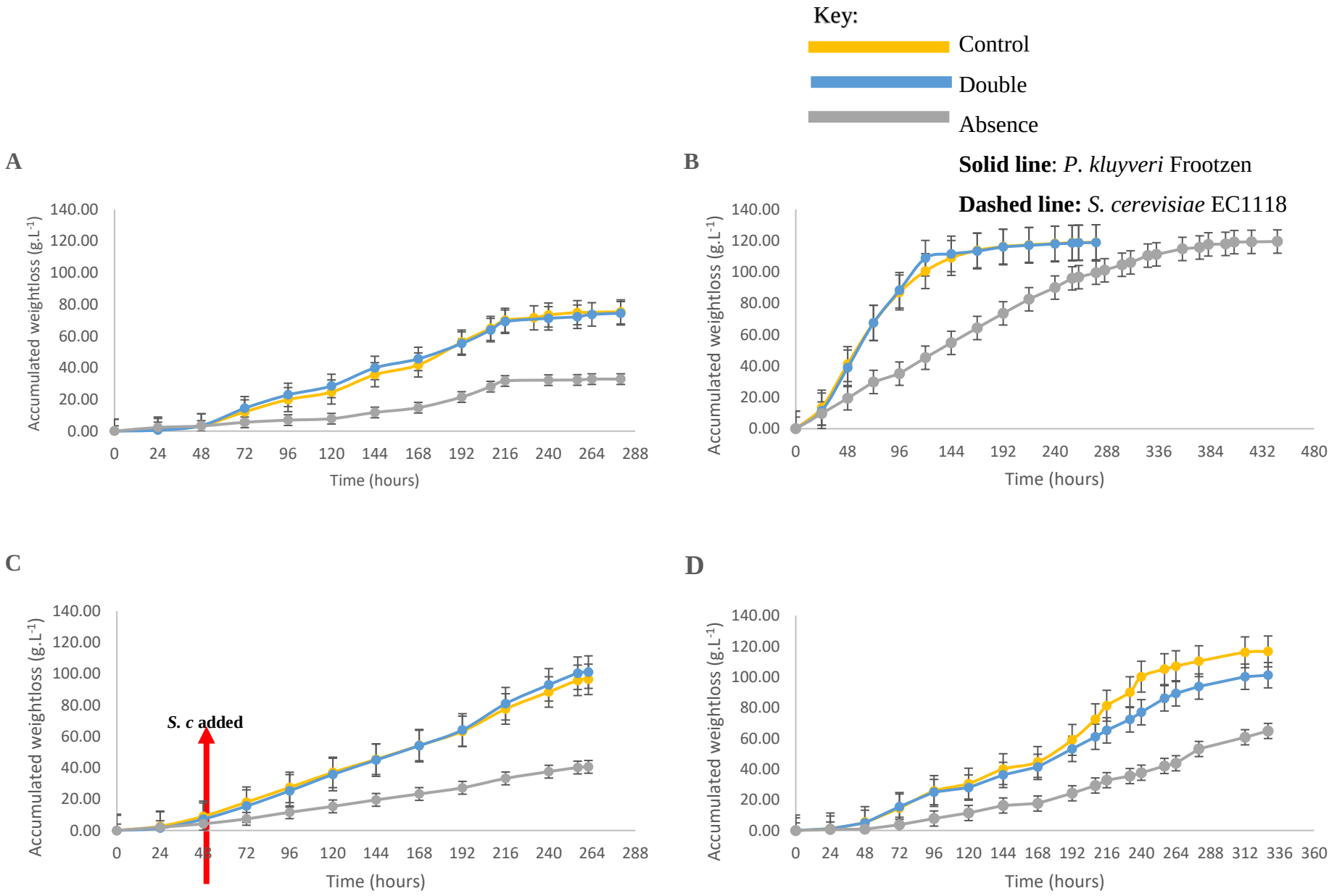

Figure 3.14: Fermentation kinetics expressed as accumulated weight loss in flasks due to CO_2-production by *P. kluyveri* Frootzen (**A**) and *S. cerevisiae* EC1118 (**B**) in monocultures respectively, as well in co-cultures (**C**: sequential; **D**: simultaneous). Error bars represent standard deviation (n=3 biological repeats).

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3.4 Discussion

3.4.1 Microtiter plate assay

This study aimed to evaluate the impact of vitamins on the growth patterns of different NS yeasts in pure culture, as well as potential competition with *S. cerevisiae* for vitamins. The impact of vitamins on the growth patterns of different yeast species was investigated with a high-throughput microtiter plate assay, initially proposed by Toussaint and Conconi (2006). The application of this protocol presented various forms of difficulty. One of the forms of difficulty was working with a minimal volume (µL) which required high precision and accuracy regarding pipetting technique. Inaccurate pipetting technique initially resulted in considerable variability between biological repeats. With the improvement in pipetting technique, reproducible results were obtained. However, in up-scale fermentations, specific differences in the growth patterns of some yeast species were observed which was not evident in the results from the microtiter plate assay. These findings suggest that the microtiter plate assay is not entirely reliable and should be rather implemented as a rapid screening protocol, with additional follow-up experiments to allow for more specific conclusions to be drawn.

There were also some limitations regarding the microtiter plate assay. The assay presented data outside the range of accuracy, which is generally between an OD of 0.1-0.8. This limits the extent to which the data can be interpreted. Apart from this, the instrument only allowed for agitation to be introduced before the automated reading, and not throughout fermentation. This could have affected sensing of nutrient availability by the yeasts, which could have had an impact on the accuracy of the results. Also, agitation may not have been optimal, resulting in some yeasts cells remaining at the bottom of the well while others are dispersed in the medium. Furthermore, the use of parafilm does not guarantee sterility during fermentation and therefore needs to be followed-up by plating out on differential media after fermentation to verify possible cross contamination.

Despite the various challenges and limitations regarding the microtiter plate assay used in this study, this high-throughput can be implemented in other studies to depict yeast growth responses towards variation in growth factors. This method presented results that concur with what has been previously recorded, but also expanded on the current knowledge of the vitamin requirements of the yeasts investigated. In addition, novel information is presented as some of the vitamin requirements by the yeasts were not previously reported on.

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Of all the yeasts investigated, *P. kluyveri* Frootzen showed the most significant differences in its growth responses towards all of the vitamins examined. In particular, double the concentration of any vitamin compared to the control allowed for improved growth. Since the control represents all vitamins and other growth factors at concentrations found in synthetic grape juice (Henschke & Jiranek, 1993), the findings suggested that the concentration of vitamins found in synthetic grape juice is limiting for *P. kluyveri* Frootzen. The concentrations of vitamins found in actual grapes are far less in comparison to what is found in synthetic grape juice (Hall *et al*., 1955). An additional loss in the vitamins found in grapes can occur due to juice extraction and vinification (Hall *et al.*, 1955). Therefore, it appears that the individual vitamins found in synthetic grape juice, and potentially actual grape juice, is limiting the growth of *P. kluyveri* Frootzen. Currently, only thiamine is allowed to be supplemented to wine fermentations (Julien *et al*., 2017). Thus, continued research into legislating the supplementation of vitamins might be necessary to improve the growth of *P. kluyveri* Frootzen or other yeasts which are not optimally supported by the concentration of vitamins in synthetic or actual grape juice.

It was surprising that only *P. kluyveri* Frootzen displayed significant differences in its growth responses towards varying concentrations of pantothenate since a recent study reported on *S. cerevisiae* EC1118 undergoing cell death when pantothenate was absent in the medium (Duc *et al*., 2017). The latter authors also used the same composition of a synthetic grape juice medium as used in this study. The difference between the findings of Duc *et al.* (2017) and this study, could be explained by taking samples over a longer duration of time than what was performed in the current study. This may have allowed for a more apparent difference to be observed by Duc *et al*. (2017). It has been reported that *H. vineae* requires pantothenate (Barnett *et al*., 2000). However, *H. vineae* IWBT Y980 investigated in the current study did not illustrate significant differences in its growth responses towards varying concentrations of pantothenate, suggesting that the requirement for pantothenate by *H. vineae* is strain specific. Other than possible strain-specificity, it could be that *H. vineae* IWBT Y980 uses β-alanine, a possible alternative to pantothenate (White *et al*., 2008).

The yeasts *S. cerevisiae* EC1118, *L. thermotolerans* Concerto, *T. delbrueckii* Biodiva and *P. kluyveri* Frootzen displayed significant differences in their growth responses towards varying concentrations of thiamine. It has been reported that *T. delbrueckii* and *P. kluyveri* does not require thiamine (Barnett *et al*., 2000). However, the findings of the current study suggest

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that thiamine is needed by strains of these yeasts, suggesting that the requirement for thiamine is strain-specific. The absence of thiamine had an adverse effect on the growth of *S. cerevisiae* EC1118, *L. thermotolerans* Concerto, *T. delbrueckii* Biodiva and *P. kluyveri* Frootzen, but increased concentrations of thiamine had different impacts on the yeasts. For *S. cerevisiae* EC1118 and *L. thermotolerans* Concerto, double the concentration of thiamine allowed for a similar growth response when compared to that of the control. These results suggested that the concentration of thiamine found in synthetic grape juice is sufficient for the growth of these yeasts. In the case of *T. delbrueckii* Biodiva, double the concentration of thiamine resulted in weaker growth when compared to that of the control. This finding proposed that the thiamine concentration found in synthetic grape juice is sufficient for the growth of *T. delbrueckii* Biodiva, but that an increase is inhibitory. The significant adverse impact on the growth of *S. cerevisiae* EC1118, *L. thermotolerans* Concerto, *T. delbrueckii* Biodiva and *P. kluyveri* Frootzen when thiamine was absent, was observed from the onset of their respective exponential growth phases. Metabolic processes including nitrogen, amino acids, nutrients and carbon catabolism take place in the exponential growth phase of yeasts- all of which thiamine plays a role in serving as an enzyme cofactor (Li *et al*., 2010). Our results are therefore in alignment with literature and emphasise the importance of thiamine for these yeasts. The same yeasts except for *L. thermotolerans* Concerto, that showed significant differences in their growth responses towards varying concentrations of thiamine, also displayed substantial differences in their growth responses towards different concentrations of pyridoxine. These findings concurred with previous reports regarding the interrelationship that exists between thiamine and pyridoxine (Zeidler *et al*., 2003).

Varying concentrations of inositol had significant impacts on the growth responses of most of the yeasts investigated in this study, including *L. thermotolerans* Concerto, *M. pulcherrima* Flavia, *H. vineae* IWBT Y980 and *P. kluyveri* Frootzen. The findings of the current study concur with the requirements of inositol described previously for *L. thermotolerans* and *H. vineae* (Barnett *et al*., 2000). However, the results of the current study are in contrast to previous literature which reported on *P. kluyveri* not requiring inositol (Barnett *et al*., 2000). This suggests that the requirement of inositol by *P. kluyveri* is strain-specific. The absence of inositol inhibited the growth of *L. thermotolerans* Concerto from 18 h onwards, resulting in a gradual decrease in cell numbers. However, for the other yeasts, only the growth rate was affected. These findings implied that *L. thermotolerans* Concerto was unable to synthesise

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inositol *de novo* and that the growth observed from 0h to 18h, was probably due to inositol from pre-culturing that was reserved within the cells of *L. thermotolerans* Concerto.

3.4.2 Selected fermentations in larger volume

3.4.2.1 *L. thermotolerans* Concerto and *S. cerevisiae* EC1118 in response to inositol

The findings for the requirement of inositol by *L. thermotolerans* Concerto in this study was confirmed and concurs with literature (Barnett *et al*., 2000). The results also provided insight regarding the requirement of inositol by *S. cerevisiae* EC1118, especially in the early and late stages of fermentation. The requirement of inositol by *S. cerevisiae* EC1118 in the early stages of fermentation infers that inositol plays a role in the adaptation of *S. cerevisiae* EC1118 to synthetic grape juice. In the later stages of fermentation, an increase in harsh conditions exists, for example, increased ethanol concentrations and osmotic stress - all which could affect the membrane integrity of a yeast. Since inositol plays a role in maintaining membrane stability (Majerus *et al*., 1986; Henry *et al*., 2014), it may explain why *S. cerevisiae* EC1118 requires inositol at the later stages of fermentation to have improved membrane stability against increasing ethanol concentrations and lack of oxygen.

The antagonistic effect between *L. thermotolerans* Concerto and *S. cerevisiae* EC1118 observed in this study concurs with what has been previously documented (Gobbi *et al*., 2013). Synthetic grape juice, represented by the control in the current study, is not sufficient to support the growth of *L. thermotolerans* and *S. cerevisiae* EC1118 when grown simultaneously, since double the concentration of inositol has shown to serve as a better support for the growth of these yeasts, especially in the early stages of fermentation. The co-culturing of *L. thermotolerans* Concerto and *S. cerevisiae* EC1118 in the absence of inositol resulted in faster fermentation rates and higher final levels of CO_2- production. This could be explained by possible autolysis of *L. thermotolerans* Concerto, resulting in the release of additional nutrients to be metabolised by *S. cerevisiae* EC1118.

3.4.2.2 *P. kluyveri* Frootzen and *S. cerevisiae* EC1118 in response to biotin

In comparison to the findings from the microtiter plate assay, non-reproducible results were obtained for the population dynamics of *P. kluyveri* Frootzen in response to varying concentrations of biotin. This could have been due to the different morphologies of *P. kluyveri* Frootzen observed microscopically (single cells, chains or clusters). It appeared that variation

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in the concentration of biotin had no impact on the growth and fermentation rate of *P. kluyveri* Frootzen, suggesting that this yeast does not require exogenous biotin. These findings agreed with what has been previously reported regarding the strain-specificity of biotin by *P. kluyveri* (Barnett *et al*., 2000). The growth of *S. cerevisiae* EC1118, when grown in monocultures, was significantly improved in response to double the concentration of biotin (maximum CFU/ml = 8.2 x 10^6) compared to the control (5.6 x 10^6) especially during exponential growth. Biotin plays a role in the central metabolic network of microorganisms (amino acid metabolism, gluconeogenesis, fatty acid synthesis) - all process known to be highly regulated during exponential growth (Dixon & Rose, 1964; Madsen *et al*., 2015). Furthermore, the absence of biotin resulted in growth responses of *S. cerevisiae* EC1118 was similar to that of the control. This suggested that *S. cerevisiae* EC1118 was able to synthesise biotin *de novo* and that biotin is a growth-enhancing rather than a growth-promoting vitamin for *S. cerevisiae* EC1118 in particular. This suggestion was emphasised with the findings regarding the fermentation kinetics, where the change in the concentration of biotin did not affect the fermentation rates of *S. cerevisiae* EC1118 in the different culturing modalities investigated. It has been reported by Tehlivets *et al*. (2007), that *S. cerevisiae* only contains the last three genes in biotin synthesis, inferring that exogenous biotin is required. However, from the findings of the present study, this was not the case since the growth of *S. cerevisiae* EC1118 in the absence of biotin was maintained at similar levels than that of the control. Other than biotin instead being a growth-enhancing vitamin for *S. cerevisiae* EC1118, the data suggest that requirement of biotin by *S. cerevisiae* is strain-specific and that genetic variety regarding biotin-synthesis amongst strains of this species exist.

3.4.2.3 *P. kluyveri* Frootzen and *S. cerevisiae* EC1118 in response to thiamine

Similar to what was found in response to biotin (**3.4.2.2**), non-reproducible results were obtained for *P. kluyveri* Frootzen regarding the population dynamics in response to varying concentrations of thiamine. Nonetheless, the absence of thiamine compared to the control resulted in the weaker growth of *P. kluyveri* Frootzen as well as *S. cerevisiae* EC1118, leading to slower fermentation rates and longer fermentation times. It has been reported that thiamine is required for successful reproduction by yeasts to facilitate processes such as amino acid and carbohydrate catabolism - all processes which are highly regulated in the early growth stages of yeasts (Li *et al*., 2010). In the absence of thiamine, slower fermentation rates by *S. cerevisiae* EC1118 was observed, but similar final levels of CO_2 was displayed. Thus, these findings

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suggest that. *S. cerevisiae* EC1118 can synthesise thiamine *de novo*, but that gene-regulation of thiamine-synthesis is only upregulated whenever the yeast senses low levels of thiamine. These findings agree with what has previously been recently reported (Julien *et al*., 2017).

It appeared that when grown sequentially, the addition of *S. cerevisiae* EC1118 led to a more prominent decrease in the growth of *P. kluyveri* Frootzen, especially when in the presence of double the concentration of thiamine, compared to that of the control. This suggested that there was competition between the two yeasts for thiamine. It was interesting to observe that although *S. cerevisiae* EC1118 displayed dominance over *P. kluyveri* Frootzen when grown simultaneously in response to varying concentrations of biotin, this dominance was reversed in favour of *P. kluyveri* Frootzen, in response to different concentrations of thiamine. Drawing any logical explanation from this observation is not possible due to non-reproducible results from the population dynamics of *P. kluyveri* Frootzen. Perhaps follow-up experiments may shed light regarding this phenomenon.

3.5 Conclusion

With the use of a microtiter plate assay, the vitamins requirements of some NS yeasts could be confirmed. This assay was successful in detecting clear differences in the growth responses of some NS yeasts towards varying concentrations of vitamins. However, non-reproducible results were obtained for that of *P. kluyveri* Frootzen, due to its different morphologies observed microscopically. Nevertheless, the findings of the assay suggest the adaptation of the vitamin concentration found in synthetic grape juice as proposed by Henschke & Jiranek (1993), due to the concentration of some vitamins either being insufficient or inhibitory for the growth of some of the yeasts investigated. Yeasts that displayed significant differences in their growth responses towards variation in the concentration of thiamine illustrated similar responses when the concentration of pyridoxine was varied. These findings supported literature reporting on the interrelationship that exists between thiamine and pyridoxine. Competition for vitamins between NS yeasts and *S. cerevisiae* EC1118 was observed in some of the findings that were selected for further investigation from the microtiter plate assays. In particular, it was observed that *S. cerevisiae* EC1118 requires inositol to adapt in the early stages of fermentation, as well as maintaining membrane integrity when conditions such as ethanol and osmotic stress increases. The vitamin requirements of yeasts and the effect of competition for vitamins have a definite impact on fermentation dynamics and kinetics, but further

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investigation into the impact of vitamins on secondary metabolites is needed. Further experiments need to be conducted to confirm the findings of this study.

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Chapter 4

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Conclusion: general discussion and future studies

4.1 General discussion

The diversification of aroma profiles with the involvement of non-*Saccharomyces* (NS) yeasts in spontaneous fermentations is a possible way to meet the ever-changing consumer demand. The extent of the contribution of NS yeasts on the diversification of aroma profiles of wine depends significantly on their persistence during fermentation. Their early decline in fermentation may in part be linked to unmet nutrient requirements. Recently, the nitrogen requirements of several important species have been investigated (Ugliano *et al*., 2009; Schnierda *et al*., 2014; Taillandier *et al*., 2014). However, the vitamin requirements of these yeasts and the possible impact on wine fermentation are still poorly understood. Therefore, this study aimed to assess the vitamin requirements of various commercial NS yeasts, as well as the competition for these vitamins when grown in different culturing modalities with *S. cerevisiae*.

This study applied a previously proposed high-throughput microtiter plate assay initially by Toussaint and Conconi (2006) to assess the vitamin requirements of the various NS yeast. Not only did the results from this assay concur with previous reports, but the assay also expanded on the existing knowledge regarding the vitamin requirements by the yeasts investigated in this study. Furthermore, the microtiter plate assay provided novel information regarding the vitamin requirements of the yeasts examined in this study and could be implemented in future studies regarding other nutrient-related topics.

Of all the yeasts investigated, non-reproducible results were obtained regarding the population dynamics of *P. kluyveri* Frootzen due to its different morphologies observed microscopically. Future studies may have to look into other methods of measuring the population dynamics of *P. kluyveri* Frootzen more accurately.

The findings of this study agree with previous reports regarding the interrelationship between thiamine and pyridoxine, by clearly indicating that the yeasts affected by variations in the concentrations of thiamine was also affected by change in the concentrations of pyridoxine (Leonian & Lilly, 1942; Schultz & Atkin, 1947; Chiao & Peterson, 1956; Bataillon *et al.*, 1996). More specifically, the study reported on the respective concentrations of thiamine and

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pyridoxine found in synthetic grape juice is sufficient for the growth of *T. delbrueckii* Biodiva, but that increasing concentrations of these vitamins can be inhibitory. Also, *S. cerevisiae* EC1118 illustrated significantly poorer growth when thiamine or pyridoxine was absent from the medium when compared to the control, which could lead to stuck fermentations. Fortunately, the supplementation of thiamine is currently legislated to prevent stuck or sluggish fermentations (Julien *et al*., 2017).

An interesting finding was presented in this study regarding the inositol requirement by *S. cerevisiae* EC1118. It appears that *S. cerevisiae* EC1118 requires inositol in adaptation to synthetic grape juice as well as for protection against increasing levels of ethanol and limiting levels of oxygen as fermentation progresses (Henry *et al*., 2014). Since *S. cerevisiae* is generally the yeast to primarily drive alcoholic fermentation, limiting amounts of inositol may impact fermentations considerably, especially when *S. cerevisiae* EC1118 has to compete with *L. thermotolerans* Concerto, which was highlighted by this study to be auxotrophic for inositol. Accordingly, this study proposes that supplementation of inositol to fermentations involving *L. thermotolerans* Concerto and *S. cerevisiae* EC1118 may be beneficial for winemaking practices. Furthermore, it might be of value of to investigate the impact of inositol on the ability of *L. thermotolerans* to produce L-lactic acid, a pleasant ‘soft’ acid which is sought after by winemakers (Jolly *et al*., 2014).

4.2 Future studies

To strengthen the results of this study, further investigations may be suggested. Other vitamins in combination with other NS yeasts other than what has been examined in the study should be investigated to obtain a more in-depth understanding of the vitamin requirements of NS yeasts. This study had a limited testing range for the vitamins examined. It could perhaps be more enlightening to increase the testing ratios of the concentrations of the vitamins in relation to other vitamins, since other concentrations of vitamins might be more beneficial for optimal yeast growth, for example, *S. cerevisiae* preferring 10% of the pantothenate concentration found in grapes (Ough *et al*., 1989). The current study did investigate 10% of the concentrations of vitamins found in synthetic grape juice (results not shown) and confirmed previous reports (Ough *et al*., 1989). Also, a combinatorial approach where two or more vitamins are changed in future work, may serve as an additional comprehensive experiment. Synthetic grape juice was used as a growth medium in this study, which could differ from real grape juice. Hence, the experiments in this study should be repeated in real grape juice to allow the research to be

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more applicable to the wine industry. However, no method exists to measure the vitamins in grape juice accurately. Once a suitable method is available, actual uptake of vitamins by yeasts and the resulting impact on fermentation over time can be monitored. Performing experiments on different grape varieties may provide fascinating insight, as it has been reported that the concentration of vitamins varies between grape varieties (Robinson, 1951; Castor, 1953; Hall *et al*., 1956; Ough & Kunkee, 1967; Peynaud & Lafourcade, 1977). The interaction between vitamins and other nutrients, for example, biotin and nitrogen, have received attention (Bohlsheid *et al*., 2007). Future studies could, therefore, investigate the interaction of vitamins with other nutrients to determine any other effects on fermentation. Lastly, future studies should examine the indirect impact of vitamins on metabolite and volatile aroma compound production to obtain more knowledge that could be subsequently presented to winemakers.

4.3 Literature cited

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