Nitrogen utilisation of selected non-*Saccharomyces* yeasts and the impact on volatile compound production

by

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Declaration

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Summary

During fermentation, nitrogenous compounds serve as nutrients for the yeasts, which enable their growth, functioning and maintenance of the yeasts cells. From a winemaking perspective, a certain amount of nitrogen is required for the yeasts in order to avoid sluggish or stuck fermentation. Moreover, nitrogen metabolism leads to the production of aroma compounds such as higher alcohols, fatty acids and esters which contribute positively to overall sensory characteristics of wine. Nitrogen metabolism (uptake of ammonium and amino acids) have been extensively studied in *Saccharomyces cerevisiae*. Nonetheless, the fairly great variances observed between strains in terms of preference for certain nitrogen sources and metabolism thereof are not so well understood. Additionally, these mechanisms nitrogen metabolism of non-*Saccharomyces* yeasts are even vaguer and simply assumed to be globally similar to those of *S. cerevisiae*.

This study aimed to investigate the uptake of nitrogen compounds (ammonium and individual amino acids) by selected non-*Saccharomyces* yeasts (*Lachancea thermotolerans* IWBT Y1240, *Torulaspora delbrueckii* Biodiva TD291, *Pichia kluyveri* FrootZen, *Metschnikowia pulcherrima* IWBT Y1123 and *Metschnikowia pulcherrima* Flavia) to assess the impact of fermentation kinetics and the production of aroma compounds during sequential fermentations with *S. cerevisiae* under different initial YAN concentrations, with 300 mg/L, 150 mg/L and 75 mg/L, respectively). Fermentations were performed in a synthetic grape juice medium with pure and sequential fermentations. The data showed that the assimilation of nitrogen compounds were species specific. For example, *L. thermotolerans* preferred alpha amino nitrogen above ammonia, where the opposite hold true for *T. delbrueckii*. Notable differences could also be identified for the uptake of certain single amino acids. Irrespective of the initial YAN concentrations during sequential fermentations, the yeasts only assimilated about half of the initial YAN. The non-*Saccharomyces* yeasts did not influence fermentation performance during sequential fermentations. However, a low initial YAN (75 mg/L) had a strong influence on the fermentation kinetics and aroma compound production. The higher uptake (compare to *S. cerevisiae*) of specific single amino acids by non-*Saccharomyces* yeasts (especially *L. thermotolerans*), can be tentatively correlated with certain aroma compounds produced at the end of fermentation. The results also revealed that agitation could impact overall fermentation performance and aroma compound production. This study contributes to an improved understanding of how different initial nitrogen concentrations affect growth, fermentation performances and aroma compound production of wine-related yeasts under fermentative conditions. Moreover, the uptake of single amino acids by selected non-*Saccharomyces* yeasts had also been identified, which is a good starting point to better understand non-*Saccharomyces* yeasts nitrogen requirements which may be used for the optimization of nitrogen source addition, during alcoholic fermentation, when used in mixed fermentations in order to ensure a complete alcoholic fermentation. To the best of our knowledge, the uptake of single amino acids and YAN consumption by selected non-*Saccharomyces* yeasts under fermentation conditions tested, have never been studied before.

Opsomming

Tydens wynfermentasies dien talle stikstof komponente as voedingstowwe vir wyngis wat hul groei, funksie en onderhoud bevorder. Van `n wynmaak perspektief word daar `n sekere hoeveelheid stikstof benodig deur die wyngis om te verhoed dat slepende of onvolledige fermentasies plaasvind. Verder lei stikstofmetabolisme na die produksie van aroma verbindings, soos hoër alkohole, vlugtige vetsure en esters wat positief bydra tot die sensoriese karaktereienskappe van wyn. Die stikstofmetabolisme (opneem ammonium en aminosure) is deeglik nagevors in die wyngis *Saccharomyces cerevisiae*, maar die klein variasies waargeneem tussen die gisras in terme van die voorkeur van sekere stikstof komponente is egter nog onduidelik. Daarbenewens is die stikstofmetabolisme nog meer onbekend in nie-*Saccharomyces* wyngis en word dit oor die algemeen aanvaar dat die werking van die stikstofmetabolisme dieselfde is as in *S. cerevisiae*.

Hierdie studie het gestreef om die opneem van stikstof komponente (ammonium en aminosure) te ondersoek van uitverkiesde nie-*Saccharomyes* gis (*Lachancea thermotolerans* IWBT Y1240, *Torulaspora delbrueckii* Biodiva TD291, *Pichia kluyveri* FrootZen, *Metschnikowia pulcherrima* IWBT Y1123 and *Metschnikowia pulcherrima* Flavia) deur te bepaal wat die impak is op die groei-kinetika en op die produksie van aroma komponente gedurende gemengde kultuur fermentasies met S. cerevisae onder verskillende aanvangs assimileerbare stikstof (300 mg/L, 150 mg/L en 75 mg/L). Fermentasies is in sintetiese druiwemos uitgevoer vir beide enkel en gemengde kultuur fermentasies. Die resultate demonstreer dat die assimilasie van stikstof ras spesifiek was. Byvoorbeeld, *L. thermotolerans* verkies alfa amino stikstof bo ammonium waar die teenoorgestelde waar is vir *T. delbrueckii*. Beduidende verskille is ook waargeneem vir die opneem van sekere individuele aminosure. Die wyngis het steeds net die helfte van die assimileerbare stikstof opgeneem gedurende gemengde kultuur fermentasies ongeag die aanvangsstikstof konsentrasies. Die nie-*Saccharomyces* gis het nie die fermentasie kinetika beïnvloed tydens gemengde kultuur fermentasies nie. Daar was egter ook waargeneem dat `n lae assimileerbare stikstof (75 mg/L) `n negatiewe invloed op die fermentasie kinetika sowel as aroma produksie gehad het. Die hoër opname (vergelyking met *S. cerevisiae*) van sekere aminosure deur nie-*Saccharomyces* gis, kan tydelik gekoppel word aan die produksie van spesifieke aroma verbindings aan die einde van fermentasies. Die resultate het ook gewys dat die toepassing van skud `n impak het op die fermentasie kinetika sowel as die produksie van aroma komponente. Die studie dra by om beter te verstaan van hoe verskillende aanvangsstikstof die groei, fermentasie kinetika en aroma produksie beïnvloed onder fermentasie kondisies. Die opneem van sekere aminosure deur nie-*Saccharomyces* gis word ook beskryf, wat `n goeie beginpunt is om beter te vertaan wat die stikstof vereistes vir die geselekteerde wyngis is, wat gebruik kan word vir die optimisering van stikstofaanvullings, sodat die risiko van probleemfermentasies verlaag sal word. So ver as wat ons kennis strek is die opneem van aminosure en die gebruik van assimileerbare stikstof deur nie-*Saccharomyces* wyngis onder fermentasie kondisies nog nie ondersoek nie.

This thesis is dedicated to

My family

Biographical sketch

Simoné de Koker was born on 22 February 1991 in Gauteng. She matriculated from High School Riebeeckrand in 2009 and in 2012 obtained a BSc-degree (Microbiology and Biochemistry) from North-West University, Potchefstroom Campus. In 2013, she enrolled at the University of Stellenbosch and completed a HonsBSc-degree in Wine Biotechnology. In 2014, Simoné commenced with a MSc in Wine Biotechnology at the University of Stellenbosch.

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Preface

This thesis is presented as a compilation of four chapters.

Chapter 1 **General Introduction and project aims**

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Chapter 1

Introduction and project aims

1.1 Introduction

Yeasts (especially *Saccharomyces cerevisiae*) play a prominent role in the successful completion of alcoholic fermentation in grape must. During this process, they produce a variety of flavour and aroma compounds which, in a large part, determine wine quality. Indeed, many primary (e.g. ethanol, CO_2, glycerol, acetic acid and acetaldehyde) and secondary metabolites (e.g. esters, higher alcohols and volatile fatty acids) are produced by the yeasts during alcoholic fermentation. The latter can be produced anabolically and catabolically (Lambrechts and Pretorius 2000; Rossouw *et al*. 2008; Styger *et al*. 2011), especially from nitrogen-containing compounds that are naturally present in grape juice. Nitrogen is an important nutrient that helps yeasts sustain growth and is used by the cell for proteins (including enzymes) and nucleic acids synthesis (Henschke and Jiranek 1993). It has also been observed in various studies that nitrogen compounds, specifically amino acids, have an impact on the final concentrations of aroma and flavour in wine via the production of higher alcohols and fatty acids via the Ehrlich pathway and their esterification (Albers *et al*. 1996; Carrau *et al*. 2008; Barbosa *et al*. 2009).

Sluggish or stuck fermentations remain one of the great challenges of wine production. The most studied factors responsible for incomplete fermentations include nitrogen deficiency as well as factors such as imbalances in vitamins, minerals, anaerobic factors, malic acid, citric acid and glucose:fructose ratio (Bisson 1999; Medina *et al*. 2012). Yeast assimilable nitrogen (YAN) concentrations in grape must have been identified the limiting factor for the completion of alcoholic fermentation in many cases (Jiranek *et al*. 1995; Bell and Henschke 2005; Jolly *et al*. 2014). Low initial YAN concentration also leads to the production of off-flavours (rotten eggs typically) due to the formation of hydrogen sulphide, an intermediate in the production of sulphur-containing amino acids. The addition of nitrogen, in the form of diammonium phosphate (DAP), is a common winemaking practice to reduce the risk of unsuccessful fermentations. However, an excess of DAP in grape must can be utilized as a nutrient for the growth of spoilage organisms and can negatively impact the fermentation performance of the fermenting yeasts in some cases by increasing in sulphur dioxide and acetic acid (Jiranek *et al*. 1995; Taillandier *et al*. 2007).

The above phenomena have been extensively studied and well described in *S. cerevisiae*. However, little is known about the requirements of non-*Saccharomyces* yeasts and their response to low or high YAN concentrations. Several studies have fairly recently concluded that the use of certain non-*Saccharomyces* yeasts in sequential use with *Saccharomyces* species can contribute positively to the overall aroma and quality of wine, selected species and strains are now commercialised (Ciani and Comitini 2011; Andorrà *et al*. 2012; Lage *et al*. 2014; Mains 2014; Taillandier *et al*. 2014; Contreras *et al*. 2015; Kock 2015; Renault *et al*. 2015). Yet, yeast strains and species differ in their nitrogen requirements and nitrogen assimilation. More research therefore needs to be conducted on nitrogen consumption of individual yeast strains

and then the impact of this uptake when used in mixed culture fermentations. This is particularly relevant in the case of sequential inoculation when the non-*Saccharomyces* yeast is inoculated 24 to 48h before *S. cerevisiae.* Indeed, the growth of inoculated non-*Saccharomyces* yeasts has been reported to negatively influence that of *S. cerevisiae* as an aftermath of the depletion of nitrogenous compounds by the former yeasts (Medina *et al*. 2012).

Understanding the effect of nitrogen on the metabolism of selected non-*Saccharomyces* yeasts used in mixed cultures is therefore of prime importance. In particular, the preference for ammonium or specific amino acids should be investigated as well as the production of aroma compounds deriving from nitrogen metabolism. Indeed, considering consumer demands for a diversity of wines, one important feature to scrutinise is the ability of non-*Saccharomyces* yeast strains to introduce diversity or complexity into the final wine organoleptic profile. Thus, from a winemaker perspective, optimizing nitrogen supplementation of musts could be useful for more effective multispecies fermentations and, to avoid sluggish or stuck fermentations, while also improving the management of the final flavour profile of wines.

1.2 Project aims

This study focused on evaluating the amount and nature of nitrogen compounds assimilated by selected non-*Saccharomyces* yeasts under various conditions (i.e. various initial YAN concentrations and with or without shaking). Most of the studies done in literature, with more or less the same fermentation conditions as in our study, shook the fermentation flasks. However, in the wine industry, little agitation are present during fermentation, thus research can investigate whether agitation have a major effect on the final product of wine with regards to aroma compound production. For the different trials, fermentation performance, uptake of nitrogenous compounds and production of major volatiles (i.e. higher alcohols, short and medium chain fatty acids and esters) were monitored.

The main aims of this project were:

1. To investigate the uptake of nitrogen (ammonium and individual amino acids) by different non-*Saccharomyces* yeast strains under fermentative conditions in synthetic grape must.
2. Investigate different YAN concentrations in fermentations sequentially inoculated with non-*Saccharomyces* yeasts and then *S. cerevisiae* in order to assess the impact on the growth and fermentation performances of both yeasts. Sugar consumption rate was used as an indication of fermentation performance and the production of major volatile compounds was evaluated in order to assess the impact of nitrogen consumption on the final wine aroma profile.

3. To examine how agitation effect the nitrogen consumption, fermentation kinetics, population dynamics and production of volatile aroma compounds in pure culture fermentations by *S. cerevisiae*.

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Chapter 2

Literature review

Nitrogen requirements of yeasts and the effect on aroma compound production during mixed cultures

2.1 Introduction

Grape must is a chemically complex and variable matrix in which yeast growth and fermentative metabolism lead to the production of wine (Ribéreau-Gayon *et al*. 2006; Viana *et al*. 2014). The composition of grape must is influenced by various factors such as grape variety, climate, soil, and ripeness stage (Viana *et al*. 2014).

Grape must contains various substances which play vital roles during alcoholic fermentation. The carbohydrates, glucose and fructose, function as energy and carbon sources to sustain growth of yeast cells, while mineral salts including phosphate, magnesium, calcium, potassium, sulphate and chloride play a fundamental physiological role during yeast growth (Pereira 1988; Walker 2004). The nitrogen sources include ammonium, amino acids, proteins and small peptides which are critical for the growth of the yeast cells (Henschke and Jiranek 1993). Vitamins and minerals which serve as growth and survival factors, e.g. as co-factors of enzymatic reactions, are essential (Ribéreau-Gayon *et al*. 2006). Furthermore, lipids (unsaturated long chain fatty acids) and sterols (mainly ergosterol) aid in maintaining the membrane integrity and function (Lafon-Lafourcade *et al*. 1979). In anaerobic fermentative conditions, all these above-mentioned compounds have been identified as essential nutrients for the yeasts and therefore for a problem free and complete alcoholic fermentation.

Monosaccharides are the most abundant components of grape must. The main sugars include glucose and fructose and occur in about the same concentrations. In general, the total concentration of sugars range between 170 and 220 g/L (Ribéreau-Gayon *et al*. 2006), but higher concentrations also occur. These carbon sources are metabolised via the glycolytic pathway during alcoholic fermentation where energy for cell growth, maintenance and functioning is obtained from substrate-level phosphorylation. The conversion of sugars to ethanol during alcoholic fermentation causes no net redox change. Indeed, during this process, NADH is first converted to NAD^+ which is then re-oxidised, when ethanol is produced from acetaldehyde, and can subsequently be used again during sugar catabolism (Aranda *et al*. 2011). However, some intermediates of this pathway, particularly pyruvate, are also metabolised in other pathways, which results in a redox imbalance. Imbalances must be corrected in order for the cell to maintain its functioning. One of the main mechanisms implemented to correct these imbalances is the production of glycerol (Hohmann 1997). During this process, NAD^+ is indeed converted to NADH.

Nitrogen content on the other hand tends to be a limiting factor during alcoholic fermentation (Ingledew and Kunkee 1985). Nitrogen metabolism in yeasts, especially *Saccharomyces cerevisiae*, has been widely studied over the years (Anderson and Kirsop 1974; Henschke and Jiranek 1993; Jiranek *et al*. 1995; Albers *et al*. 1996a; Arias-Gil *et al*. 2007; Carrau *et al*. 2008; Bergdahl *et al*. 2012; Crépin *et al*. 2012; Casalta *et al*. 2013). A complex mixture of nitrogen-

containing compounds is present in grape juice. The concentrations and composition within grape varieties vary according to factors including rootstock, grape cultivar, seasonal conditions, levels of maturity and vine nutrient management (Beltran *et al*. 2004; Bell and Henschke 2005; Barbosa *et al*. 2012).

Yeasts have the ability to use a wide range of nitrogen-containing compounds as sole nitrogen sources, but the yeast growth and fermentative activity are not always consistently supported by these compounds. Indeed, *S. cerevisiae* preferentially uses some nitrogen sources (Cooper 1982; Barbosa *et al*. 2012). This discernment is made possible through a mechanism known as nitrogen catabolite repression (NCR). During this process, the expression of genes which are required for the uptake and degradation of less preferred nitrogen sources is inhibited when cells are provided with preferred ones (Beltran *et al*. 2004; Barbosa *et al*. 2012).

Free amino acids and ammonium ions are the major nitrogen-containing compounds which may constitute on average 40% of the nitrogenous compounds in grape juice and between 51 and 92% of the total assimilable nitrogen in must (Bell and Henschke 2005), with amino acids representing the largest part (Ugliano *et al*. 2007). Previous research suggests that insufficient yeast assimilable nitrogen (YAN) can lead to sluggish or stuck fermentations (Mendes Ferreira *et al*. 2004; Vilanova *et al*. 2007). Furthermore, the initial nitrogen concentration of grape juice correlates with the amount of biomass that is produced. This connection is the strongest when the nitrogen concentration present in the medium is lower than 300 mg/L (Agenbach 1977; Bely *et al*. 1990; Mendes Ferreira *et al*. 2004; Martínez-Moreno *et al*. 2012). The minimum YAN recommended for completion of alcoholic fermentation by wine yeasts has so far only been determined for *S. cerevisiae*, and is between 120-140 mg/L (Bely *et al*. 1990; Jiranek *et al*. 1995). Yet, this can only be used as a guideline, since this threshold value is must- and strain-dependent (Crépin *et al*. 2014). Some winemakers manage nitrogen concentrations by adding a standard solution of diammonium phosphate (DAP) at inoculation, without measuring the nitrogen concentration beforehand. This practice is however risky as it can in turn result in an excessive YAN concentration (Ugliano *et al*. 2007). In addition, high concentrations of DAP can lead to acidification of juices and excessive wine phosphate content (Taillandier *et al*. 2007).

Stuck fermentation is no new term in winemaking, as it has been used since the beginning of the 20th century (Ribéreau-Gayon *et al*. 2006). Various factors may lead to sluggish and stuck fermentations. It is therefore difficult to establish a clear cause-to-effect correlation and to recommend possible solutions. These factors include high sugar concentration, excessive temperature, initial low temperature, anti-fungal substances and nutritional deficiencies (Ribéreau-Gayon *et al*. 2006). The latter, and in particular nitrogen limitations, are the main cause of sluggish or stuck fermentations.

Saccharomyces cerevisiae has been well studied with regards to the impact of nitrogen sources on growth and completion of alcoholic fermentation (Albers *et al*. 1996b; Godard *et al*. 2007; Gutiérrez *et al*. 2012; Gutiérrez *et al*. 2013). However, very little is known about nitrogen utilization and the fermentation abilities of non-*Saccharomyces* yeasts. Yet, the use of non-*Saccharomyces* yeasts as wine starter cultures is gaining increasing attention. Further research is therefore needed. This review will discuss the general nitrogen requirements of wine yeast species and the impact of the nature and availability of nitrogenous compounds on yeast growth and production of aroma.

2.2 General nitrogen requirements of *S. cerevisiae* to complete alcoholic fermentation

2.2.1 Nitrogen uptake and metabolism

2.2.1.1 Transport of nitrogenous compounds

Active transport systems (proton symport) make it possible for most nitrogenous compounds to be assimilated by the cells (Fig. 1). One general amino acid permease as well as numerous specific permeases have been identified for different amino acids in *S. cerevisiae*. Moreover, three transporters with varying affinities have been identified (Mep1p, Mep2p and Mep3p) for ammonia where the active transport of the protonated species requires the presence of glucose (Henschke and Jiranek 1993; Aranda *et al*. 2011). The permease Gap1p has been identified as the sole general amino acid transporter. It allows the uptake of all L-amino acids and is the only permease known to transport the amino acids glycine and alanine (Crépin *et al*. 2012). Permeases that are specific to single amino acids include Lyp1p (lysine), Hip1p (histidine), Mup1p (methionine), Can1p (arginine) and Put4p (proline). Furthermore, other so-called specific amino acid permeases can transport dedicated groups of amino acids. These include Tat1p and Tat2p (aromatic amino acids), Bap2p and Bap3p (branched-chain amino acids), Dip5p (acidic amino acids), and Agp1p and Gnp1 (threonine, glutamine and serine) (Marini *et al*. 1997). Specific factors (including requirements for each compound, efficiency of transport and possible conversion into ammonia or glutamate) influence the specific order of uptake of nitrogen compounds, which are generally assimilated within the first day of alcoholic fermentation (Monteiro and Bisson 1991).

2.2.1.2 Overview of the intracellular metabolism of nitrogenous compounds

Once across the plasma membrane, amino acids and other nitrogenous compounds undergo deamination to generate ammonium, which can be directly used in biosynthetic processes or can serve as substrates for transaminases to form glutamate (Fig. 1). Ammonia and glutamate play a key role during nitrogen metabolism in yeasts. Glutamate is produced from an ammonium ion and an α-ketoglutarate molecule (which is synthesised from acetyl CoA and oxaloacetate via the tricarboxylic acid cycle), a reaction catalysed by $NADP^+$ glutamate

dehydrogenase, encoded by *GDH1*. NAD^+ glutamate dehydrogenase, encoded by *GDH2*, is involved in the oxidative catabolism of glutamate for the synthesis of glutamine (Bell and Henschke 2005; Ribéreau-Gayon *et al*. 2006). Glutamate supplies about 85% of the cell's requirement for nitrogen, and the remaining 15% is supplied by the amide group of glutamine. This reaction contributes to the synthesis of purines, pyrimidines and several amino acids (Cooper 1982; Magasanik and Kaiser 2002). In addition, during yeast growth, a proportion of amino acids can be stored in the vacuole. This storage allows the regulation of amino acids for efficient protein synthesis. Moreover, the nature of the nitrogen compound is important for the transport into the vacuolar compartment. Transport of substances across the membrane occurs by either H^+ - antiport or H^+ - symport, in or out, respectively. Importantly, cells have discrete pools of amino acids, where for example the basic amino acids including histidine, arginine and lysine are compartmentalised in the vacuole, whereas other amino acids, particularly aspartate and glutamate, are found in the cytoplasm (Aranda *et al*. 2011; Ljungdahl and Daignan-Fornier 2012; Crépin *et al*. 2014).

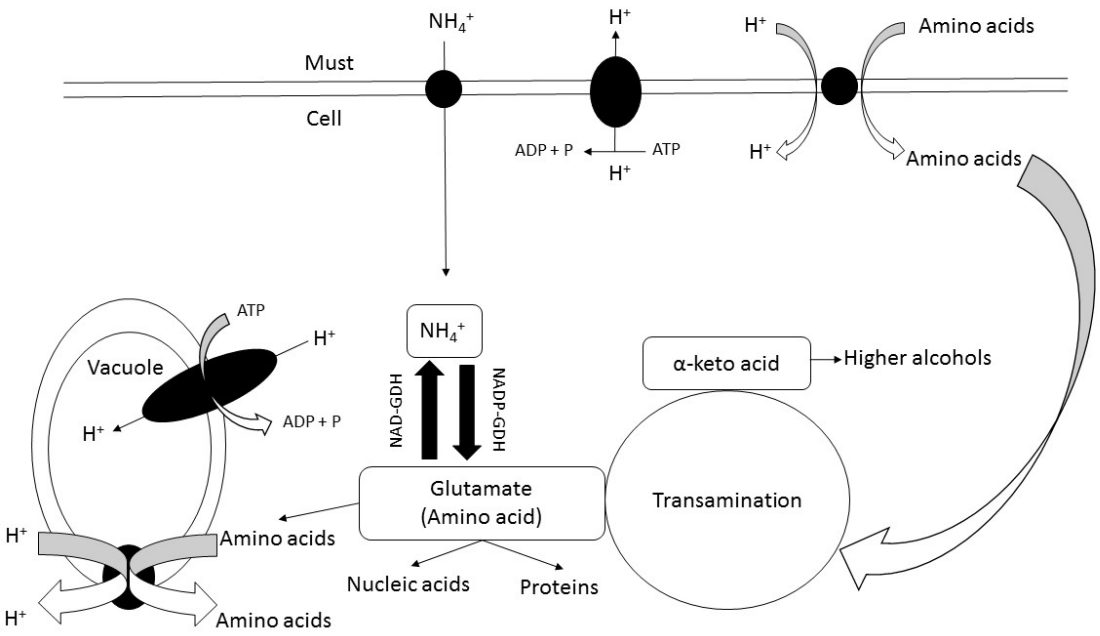

Figure. 1 Diagram of the uptake and metabolism of nitrogenous compounds in yeasts (Adapted from (Aranda *et al*. 2011).

2.2.1.3 Cellular regulation of nitrogen transport and metabolism

Nitrogen catabolite repression (NCR) and Ssy1p-Ptr3p-Ssy5p (SPS) are two separate regulatory systems that control amino acid permeases. The SPS is a plasma membrane sensor. Itis made up of three core plasma membrane proteins Ssy1p, Ptr3d and Ssy5p which are responsible for sensing amino acids in the external environment as well as regulating genes encoding amino acid transporters (Forsberg and Ljungdahl 2001; Ljungdahl and Daignan-Fornier 2012). The NCR regulates the uptake, utilisation and biosynthesis of amino acids. The

permeases that are controlled via the NCR include Mep1p, Mep2p, Mep3p (ammonium permeases), Gap1p (general permease), Agp1p (arginine permease), and Put4p (proline permease). The NCR system helps distinguish between efficient and less efficient amino acids. The in-depth mechanism of these regulatory systems have been discussed extensively in literature (Bell and Henschke 2005; Crépin *et al*. 2012; Ljungdahl and Daignan-Fornier 2012) and will therefore not be detailed further in this review.

A significant variability between biomass production and YAN consumption is observed among *S. cerevisiae* strains under nitrogen-limiting conditions. Insufficient nitrogen leads to low biomass yield and a slow fermentation rate (Tesnière *et al*. 2015). A study conducted by Crépin *et al.* (2014) investigated high and low biomass-producing strains in relation to initial nitrogen concentration as a possible cause for sluggish or stuck fermentation. The study showed that when an ample amount of nitrogen is available, the yeasts selectively assimilate preferred nitrogen sources (as a result of NCR). However, this selectivity is strain-specific and is thus the consequence of various mechanisms (including differences in YAN uptake, involving variations in ATPase activity or global regulatory mechanisms that control the access of all nitrogen sources to the cell) that may contribute to this variability. This could explain the different rate of YAN uptake between strains.

Moreover, when nitrogen sources are limited, especially when the ammonium concentration is low or only non-preferred sources are available, the regulatory proteins are transferred to the nucleus and this phenomenon consequentially leads to the up-regulation of the *MEP* genes. Nonetheless, when preferred nitrogen sources become available again, the proteins return to the cytoplasm (Marini *et al*. 1997) Because genetic background affects the efficiency of NCR control, difference in the rate of ammonium consumption between high and low biomass-producers may be due to differences of *MEP* genes (regulated in NCR) (Georis *et al*. 2009).

Despite years of research on this topic and the fact that the nitrogen metabolic pathways are known, there is still very little understanding of the reasons behind the differences observed between strains. (Camarasa *et al*. 2011).

2.2.2. Nitrogen requirements to ensure successful alcoholic fermentation

2.2.2.1 Nitrogen needs to complete alcoholic fermentation of grape must

From as early as 1977, researchers began to investigate the importance of assimilable nitrogen to sustain yeast growth and functioning (Jiranek *et al*. 1995). The nitrogen needs of *S. cerevisiae* in particular have been extensively studied (Cooper 1982; Bisson and Kunkee 1991; Henschke and Jiranek 1993; Albers *et al*. 1996a; Beltran *et al*. 2004; Bell and Henschke 2005). As mentioned in the previous paragraph, yeasts prefer certain nitrogen sources above others. During wine fermentation, this selectivity is influenced by the nitrogen composition of grape must and the environmental conditions, among which the limitation in oxygen availability plays a

critical role. For example, ammonium, glutamine and asparagine are accumulated preferentially to other amino acids such as arginine, alanine, aspartic acid, glutamate, glycine and proline (Henschke and Jiranek 1993; Bell and Henschke 2005). The dominant amino acid in grape must, proline is poorly utilised because the absence of oxygen prevents its metabolism. Studies reported that ranges between 300 mg/L YAN and 429 mg/L YAN are sufficient for *S. cerevisiae* to complete alcoholic fermentation, with a recommended minimum amount of 120-140 mg/L YAN (Bely *et al*. 1990).

It is important to note that most of the nitrogen sources are assimilated at the start of fermentation, although some amino acids are metabolized until the end of fermentation. For example, two different studies performed mixed culture fermentations with non-*Saccharomyces* yeasts and *S. cerevisiae* and measured YAN consumption during alcoholic fermentation. The authors showed that most YAN was consumed during the first 3 days (Medina *et al*. 2012; Taillandier *et al*. 2014). In addition, another study which compared amino acid uptake at the beginning and end of fermentation by pure *S. cerevisiae* strains reported that phenylalanine, lysine, threonine, arginine and serine were mainly consumed at the beginning of fermentation, whereas alanine and methionine were consumed mostly at the end of fermentation (Barrajón-Simancas *et al*. 2011). These pieces of information are important for winemakers to supplement at the right time during alcoholic fermentation to prevent nitrogen limited conditions.

2.2.2.2 Impact of the initial amount of nitrogen on fermentation performance

A direct relationship between nitrogen and sugar consumption exists. Indeed, the more nitrogen available, the higher the fermentation rate but only to a certain amount of nitrogen (Ingledew and Kunkee 1985). This is supported in a study performed by Arias-Gil *et al* (2007), in which different amino acid concentrations (0, 45, 120, 250 and 450 mg/L) were added to the must during spontaneous alcoholic fermentation to investigate the effect on yeast nitrogen metabolism during alcoholic fermentation and to evaluate the role of the nitrogen sources added. Fermentations were carried out in *Mazuelo* must which is poor in nitrogen compounds (40 mg/L YAN in this experiment). One interesting finding was that the addition of 45, 120 and 250 mg/L of amino acids to the must, resulted in improved fermentation kinetics to such an extent that the fermentations finished 6-7 days earlier (Arias-Gil *et al*. 2007). In addition, Vilanova *et al*. (2007) reported that the duration of the fermentation in response to different initial YAN concentrations was roughly the same for the *S. cerevisiae* strains used and was reduced from 20 days at low nitrogen (117 mg/L and 150 mg/L) to 16 – 17 days at 200 mg/L initial YAN and 12 days at higher initial YAN concentrations, which were 250 mg/L to 500 mg/L.

In the event of stuck fermentation caused by nitrogen limitation, both ammonium and amino acids have been shown to be needed by the yeast cells to help restart growth (Jiménez-Marí *et al*, 2007). Yet, not all nitrogen sources equally support the yeast cells in these limiting conditions

efficiently (Godard *et al*. 2007). Supporting these findings, and in addition to the above-mentioned studies, a study on alcoholic fermentation during which ammonia and amino acids were added, was performed examining gene expression patterns in yeast cells (*S. cerevisiae*) (Jiménez-Martí and Del Olmo 2008). The data demonstrated that during the first hours following the additions, gene expression reprogramming occurs. Indeed, the addition of ammonia resulted in higher expression of genes involved in amino acid biosynthesis. Moreover, the addition of amino acids led to higher transcription of genes related to translational machinery.

The supplementation of mixtures of amino acids and ammonium into the grape must can enhance the fermentation performance, since amino acids can be incorporated directly into the yeast protein synthesis (Arias-Gil *et al*. 2007). However, a recent study has shown that the amount of amino acids directly incorporated into proteins is very limited (Crépin *et al*. 2014). It is also important that the residual nitrogen content of wine at the end of fermentation is low, because high amounts of residual nitrogen could possibly cause microbial instability and lead to the development of microorganisms responsible for the formation of compounds such as acetic acid, biogenic amines and ethyl carbamate, which negatively influence wine quality (Ugliano and Henschke 2009; Garde-Cerdán *et al*. 2011).

2.3 Assimilation of nitrogenous compounds by selected non-*Saccharomyces* yeasts

2.3.1 Nitrogen requirements of individual non-*Saccharomyces* yeasts

Considering the recent attention given to non-*Saccharomyces* yeasts, it is essential to identify whether these yeasts have similar nitrogen requirements as *S. cerevisiae*. Yet, only a few studies have investigated these requirements and information is therefore limited in literature.

A recent study by (Kemsawasd *et al*. 2015) investigated the influence of different single and mixed amino acids on the growth and fermentation performance of four wine related non-*Saccharomyces* yeast species. This study concluded that the nitrogen sources with beneficial effects on the performance of the yeasts were serine (for *L. thermotolerans*), alanine (for *H. uvarum*), alanine and asparagine (for *M. pulcherrima*), arginine, asparagine, glutamine, isoleucine and mixture of 19 amino acids (for *T. delbrueckii*), respectively. Some of these amino acids, including alanine, arginine, glutamine and serine, also gave the best fermentation performance when used as sole nitrogen source by *S. cerevisiae* (Mckinnon 2013).

Another study comparing *T. delbrueckii* and *S. cerevisiae* commercial strains revealed that *T. delbrueckii* fermented to dryness independently of the initial nitrogen concentration (170 mg/L or 300 mg/L YAN) of the fermentation. Nevertheless, with 170 mg/L, the fermentation took 37 hours longer than the fermentation with MS300 (300 mg/L YAN) (Taillandier *et al*. 2014). The former study also showed that overall *T. delbrueckii* consumed less nitrogen than *S. cerevisiae*. This is in agreement with a previous study conducted on the same yeast species (Bely *et al*. 2008).

2.3.2 YAN consumption by mixed yeast cultures

An experiment was performed in synthetic grape must (MS300) using *T. delbrueckii* and *S. cerevisiae* in mixed and sequential fermentation to study interactions between these yeasts under two nitrogen regimes (324 mg/L and 176 mg/L initial YAN) (Taillandier *et al*. 2014). Interestingly, during sequential fermentations with 176 mg/L initial YAN, *S. cerevisiae*'s growth was reduced compared to that observed in pure culture fermentations. The authors concluded that, in the presence of *T. delbrueckii*, the initial nitrogen content of the must does not support optimal growth for the *S. cerevisiae* as the non-*Saccharomyces* yeast consumed most of the nitrogen and too little remained available for *S. cerevisiae* at the time of its inoculation, even though the viability of *S. cerevisiae* did not decrease during the fermentation. The YAN consumption profile of two non-*Saccharomyces* yeast species, *H. vineae* and *M. pulcherrima*, was also investigated to study the impact on *S. cerevisiae*'s fermentation ability in mixed cultures (Medina *et al*. 2012). Both non-*Saccharomyces* species consumed nitrogen rapidly (*M. pulcherrima* consumed more YAN than *H. vineae*), within the first 3 days. *M. pulcherrima*'s growth was faster than that of *H. vineae*, which may explain the higher consumption of YAN with the inoculation of *S. cerevisiae* 3 days after *H. vineae* and *M. pulcherrima*, resulting in sluggish fermentations compared with fermentations using pure cultures of *S. cerevisiae*. Competition for nutrients, in particular nitrogen compounds, was identified by the authors of this study as the main factor leading to these delayed fermentations.

By comparison, a study conducted on *Hanseniaspora guilliermondii* suggested that this yeast negatively affects the growth and fermentation performance of *S. cerevisiae* in mixed cultures (Lage *et al*. 2014). The authors speculated that competition for nutrients may not be the main reason for delayed fermentations. Indeed, some studies suggested that the interference in glycolytic enzymes of *S. cerevisiae* and the depletion of certain vitamins from the grape juice by non-*Saccharomyces* yeasts can be a possible cause of the delayed fermentations (Milanovic *et al*. 2012).

What is more, a recent study involving *S. cerevisiae* and *H. guiliermondii* focused on yeast-yeast interactions on a molecular level in natural grape must (Barbosa *et al*. 2015). The authors found that *GAP1*, *AGP1* and *PUT4*, encoding general amino acid transporters for the former two and a proline permease for the latter, were more highly expressed in *S. cerevisiae* in the single-culture, compared to the mixed culture. Furthermore, *MEP1* and *MEP2*, which encode ammonium permeases, were also more highly expressed in single-culture fermentation than in mixed culture fermentation. These results suggest that the presence of *H. guilliermondii* could be limiting the efficient nitrogen assimilation in grape-juice by *S. cerevisiae* or the expression is simply lower since there are less amino acids and ammonium available that can be taken up so the yeast conserves energy by not overproducing transporters (Barbosa *et al*. 2015). The molecular mechanisms behind this observation are however yet to be investigated.

On the whole, the above-mentioned studies give good insight into nitrogen requirements and consumption by yeasts and how fermentation performance is influenced by the addition of different initial concentrations of YAN and amino acids. Considering consumer demands for wines with more diverse characteristics, mixed culture fermentations can introduce complexity into the final wine flavour. However, differences are detected in aroma compound production of mixed culture fermented wines compared to *S. cerevisiae* pure culture fermented wines. Furthermore, the medium should contain enough YAN to allow the development of *S. cerevisiae* and for a good fermentation performance.

2.4 Production of Nitrogen-derived fermentative aroma compounds

2.4.1 Brief overview of the yeast contribution to the aromatic profile of wine

2.4.1.1 Release of primary or varietal aroma from grapevine-produced precursors

Grape derivatives are responsible for the primary aromas (Lambrechts and Pretorius 2000). Terpenes and thiols are an example amongst the most important grape derivatives and precursors which significantly contribute to wine aroma. Some non-*Saccharomyces* yeasts (*Debaryomyces*, *Hansenula*, *Candida*, *Pichia* and *Kloeckera*) have been shown to release varietal aroma compounds from grapevine precursors, as listed in Table 2.3. However, these compounds are not produced de novo and this aspect of non-*Saccharomyces* yeasts' contribution to wine aroma will not be discussed further in this review.

2.4.1.2. De novo production of secondary or fermentative aroma

Secondary aroma compound production occurs during alcoholic fermentation as part of the yeast metabolism. Higher alcohols, esters, volatile fatty acids and aldehydes have a great contribution to secondary aroma production (Manzanares *et al*. 2011). The Ehrlich and glycolytic pathways are the two main routes via which these compounds are produced. Amino acids metabolised via the Ehrlich pathway (Fig. 2) (valine, leucine, isoleucine, tryptophan, tyrosine and phenylalanine) undergo an initial transamination reaction to the corresponding α-keto acid (Hazelwood *et al*. 2008). α-Keto acids can also be formed from sugars via pyruvate, then decarboxylated to yield an aldehyde which is further reduced to corresponding alcohols or fatty acids, which occur anabolically (Dickinson *et al*. 1998; Lambrechts and Pretorius 2000). Higher alcohols are the largest aromatic compound group (Amerine and Cruess 1980). It has been reported that when found in concentrations lower than 300 mg/L a desirable level of complexity and fruity notes can be produced, whereas levels exceeding 400 mg/L can negatively influence wine quality (Rapp and Versini 1996). The initial nitrogen concentration in must has a strong impact on the production of higher alcohols. In general, when the nitrogen concentration in must is low, the production of higher alcohols are also low, whereas with sufficient nitrogen concentrations an inverse relationship with higher alcohols prevails (Äyräpää 1971). Additionally, strains also vary greatly in terms of the higher alcohols they produce

(Romano *et al*. 1992; Zironi *et al*. 1993). Wine esters, which contribute to wine fruitiness, are largely derived from the sugar and amino acid metabolism by yeasts, while some esters are produced from grape glycosides (Lambrechts and Pretorius 2000; Swiegers and Pretorius 2005). Esters are synthesized enzymatically by yeast from alcohols and fatty acids, in a reaction dependent on the availability of coenzyme A (CoA) (Lambrechts and Pretorius 2000).

In general, the application of nitrogen in the vineyard results in higher production of esters in wine. Additionally, the different nitrogen requirements of yeasts can also affect ester production, because a strain with a high nitrogen demand produces higher total esters than strains with lower nitrogen demand (Torrea *et al*. 2003). Ninety percent of the volatile fatty acids consists of acetic acid, which is of great importance to the quality of wine (Henschke and Jiranek 1993). Acetic acid levels of 0.2 – 0.7 g/L are considered as acceptable, while levels exceeding 0.7 g/L may give wine a vinegar-like character (Corison *et al*. 1979). Nitrogen is one of the factors which has a major impact on acetic acid production. Other factors include yeast strain, inoculation rate, pH, fermentation temperature and aeration. Nitrogen limiting conditions (below 140 mg/L) induce an increase in the production of acetic acid, while an inverse relationship is found for nitrogen concentrations lower than 450 mg/L (Bely *et al*. 2003). Medium chain fatty acids (hexanoic, octanoic and decanoic acids) occur in low concentrations in wine, but contribute to the aroma profile of Chardonnay for example (Smyth *et al*. 2005).

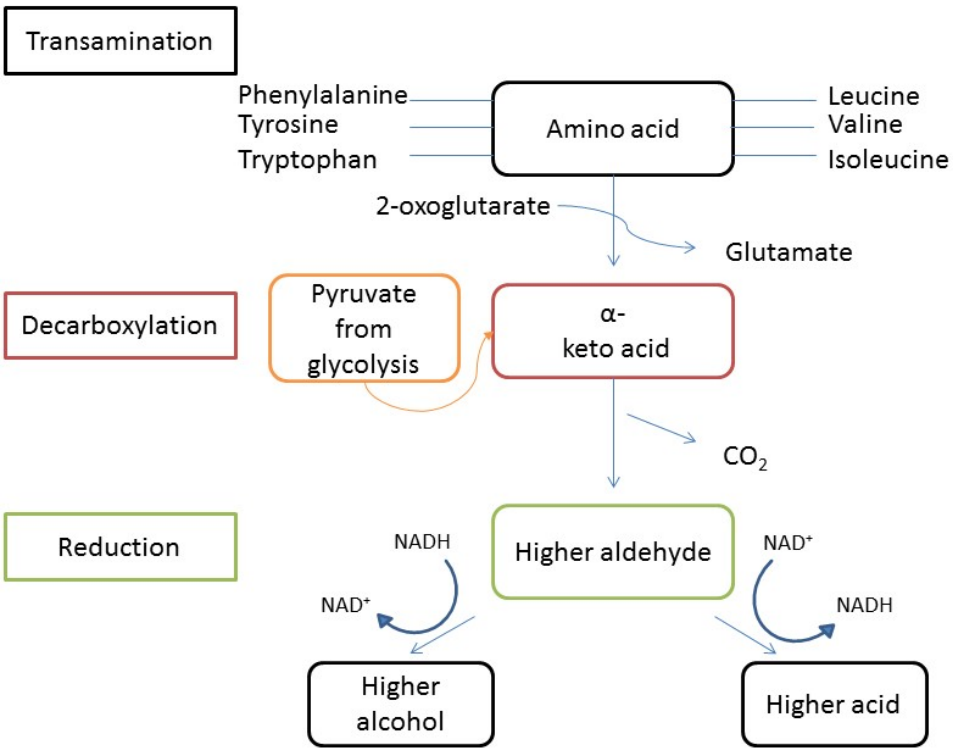

Figure. 2 Illustration of a simplified Ehrlich pathway (Adapted from (Hazelwood *et al*. 2008).

2.4.2. Overview of positive attributes of non-*Saccharomyces* yeasts in mixed culture with *S. cerevisiae*

A study carried out in synthetic grape must, comparing a variety of pure and mixed cultures, investigated the effect of yeast populations on the aroma profile. The authors found that isoamyl alcohol showed significantly lower levels in mixed cultures (*S. cerevisiae* and *H. uvarum*, and *S. cerevisiae* and *C. zemplinina*) compared to pure cultures (*S. cerevisiae*, *H. uvarum* and *C. zemplinina*) (Andorrà *et al*. 2012). This study also found that acetate esters were more affected by the presence of the non-*Saccharomyces* species (*H. uvarum*) as isoamyl acetate increased during mixed culture fermentations. This was also true for studies conducted on *Pichia* species in mixed fermentations with *S. cerevisiae* which revealed an increase in isoamyl acetate concentrations (Kurita 2008; Anfang *et al*. 2009). Another interesting finding from the former study was that 2-phenylethanol acetate, the only acetate ester that is highly produced by *S. cerevisiae*, was significantly reduced in the presence of non-*Saccharomyces* yeasts. This is in contrast to other studies where mixed fermentations with *Hanseniaspora osmophila* and *H. uvarum* positively increased the 2-phenylethanol acetate production (Ciani *et al*. 2006; Bely *et al*. 2008; Viana *et al*. 2009; Comitini *et al*. 2011; Moreira *et al*. 2011). Sequential fermentations carried out with *Candida stellata* (*Starmerella bacillaris*) and *S. cerevisiae* demonstrated an enhancement in the chemical profile of the wine with higher levels of glycerol and succinic acid and lower acetic acid levels (Ciani and Ferraro 1998; Ferraro *et al*. 2000). Another study conducted by Comitini *et al*. (2011), also found a reduction in the volatile acidity and increased glycerol production when *L. thermotolerans and S. cerevisiae* were used in mixed culture fermentations. These studies highlights the similarities and differences found amongst different species or sometimes even within species.

Indeed, differences between strains with regards to aroma compound production were also identified. For example, the yeast *T. delbrueckii*, one of only a few non-*Saccharomyces* yeast species currently commercialized, has been described as having a positive influence on the organoleptic quality of wines, because of its low production of compounds such as acetic acid, ethyl acetate, acetoin, acetaldehydes and hydrogen sulphide (Ciani and Ferraro 1998; Renault *et al*. 2009). Furthermore, when *T. delbrueckii* was used in mixed cultures with *S. cerevisiae* the production of terpenols, lactones and 2-phenylethanol increased (Herraiz and Reglero 1990; Comitini *et al*. 2011; Azzolini *et al*. 2012; Sadoudi *et al*. 2012). Results concerning ester production in sequential fermentations by *T. delbrueckii* and *S. cerevisiae* remain conflicting. Some studies showed that the total ester production of mixed fermentations was lower than that of pure culture *S. cerevisiae* with a significant reduction in acetate esters (Comitini *et al*. 2011; Sadoudi *et al*. 2012). In a recent paper, (Renault *et al*. 2015) showed that some esters produced by pure *T. delbrueckii* were different than those produced by *S. cerevisiae*, as ethyl propanoate, ethyl isobutanoate and ethyl dihydrocinnamate are specifically produced by *T.*

delbrueckii in higher concentrations than what is observed for *S. cerevisiae*. Conversely, (Herraiz and Reglero 1990) showed that mixed culture fermentation (*S. cerevisiae* and *T. delbrueckii*) allows an increase in the production of esters compared to pure cultures of *S. cerevisiae* and *T. delbrueckii*.

High alcohol concentrations negatively influence the complexity of wine sensory properties. Nonetheless, in another study, certain non-*Saccharomyces* yeasts (*T. delbrueckii* and *Zygosaccharomyces bailii*) showed a reduction in the production of ethanol in mixed cultures (Contreras *et al*. 2015). In brief, non-*Saccharomyces* yeasts produce positive aroma compounds, under desirable fermentation conditions (for example temperature and initial YAN concentrations).

2.5 Conclusion

Yeasts play a vital role in winemaking. The ideal for winemakers is to achieve the completion of fermentation with optimal aroma and complexity. Given the direct implication of nitrogen metabolism in achieving this goal, nutritional support for the yeast cells is of great importance. The intricacies and variability of natural grape must, however, make it difficult to ensure a problem free fermentation. The concentrations of sugars, nitrogen and micronutrients differ from vintage to vintage (Viana *et al*. 2014). Hence, understanding the nitrogen metabolism of different yeast species can assist the winemaker to select the most suitable supplementation for the yeast strains utilised. In order to optimize grape must to such an extent that sluggish or stuck fermentation become a rare event, future work should include studies on a cellular and molecular level using sequential fermentations to understand both non-*Saccharomyces* and *Saccharomyces* stains' nutrition requirements and preferences. Moreover, if a balance can be reached between nitrogen supplementation during sequential fermentations, wines with more complexity and favourable aromas can be produced.

Overall, the use of non-*Saccharomyces* yeasts in controlled mixed/sequential culture fermentations with *S. cerevisiae* can indeed create wines with more complexity and favourable aromas (Table 2.3). To further enhance the contribution of non-*Saccharomyces* yeasts with regards to aroma production, a better understanding of these yeasts' nitrogen requirements will be advantageous to ensure optimal growth and survival to the cells which will lead to an overall improvement of fermentation performance.

Table 2.3. Fermentation characteristics of non-*Saccharomyces* and *Saccharomyces cerevisiae* strains in mixed culture fermentations (Ciani and Comitini 2011; Jolly *et al*. 2014).

Non-*Saccharomyces* yeast species	**Distinguishing behaviour of pure culture**	**Effects of mixed fermentation production between *S. cerevisiae*, and pure *S. cerevisiae* culture**
Starmerella bombicola	High acetaldehyde producer High acetoin producer Low ethanol yield	No increase No increase Reduction in final [ethanol]
Lachancea thermotolerans	Low acetaldehyde producer Increased 2-phenylethanol (strain specific)	Reduction in final acetaldehyde formation Increase in titratable acidity
Hanseniaspora uvarum	High acetic acid producer High ethyl acetate producer	No increase in acetic acid production Slight increase in ethyl acetate production Increased production of higher alcohols, acetate ethyl esters and medium-chain fatty acids
Torulaspora delbrueckii	Low acetic acid producer Linalool producer (strain specific)	Reduction in acetic acid production
Hanseniaspora vineae and *Hanseniaspora guilliermondii*	High 2-phenyl-ethyl producer	Increase in 2-phenyl ethyl acetate
Pichia kluyveri	High producer of varietal thiols (especially 3-mercaptohexyl acetate)	Increase in thiols content
Debaryomyces variji	High levels of β-glucosidase activity	Increase in terpenols content
Metschnikowia pulcherrima	Terpenes and volatile thiol producer High ester producer	

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Chapter 3

Research results

Nitrogen utilisation of selected non-*Saccharomyces* yeasts and the impact on volatile compound production

3.1 Introduction

Wine results from the interactions of diverse microorganisms (fungi, yeasts, lactic acid bacteria and acetic acid bacteria) in grape juice, a chemically complex and non-sterile matrix (Fleet 2003). Grape sugars are converted to ethanol, carbon dioxide and hundreds of other secondary compounds, mediated by yeasts species amongst which *Saccharomyces cerevisiae* plays a dominant role (Jolly *et al*. 2006; Ciani *et al*. 2010). The uptake and catabolism of nitrogenous compounds also contributes to the production of secondary metabolites of critical oenological relevance. Through the production of these numerous secondary compounds, yeasts have a major impact on wine flavour and quality. In oenology, these yeasts are traditionally separated into two groups: *Saccharomyces* spp. (largely dominated by *S. cerevisiae*) and the non-*Saccharomyces* yeasts. For a long time, the latter were often considered as spoilage yeasts due to the fact that they were frequently isolated from stuck or sluggish fermentations (Ciani *et al*. 2010). Others were often regarded as of negligible interest because of their poor fermentation abilities or poor adaptation to the environmental conditions (e.g. increasing amount of ethanol, absence of oxygen, low pH) governing grape juice fermentation. However, recent studies corrected this misperception and reported that the presence of certain non-*Saccharomyces* yeasts can contribute positively to overall sensory characteristics of wine (Medina *et al*. 2012), especially when inoculated at a high cell density.

In spontaneous fermentations, non-*Saccharomyces* yeasts are thought to be active in the beginning of alcoholic fermentation, when the ethanol concentration is still low. Indeed, according to literature, most non-*Saccharomyces* yeasts are not as well adapted as *S. cerevisiae* to withstand the ethanol concentrations up to the end of alcoholic fermentations and are progressively outcompeted by species such as *S. cerevisiae* which are more ethanol tolerant (Taillandier *et al*. 2014). This long-standing belief is however being challenged by a few fairly recent studies which have shown that the lack of oxygen plays a stronger role in the decline of non-*Saccharomyces* than the increasing ethanol concentration (Holm Hansen *et al*. 2001; Nissen *et al*. 2004). Regardless of the reasons pertaining to their decline, non-*Saccharomyces* yeasts cannot on their own complete alcoholic fermentation under typical winemaking conditions. Therefore, in order to increase diversity in wines and achieve enhanced aroma and flavour complexity, research is being conducted on co-inoculations of non-*Saccharomyces* yeasts in association with *S. cerevisiae*. Several reports indeed demonstrate that this strategy allows non-*Saccharomyces* yeasts to positively impact on the final wine composition while ensuring the smooth completion of fermentation (Jolly *et al*. 2014).

Grapes comprise a selection of nitrogenous compounds of which primary or alpha amino acids, ammonium ion and small peptides are key components (Ugliano *et al*. 2007). These can be assimilated by the yeasts to sustain biomass production and ensure cellular functioning and maintenance. For this reason, these compounds all together are referred to as Yeast

Assimilable Nitrogen (YAN). Moreover, it has been observed in previous studies that nitrogen compounds, specifically amino acids, have a major impact on the final concentration of both primary and secondary flavour and aroma compounds in wine (Albers *et al*. 1996; Miller *et al*. 2007). Nitrogen is a key macronutrient that plays a significant part in the growth of yeasts, which is used by the cell for the synthesis of proteins, enzymes and nucleic acids (Henschke and Jiranek 1993). Amino acids are an efficient nitrogen source due to the fact that the direct uptake of amino acids from the external environment reduces the energy required for amino acid biosynthesis (Godard *et al*. 2007). Although all amino acids are required by the cell for the biosynthesis of nitrogenous compounds, not all amino acids are consistently suitable for supporting optimal yeast growth and fermentative activity (Cooper 1982; Barbosa *et al*. 2012; Ljungdahl and Daignan-Fornier 2012). Indeed, *S. cerevisiae* cells have the ability to use efficient or so-called “good” nitrogen sources preferentially to those that are less efficient or “poor” (Cooper 1982; Barbosa *et al*. 2012). This selectivity is accomplished through the nitrogen catabolite repression mechanism (NCR), which represses and activates the transcription of catabolic genes depending on the nitrogen requirements of the cell and availability of nitrogen sources (Magasanik and Kaiser 2002), thus inhibiting the genes required for the uptake and degradation of poor nitrogen sources when cells are provided with good ones (Beltran *et al*. 2004). Previous research has shown that *S. cerevisiae*’s most preferred nitrogen sources include ammonium, glutamine, glutamate, alanine, asparagine, aspartate and serine while the other nitrogen sources are categorized into different groups of inefficiency (Ljungdahl and Daignan-Fornier 2012; Crépin *et al*. 2014).

Although previous studies have been conducted on the impact of nitrogen on aroma character of wine and possible solutions by the addition of either di-ammonium phosphate (DAP) or mixtures of amino acids to nitrogen-deficient grape musts, only a few comparative studies have investigated the impact of different initial nitrogen sources (Barbosa *et al*. 2012). Such a study suggested that adjustment of the initial nitrogen concentration could direct the production of specific metabolites thereby producing wines with particular flavour profiles (Barbosa *et al*. 2012). Fermentation-derived volatile aroma compounds (i.e. volatile fatty acids, higher alcohols and their corresponding esters) and their respective metabolic pathways have been studied broadly (Lambrechts and Pretorius 2000; Swiegers and Pretorius 2005; Styger *et al*. 2011). Their production occurs catabolically (Ehrlich pathway) or anabolically (*de novo* formation) (Smit 2013). Because of the various interconnected metabolic pathways, regulation occurs on a genetic level and volatile compounds production is consequently strain dependent in *S. cerevisiae* (Lambrechts and Pretorius 2000; Lilly *et al*. 2006; Rossouw *et al*. 2008; Styger *et al*. 2011). Nevertheless, because of the previous absence of interest for non-*Saccharomyces* yeasts, these pathways and their regulation mechanisms have all been studied in *S. cerevisiae* and very little information is available for the former yeasts.

This study evaluated the uptake of nitrogen sources (amino acids and ammonium) by selected non-*Saccharomyces* yeast strains and the relevance of these findings on fermentation performance and aroma compounds in comparison with *S. cerevisiae*. To our knowledge, this study is one of very few that investigated the utilisation of amino acids by non-*Saccharomyces* yeast strains. Furthermore, the impact of the assimilation of nitrogen sources by non-*Saccharomyces* yeasts on general fermentation performance was also investigated in a sequential inoculation scenario. This study also investigated the aroma production by non-*Saccharomyces* yeasts in combination with *S. cerevisiae* under different initial nitrogen concentrations.

3.2 Materials and methods

3.2.1 Yeast species used in this study

The wine yeast strains used in this study are listed in Table 1. The commercial strain of *S. cerevisiae*, Lalvin® EC1118 (Lallemand, Blagnac, France), was used in all fermentations as a control in pure culture fermentations and in sequential fermentations in combination with the following non-*Saccharomyces* yeast species: *Pichia kluyveri* Viniflora® FootZen™ (Chr. Hansen, Hørsholm, Denmark), *Torulaspora delbrueckii* Biodiva™ TD291 (Lallemand), *Metschnikowia pulcherrima* Flavia® MP346 (Lallemand) as well as *L. thermotolerans* Y1240 and *M. pulcherrima* Y1123 that originate from the culture collection of the Institute for Wine Biotechnology (Stellenbosch, South Africa).

Table 1 Yeast strains used during this study

Yeast species	Strain/Commercial name	Description	Collection/Manufacturer
Saccharomyces cerevisiae	Lalvin® EC1118	Isolated from Champagne (France)	Lallemand[a]
Lachancea thermotolerans	Y1240	Isolated from Muscat d`Alexandrie (Jason's Hill, Rawsonville, South Africa, 2009)	IWBT[b]
Torulaspora delbrueckii	Biodiva™ TD291	Isolated from white wine (North America).	Lallemand[a]
Pichia kluyveri	Viniflora® FootZen™	Isolated from a spontaneously fermenting Chardonnay must (Auckland University, New Zealand)	Chr. Hansen[c]
Metschnikowia pulcherrima	Y1123	Isolated from Sauvignon blanc juice (Stonewall, Somerset West, 2009)	IWBT[b]
Metschnikowia pulcherrima	Flavia® MP346	Isolated and selected by the Universitad de Santiago de Chile	Lallemand[a]

[a]Lallemand: Lallemand SAS (Blagnac, France)
[b]IWBT: Institute for Wine Biotechnology (Stellenbosch, South Africa)
[c]Chr Hansen: Chr Hansen A/S Bøge Allé (Hørsholm, Denmark)

3.2.2 Pre-culture and Fermentation conditions

Pure culture as well as sequential culture fermentations were all performed in triplicate (Table 2). The pre-culture approach for all the fermentations, pure and sequential, performed in this project started by inoculating a single colony of the various yeast cultures into 50 mL Yeast Nitrogen Base (YNB) medium (20 g/L glucose, 1.7 g/L YNB base without amino acids and ammonium (Difco Laboratories), 1 g/L ammonium sulphate) for 24 h at 30°C. Thereafter, the cells were harvested after the cultures reached a concentration of 10^6 CFU/mL, OD_{600nm} of 0.1 and inoculated into 100 mL of 50% synthetic grape juice-like medium (Table 3) for 12 – 15 h at 30°C. When cultures reached an OD_{600nm} of approximately 1-2, cells were harvested and inoculated at an OD_{600nm} of 0.1 into 200 mL 100 % synthetic grape juice-like medium. The composition of the synthetic medium was adapted from (Bely *et al*. 1990; Henschke and Jiranek 1993) as described in Tables 3 and 4. The pH of the synthetic medium was adjusted to 3.3 with potassium hydroxide (Saarchem, Krugersdorp, South Africa). The trace elements, vitamins, nitrogen sources and anaerobic factors were filtered through a 0.22-µm syringe filter (Starlab Scientific, Cape Town, South Africa) and added into the autoclaved synthetic medium. The small scale fermentations were performed in 250-mL Erlenmeyer flasks with stoppers and fermentation caps. All fermentations were carried out at 25°C. Furthermore, for the sequential fermentations, three different nitrogen concentrations were applied: 300 mg/L, 150 mg/L and 75 mg/L (using the same ratios between the different nitrogen sources).

Table 2 Fermentation combinations

Fermentation type	Species combination	Abbreviation	CFU/mL
Pure cultures	*Saccharomyces cerevisiae* (Control)	Sc	2 x 10^6
	Lachancea thermotolerans	Lt	
	Torulaspora delbrueckii	Td	
	Pichia kluyveri	Pk	
	Metschnikowia pulcherrima (Y1123)	MpY	
	Metschnikowia pulcherrima (Flavia)	MpF	
Sequential cultures[a]	*Saccharomyces cerevisiae* (Control)	Sc	1 x 10^6 of each
	Lachancea thermotolerans + *Saccharomyces cerevisiae*	LtSc	
	Torulaspora delbrueckii + *Saccharomyces cerevisiae*	TdSc	
	Pichia kluyveri + *Saccharomyces cerevisiae*	PkSc	
	Metschnikowia pulcherrima + *Saccharomyces cerevisiae*	MpYSc	
	Metschnikowia pulcherrima + *Saccharomyces cerevisiae*	MpFSc	

[a] *S. cerevisiae* was inoculated 48 h after the non-*Saccharomyces* species

Table 3 Chemically Defined Grape Juice-like Medium formerly described by Henschke and Jiranek (1993) including carbon sources, acids, salts, trace elements, vitamins and lipid sources. The amounts are given for 1 L of medium. Ammonium chloride concentration is given for a total YAN concentration of 300 mg/L. For fermentations using 150 and 75 mg/L YAN, the concentration was adjusted accordingly.

		100% AWRI per litre
Carbon sources	Glucose	*115 g
	Fructose	*115 g
Acids	Potassium L-Tartrate	2.5 g
	L-Malic acid	3.0 g
	Citric acid	0.2 g
Salts	Potassium hydrogen phosphate (K_2HPO_4)	1.14 g
	Magnesium sulphate heptahydrate ($MgSO_4.7 H_20$)	0.44 g
	Calsium chloride dehydrate ($CaCl_2.2 H_2O$)	1.23 g
Trace elements (Made in 1L stock solution)	Manganese(II) chloride tetrahydrate ($MnCl_2.4 H_2O$)	200 µg
	Zink chloride ($ZnCl_2$)	135 µg
	Iron(II) chloride ($FeCl_2$)	30 µg
	Copper(II) chloride ($CuCl_2$)	15 µg
	Boric acid (H_3BO_3)	5 µg
	Cobalt(II) nitrate hexahydrate ($Co(NO_3)_2.6 H_2O$)	30 µg
	Sodium molybdate dehydrate ($NaMoO_4.2 H_2O$)	25 µg
	Potassium iodate (KIO_3)	10 µg
Vitamins (Made in 1L stock solution)	Myo-inositol	100 mg
	Pyridoxine hydrochloride	2 mg
	Nicotinic acid	2 mg
	Calcium pantothenate	1 mg
	Thiamin hydrocloride	0.5 mg
	PABA.K	0.2 mg
	Riboflavin	0.2 mg
	Biotin	0.125 mg
	Folic acid	0.2 mg
Anaerobic factors	Ergosterol	10 mg
	Tween 80	0.5 mL
Nitrogen sources	Ammonium chloride	0.46 g
	Amino acids **(Table 4)**	

*Modified value

Table 4 Nitrogen supplementation as described by Bely *et al*. (1990). The concentrations are given for a total YAN concentration of 300 mg/L. For fermentations using 150 and 75 mg/L YAN, the concentrations were adjusted accordingly.

Nitrogen sources (prepared in 100X stock solution)	Amino acids	Amount per litre
	Tyrosine	18.326 mg
	Tryptophane	179.333 mg
	Isoleucine	32.725 mg
	Aspartic acid	44.506 mg
	Glutamic acid	120.428 mg
	Arginine	374.374 mg
	Leucine	48.433 mg
	Threonine	75.922 mg
	Glycine	18.326 mg
	Glutamine	505.274 mg
	Alanine	145.299 mg
	Valine	44.506 mg
	Methionine	31.416 mg
	Phenylalanine	37.961 mg
	Serine	78.54 mg
	Histidine	32.725 mg
	Lysine	17.017 mg
	Cysteine	13.09 mg
	Proline	612.612 mg

3.2.3 Sampling and assessing of fermentation kinetics

All pure and sequential fermentations were sampled (5 mL) in triplicate every 24 h for the first 3 days and then every second day until the end of fermentation (i.e. sugar concentration lower than 2 g/L or not decreasing anymore). Thereafter, the samples were spun at 5000 rpm for 5 min, after which the supernatants were filtered through a 0.22-µm syringe filter (Starlab Scientific, Cape Town, South Africa) and stored at -20°C for further chemical analysis. The yeast cell populations were monitored by plating onto Wallerstein Laboratory (WL) nutrient agar (Fluka Analytical, Sigma Aldrich, Aston Manor, South Africa) during pure and sequential fermentations. Plates were incubated at 30°C, generally for 3-4 days, until colonies were formed. The accumulated weight losses were also monitored. For the residual glucose, fructose, ammonia and alpha amino nitrogen concentrations, 1.5 mL of filtered sample was enzymatically analysed using the Arena 20XT (Thermo Fisher Scientific, Waltham, MA) which makes use of automated spectrophotometric readings to determine the concentrations of the various compounds. The different enzymatic assay kits are listed in Table 5.

Table 5 Summary of the methods used during enzymatic assay

D-Glucose	
Kit used	• EnzytecTM Fluid D-Glucose Id-No: 5140 Manufacturer: Thermo Fisher Scientific. Distributed by: R-Biopharm AG, Germany
D-Fructose	
Kit used	• EnzytecTM Fluid D-Fructose Id-No: 5120 Manufacturer: Thermo Fisher Scientific Distributed by: R-Biopharm AG
Ammonia	
Kit used	• EnzytecTM Fluid Ammonia Id-No: 5390 Manufacturer: Thermo Fisher Scientific Distributed by: R-Biopharm AG
Alpha amino nitrogen	
Kit used	Megazyme Primary Amino Nitrogen (PAN) Code: K-PANOPA 07/12 Manufacturer: Megazyme, Bray, Ireland. Distributed by: Megazyme

3.2.4 Analysis of Major volatile compound via Gas Chromatographic

The quantification of 32 major volatiles (Table 6) was carried out by gas chromatography equipped with a flame ionization detector (GC-FID) using the Agilent GC System HP 6890 Series (Agilent, Little Falls, Wilmington, USA) as described by (Louw *et al*. 2009) with minor modifications. Five millilitres of each of the filtered samples were used with 100 µL of 4-methyl-2-pentanol (internal standard). Diethyl ether (1 mL) was added to the mixture were it was then placed in an ultrasonic bath for 5 min to extract the volatile compounds. Thereafter, the samples were centrifuged at 4000 rpm for 3 min. Sodium sulphate was added to remove any water from the non-polar layer. The samples that were analysed originated from sequential fermentations end points.

Table 6 Volatile compounds analysed in this study

Acetate esters	
Ethyl acetate	Hexyl acetate
Isoamyl acetate	2-Phenylethyl acetate
Volatile fatty acids	
Iso-butyric acid	Butyric acid
Iso-valeric acid	Valeric acid
Hexanoic acid	Octanoic acid
Decanoic acid	Propionic acid
Ethyl esters	
Ethyl hexanoate	Diethyl succinate
Ethyl caprate	Ethyl caprylate
Ethyl lactate	Ethyl butyrate
Ethyl phenylacetate	Ethyl-3-hydroxybutanoate
Higher alcohols	
2-Phenylethanol	Hexanol
Methanol	Propanol
Isobutanol	Butanol
Isoamyl alcohol	Pentanol
3-Ethoxy-1-propanol	
Carbonyl compound	
Acetoin	

3.2.5 Quantification of individual amino acids via HPLC

Amino acids quantification was performed by high performance liquid chromatography (HPLC), Agilent 1100 (Agilent Technologies, Waldbronn, Germany) by pre-column derivatization and fluorescence detection based upon a method previously described (Henderson and Brooks 2010) with some modifications to the derivatisation and injection. A Zorbax Eclipse plus C18 Rapid Resolution column (4.6 x 150 mm, 3.5 µm particle size; Agilent Technologies) was used following derivatisation of the amino acids. Derivatisation was performed using three different reagents: iodoacetic acid (Sigma Aldrich) for cysteine, o-phthaldialdehyde (OPA, Sigma Aldrich) for primary amino acids and fluorenylmethyloxycarbonyl chloride (Sigma Aldrich) for secondary amino acids. Internal standards, norvaline (Sigma Aldrich) and sarcosine (Sigma Aldrich) were spiked to each sample prior to derivatisation. One millilitre of each filtered sample was used for time point 0 h, 24 h, 48 h and end point. These samples were taken from pure culture fermentations.

3.2.6 Statistical analysis

Principal component analysis (PCA) was performed using METABOANALYST software 3.0, a Web-based platform for the analysis of quantitative metabolomics data (Xia and Wishart 2011), to illustrate the distribution of the fermentation cultures based on different initial yeast assimilable nitrogen and aroma compounds.

3.2.7 Killer activity screening

Yeast strains tested for killer activity and sensitivity were *Saccharomyces cerevisiae* EC1118 and *Pichia kluyveri* FrootZen (Table 1), respectively. Pre-cultures of these yeasts were grown in 5 mL YPD medium (Biolab-Merck) at 30ºC on a test tube rotator for 24 h with agitation. Five microlitres of the pre-culture were inoculated into 5 mL YPD medium and incubated at 30ºC overnight again on a test tube rotator with agitation. Killer activity screening was performed using the seeded agar method on synthetic grape must (MS300). The medium were adjusted to pH 3.3 with potassium hydroxide (Saarchem, Krugersdorp, South Africa). The yeast strain *S. cerevisiae* was inoculated as potentially sensitive cells at concentration of 10^6 cfu/mL in 7.5 mL of the pH adjusted media. Bacteriological agar, 25 mL, was kept at 50ºC and was mixed with the inoculated medium to a final volume of 10 mL and after brief vortexing, the medium was poured into sterile Petri dishes. Five microlitres of the killer yeast strain, *P. kluyveri*, were spotted on the surface of the solidified agar plate. The plates were incubated at 25ºC for 2-3 days. Killer assays were performed in triplicate.

3.3 Results

3.3.1 Impact of initial Yeast Assimilable Nitrogen concentration on the fermentation dynamics of single and mixed yeast cultures and on the uptake of amino acids by selected yeast species.

3.3.1.1 Fermentation kinetics, yeast population dynamics and nitrogen consumption in pure culture fermentations

Synthetic grape must containing 300 mg/L yeast assimilable nitrogen was inoculated with pure cultures of different yeast species in order to compare their pattern of nitrogen consumption with that of *S. cerevisiae*.

Fermentation kinetics

The pure culture of *S. cerevisiae*, which served as a reference, fermented to dryness (i.e less than 2 g/L of residual sugars) in 11 days (Fig. 1A). In contrast, none of the non-*Saccharomyces* yeasts was able to ferment to dryness under the conditions tested (Fig. 1A, B). Indeed, the fermentations stopped with residual sugar concentrations of 44 g/L, 80 g/L, 164 g/L, 170 g/L and 150 g/L for *L. thermotolerans*, *T. delbrueckii*, *M.pulcherrima* IWBT Y1123, *M.pulcherrima* Flavia and *P. kluyveri*, respectively.

The sugar consumption pattern indicated that all the above mentioned non-*Saccharomyces* yeasts were glucophilic. Indeed, glucose was consumed faster and in greater amounts than fructose. In the first few days of fermentation, *L. thermotolerans* and *T. delbrueckii* displayed a similar consumption rate of total sugars as *S. cerevisiae*, whereas *M. pulcherrima* IWBT Y1123, *M. pulcherrima* Flavia and *P. kluyveri* differed significantly. Regardless of the non-*Saccharomyces* yeast, the consumption rate dropped more rapidly than for *S. cerevisiae* and the uptake of sugars ceased prematurely after 60 h for *M. pulcherrima* IWBT 1123, *M. pulcherrima* Flavia, *P. kluyveri* and after 264 hours for *L. thermotolerans* and *T. delbrueckii*.

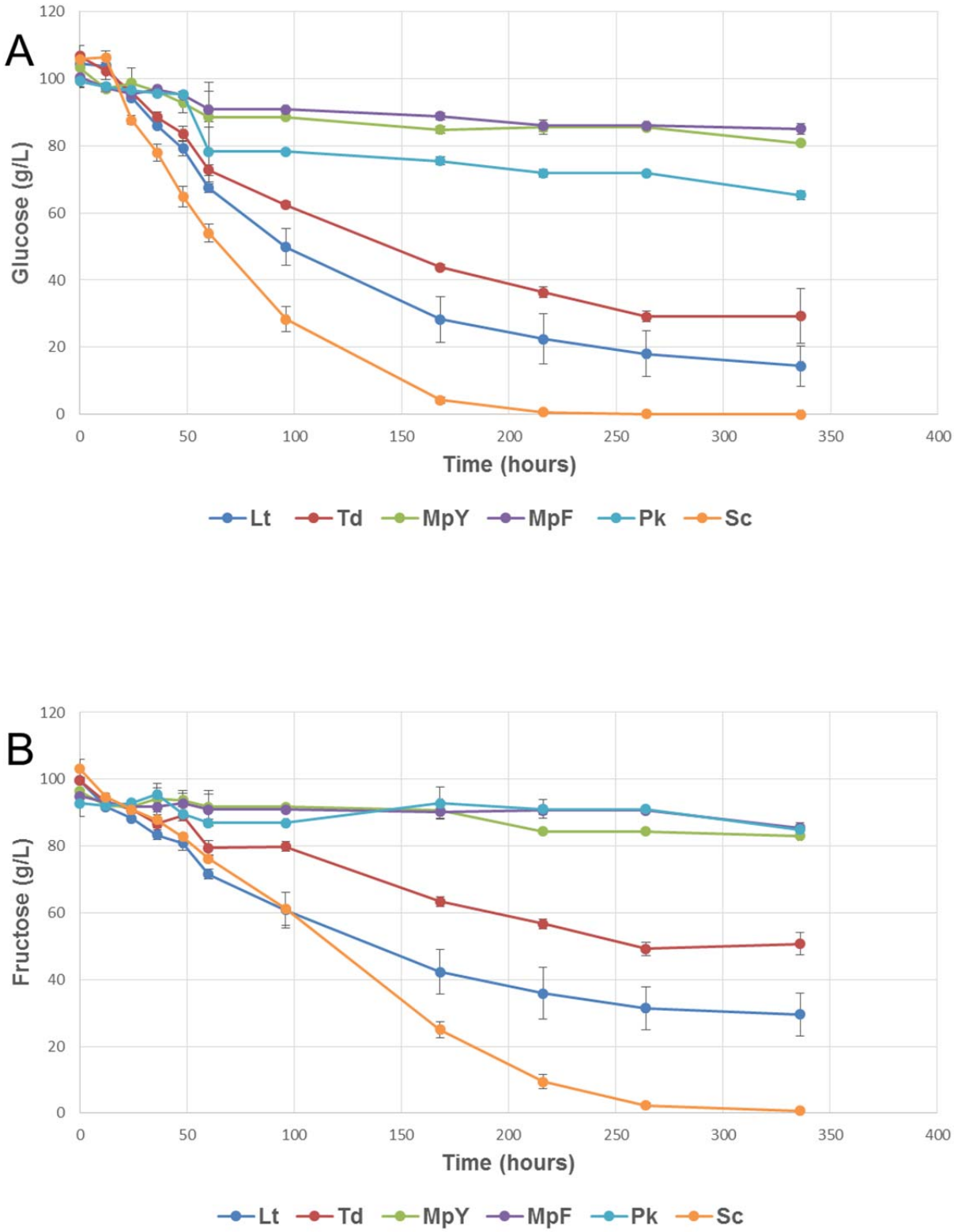


Figure. 1 Glucose (A) and fructose (B) consumption throughout fermentation. Lt: *L. thermotolerans*; Td: *T. delbrueckii*; MpY: *M. pulcherrima* IWBT Y1123; MpF: *M. pulcherrima* Flavia; Sc: *S. cerevisiae*.

Population dynamics

Of all strains, *M. pulcherrima* IWBT1123 and Flavia had (reaching a maximum of 1 x 10^7 cfu/mL, respectively) the lowest cell counts throughout the fermentation and started to decline sharply at 96 h of the fermentation (Fig. 2). Conversely, *T. delbrueckii*, *L. thermotolerans* and *S. cerevisiae* reached the highest cell density (reaching a maximum of about 4 x 10^7, 4 x 10^7 and 3 x 10^7 cfu/mL, repectively). *T. delbrueckii* and *L. thermotolerans* populations started to decline after 96 h and 168 h (they persisted at a cell count of about 1 x 10^6 cfu/mL), respectively, but not as sharply as *M. pulcherrima*. This coincides with the time at which sugar consumption ceased for *M. pulcherrima*, but for species *T. delbrueckii* and *L. thermotolerans* sugar consumption only ceased a few days after the population started dropping. *S. cerevisiae* cell count however only started to decline after 216 h of fermentation when alcoholic fermentation was almost completed. *P. kluyveri* reached a cell count of approximately 2 x 10^7 cfu/mL and started to decline at 168 h of fermentation, whereafter the cell count was stable at 1 x 10^7 cfu/mL until the end of fermentation.

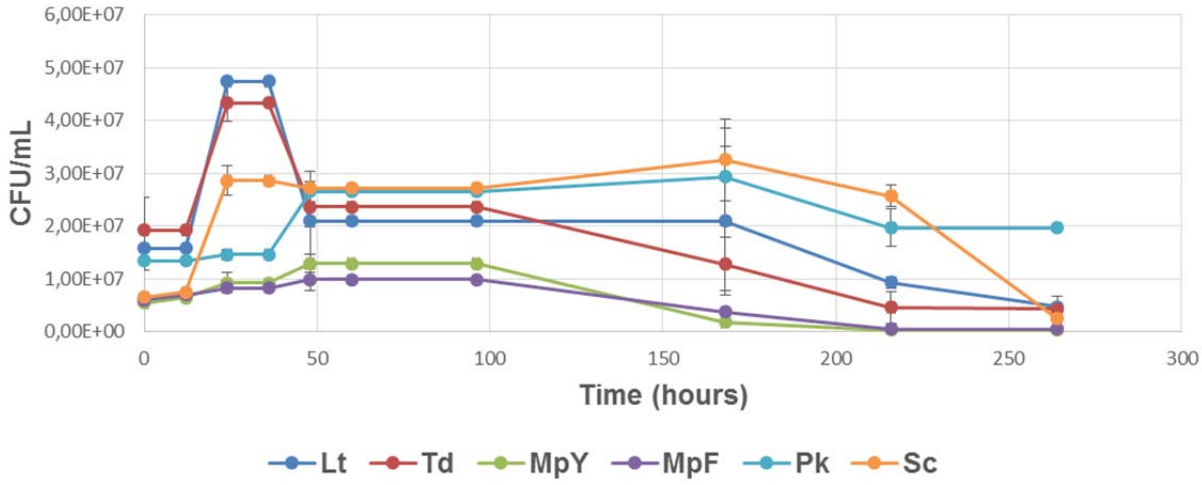


Figure. 2 Cell count throughout fermentation. Lt: *L. thermotolerans*; Td: *T. delbrueckii*; MpY: *M. pulcherrima* IWBT Y1123; MpF: *M. pulcherrima* Flavia; Sc: *S. cerevisiae*.

Nitrogen consumption

Most ammonia and alpha amino nitrogen consumption occurred within the first 60 h for all of the yeast species studied (Fig. 3A, B). At that time, *S. cerevisiae* had consumed more ammonia and alpha amino nitrogen (total YAN of 118 mg/L) than the other yeasts. *T. delbrueckii* and *L. thermotolerans* followed similar consumption patterns as *S. cerevisiae* for nitrogen compounds overall with a total YAN consumption of 77 mg/L and 65 mg/L, respectively, but ammonia appears preferred over alpha amino nitrogen by *T. delbrueckii* (41 mg/L and 35 mg/L, respectively), whereas *L. thermotolerans* (45 mg/L and 20 mg/L, respectively) clearly preferred alpha amino nitrogen over ammonia. *M. pulcherrima* IWBT1123, *M. pulcherrima* Flavia and *P. kluyveri* did not consume much ammonia and alpha amino nitrogen. This could be correlated to

their very low consumption of sugars as well their lower population throughout fermentation (Fig. 2).

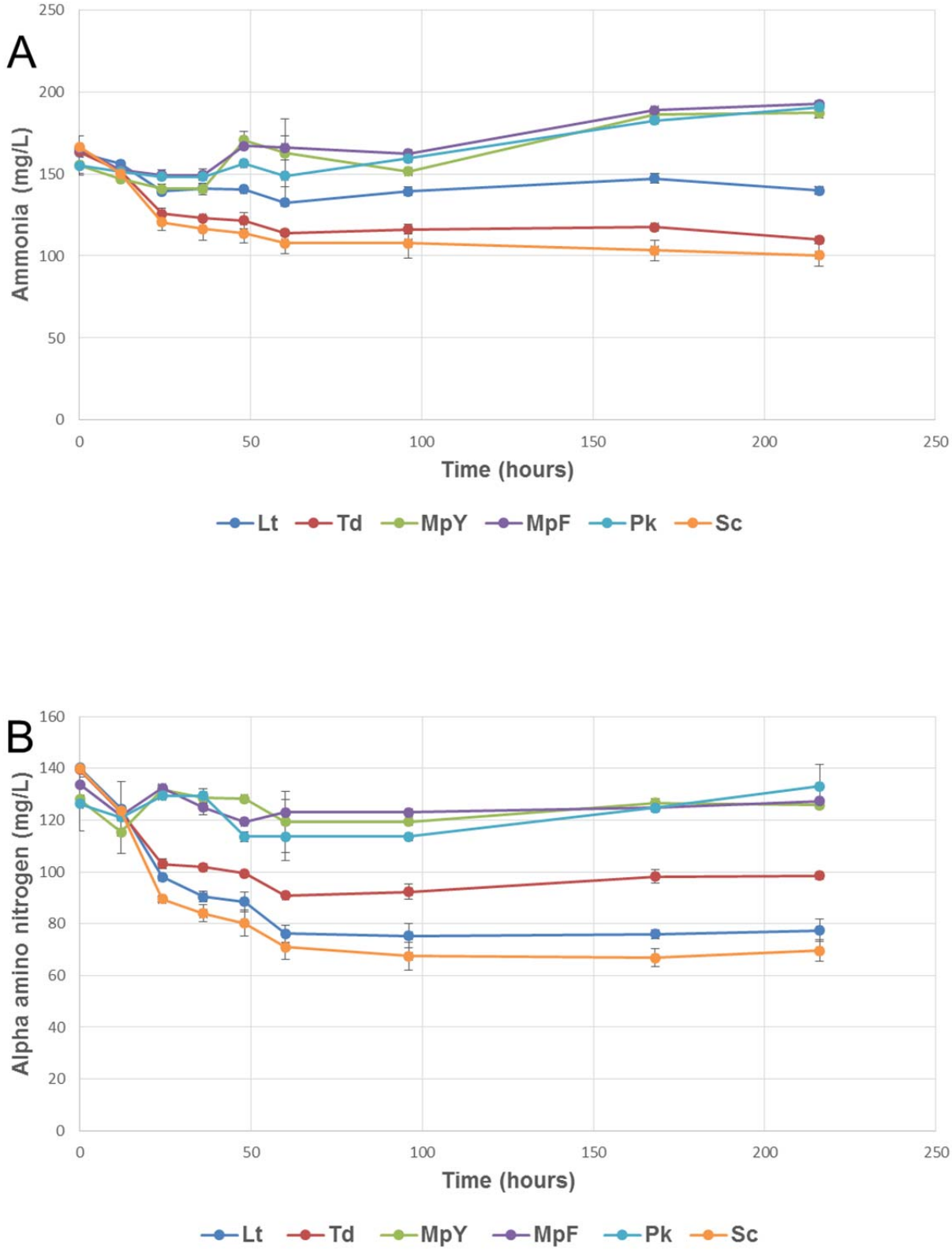


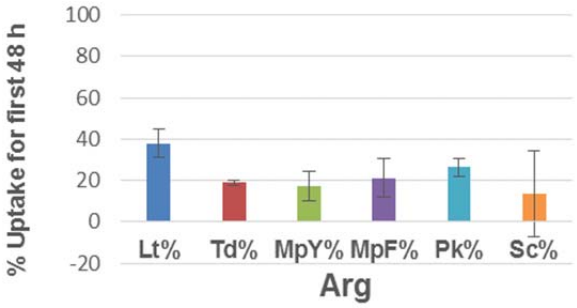
Figure. 3 (A) Ammonia and (B) Alpha amino nitrogen consumption throughout the fermentation

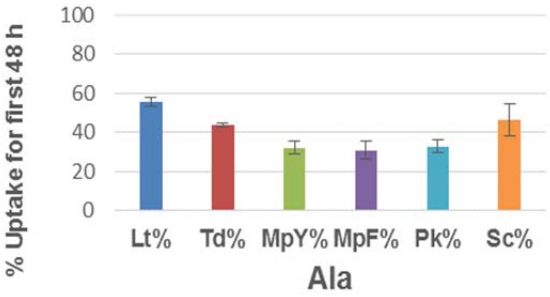
3.3.1.2 Uptake of amino acids by selected non-*Saccharomyces* yeasts under fermentative conditions

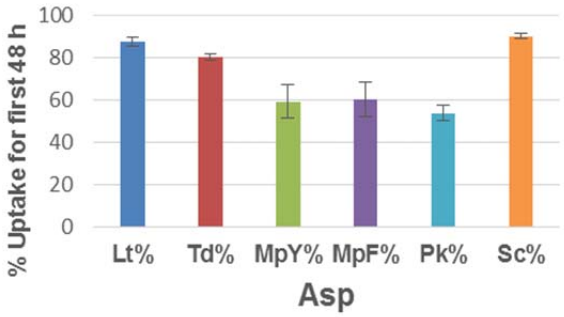
The percentage uptake during the first 48 h of single amino acids are presented in Fig. 4. *S. cerevisiae*, assimilated amino acids to varying degrees, with Leu, Ile and Asp being taken up above 90%, whereas Glu, Ser, Val and Met were consumed to around 70% - 80%. Other yeast showed significantly different uptake preferences. In *L. thermotolerans*, Leu and Phe were taken up above 90%, whereas Asp, Glu, Val, Met and Ile were assimilated between 70% - 80%. His and Gly were not taken up by the different yeasts tested in our study, with the exception of *L. thermotolerans* and *S. cerevisiae*, but even then, very low amounts were assimilated, between 7% - 37%. The two *M. pulcherrima* strains and *P. kluyveri*, assimilated the least amount of single amino acids compared to *S. cerevisiae*, which correlates with the results shown in Fig. 3. The uptake by *L. thermotolerans* was close to that of *S. cerevisiae* for single amino acids Asp, Glu, Gln, Tyr, Leu and His.

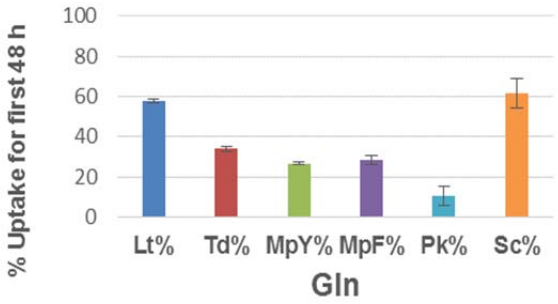
Figure 4 clearly shows differences in the percentage uptake between species for each amino acid analysed. For amino acids Asp, Glu, Ser, Gln, His, Thr, Val and Trp, a general trend for the percentage uptake was observed where *S. cerevisiae* took up the most followed by *L. thermotolerans*, *T. delbrueckii*, *M. pulcherrima* Y1123, *M. pulcherrima* Flavia and *P. kluyveri.* The percentage uptake for Glu, Tyr and Pro showed the least variation between species. Indeed, they differed by less than 20% between the highest and lowest uptake. Nevertheless, the percentage uptakes of Gln, Ile, Phe and Ser differed more than 50% between the strain that took up the most and least of this specific amino acid. For example, *S. cerevisiae* took up about 6-fold more of Gln and 3-fold more Ser as *P. kluyveri*, whereas the former took up 4-fold more Ile as *M. pulcherrima* Flavia. Moreover, *L. thermotolerans* took up about 3-fold more Phe than *M. pulcherrima* Flavia. The negative values observed for single amino acids His and Gly by different species were probably because this values were below detection level on the HPLC.

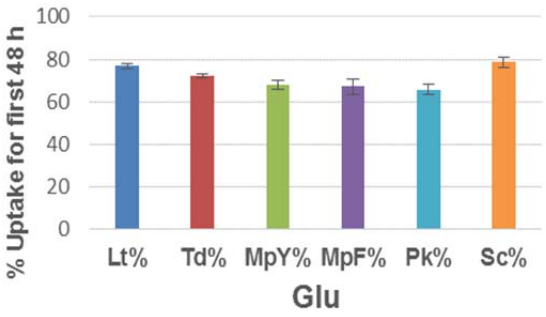
The concentrations of single amino acids were also monitored over time (24 h, 48 h and end point) (Supplementary Table 1). It confirmed the results shown in Fig. 3. Indeed, the amino acids were assimilated during the first 48 h. Furthermore, there was barely any difference in the amino acid uptake when comparing 24h and 48h. At end point, an increase was observed for most of the single amino acids by all yeasts species. This increase is probably due to yeasts autolysis. This trend also correlated to the total alpha amino nitrogen consumption during pure culture fermentation (Fig. 3).

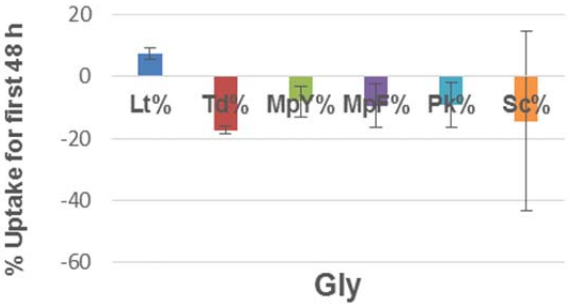


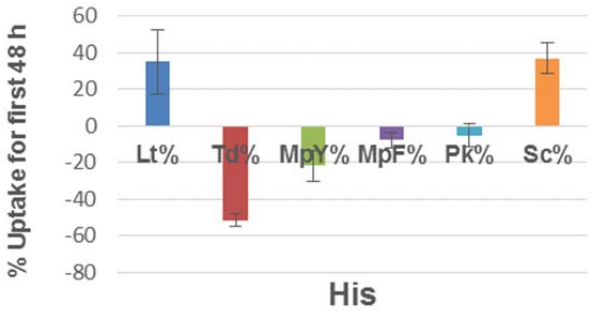


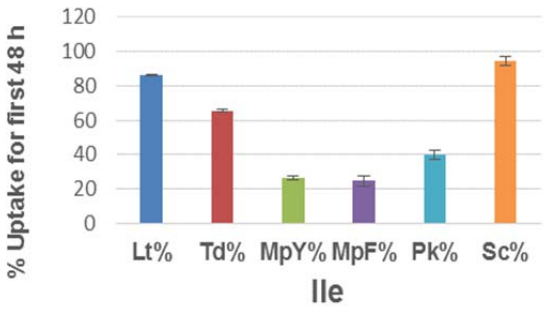


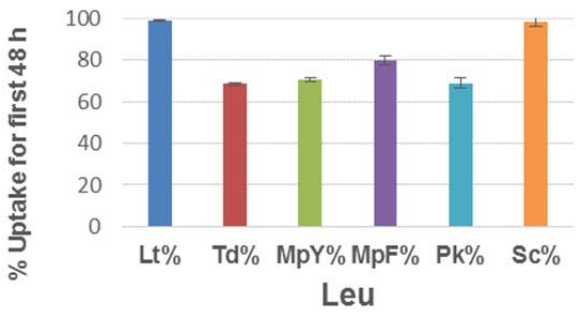


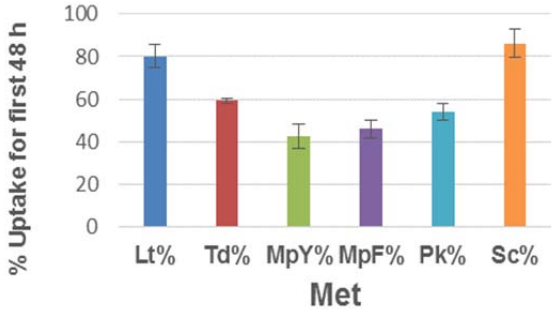


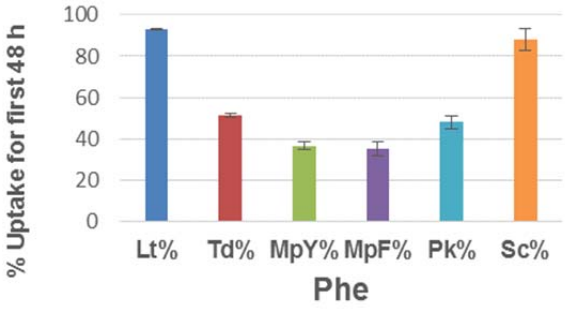


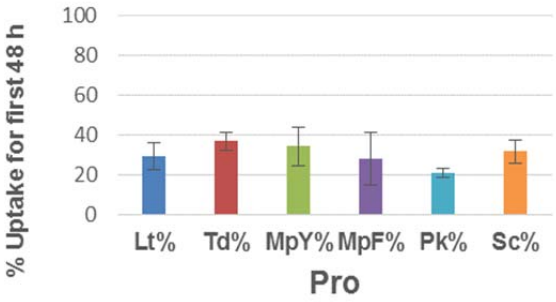


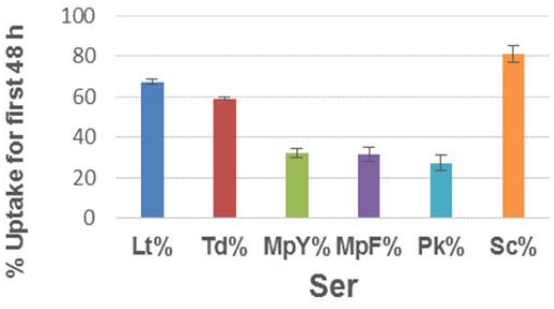


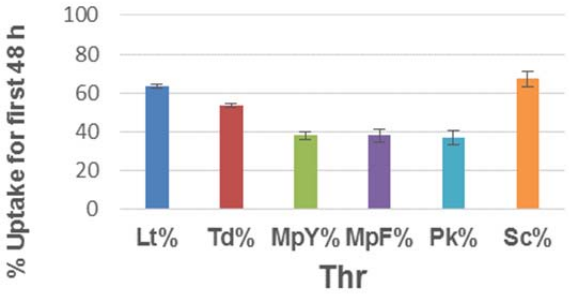


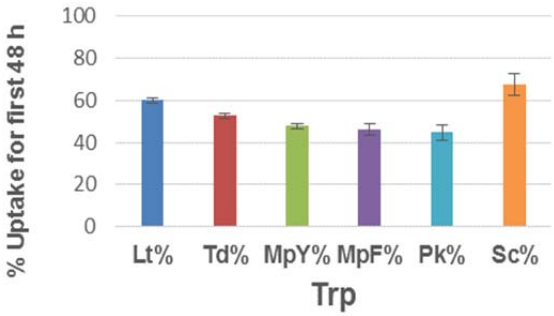


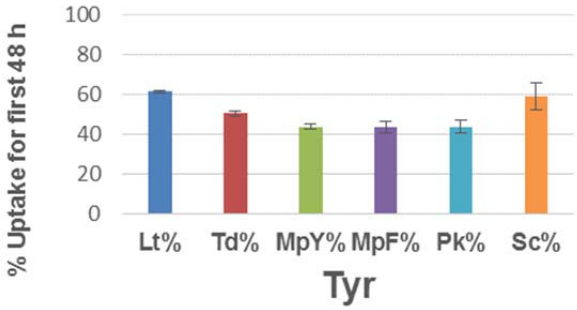












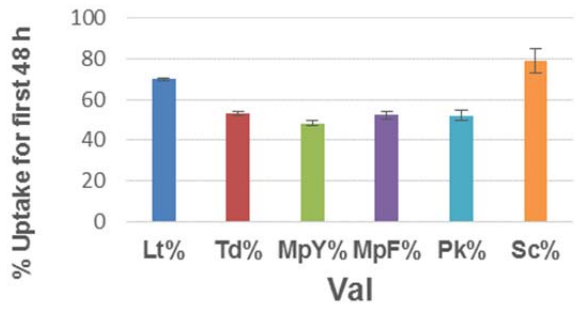


Figure. 4 Single amino acid uptake (%) during pure culture fermentation for the first 48 h by Lt: *L. thermotolerans*; Td: *T. delbrueckii*; MpY: *M. pulcherrima* IWBT Y1123; MpF: *M. pulcherrima* Flavia; Pk: *P. kluyveri*; Sc: *S. cerevisiae* for amino acids Arg: arginine; Ala: alanine; Asp: aspartic acid; Gln: glutamine; Glu; glutamic acid; Gly: glycine; His: histidine; Ile: isoleucine; Leu: leucine; Met: methionine; Phe: phenylalanine; Pro: proline; Ser: serine; Thr: threonine; Trp: tryptophan; Tyr: tyrosine; Val: valine.

3.3.1.3 Fermentation kinetics, population dynamics and nitrogen consumption patterns of mixed (sequential) culture fermentations

Synthetic grape juice was sequentially inoculated with the same yeast species described in the previous paragraph, *S. cerevisiae* being inoculated 48 h after the non-*Saccharomyces* yeasts. Three different YAN concentrations were studied in synthetic grape juice under winemaking conditions. The nitrogen concentrations applied were 300 mg/L (MS300), 150 mg/L (MS150) and 75 mg/L (MS75) keeping in mind that the lowest concentration of nitrogen required for completion of alcoholic fermentation by *S. cerevisiae* strains is generally around 140 mg/L total YAN (Agenbach 1977; Bely *et al*. 1990; Bell and Henschke 2005).

Sugar consumption

Glucose and fructose concentrations were monitored throughout fermentation to determine the rate of sugar consumption (Fig. 5). All sugars for *S. cerevisiae* (control) were completely depleted after 336 h, with glucose being completely consumed in 216 h in treatments MS300 and MS150. However, when the initial YAN concentration was only 75 mg/L, the fermentation was very sluggish. After 840 h, the fermentation was stopped with a residual fructose concentration of 14 g/L, glucose having been completely consumed after 576 h of fermentation. Co-cultures of MpYSc and MpFSc were very similar to each other with regards to sugar consumption and achieved dryness after 408 h in treatments MS300 and MS150 (Fig. 5A, B), one day earlier than the co-cultures LtSc and TdSc. The stalling or even slight increase in sugar concentration for MpYSc and MpFSc measured at 200 h is most probably due to a technical fault during the enzymatic assays. Co-cultures of *P. kluyveri* and *S. cerevisiae* took the longest to complete the fermentation under treatments MS300 and MS150. It reached dryness only after 504 h.

The reduction of YAN concentration from 300 to 150 mg/L resulted in a slower fermentation kinetics. Moreover, the low amount of YAN in MS75 drastically impacted on the fermentation

duration (Fig. 5C). Indeed, co-cultures LtSc, TdSc, PkSc, MpYSc and MpFSc under conditions MS75 fermented very slowly and resulted in sluggish fermentations. Fermentations were terminated at 840 h with residual sugar concentrations of 69 g/L, 76 g/L, 72 g/L, 106 g/L and 111 g/L, respectively.

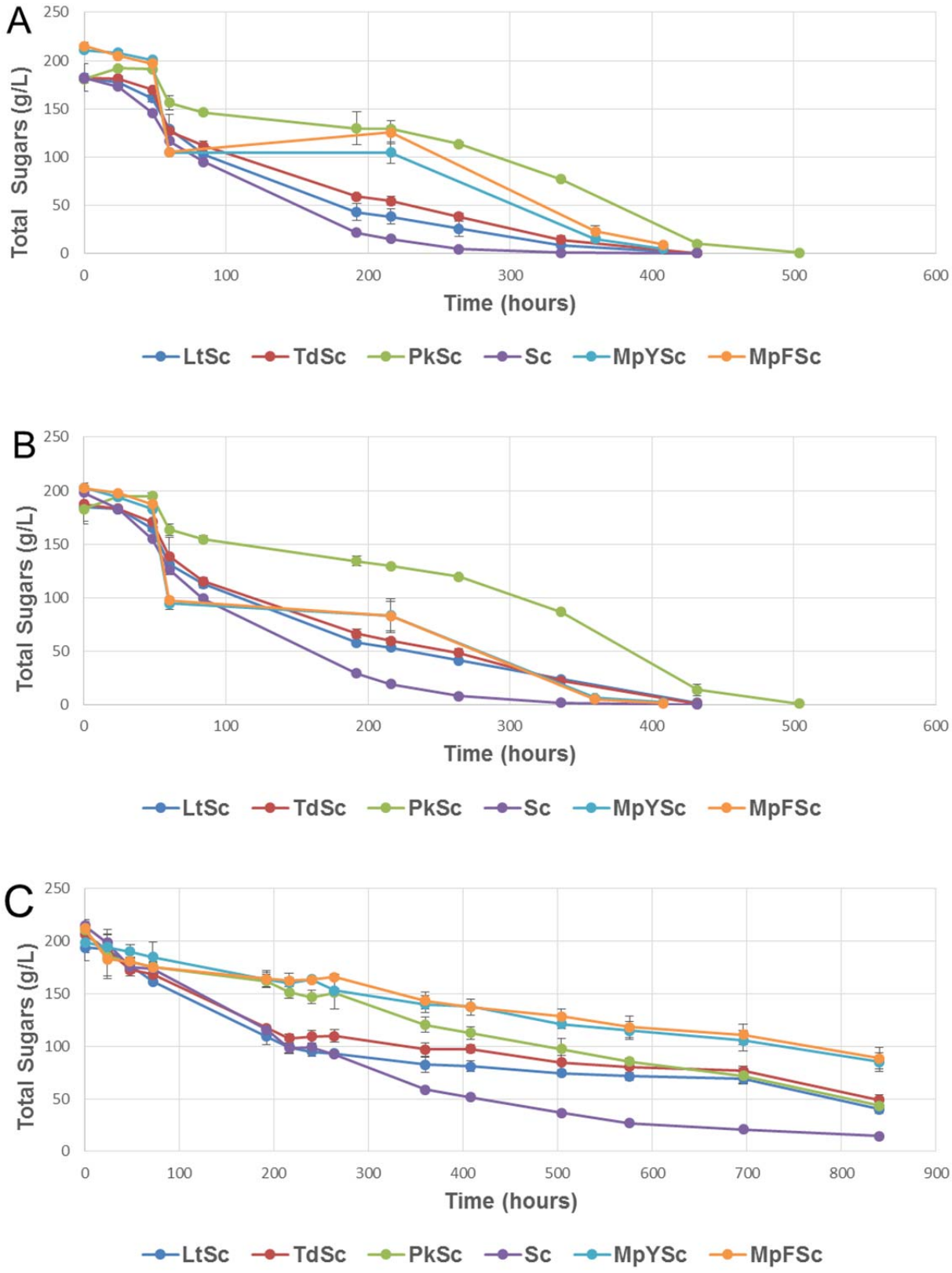
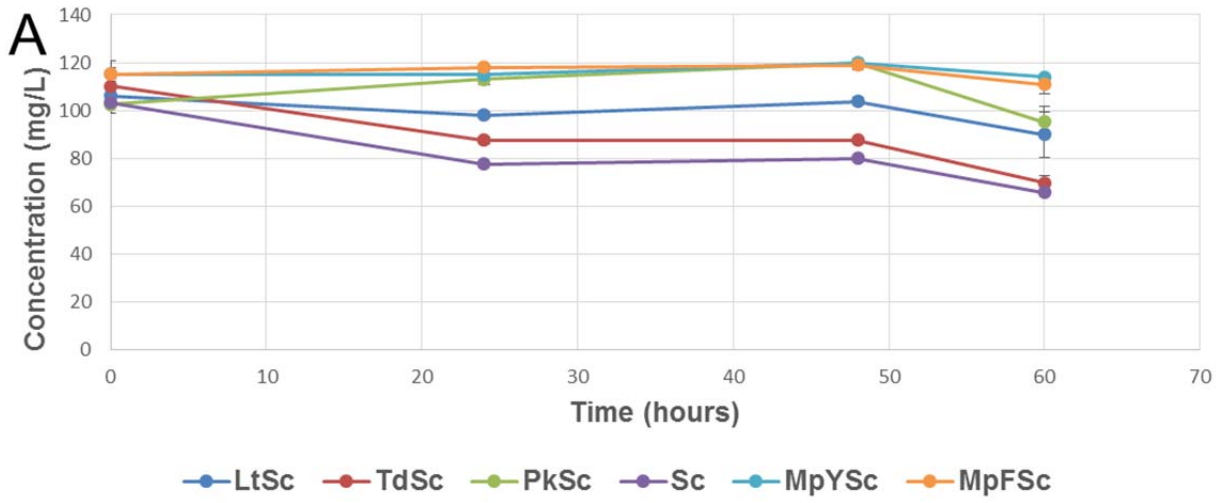
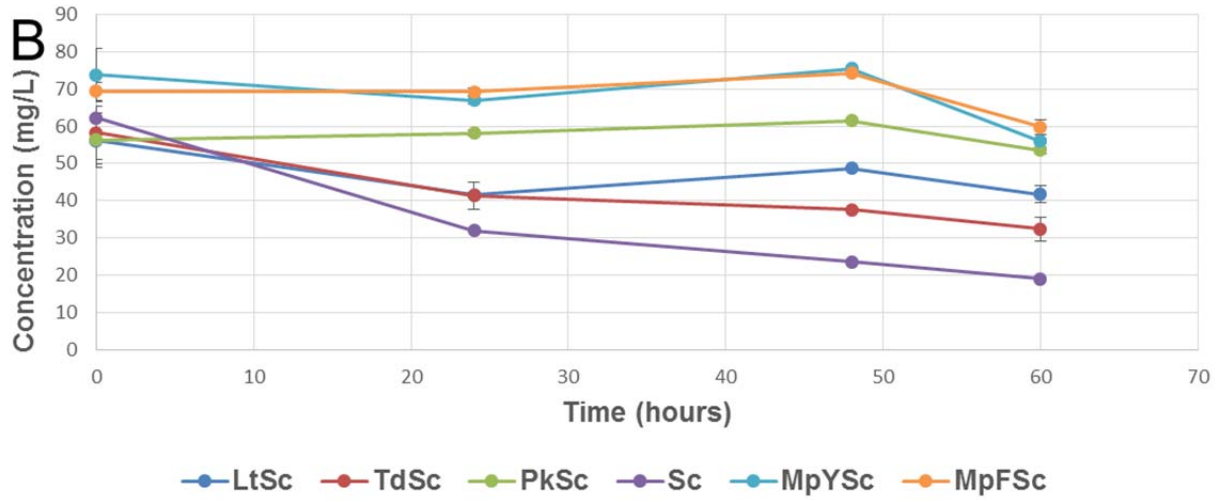


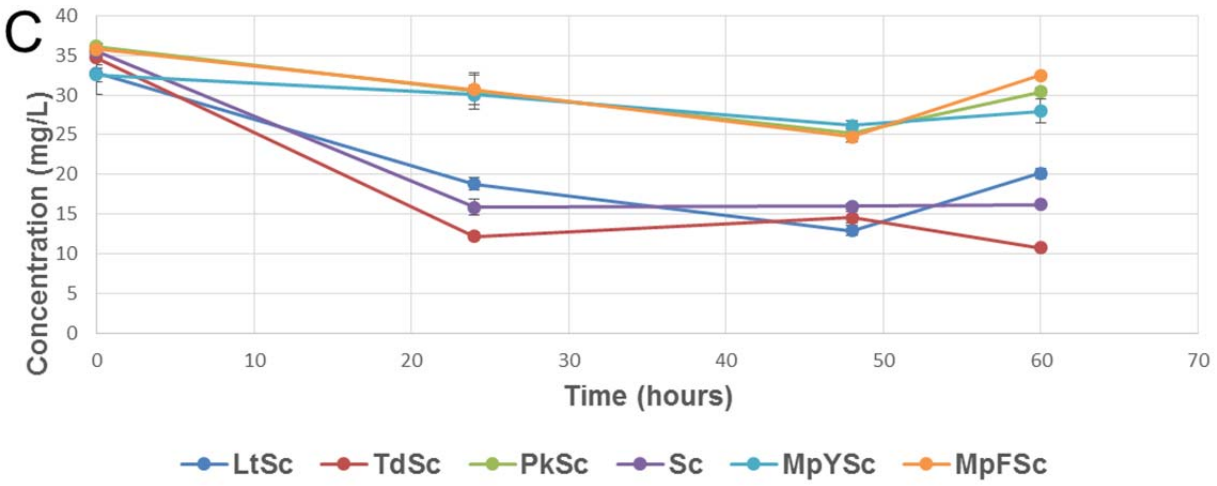
Figure 5. Total sugar consumption for different initial nitrogen treatments: (A) MS300, (B) MS150, (C) MS75.

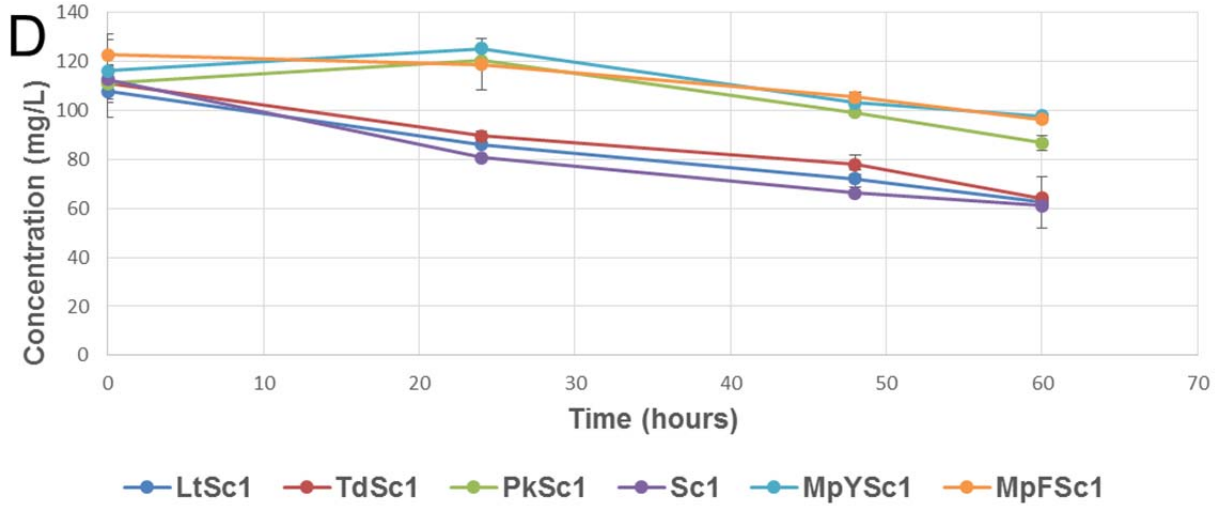
Consumption of nitrogen compounds

The concentrations of ammonia and alpha amino nitrogen were also monitored throughout the fermentation (Fig. 6). Irrespective of the initial nitrogen concentration, most of the YAN was assimilated within the first 60 h for all co-cultures. This was also observed for all pure culture fermentations, as mentioned in the previous paragraph. The total consumption for ammonia within the first 60h by, LtSc, TdSc, PkSc, Sc, MpYSc and MpFSc ranged between 1 – 41 mg/L for treatment MS300, 3 – 43 mg/L for treatment MS150 and 3 – 24 mg/L for treatment MS75. The total consumption for alpha amino nitrogen within the first 60h by LtSc, TdSc, PkSc, Sc, MpYSc and MpFSc ranged between 24 – 51 mg/L for treatment MS300, 18 – 55 mg/L for treatment MS150 and 12 – 24 mg/L for treatment MS75. From the above mentioned data, YAN were assimilated more when more nitrogen were available. For example, LtSc and TdSc consumed more ammonia and alpha amino nitrogen during treatment MS300 compared to treatment MS75 (total YAN consumption of 61 mg/L compared to 37 mg/L for former and 88 mg/L compared to 45 mg/L for latter) Figure 6 showed that co-cultures LtSc and TdSc behaved the same as the control (with similar consumption rates), whereas co-cultures MpYSc, MpFSc and PkSc behaved differently with regards to both ammonia and alpha amino nitrogen under the different initial YAN treatments. A slight decrease was observed after the inoculation of *S. cerevisiae* (at 48h) for the different co-cultures (Fig 6A, B, D, E, and F).









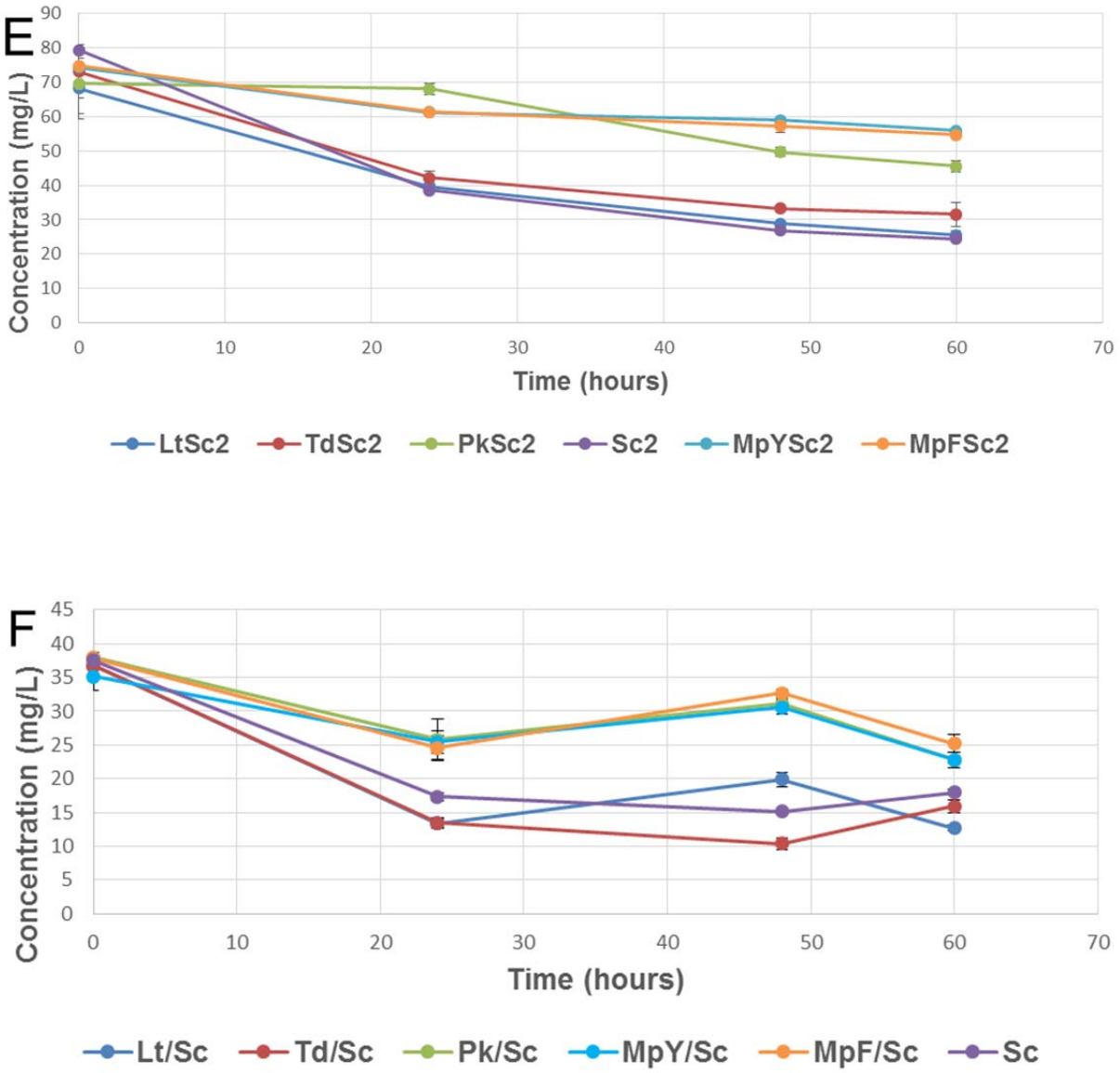


Figure 6. Ammonia consumption for the first 60 h (most of the ammonia and alpha amino nitrogen were taken up in the first 60 h, thereafter the graph plateaued): (A) MS300, (B) MS150, (C) MS75 as well as alpha amino nitrogen consumption: (D) MS300, (E) MS150, (F) MS75.

3.3.2 Production of nitrogen-containing volatile aroma compounds

A total of 32 major volatile aroma compounds were quantified via GC-FID during sequential fermentations for treatments MS300, MS150 and MS75. The final concentrations of volatile aroma compounds are presented in Supplementary Table 3.

The fermentations carried out with an initial YAN concentration of 75 mg/L were characterised by high concentrations of acetic acid, propionic acid, ethyl acetate, diethyl succinate and ethyl butyrate and on the contrary low concentrations of butyric acid, acetoin, higher alcohols (with the exception of isobutanol) and fatty acids. For example, *S. cerevisiae* produced more acetic acid for treatment MS75 (917 mg/L) than treatments MS300 (639 mg/L) and MS150 (647 mg/L) (Fig. 7). The same trend was observed for all co-cultures in different ratios, with the exception of MpYSc and MpFSc. The high total concentration of acetate esters produced is mainly attributed

to the high production of ethyl acetate (Fig. 8). Interestingly, diethyl succinate was not produced for treatments MS300 and MS150, but was detected in all co-cultures for treatment MS75 (LtSc: 0.6 mg/L; TdSc: 0.6 mg/L; PkSc: 0.6 mg/L; MpYSc: 1.2 mg/L; MpFSc: 1.2 mg/L; Sc: 0.9 mg/L).

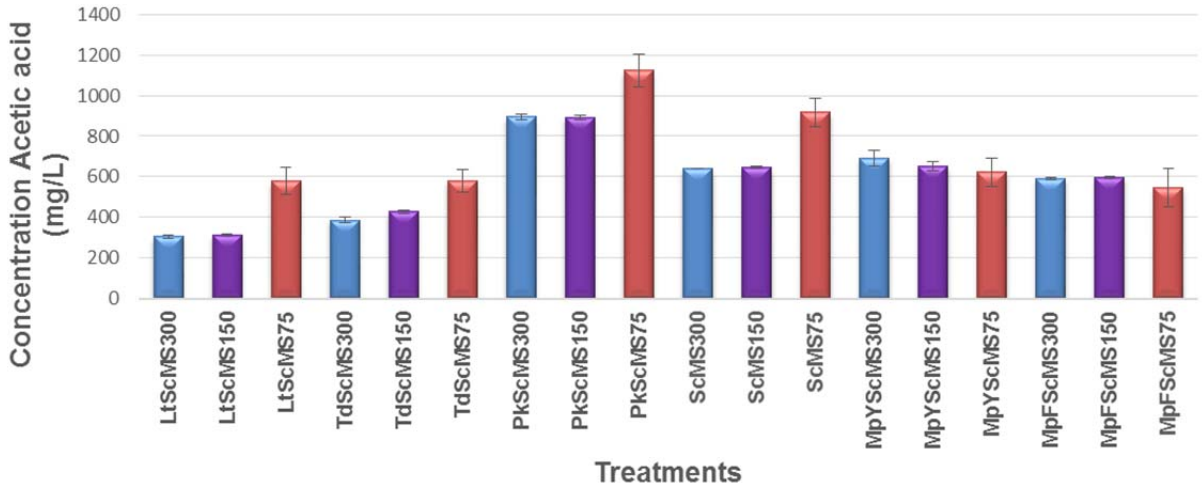


Figure. 7 Concentrations of acetic acid for different initial nitrogen concentrations.

Overall, the fermentations carried out with treatments MS300 and MS150 showed similar results for the production of different aroma compounds measured. However, differences in species behaviour was observed (Supplementary Table 2). For example, LtSc and TdSc produced less acetic acid (about 310 mg/L and 408 mg/L, respectively) and more butanol (about 2 mg/L and 0.8 mg/L) and propanol (about 101 mg/L and 93 mg/L) compare to the other species (i.e. *S. cerevisiae* produced on average (between MS300 and MS150) 643 mg/L acetic acid, 0.52 mg/L butanol and 11 mg/L propanol). The total concentration of higher alcohols produced by the co-cultures was higher than that of *S. cerevisiae* pure culture for all the treatments (i.e. MS300, MS150 and MS75), whereas the total medium chain fatty acids by *S. cerevisiae* was highest compare in all other fermentations. However, under the different co-cultures, MpYSc (MS300: 6 mg/L; MS150: 6 mg/L; MS75: 4 mg/L), MpFSc (MS300: 5 mg/L; MS150: 6 mg/L; MS75: 4 mg/L) and PkSc (MS300: 4 mg/L; MS150: 4 mg/L; MS75: 4 mg/L) under all treatments produced the highest concentration of medium chain fatty acids. From the different co-cultures, MpYSc, MpFSc and PkSc in all treatments exhibited the highest concentration of esters (MpYSc: MS300: 64 mg/L; MS150: 58 mg/L; MS75: 139 mg/L, MpFSc: MS300: 53 mg/L; MS150: 63 mg/L; MS75: 134 mg/L, PkSc: MS300: 15 mg/L; MS150: 141 mg/L; MS75: 169 mg/L, respectively).

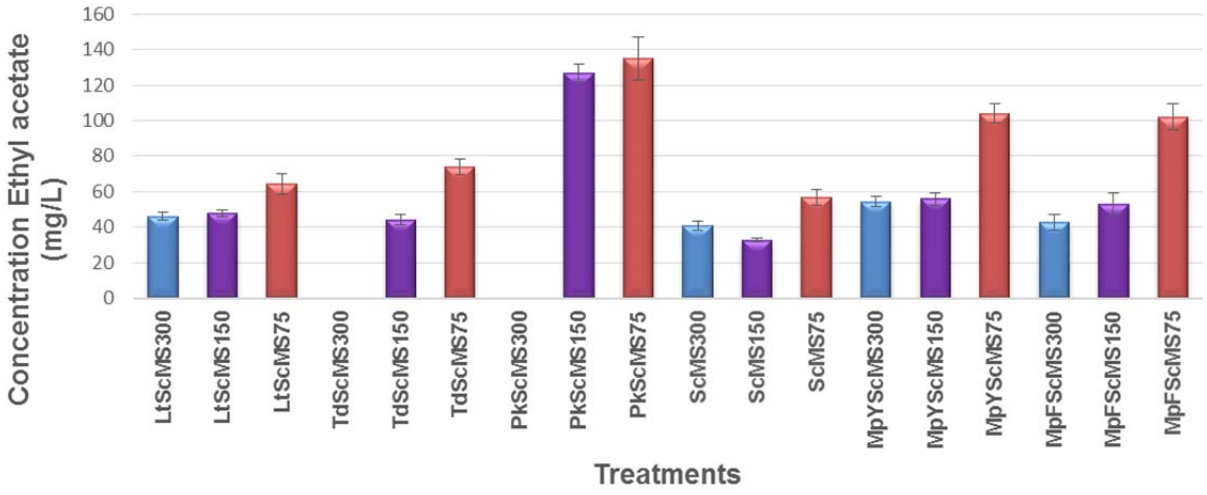


Figure. 8 Concentrations of ethyl acetate for different initial nitrogen concentrations.

Principal component analysis

A principal component analysis (PCA) model with two PCs explaining 46.4% of the variance provides an overview of the results (Fig. 9). The biological repeats grouped well together, which showed high reproducibility of the experimental technique. It was possible to observe separation between different initial YAN concentrations from PC1, which correspond to the above paragraph where MS75 behaved differently with regards to aroma compound production compared to MS300 and MS150. For example, treatment MS75 in all co-cultures correlated strongly positively with isobutanol, propionic acid, ethyl acetate, ethyl lactate and diethyl succinate. Nevertheless, PC2 allows a greater separation between certain species. For example, treatments LtScMS300, LtScMS150, TdScMS300 and TdScMS150 showed a strong positive correlation towards propanol and butanol. Nevertheless, the rest of the co-cultures under treatments MS300 and MS150 grouped closely together and showed strong positive correlation towards the volatile fatty acids. These results seem to confirm significant differences between initial nitrogen concentrations and the impact on the production of volatile compounds within different co-culture species from the above paragraph in this section.

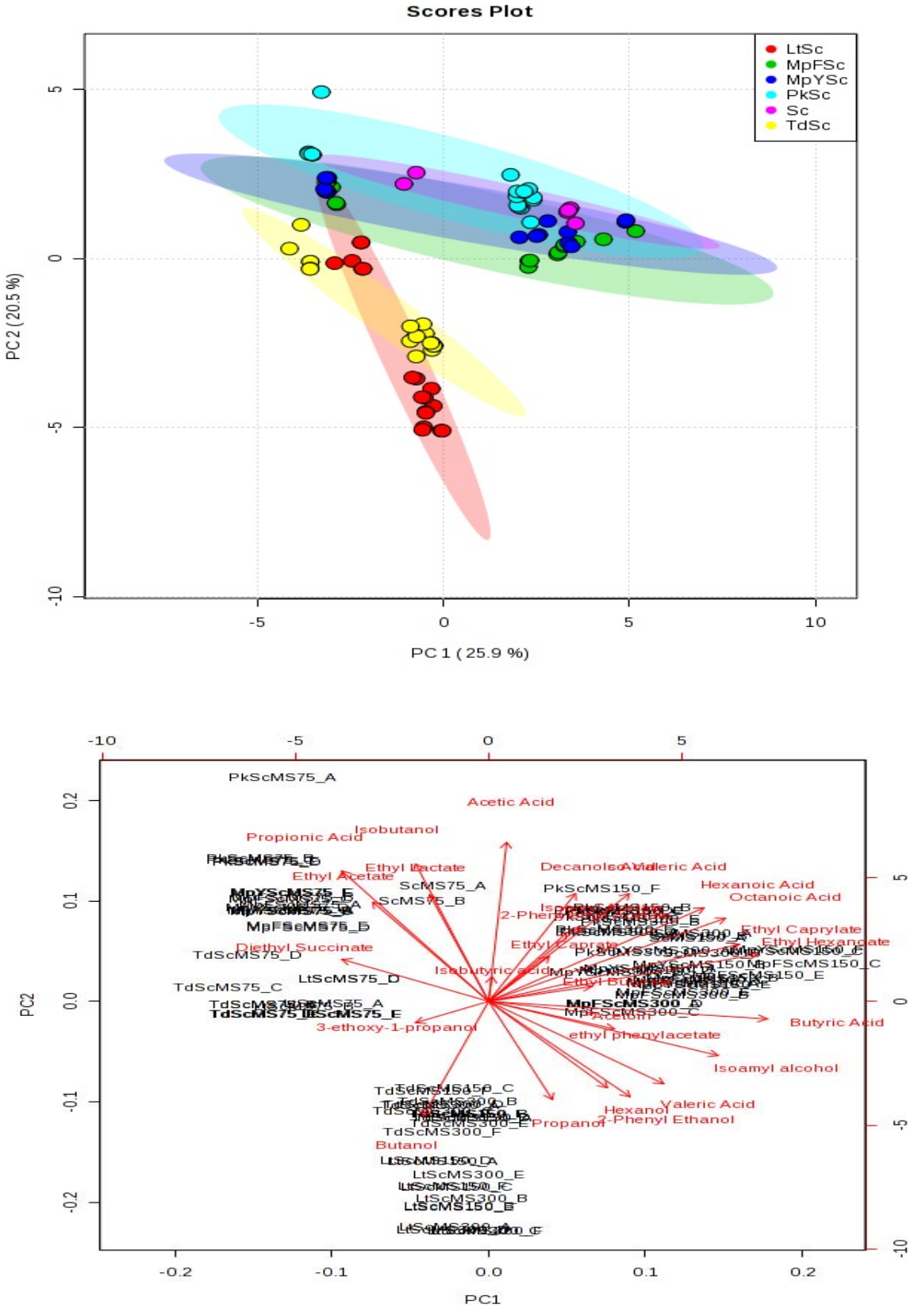


Figure. 9 Distribution of the different fermentations with different initial nitrogen concentrations according to PCA analysis. In the score plot for the first two principal components, PC1 explains 25.9 % of the variation while PC2 explains 20.5 %. The fermentations were represented as LtSc (MS300, MS150, MS75); TdSc (MS300, MS150, MS75); PkSc (MS300, MS150, MS75); MpYSc (MS300, MS150, MS75); MpFSc (MS300, MS150, MS75); Sc (MS300. MS150, MS75).

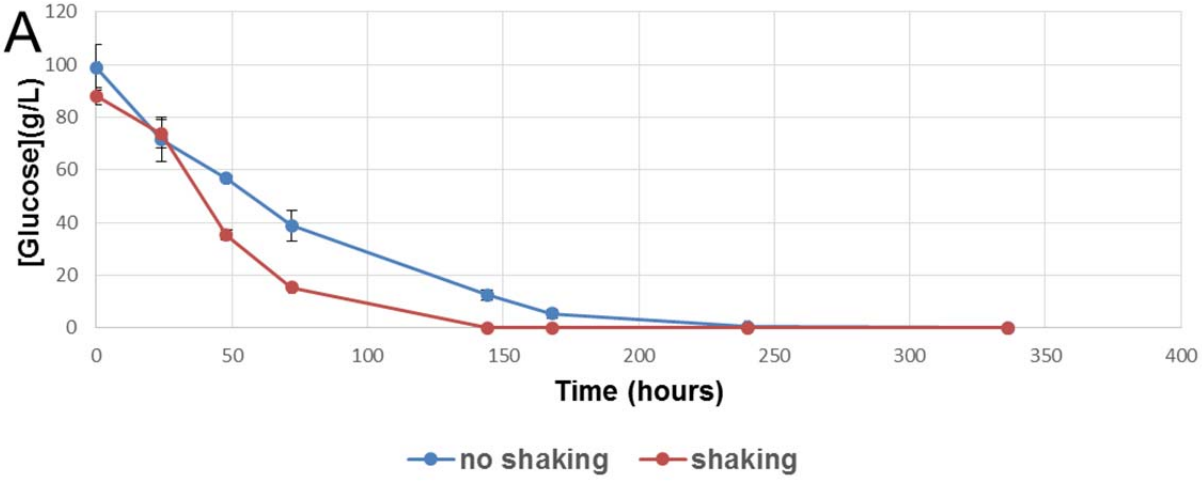
3.3.3 Impact of agitation on nitrogen consumption, fermentation kinetics, population dynamics and production of volatile aroma compounds by pure culture *S. cerevisiae*

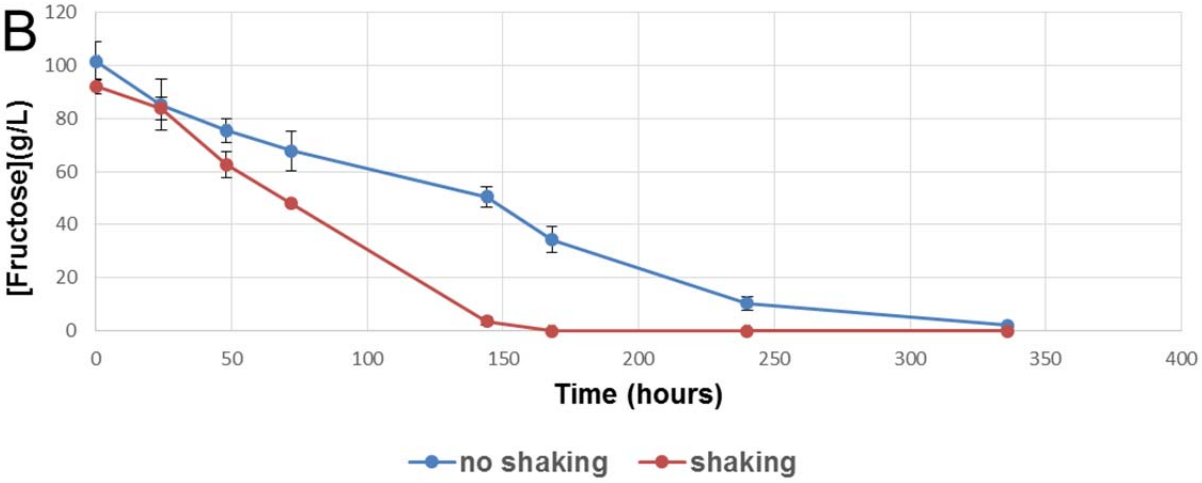
In all the fermentations described above, pure and sequential cultures, the yeasts assimilated only about half of the YAN provided irrespectively of the initial YAN concentrations. These results contradicted what is described in most of the studies reported in literature, where almost all YAN is consumed within the first 3 days of fermentation. The only major distinct parameter between these studies and ours was the application of stirring. Indeed, in an attempt to mimic an industrial fermentation, no stirring was applied in our study, but in the experiments described in literature, the medium is always agitated. In order to investigate whether this could explain the difference in nitrogen consumption, the influence of agitation was tested in the following paragraph.

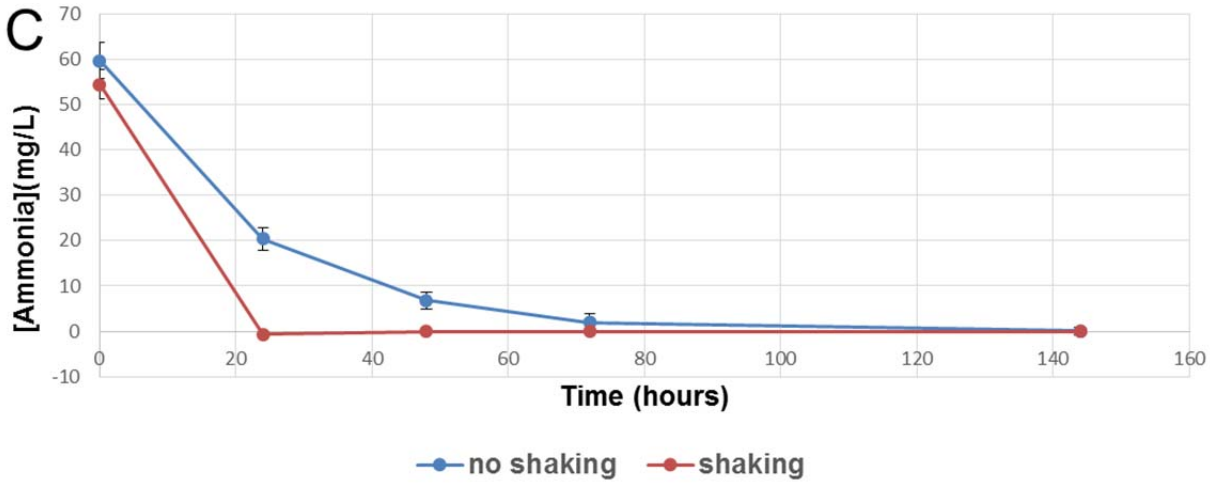
Fermentation kinetics

A pure culture fermentation was performed with the *S. cerevisiae* EC1118 strain using an initial YAN concentration of 150 mg/L. Three flasks were fermented in a static position, as carried out in the previous paragraphs and three flasks were shaken continuously at 140 rpm until fermentation was complete. No oxygen was allowed to enter into the flasks as they were fitted with fermentation caps.

Glucose, fructose, ammonia and alpha amino nitrogen were monitored throughout the fermentation (Fig. 10). In the static flasks, fermentation took 336 h to reach dryness, with glucose being fully depleted after 240 h. On the contrary, in the flasks that were shaken vigorously, *S. cerevisiae* completed the fermentation in just 168 h after glucose was completely consumed in 144 h (Fig. 10A, B). A major difference was also observed for ammonia and alpha amino nitrogen consumption (Fig. 10C, D). The control took 144 h to completely consume the ammonia, whereas in the fermentation where shaking was applied, ammonia was consumed within only 24 h. Moreover, alpha amino nitrogen consumption also showed major differences: where a total concentration of 42.14 mg/L was assimilated in the first day in the static fermentations, 66.79 mg/L was assimilated by the agitated yeast cells.







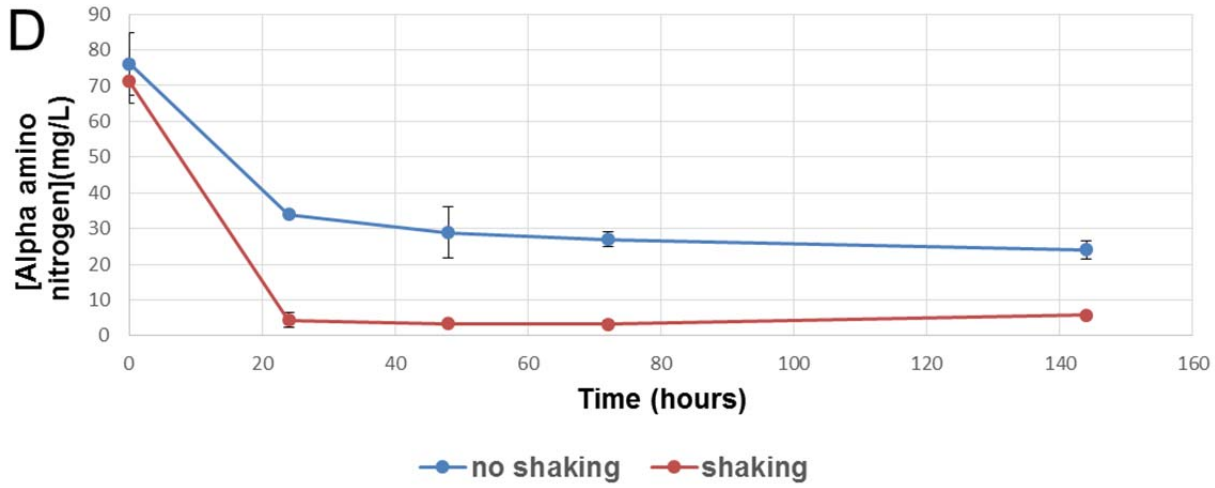


Figure 10. (A) Glucose, (B) fructose, (C) ammonia and (D) alpha amino nitrogen consumption throughout the fermentation.

Volatile aroma compounds

The final concentrations of volatile compounds are represented in Supplementary Table 4. The total higher alcohol concentration for the static fermentation (control) was significantly lower than the fermentation where agitation was applied, with values 99 mg/L and 270 mg/L, respectively. The production of more higher alcohols in the agitated fermentation can in particular be attributed to the production of isobutanol, isoamyl alcohol and 2-phenyl ethanol.

The production of volatile acidity is largely dominated by acetic acid. The agitated fermentation produced the highest amount of acetic acid. Overall, differences observed between the static fermentation and agitated fermentation with regards to short chain fatty acids and medium chain fatty acids were not large, except for isobutyric acid where the agitated fermentation produced significantly more than the static fermentation (3 mg/L and 1 mg/L) and for iso-valeric acid where the opposite was true (0.4 mg/L and 0.9 mg/L). Yet, both isobutyric and iso-valeric acid are constitutes from the Ehrlich pathway and are results from the catabolism of valine and leucine.

Ethyl acetate was one of the most prominent esters produced. The agitated fermentation produced 63 mg/L, whereas the static fermentation produced 36 mg/L respectively. Nevertheless, the static fermentation produced more ethyl lactate, ethyl hexanoate, ethyl caprylate and 2-phenylethyl acetate, whereas fermentation with agitation produced more ethyl phenylacetate, ethyl butyrate, diethyl succinate and isoamyl acetate.

3.4 Discussion

3.4.1 Fermentation performance

Overall, the fermentation kinetics observed in this study are in agreement with those previously reported (Jolly *et al*. 2003; Ciani *et al*. 2010; Mains 2014; Kock 2015). However, a study

conducted with *T. delbrueckii* in pure culture fermentation found no residual sugars at the end of day 11 of the fermentation (Taillandier *et al*. 2014). This could possibly reveal that fermentation performance is strain dependent within this species and/or that other environmental parameters (e.g. stirring in Taillandier *et al* (2014) vs no stirring in our study) play a role. The availability of nitrogen is well known to affect the growth and fermentation performance of *S. cerevisiae*, yet its effect on non-*Saccharomyces* yeast strains has never been thoroughly investigated. From our data, it seems as if the stronger fermenters within the non-*Saccharomyces* yeast species studied (i.e. *T. delbrueckii* and *L. thermotolerans*) followed the same pattern as *S. cerevisiae* for consumption of ammonia and alpha amino nitrogen, except for their slightly different preferences for either ammonia or alpha amino nitrogen. This relevant information could be key to the promotion of these yeasts' growth through the formulation of adapted nitrogen supplementation. *T. delbrueckii* and *L. thermotolerans* consumed a lesser but still significant amount of nitrogen than *S. cerevisiae* within the first 48 h of fermentation. Unlike *T. delbrueckii* and *L. thermotolerans*, *M. pulcherrima* and *P. kluyveri* did not consume much nitrogen compounds and the impact on *S. cerevisiae* is likely to be minimal with regards to competition of nitrogen compounds.

Amino acid uptake in this study was species specific, since differences in the amount of individual amino acid uptake vary between certain species. It has been reported in literature that amino acid uptake is strain-dependent (Ough *et al*. 1991; Jiranek *et al*. 1995; Torrea *et al*. 2003; Barrajón-Simancas *et al*. 2011). To our knowledge, the uptake of single amino acids by non-*Saccharomyces* yeasts has only be investigated in a recent study by (Kemsawasd *et al*. 2015). The above mentioned study found that, the nitrogen sources that improve overall performance of the yeast species are: Ala, Arg, Asn, Asp, Gln, Ile, Ser and Val (for *S. cerevisiae*), Ser (for *L. thermotolerans*), Ala and Asn (for *M. pulcherrima*) and Arg, Asn, Gln and Ile (for *T. delbrueckii*). Furthermore, the former study also observed that *T. delbrueckii* nitrogen source profile were very much the same as *S. cerevisiae*. One can speculate that with the selected non-*Saccharomyces* yeasts used in our study, *L. thermotolerans* and to a lesser extent *T. delbrueckii* can be competition for *S. cerevisiae* when used in co-culture fermentation with regards to nitrogen consumption, since the former yeasts assimilated similarly certain amino acids (Asp, Glu, Gln, Tyr, Leu and His). On the other hand, the two *M. pulcherrima* strains and *P. kluyveri* percentage uptake of single amino acids were overall low (which correlates with the low biomass production and the poor fermentation performance), with the exception of the following amino acids: Asp, Glu, Leu and Trp, which could indicate that these amino acids are essential for these species. Thus, when *S. cerevisiae* is sequentially inoculated with *L. thermotolerans* and *T. delbrueckii*, nitrogen sources may become limited since these non-*Saccharomyces* yeast species already assimilated some of the important nitrogen sources in the first 48 h, before *S. cerevisiae* are inoculated. So for this combination of species, more nitrogen should perhaps be added initially. On the other hand, because the nitrogen uptake of

strains *M. pulcherrima* and *P. kluyveri* is much lower, this nitrogen limited condition will not occur, since enough nitrogen sources will be available for *S. cerevisiae* to complete alcoholic fermentation. The nature and amount of nitrogen consumed could impact on *S. cerevisiae*'s overall fermentation performance in the case of sequential inoculation, when *S. cerevisiae* is typically inoculated 48 h after the non-*Saccharomyces* yeast.

This study is a good starting point to further investigate the nitrogen requirements of individual non-*Saccharomyces* yeast species, since some studies suggested that commercialized yeasts strains require more nitrogen than wild yeasts strain (Barrajón-Simancas *et al*. 2011).

For the co-cultures LtSc and TdSc in both MS300 and MS150, total sugar consumption was similar to that of pure culture *S. cerevisiae*. This is in agreement with a similar study performed with *T. delbrueckii* and *S. cerevisiae*, in which the fermentation finished only one day after that of the pure *S. cerevisiae* (Taillandier *et al*. 2014). Nonetheless, the sugar consumption rate of co-cultures MpYSc and MpFSc may be attributed to the fact that, unlike with *L. thermotolerans* and *T. delbrueckii*, *S. cerevisiae* rapidly outcompeted *M. pulcherrima*, a phenomenon also observed in other studies (Mains 2014; Kock 2015). The sluggish trend for PkSc could be a possible antagonistic effect of *P. kluyveri* on *S. cerevisiae*. Indeed, literature reports that killer toxins (zymocins) produced by *P. kluyveri* can inhibit certain strains of *S. cerevisiae* (Middelbeek *et al*. 1980). In order to verify this hypothesis, a killer plate assay was carried out using *S. cerevisiae* EC1118 as a sensitive organism and *P. klyveri* Frootzen as a potential killer yeast. No zone of growth inhibition was observed around *P. kluyveri* (data not shown), suggesting that *P. kluyveri* Frootzen does not exhibit killer activity against *S. cerevisiae* EC1118. However, plate assays do not always reflect phenotypes observed in liquid medium and killer activity cannot be totally excluded. Further investigation would thus need to be conducted to identify the nature of the antagonistic interactions between *P. kluyveri* and *S. cerevisiae*. Since alcoholic fermentation was completed in treatments MS300 and MS150, one can assume that these initial nitrogen concentrations were sufficient for the yeasts cells (especially *S. cerevisiae*) to survive until the end of fermentation. This was expected as previous studies observed that only at an initial nitrogen concentration of less than 140 mg/L the fermentation were sluggish or got stuck (Bely *et al*. 1990; Lage *et al*. 2014; Taillandier *et al*. 2014). But, some delay with regard to nitrogen consumption could have been expected, because of the competition between yeasts. One study also showed that during sequential fermentations (with an initial YAN of 175 mg/L) with non-*Saccharomyces* and *S. cerevisiae* yeasts, the non-*Saccharomyces* yeasts negatively affected *S. cerevisiae*'s growth and fermentation rate (Lage *et al*. 2014). However, no increase in the amount of nitrogen consumed was observed and the authors concluded that competition between strains for nutrients was not necessarily the factor responsible for the poor fermentation performance of *S. cerevisiae*. Nevertheless another study conducted (Medina *et al*. 2012) on mixed culture fermentations with *S. cerevisiae* and the *M. pulcherrima* and *H.*

vineae, resulted in sluggish fermentations. Thus, competition for nutrients (YAN) had a negative effect on the fermentation kinetics in this study. This can explain why treatment MS75 resulted in sluggish fermentation. Yet, neither the ammonia nor the alpha amino nitrogen was completely consumed in any of these fermentations, including in the control. In fact, the same general trend was observed in all treatments (MS300, MS150 and MS75) with regards to ammonia and alpha amino nitrogen: the yeasts only assimilated about half of the total YAN that was present at the beginning of the fermentation. In studies using similar fermentation setups (Crépin *et al*. 2014; Lage *et al*. 2014; Rollero *et al*. 2014; Taillandier *et al*. 2014; Viana *et al*. 2014) ammonia and YAN are consumed almost fully. The main differing parameter was the presence/absence of stirring. Our study aimed to keep the conditions as close as possible to the conditions in the wine industry, therefore the flasks were only stirred once a day, immediately before sampling. The impact of agitation with regards to fermentation kinetics and production of aroma compounds was therefore deemed worthy of investigation.

3.4.2 Volatile compound production

Differences with regards to aroma compound production were observed between different initial YAN concentrations (MS300, MS150 and MS75), but to a lesser extent amongst MS300 and MS150. Fermentations with an initial YAN of 75 mg/L were characterised by high organic and ester production and low fatty acid and higher alcohol production. The differentiation between mixed culture fermentations observed in this study can be to maintain the redox balance of the cell, since most higher alcohols are produced through the Ehrlich pathway. NADH is released when a higher alcohol is produced whereas NAD^+ is released if the corresponding acid is produced. The high concentrations of butanol and propanol observed for *L. thermotolerans* are in agreement with previous studies (Gobbi *et al*. 2013; Mains 2014; de Kock 2015). The high acetic acid production in all co-cultures (especially during treatments MS75) can possibly be explained by the metabolic pathway leading to acetic acid production. When there is a sufficient amount of nitrogen available for the yeasts to use, a reduced need to generate NADH through other redox reactions, such as the oxidation of acetaldehyde to acetic acid, results in reduced levels of volatile acidity (Bely *et al*. 2003; Fairbairn 2012). Thus, when the yeasts need to withstand growth limited conditions, such as low nitrogen concentrations, acetic acid production will increase (Lambrechts and Pretorius 2000). Acetic acid levels exceeding 0.7 g/L may impart a vinegar-like character, while levels of 0.2-0.7 g/L are considered positive (Dubois 1994). Thus, in this study, co-culture PkSc in all treatments exceeded the acceptable level (890 – 1100 mg/L). This can be explained by the sluggish trend of PkSc observed during sequential fermentation. Treatment MS75 for pure *S. cerevisiae* also exceeded this level (951.06 mg/L). In this study, MpYSc, MpFSc and Sc exhibited high concentrations of fatty acids under all treatments. Studies carried out by Sadoudi *et al* (2012) and Mains (2014) reported similar results with regards to an increase in total production of volatile fatty acids for *M. pulcherrima*

and *S. cerevisiae* in sequential use. The co-culture TdSc produced the least ethyl acetate in all different treatments. This result is similar than what was found in literature, where co-cultures of *T. delbrueckii* and *S. cerevisiae* were used (Barrajón-Simancas *et al*. 2011; Sadoudi *et al*. 2012). Studies have reported a direct relation between the formation of acetate and the must nitrogen concentration, especially in the case of ethyl acetate, which is positively related to the nitrogen concentration in the medium (Burin *et al*. 2015). A study conducted on non-*Saccharomyces* yeasts found that the ester production of the genera *Pichia* and *Hanseniaspora* was more than that of the other non-*Saccharomyces* yeasts (Bisson and Kunkee 1991). This was also observed in our study for *Pichia kluyveri* co-cultures which resulted in the highest ester production compared with the other co-cultures and the control.

Another interesting observation was the diethyl succinate production between the different treatments. Treatments MS300 and MS150 did not produce any diethyl succinate. Nevertheless, during treatment MS75 all co-cultures produced diethyl succinate. When preferred amino acids are present in the medium, less succinic acid will be formed, because these amino acids take directly part in transamination reactions to form other amino acids, instead of being transformed to α-ketoglutarate. It could be argued that higher alcohols are produced primarily from the available carbon skeletons, possibly to maintain redox balancing (Jain *et al*. 2011). This could be the case for the present study, since co-cultures for treatments MS300 and MS150 produced more higher alcohols and less esters compared to co-cultures in treatment MS75. Treatments MS75 is nitrogen-limiting for the yeasts cells and the production of diethyl succinate was likely to ensure redox balancing. It could be that the remaining α-ketoglutarate, from the branched-chain amino acids, causes an increase of the enzyme activities of the oxidative branch of the tricarboxylic acid cycle, subsequent in the production of succinate.

Since the non-*Saccharomyces* yeasts fermented as pure cultures for the first 48 h during sequential fermentation, one can speculate between possible single amino acid uptake and probable aroma compounds that were produced during the first 48 h by the non-*Saccharomyces* yeasts. For example, the single amino acids Phe and Leu were taken up the most by *L. thermotolerans* during pure culture fermentation, thus aroma compounds including isoamyl alcohol, isovaleric acid, phenylethanol and phenylacetic acid were probably produced by *L. thermotolerans* in greater amounts than what *S. cerevisiae* had produced during the rest of alcoholic fermentation. Indeed, LtSc showed the highest concentration of phenylethanol (Supplementary Table 1). The non-*Saccharomyces* yeasts *P. kluyveri* and the two *M. pulcherrima* strains showed the highest percentage uptake for Leu, Asp, Glu and Val, especially Leu. Thus, these yeasts contribute to the aroma compounds such as isoamyl alcohol and isovaleric acid (from Leu), and some volatile fatty acids (from Val). Another way to look at this, was to calculate the amino acids per sugars consumed for each yeasts species (Supplementary

Table 3). Interestingly, Leu and Val showed the highest uptake per sugars consumed by *P. kluyveri* and the two *M. pulcherria* strains, which could explain why PkSc, MpYSc and MpFSc exhibited the highest concentrations of isoamyl alcohol and isobutanol than the other yeasts. However tryptophol wasn't measured in this study, one can speculate that since *P. kluyveri* and the two *M. pulcherrima* strains took up the most Trp per sugars consumed, the former compound would have been produced by the former yeasts.

3.4.3 Shaking vs no shaking

The sugar and YAN rate were different between the agitation vs no-agitation. The rate of sugar consumption, for the agitated fermentation, is in agreement with previous studies (Taillandier *et al*. 2014; Rollero *et al*. 2014). When agitation was applied, the yeasts assimilated much more YAN, possibly because shaking enhanced availability of nutrients for the yeasts.

Overall, the agitated fermentation produced more higher alcohols as well as esters. The production of more higher alcohols can possibly be explained by the process quorum sensing. Quorum sensing (QS) is a process of intercellular communication where yeasts communally adapt their metabolism at high cell density or under low nutrient conditions by secretion of aromatic alcohols. 2-Phenyl ethanol have been reported to have some QS roles under low nitrogen conditions in *S. cerevisiae* (Zupan *et al*. 2013). In the same study, the authors reported that agitation stimulated growth of yeasts and that the production of 2-phenyl ethanol and tyrosol were higher than when no agitation was applied (Zupan *et al*. 2013). This can explain some of the results obtained in this study where the increased production in biomass leading to the increase in YAN uptake during the agitated fermentation resulted in the increase of higher aalcohol production possibly due to the QS effect in cells of *S. cerevisiae*, specifically for the production of 2-phenyl ethanol. Thus, agitated fermentations could produce wines which are more complex and fruity if the concentration of higher alcohols produced are under the odour threshold (less than 300 mg/L) (Lambrechts and Pretorius 2000) The agitated fermentation produced significantly more total esters than the static fermentation. This can be attributed to the production of ethyl acetate. Thus, agitation contribute positively to overall higher alcohol and ester production which will contribute to the wines complexity and fruitiness.

3.5 Conclusion

This study confirmed that non-*Saccharomyces* yeasts, during single culture fermentations, have lower fermentation abilities than *S. cerevisiae*. Nevertheless, some non-*Saccharomyces* yeasts, *L. thermotolerans* and *T. delbrueckii*, showed potential as "strong" fermenters under the conditions tested. Moreover, ammonium and alpha amino nitrogen were species specific. During sequential fermentations, treatments with initial YAN concentrations of 300 and 150 mg/L, finished alcoholic fermentation. However, a sluggish fermentation was observed for

sequential fermentation with 75 mg/L initial YAN for all co-cultures including pure culture *S. cerevisiae*.

The single amino acid uptake data gave a good starting point to better understand some selected non-*Saccharomyces* yeasts nitrogen uptake needs in the first 48 h. It was observed that the percentage uptake of *L. thermotolerans* and *T. delbrueckii* (to a lesser extent) compared well with the percentage uptake of *S. cerevisiae*. One can speculate that nitrogen competition can occur when these species are used in co-culture fermentations. Furthermore, *L. thermotolerans* assimilated most of some single amino acids compared to *S. cerevisiae* during the first 48 h, which could mean that the former produced most of the aroma compounds from the specific amino acid before *S. cerevisiae* should have been added.

From the sequential fermentations the production of specific desirable aromatic profiles under different initial assimilable nitrogen conditions by non-*Saccharomyces* yeasts were confirmed. Both fermentations with 300 and 150 mg/L initial YAN showed positive contributions towards aroma compounds. For example, *L. thermotolerans* and *T. delbrueckii* reduced the production of acetic acid, the mouthfeel of wine can be increased by *M. pulcherrima* strains, since these yeasts produced high levels of medium chain fatty acids and fruitier wines can be produced with *P. kluyveri* in sequential use with *S. cerevisiae* due to its high ester production. Nevertheless, during sequential fermentation with treatment MS75, the following was observed regardless of the yeasts species: more organic acids (e.g. acetic acid (except for the *M. pulcherrima* strains) and propionic acid, which originate from the decarboxylation of succinic acid) and esters of organic acids (e.g. ethyl acetate, ethyl lactate, diethyl succinate) were produced. Moreover, less acetoin, butyric acid, fatty acids (both medium and short) and higher alcohols (except for isobutanol) were produced. Thus, it seemed as if the cells accumulated (and excreted) compounds from the carbon metabolism pathway (with a halt of certain pathways, e.g. those leading to acetoin, and butyric acid) and produced significant less compounds originating from the nitrogen metabolism. Perhaps there was not enough nitrogen available to convert alpha-keto glutarate to glutamate, hence the accumulation of significantly higher amounts of succinic and propionic acids.

Furthermore, the greater uptake of specific amino acids by certain non-*Saccharomyces* yeasts could be tentatively correlated with greater concentrations of specific aroma compounds at the end of fermentation, thereby demonstrating how the nitrogen metabolism of non-*Saccharomyces* yeasts can influence the final organoleptic profile of wines. Yet, this is preliminary results. Further studies, with more strains, can measure aroma compounds production during pure culture fermentations, when providing a single amino acid as sole nitrogen source.

It was evident in this study that single culture fermentation with agitation speed up fermentation kinetics and resulted in higher biomass production. In addition, all yeasts species consumed about all the YAN in 24 hours. Moreover, agitation during fermentation resulted in greater production of higher alcohol and esters. However, there are only little agitation in an industrial tank. Thus, studies in laboratories can try to mimic what happens in a real tank and redo this experiment at a realistic agitation speed and analyse at what speed aroma compound production will enhance the flavour and complexity of wine.

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3.7 Supplementary data

Supplmentary Table 1 Concentrations of single amino acids (mg/L) at different time point during pure culture fermentation. (A): 24 h; (B): 48 h; (C) end point.

A

	Time 0	LtYt24			Tdt24			Sct24			Pkt24			MpYt24			MpFt24		
Asp	44,506	7,85	±	0,79	8,67	±	0,67	5,19	±	0,27	17,40	±	3,68	19,07	±	1,98	16,36	±	2,49
Glu	120,428	30,21	±	2,55	31,70	±	1,77	25,73	±	0,83	41,11	±	4,61	40,48	±	2,61	37,76	±	1,60
Asn	0	nd	±		nd	±		nd	±		nd	±		nd	±		nd	±	
Ser	78,54	29,53	±	1,65	29,06	±	1,09	16,95	±	0,40	54,61	±	6,67	54,25	±	4,01	50,53	±	3,35
Gln	505,274	245,92	±	9,34	305,40	±	8,98	201,27	±	5,89	469,50	±	57,21	399,53	±	27,89	372,12	±	27,87
His	32,725	25,92	±	5,35	34,50	±	7,63	21,59	±	1,10	43,65	±	8,28	39,62	±	2,38	34,10	±	9,29
Gly	18,326	20,92	±	1,83	18,98	±	0,85	18,57	±	1,38	20,37	±	2,90	20,00	±	1,55	20,14	±	2,02
Thr	75,922	29,25	±	1,72	32,45	±	1,02	23,66	±	1,03	46,97	±	5,53	47,91	±	3,18	44,26	±	2,50
Arg	374,374	253,33	±	16,78	249,59	±	21,59	287,06	±	18,12	304,75	±	43,00	308,33	±	19,47	282,34	±	41,50
Ala	145,299	75,78	±	5,55	73,70	±	2,93	78,08	±	3,15	93,58	±	10,67	98,42	±	6,75	92,94	±	4,69
GABA	0	nd	±		nd	±		nd	±		nd	±		nd	±		nd	±	
Tyr	18,326	7,90	±	0,39	8,34	±	0,18	7,75	±	0,32	10,25	±	1,30	10,56	±	0,72	9,78	±	0,68
Cy2	0	15,28	±	1,24	16,32	±	0,32	14,94	±	1,27	23,23	±	3,51	22,17	±	2,06	19,21	±	1,40
Val	44,506	17,18	±	0,96	19,46	±	0,35	13,13	±	0,93	24,10	±	2,73	24,94	±	1,08	22,11	±	1,21
Met	31,416	5,98	±	0,32	3,61	±	0,13	6,25	±	0,74	17,00	±	2,74	16,98	±	1,44	15,38	±	2,89
Phe	37,961	5,07	±	0,35	17,29	±	0,31	8,28	±	0,43	22,88	±	2,77	25,42	±	1,67	23,76	±	1,44
Ile	32,725	6,69	±	0,26	10,63	±	0,25	4,74	±	0,36	17,63	±	2,12	16,44	±	1,22	12,73	±	0,72
Leu	48,433	2,44	±	0,30	14,34	±	0,38	3,27	±	0,55	26,07	±	3,21	24,58	±	1,84	18,56	±	1,01
Pro	612,612	396,39	±	57,73	351,53	±	93,30	328,77	±	15,24	331,60	±	43,87	354,69	±	21,11	349,38	±	69,61
Trp	179,333	88,01	±	4,97	85,85	±	1,18	75,24	±	4,66	106,12	±	14,73	98,50	±	6,08	98,31	±	6,36
Lys	17,017	nd	±		nd	±		nd	±		nd	±		nd	±		nd	±	
Cys	13,09	nd	±		nd	±		nd	±		nd	±		nd	±		nd	±	

[a]nd: not detected; Lt: *L. thermotolerans*; Td: *T. delbueckii*; Pk: *P. kluyveri*; Mp: *M. pulcherrima*; Sc: *S. cerevisiae*; T24: measured at time point 48 h during pure culture fermentation.

B

	Time 0	LtYt48			Tdt48			Sct48			Pkt48			MpYt48			MpFt48		
Asp	44,506	5,58	±	0,96	8,72	±	0,69	4,23	±	0,53	20,52	±	1,62	18,09	±	3,57	17,58	±	3,60
Glu	120,428	27,87	±	1,45	33,38	±	0,98	25,54	±	2,95	41,12	±	2,82	38,64	±	2,58	39,59	±	4,30
Asn	0	nd	±		nd	±		nd	±		nd	±		nd	±		nd	±	
Ser	78,54	25,61	±	1,21	32,22	±	0,52	14,75	±	3,23	57,14	±	3,10	53,45	±	1,70	53,74	±	2,89
Gln	505,274	212,43	±	5,37	332,83	±	6,37	193,94	±	36,63	451,98	±	24,86	370,12	±	2,32	361,88	±	12,39
His	32,725	21,30	±	5,67	49,60	±	1,12	20,70	±	10,12	34,40	±	2,17	39,82	±	9,25	35,24	±	11,98
Gly	18,326	16,97	±	0,34	21,51	±	0,23	20,95	±	5,32	20,01	±	1,32	19,79	±	0,89	20,07	±	1,29
Thr	75,922	27,76	±	0,62	35,27	±	0,88	24,91	±	3,18	47,77	±	2,62	46,90	±	1,56	47,03	±	2,66
Arg	374,374	232,51	±	25,26	303,28	±	4,46	323,51	±	30,49	276,01	±	15,75	309,93	±	26,02	295,36	±	34,54
Ala	145,299	64,84	±	3,16	81,50	±	1,58	77,60	±	11,72	97,36	±	5,20	98,31	±	4,36	100,42	±	6,50
GABA	0	nd	±		nd	±		nd	±		nd	±		nd	±		nd	±	
Tyr	18,326	7,07	±	0,13	9,09	±	0,21	7,49	±	1,24	10,32	±	0,59	10,29	±	0,25	10,31	±	0,50
Cy2	0	13,73	±	0,74	16,84	±	1,13	14,43	±	1,65	16,95	±	1,57	18,78	±	0,74	18,50	±	0,69
Val	44,506	13,38	±	0,33	20,84	±	0,45	9,38	±	2,78	21,27	±	1,13	23,04	±	0,56	21,23	±	0,86
Met	31,416	6,20	±	1,66	12,82	±	0,40	4,31	±	2,10	14,46	±	1,31	18,01	±	1,83	17,01	±	1,39
Phe	37,961	2,62	±	0,11	18,45	±	0,38	4,47	±	1,98	19,75	±	1,14	24,05	±	0,73	24,61	±	1,23
Ile	32,725	4,51	±	0,20	11,28	±	0,22	1,80	±	0,89	19,75	±	0,86	24,05	±	0,46	24,61	±	0,99
Leu	48,433	0,54	±	0,05	15,20	±	0,31	0,88	±	1,05	15,03	±	1,11	14,18	±	0,43	9,80	±	1,17
Pro	612,612	435,29	±	41,15	388,35	±	26,08	419,83	±	36,54	485,12	±	14,60	402,60	±	59,45	441,15	±	81,65
Trp	179,333	71,57	±	2,32	85,04	±	1,91	58,57	±	9,03	98,98	±	6,62	93,77	±	1,85	96,29	±	4,96
Lys	17,017	nd	±		nd	±		nd	±		nd	±		nd	±		nd	±	
Cys	13,09	nd	±		nd	±		nd	±		nd	±		nd	±		nd	±	

[a]nd: not detected; Lt: *L. thermotolerans*; Td: *T. delbueckii*; Pk: *P. kluyveri*; Mp: *M. pulcherrima*; Sc: *S. cerevisiae*; T48: measured at time point 48 h during pure culture fermentation.

C

	Time 0	LtY end			Td end			Sc end			Pk end			MpY end			MpF end		
Asp	44,506	4,94	±	1,08	9,91	±	4,45	6,33	±	0,82	18,43	±	4,63	19,94	±	0,39	20,67	±	0,84
Glu	120,428	32,69	±	2,48	36,52	±	11,88	32,73	±	3,99	41,97	±	6,99	41,75	±	0,75	42,44	±	1,29
Asn	0	0,42	±	0,42	0,93	±	0,93	6,95	±	6,95	0,47	±	0,63	nd	±		nd	±	
Ser	78,54	25,34	±	2,39	32,20	±	11,42	13,20	±	1,27	54,96	±	11,75	53,75	±	0,40	58,54	±	2,19
Gln	505,274	150,13	±	10,57	240,00	±	26,84	149,03	±	6,61	169,18	±	28,42	159,10	±	1,57	210,06	±	6,17
His	32,725	23,07	±	2,64	40,90	±	19,21	10,12	±	1,18	57,59	±	8,38	55,18	±	3,35	81,34	±	7,12
Gly	18,326	24,58	±	1,18	25,97	±	0,33	21,19	±	0,54	14,31	±	0,45	9,86	±	0,96	13,40	±	1,47
Thr	75,922	33,12	±	0,96	39,39	±	6,66	28,93	±	0,61	49,37	±	6,46	49,94	±	0,63	46,40	±	1,54
Arg	374,374	243,25	±	17,08	345,33	±	37,50	265,39	±	1,55	377,38	±	14,36	376,23	±	12,11	404,53	±	17,88
Ala	145,299	82,73	±	5,23	104,87	±	35,90	89,47	±	6,12	107,49	±	11,06	104,70	±	1,69	103,20	±	3,57
GABA	0	3,88	±	0,57	2,35	±	0,34	9,57	±	2,50	29,44	±	2,34	nd	±		nd	±	
Tyr	18,326	7,95	±	0,14	8,32	±	4,20	7,03	±	0,99	9,92	±	2,85	11,58	±	0,42	11,41	±	0,42
Cy2	0	14,02	±	0,16	18,36	±	4,20	17,17	±	0,68	13,80	±	0,31	13,04	±	0,48	10,58	±	2,02
Val	44,506	13,57	±	0,44	14,00	±	11,73	6,50	±	2,18	43,57	±	9,76	42,48	±	0,70	47,83	±	3,15
Met	31,416	3,17	±	0,46	6,78	±	3,08	2,74	±	0,15	9,39	±	5,66	8,26	±	1,13	5,68	±	0,46
Phe	37,961	2,43	±	0,41	15,35	±	10,76	1,54	±	0,42	28,12	±	10,05	27,80	±	0,89	22,45	±	0,68
Ile	32,725	4,67	±	0,60	6,48	±	7,29	-0,10	±	0,48	15,78	±	7,60	10,53	±	0,54	15,95	±	0,83
Leu	48,433	1,42	±	0,40	7,55	±	10,09	0,55	±	0,31	25,62	±	10,35	16,68	±	0,71	23,59	±	0,45
Pro	612,612	508,46	±	46,73	456,34	±	53,97	553,02	±	17,51	403,27	±	14,23	315,44	±	1,22	308,10	±	0,64
Trp	179,333	55,08	±	3,79	50,01	±	19,99	43,42	±	3,48	36,07	±	24,35	41,08	±	83,98	42,54	±	29,56
Lys	17,017	nd	±		nd	±		nd	±		nd	±		nd	±		nd	±	
Cys	13,09	nd	±		nd	±		nd	±		nd	±		nd	±		nd	±	

[a]nd: not detected; Lt: *L. thermotolerans*; Td: *T. delbueckii*; Pk: *P. kluyveri*; Mp: *M. pulcherrima*; Sc: *S. cerevisiae*; End: measured at end point of pure culture fermentation.

Supplentary Table 2 Concentrations of major volatile compounds (mg/L) at different initial YAN concentrations at the end of fermentation. (A): 300 mg/L YAN; (B): 150 mg/L YAN; (C) 75 mg/L YAN

A

	LtSc MS300	TdSc MS300	PkSc MS300	MpYSc MS300	MpFSc MS300	Sc MS300
∑ Higher alcohols	**220,33**	**131,88**	**143,65**	**200,56**	**196,06**	**91,89**
Methanol	nd	nd	nd	nd	nd	nd
Propanol	95,34 ± 16,64	nd ±	nd	59,71 ± 6,45	53,65 ± 10,63	nd
Isobutanol	12,84 ± 1,04	16,15 ± 1,33	23,90 ± 0,96	23,63 ± 0,89	25,50 ± 0,76	11,39 ± 0,13
Butanol	2,16 ± 0,43	0,73 ± 0,03	1,09 ± 0,04	0,65 ± 0,01	0,66 ± 0,02	0,51 ± 0,02
Isoamyl alcohol	84,66 ± 2,92	86,19 ± 3,24	96,69 ± 1,41	89,89 ± 6,32	87,94 ± 9,57	62,26 ± 0,05
Pentanol	nd	nd	nd	nd	nd	nd
3-methyl-1-pentanol	nd	nd	nd	nd	nd	nd
Hexanol	0,26 ± 0,001	0,25 ± 0,002	nd ±	0,32 ± 0,0001	0,31 ± 0,0005	0,25 0,001
3-ethoxy-1-propanol	3,40 ± 0,48	11,98 ± 0,50	5,67 ± 0,17	7,27 ± 0,24	7,29 ± 0,36	6,29 ± 0,06
2-Phenyl Ethanol	21,69 ± 1,05	16,57 ± 0,51	16,30 ± 0,27	19,10 ± 0,80	20,69 ± 0,37	11,20 ± 0,0004
∑ Short chain fatty acids	**3,25**	**4,31**	**6,25**	**4,50**	**4,32**	**4,14**
Propionic Acid	1,06 ± 0,12	1,16 ± 0,06	1,68 ± 0,02	1,54 ± 0,16	1,45 ± 0,14	1,24 ± 0,001
Isobutyric acid	0,79 ± 0,04	1,60 ± 0,09	2,22 ± 0,07	0,79 ± 0,05	0,77 ± 0,02	0,89 ± 0,04
Butyric Acid	0,40 ± 0,02	0,47 ± 0,004	0,68 ± 0,01	0,74 ± 0,04	0,70 ± 0,02	0,71 ± 0,001
Iso-Valeric Acid	0,53 ± 0,02	0,74 ± 0,004	1,23 ± 0,01	1,05 ± 0,07	1,02 ± 0,09	0,95 ± 0,001
Valeric Acid	0,48 ± 0,01	0,34 ± 0,01	0,43 ± 0,01	0,38 ± 0,04	0,37 ± 0,03	0,35 ± 0,001
∑ Medium chain fatty acids	**2,44**	**2,38**	**4,08**	**5,63**	**5,13**	**7,13**
Hexanoic Acid	0,50 ± 0,02	0,55 ± 0,04	1,07 ± 0,03	1,44 ± 0,11	1,31 ± 0,10	1,95 ± 0,004
Octanoic Acid	0,64 ± 0,04	0,85 ± 0,05	1,45 ± 0,04	2,12 ± 0,17	1,90 ± 0,12	2,79 ± 0,003
Decanoic Acid	1,30 ± 0,09	0,98 ± 0,14	1,55 ± 0,11	2,08 ± 0,12	1,92 ± 0,06	2,40 ± 0,002
∑ Ethyl esters	**0,93**	**5,54**	**6,56**	**9,35**	**9,35**	**6,93**
Ethyl phenylacetate	0,85 ± 0,01	0,16 ± 0,02	0,23 ± 0,01	0,70 ± 0,17	0,45 ± 0,09	0,82 ± 0,00001
Ethyl Lactate	nd ±	5,34 ± 0,03	5,88 ± 0,29	8,12 ± 0,44	8,45 ± 0,91	5,20 ± 0,01
Ethyl Caprate	0,08 ± 0,01	nd ±	0,13 ± 0,02	0,13 ± 0,02	0,11 ± 0,03	0,34 ± 0,0003
Ethyl Hexanoate	nd	0,04 ± 0,07	0,19 ± 0,01	0,26 ± 0,02	0,24 ± 0,03	0,27 ± 0,0001
Ethyl Caprylate	nd	nd ±	0,12 ± 0,01	0,14 ± 0,02	0,11 ± 0,03	0,30 ± 0,0003
Ethyl Butyrate	nd	nd	nd	nd	nd	nd
Ethyl-3-hydroxybutanoate	nd	nd	nd	nd	nd	nd
Diethyl Succinate	nd	nd	nd	nd	nd	nd
∑ Acetate esters	**46,54**	**0,34**	**8,40**	**55,07**	**43,39**	**41,09**
Ethyl Acetate	46,23 ± 2,22	nd ±	nd ±	54,47 ± 2,74	42,78 ± 4,25	40,72 ± 2,53
Isoamyl Acetate	0,04 ± 0,02	nd ±	3,75 ± 0,08	0,27 ± 0,03	0,27 ± 0,06	0,04 ± 0,002
Hexyl Acetate	nd	nd	nd	nd	nd	nd
2-Phenylethyl Acetate	0,27 ± 0,13	0,34 ± 0,003	4,65 ± 0,11	0,34 ± 0,003	0,35 ± 0,01	0,32 ± 0,0004
Carbonyl compound						
Acetoin	24,43 ± 8,60	38,65 ± 6,14	44,55 ± 6,98	50,27 ± 17,03	113,93 ± 11,09	3,86 ± 0,20
Acetic Acid	304,99 ± 6,26	387,65 ± 14,59	896,21 ± 13,68	693,31 ± 39,52	593,27 ± 5,00	639,35 ± 0,001

[a]nd: not detected; Lt: *L. thermotolerans*; Td: *T. delbueckii*; Pk: *P. kluyveri*; Mp: *M. pulcherrima*; Sc: *S. cerevisiae*; MS300: 300 mg/L YAN initially

B

	LtSc MS150	TdSc MS150	PkSc MS150	MpYSc MS150	MpFSc MS150	Sc MS150
∑ Higher alcohols	**240,20**	**227,36**	**146,81**	**216,21**	**236,61**	**88,03**
Methanol	nd	nd	nd	nd	nd	nd
Propanol	108,22 ± 6,99	93,27 ± 5,51	nd	67,18 ± 7,54	66,41 ± 10,51	nd
Isobutanol	15,34 ± 0,43	15,58 ± 0,31	23,02 ± 0,41	24,53 ± 4,41	30,56 ± 1,42	11,17 ± 0,13
Butanol	2,09 ± 0,23	0,84 ± 0,02	1,10 ± 0,04	0,60 ± 0,05	0,64 ± 0,05	0,53 ± 0,02
Isoamyl alcohol	87,85 ± 1,80	88,29 ± 2,03	100,02 ± 2,76	98,14 ± 9,42	108,35 ± 13,17	58,88 ± 0,05
Pentanol	nd	nd	nd	nd	nd	nd
3-methyl-1-pentanol	nd	nd	nd	nd	nd	nd
Hexanol	0,24 ± 0,001	0,24 ± 0,003	0,28 ± 0,002	0,30 ± 0,001	0,33 ± 0,02	nd
3-ethoxy-1-propanol	4,95 ± 0,34	12,99 ± 0,29	5,06 ± 0,33	7,10 ± 0,79	7,27 ± 0,77	6,41 ± 0,06
2-Phenyl Ethanol	21,49 ± 0,64	16,14 ± 0,45	17,32 ± 0,13	18,35 ± 0,12	23,06 ± 0,80	11,04 ± 0,0004
∑ Short chain fatty acids	**3,30**	**4,63**	**6,37**	**4,74**	**4,89**	**4,03**
Propionic Acid	1,14 ± 0,03	1,34 ± 0,01	1,84 ± 0,06	1,68 ± 0,11	1,63 ± 0,12	1,25 ± 0,001
Isobutyric acid	0,83 ± 0,03	1,65 ± 0,09	2,18 ± 0,09	0,84 ± 0,03	0,92 ± 0,07	0,86 ± 0,04
Butyric Acid	0,40 ± 0,01	0,49 ± 0,01	0,67 ± 0,01	0,74 ± 0,05	0,76 ± 0,03	0,69 ± 0,001
Iso-Valeric Acid	0,53 ± 0,01	0,81 ± 0,01	1,29 ± 0,05	1,08 ± 0,08	1,18 ± 0,12	0,89 ± 0,001
Valeric Acid	0,40 ± 0,01	0,34 ± 0,01	0,38 ± 0,005	0,40 ± 0,03	0,41 ± 0,01	0,34 ± 0,001
∑ Medium chain fatty acids	**2,17**	**1,81**	**4,21**	**6,02**	**5,87**	**7,47**
Hexanoic Acid	0,47 ± 0,01	0,55 ± 0,02	1,04 ± 0,02	1,57 ± 0,12	1,56 ± 0,04	2,04 ± 0,004
Octanoic Acid	0,62 ± 0,01	0,64 ± 0,01	1,52 ± 0,03	2,35 ± 0,18	2,32 ± 0,08	2,89 ± 0,003
Decanoic Acid	1,08 ± 0,10	0,61 ± 0,07	1,65 ± 0,06	2,11 ± 0,11	1,99 ± 0,15	2,54 ± 0,002
∑ Ethyl esters	**6,29**	**6,05**	**6,19**	**1,42**	**9,36**	**1,78**
Ethyl phenylacetate	0,81 ± 0,03	0,25 ± 0,02	0,29 ± 0,02	0,77 ± 0,22	0,73 ± 0,06	0,92 ± 0,00001
Ethyl Lactate	5,41 ± 0,03	5,55 ± 0,03	5,48 ± 0,29	nd ±	8,01 ± 0,06	nd ±
Ethyl Caprate	0,06 ± 0,002	0,06 ± 0,001	0,11 ± 0,01	0,18 ± 0,04	0,16 ± 0,02	0,29 ± 0,0003
Ethyl Hexanoate	nd	0,12 ± 0,002	0,19 ± 0,01	0,28 ± 0,03	0,29 ± 0,03	0,27 ± 0,0001
Ethyl Caprylate	nd	0,07 ± 0,001	0,12 ± 0,003	0,18 ± 0,04	0,18 ± 0,03	0,30 ± 0,0003
Ethyl Butyrate	nd	nd	nd	nd	nd	nd
Ethyl-3-hydroxybutanoate	nd	nd	nd	nd	nd	nd
Diethyl Succinate	nd	nd	nd	nd	nd	nd
∑ Acetate esters	**48,26**	**44,49**	**135,09**	**56,52**	**53,67**	**33,01**
Ethyl Acetate	47,87 ± 1,71	44,15 ± 2,79	126,58 ± 4,98	55,89 ± 3,59	52,99 ± 6,38	32,67 ± 2,53
Isoamyl Acetate	0,05 ± 0,01	0,01 ± 0,01	3,87 ± 0,18	0,30 ± 0,05	0,33 ± 0,07	0,01 ± 0,002
Hexyl Acetate	nd	nd	nd	nd	nd	nd
2-Phenylethyl Acetate	0,33 ± 0,01	0,34 ± 0,004	4,64 ± 0,11	0,33 ± 0,01	0,35 ± 0,01	0,32 ± 0,0004
Carbonyl compound						
Acetoin	23,58 ± 1,71	28,54 ± 5,01	50,37 ± 4,99	20,84 ± 11,93	22,38 ± 12,75	5,87 ± 0,20
Acetic Acid	313,75 ± 4,69	427,07 ± 8,30	891,73 ± 10,05	650,63 ± 22,39	597,58 ± 4,51	645,60 ± 0,001

[a]nd: not detected; Lt: *L. thermotolerans*; Td: *T. delbueckii*; Pk: *P. kluyveri*; Mp: *M. pulcherrima*; Sc: *S. cerevisiae*; MS150: 150 mg/L YAN initially

C

	LtSc MS75	TdSc MS75	PkSc MS75	MpYSc MS75	MpFSc MS75	Sc MS75
∑ Higher alcohols	**109,34**	**107,84**	**100,99**	**112,56**	**116,93**	**96,00**
Methanol	nd	nd	nd	nd	nd	nd
Propanol	nd	nd	nd	nd	nd	nd
Isobutanol	27,5 ± 4,86	24,72 ± 1,02	31,98 ± 0,17	30,98 ± 0,41	33,84 ± 0,68	26,84 ± 1,41
Butanol	1,5 ± 0,02	0,75 ± 0,03	0,83 ± 0,06	0,95 ± 0,08	0,89 ± 0,05	0,67 ± 0,04
Isoamyl alcohol	62,0 ± 3,83	51,38 ± 2,69	53,22 ± 1,63	58,16 ± 3,93	58,04 ± 2,99	51,20 ± 2,63
Pentanol	nd	nd	nd	nd	nd	nd
3-methyl-1-pentanol	nd	nd	nd	nd	nd	nd
Hexanol	0,33 ± 0,01	0,32 ± 0,003	nd	nd	nd	nd
3-ethoxy-1-propanol	4,27 ± 0,69	18,67 ± 2,13	4,10 ± 0,18	7,39 ± 0,82	6,37 ± 0,43	5,41 ± 0,56
2-Phenyl Ethanol	13,77 ± 0,67	12,00 ± 0,47	10,86 ± 0,22	15,08 ± 0,63	17,80 ± 0,83	11,87 ± 0,21
∑ Short chain fatty acids	**3,90**	**4,86**	**5,37**	**4,17**	**4,04**	**3,78**
Propionic Acid	1,61 ± 0,23	1,93 ± 0,07	2,95 ± 0,08	3,45 ± 0,32	3,35 ± 0,23	2,81 ± 0,29
Isobutyric acid	1,18 ± 0,15	1,81 ± 0,11	1,27 ± 0,06	0,71 ± 0,01	0,70 ± 0,01	0,96 ± 0,03
Butyric Acid	nd	nd	nd	nd	nd	nd
Iso-Valeric Acid	0,76 ± 0,14	0,78 ± 0,02	0,82 ± 0,02	nd	nd	nd
Valeric Acid	0,36 ± 0,02	0,34 ± 0,001	0,32 ± 0,01	nd	nd	nd
∑ Medium chain fatty acids	**3,67**	**2,75**	**3,65**	**3,62**	**3,60**	**4,76**
Hexanoic Acid	0,61 ± 0,06	0,57 ± 0,02	0,74 ± 0,01	0,94 ± 0,10	0,94 ± 0,12	0,92 ± 0,03
Octanoic Acid	0,78 ± 0,09	0,64 ± 0,04	1,07 ± 0,09	1,00 ± 0,08	0,98 ± 0,05	1,71 ± 0,14
Decanoic Acid	2,27 ± 0,14	1,53 ± 0,04	1,84 ± 0,04	1,69 ± 0,12	1,67 ± 0,10	2,13 ± 0,12
∑ Ethyl esters	**21,78**	**23,13**	**30,34**	**34,67**	**31,63**	**25,64**
Ethyl phenylacetate	0,69 ± 0,05	0,24 ± 0,02	0,33 ± 0,03	0,23 ± 0,01	0,19 ± 0,04	0,75 ± 0,07
Ethyl Lactate	7,99 ± 0,34	8,33 ± 0,30	8,87 ± 0,40	8,04 ± 0,73	7,68 ± 0,68	10,39 ± 0,34
Ethyl Caprate	nd	0,08 ± 0,002	0,09 ± 0,001	0,10 ± 0,01	0,10 ± 0,01	0,09 ± 0,001
Ethyl Hexanoate	nd	nd	nd	nd	nd	nd
Ethyl Caprylate	nd	nd	nd	nd	nd	0,12 ± 0,01
Ethyl Butyrate	12,52 ± 2,12	13,87 ± 1,33	20,49 ± 0,90	25,14 ± 1,81	22,48 ± 1,57	13,40 ± 1,71
Ethyl-3-hydroxybutanoate	nd	nd	nd	nd	nd	nd
Diethyl Succinate	0,58 ± 0,23	0,60 ± 0,29	0,56 ± 0,28	1,16 ± 0,01	1,17 ± 0,05	0,90 ± 0,25
∑ Acetate esters	**64,48**	**73,72**	**138,63**	**104,11**	**102,02**	**56,55**
Ethyl Acetate	64,48 ± 5,76	73,72 ± 4,57	135,00 ± 12,08	104,11 ± 5,13	102,02 ± 7,22	56,55 ± 4,52
Isoamyl Acetate	nd	nd	1,29 ± 0,17	nd	nd	nd
Hexyl Acetate	nd	nd	nd	nd	nd	nd
2-Phenylethyl Acetate	nd	nd	2,33 ± 0,20	nd	nd	nd
Carbonyl compound						
Acetoin	17,17 ± 1,48	14,98 ± 1,91	13,25 ± 3,59	13,95 ± 2,81	11,77 ± 3,87	25,40 ± 6,51
Acetic Acid	579,23 ± 65,80	580,95 ± 56,99	1124,24 ± 80,35	621,77 ± 69,86	546,93 ± 95,60	917,12 ± 70,56

[a]nd: not detected; Lt: *L. thermotolerans*; Td: *T. delbueckii*; Pk: *P. kluyveri*; Mp: *M. pulcherrima*; Sc: *S. cerevisiae*; MS75: 75 mg/L YAN initially

Supplementary Table 3 Representation of amino acids consumed per total sugars consumed (mg/g) during pure culture fermentation.

	LtYt48	Tdt48	Sct48	Pkt48	MpYt48	MpFt48
Asp	0,8844	0,25926	0,65775	3,35723	2,02391	3,64791
Glu	2,10275	0,99216	1,54945	11,0999	6,26654	10,9533
Asn	0	0	0	0	0	0
Ser	1,20233	0,95765	1,04164	2,99564	1,92206	3,36095
Gln	6,65253	9,89294	5,08412	7,45865	10,3556	19,4295
His	0,25964	1,47428	0,19644	-0,2339	-0,5433	-0,341
Gly	0,03076	0,63935	-0,0428	-0,2354	-0,1122	-0,236
Thr	1,09416	1,04827	0,83299	3,94058	2,22374	3,91536
Arg	3,22266	9,01475	0,83068	13,7671	4,93776	10,7061
Ala	1,82788	2,42261	1,10557	6,70919	3,59995	6,08122
GABA	0	0	0	0	0	0
Tyr	0,25577	0,27008	0,17698	1,12116	0,61566	1,08638
Cy2	-0,3118	0,50045	-0,2356	-2,3729	-1,4387	-2,5073
Val	0,707	0,61935	0,57358	3,2526	1,64502	3,15378
Met	0,5728	0,38092	0,44272	2,37279	1,02739	1,95138
Phe	0,80286	0,54851	0,54697	2,549	1,06575	1,80872
Ile	0,64107	0,33533	0,50496	1,81615	0,66458	1,09924
Leu	1,08807	0,45167	0,77649	4,67508	2,62465	5,23547
Pro	4,02816	11,5432	3,14821	17,8445	16,0906	23,2337
Trp	2,44809	2,52761	1,97205	11,2471	6,55553	11,2528
Lys	0,38658	0	0,27789	2,38178	1,30382	2,30583
Cys	0,29737	0	0,21376	1,83214	1,00294	1,77371

Supplementary Table 4 Concentrations of major volatile compounds between static vs agitation for end point fermentation samples (mg/L)

	ScC			ScSh		
∑ Higher alcohols	**98,69**			**269,78**		
Methanol	nd			nd		
Propanol	nd			nd		
Isobutanol	15,06	±	0,77	45,25	±	1,76
Butanol	0,64	±	0,04	0,61	±	0,05
Isoamyl alcohol	60,48	±	4,49	169,87	±	13,31
Pentanol	nd			0,09		0,002
3-methyl-1-pentanol	nd			nd		
Hexanol	nd			nd		
3-ethoxy-1-propanol	6,46	±	0,20	3,81	±	0,03
2-Phenyl Ethanol	16,05	±	0,40	50,14	±	7,14
∑ Short chain fatty acids	**6,16**			**8,52**		
Propionic Acid	1,91	±	0,19	2,95	±	0,07
Isobutyric acid	0,78	±	0,07	3,45	±	0,04
Butyric Acid	2,13	±	0,09	1,19	±	0,18
Iso-Valeric Acid	0,92	±	0,10	0,36	±	0,04
Valeric Acid	0,41	±	0,10	0,57	±	0,01
∑ Medium chain fatty acids	**7,36**			**4,28**		
Hexanoic Acid	1,81	±	0,06	1,11	±	0,03
Octanoic Acid	2,67	±	0,13	1,20	±	0,02
Decanoic Acid	2,89	±	0,07	1,97	±	0,07
∑ Ethyl esters	**13,87**			**16,19**		
Ethyl phenylacetate	1,32	±	0,07	1,87	±	0,02
Ethyl Lactate	6,70	±	0,14	6,22	±	0,01
Ethyl Caprate	0,12	±	0,01	0,12	±	0,001
Ethyl Hexanoate	0,28	±	0,01	0,21	±	0,01
Ethyl Caprylate	0,25	±	0,01	0,11	±	0,013
Ethyl Butyrate	4,68	±	0,18	5,07	±	0,10
Ethyl-3-hydroxybutanoate	nd			nd		
Diethyl Succinate	0,52	±	0,02	2,58	±	0,19
∑ Acetate esters	**37,09**			**63,98**		
Ethyl Acetate	36,34	±	1,86	63,15	±	10,39
Isoamyl Acetate	0,26	±	0,02	0,37	±	0,03
Hexyl Acetate	nd			nd		
2-Phenylethyl Acetate	0,48	±	0,01	0,46	±	0,030
Carbonyl compound						
Acetoin	5,65	±	0,79	34,02	±	11,83
Acetic Acid	898,77	±	38,56	1151,99	±	20,19

[a]nd: not detected; ScC: *S. cerevisiae* control; ScSh: *S. cervisiae* shaking

Chapter 4

General discussion and conclusions

4.1 General discussion

From as early as 1977, studies began to evaluate the importance of assimilable nitrogen to yeast growth and metabolism during wine fermentation and in particular the effect that nitrogen consumption has on fermentation performance and aroma compound production (Jiranek *et al*. 1995). These studies, however, were limited to the genus *Saccharomyces* and focussed mostly on optimizing Yeast Assimilable Nitrogen (YAN) concentrations in grape musts. These alterations included nitrogen additions at different times before and throughout fermentation, the supplementation of diammonium phosphate and/or the addition of complex sources of amino acids (Beltran et al. 2005; Hernández-Orte *et al*. 2006; Jiménez-Martí *et al*. 2007; Vilanova *et al*. 2007; Carrau *et al*. 2008; Garde-Cerdán and Ancín-Azpilicueta 2008; Barbosa *et al*. 2009). The sequential use of non-*Saccharomyces* yeasts together with *Saccharomyces cerevisiae* has been proposed to enhance possible positive contributions from the former towards wine complexity and aroma compound production. This interest in non-*Saccharomyces* yeasts shifted research investigations towards the influence that non-*Saccharomyces* yeast growth has on nitrogen availability for *S. cerevisiae* (Medina *et al*. 2012), since sluggish and stuck fermentations are still an occasional problem for winemakers. Thus, there is indeed a need investigate this issue, since very few authors have investigated the topic so far. In this study, we aimed to investigate nitrogen requirements of selected non-*Saccharomyces* yeasts to determine how much nitrogen is sufficient when using certain non-*Saccharomyces* yeasts together with *S. cerevisiae*, since nitrogen consumption is strain specific and also to link nitrogen consumption with production of major volatile compounds. To our knowledge, only one study hasinvestigated the actual amino acid preferences of non-*Saccharomyces* yeast (Kemsawasd *et al*. 2015). Thus, more research is needed to investigate nitrogen requirements of non-*Saccharomyces* yeasts and the impact on fermentation performance and growth of *S. cerevisiae* when used in mixed cultures.

As expected, our data confirmed that variations in nitrogen consumption between selected yeasts were observed. This finding has been reported in previous studies (Jiranek *et al*. 1995; Barrajón-Simancas *et al*. 2011). Some non-*Saccharomyces* yeasts preferred ammonia above alpha amino nitrogen during pure culture fermentation and vice versa. Overall, *Lachancea thermotolerans* and *Torulaspora delbrueckii* followed a similar YAN consumption pattern as *S. cerevisiae,* where *T. delbrueckii* consumed approximately the same amount of ammonia as *S. cerevisiae* and *L. thermotolerans* approximately the same amount of alpha amino nitrogen. This could mean that when these yeasts are used in co-culture with *S. cerevisiae*, direct competition for the same nitrogen sources most certainly occurs. Nonetheless, the two *Metschnikowia pulcherrima* strains and *Pichia kluyveri* barely consumed any YAN, which could mean that less competition for

nitrogenous compounds would exist when used sequentially with *S. cerevisiae*. This low consumption of YAN by the above-mentioned species might be linked to lower biomass production.

It was observed that the uptake of individual amino acids between the non-*Saccharomyces* yeasts was species specific. Furthermore, single amino acids were assimilated during the first 24 h, as observed during pure culture fermentations. This highlights the importance for optimizing nitrogen sources before the sequential inoculation of *S. cerevisiae* 24 - 48h after the inoculation of non-*Saccharomyces* yeasts by winemakers to avoid nitrogen limited conditions which could lead to poor growth and fermentation performance by *S. cerevisiae* which could result in sluggish or stuck fermentations. The single amino acid data provide a good beginning to better understand the uptake of individual amino acids by selected non-*Saccharomyces* yeasts.

Non-*Saccharomyces* yeasts can be favorable during wine fermentations, but due to their low fermentation abilities, they must be inoculated in mixed cultures with *S. cerevisiae* (Ciani *et al*. 2006; Comitini *et al*. 2011; Taillandier *et al*. 2014). In our study, sequential fermentations were therefore performed. Irrespective of the initial YAN concentrations, most of the ammonia and alpha amino nitrogen were assimilated within the first 60 h for all cultures. For treatments with an initial YAN of 300 and 150 mg/L, alcoholic fermentation was completed, although the fermentation rates for treatment MS150 were slightly slower than treatment MS300. As expected, an initial YAN concentration of 75 mg/L resulted in sluggish fermentation for all mixed cultures. Under our experimental conditions, it seemed as if the presence of non-*Saccharomyces* yeasts did not impact the fermentation kinetics when the initial YAN was lower, because the pure *S. cerevisiae* also resulted in sluggish fermentation with a low initial YAN. This is in disagreement with a previous study (Medina *et al*. 2012), which was conducted with different yeast species.

Since the assimilation of nitrogen sources influences the volatile aroma profile of wine (Jiménez-Martí *et al*. 2007; Vilanova *et al*. 2007; Carrau *et al*. 2008), our study also investigated volatile aroma compound production during sequential fermentations. All the mixed cultures for all treatments (MS300, MS150 and MS75) exhibited higher production of higher alcohols compared to pure *S. cerevisiae*. Co-culture LtSc produced the most higher alcohols, because of its high production of propanol and butanol. Acetic acid produced was highest in nitrogen limited conditions (MS75) for all cultures. The co-cultures MpYSc, MpFSc and PkSc exhibited high concentrations of fatty acids, but *S. cerevisiae* produced the most fatty acids. Cultures MpYSc, MpFSc and PkSc exhibited by far the highest concentration of esters. When comparing the different initial YAN concentrations, the production of higher alcohols and esters was overall higher under nitrogen limited conditions (MS75).

The differences between initial YAN treatments with regards to aroma compound production could be due to the maintenance of redox balance of the cell, since most higher alcohols are produced through the Ehrlich pathway. Moreover, the high production of specific compounds, especially higher alcohols, could be tentatively attributed to the uptake of specific amino acids. For example, LtSc showed the highest concentration of phenylethanol which could be correlated with the fact that *L. thermotolerans* assimilated the highest concentration of phenylalanine amongst the non-*Saccharomyces* yeasts studied. Likewise, MpYSc, MpFSc, and PkSc exhibited the highest concentrations of isoamyl alcohol and isobutanol. This result could be attributed to a higher uptake of leucine and valine per sugars consumed by the two *M. pulcherrima* strains and *P. kluyveri* compared to the other pure culture yeasts. Considering the specific nitrogen needs of selected yeasts tested in our study, these above-mentioned results increased our understanding of amino acid uptake in some non-*Saccharomyces* yeasts during the first 48 h, which can help winemakers for the optimization of amino acid additions to their alcoholic fermentations, considering the specific nitrogen needs of the yeast inoculated.

Neither the alpha amino nitrogen nor the ammonia was completely consumed in any of the treatments for all co-cultures. Indeed, regardless of the initial amount of YAN provided, the yeasts consumed about half of this amount. This is in contrast with studies carried out using similar fermentation setups (Crépin *et al*. 2014; Lage *et al*. 2014; Rollero *et al*. 2014; Taillandier *et al*. 2014; Viana *et al*. 2014). The major discrepancy between our study and the latter was the fact that in literature, the fermentation medium is constantly stirred. Indeed, in our setup, the flasks were only shaken at sampling times, to ensure sample homogeneity. A pure culture fermentation was therefore carried out with *S. cerevisiae* and the effect of static vs shaken fermentations was assessed. The results showed that with the agitated fermentation, the YAN was completely consumed within 24 h, as observed in literature. Once again, in the static fermentation, only half was consumed. Moreover, an overall improvement in fermentation kinetics as well as biomass production were observed. The differences in volatile aroma production between the two fermentation setups were also striking. This could mean that greater availability of nitrogen sources during the first 24 h is important, because it would suggest that even if nitrogen is still available after 24 h, the yeasts do not assimilate it; the first 24-48 h are therefore critical. However, the shaking speed (140 rpm) used during our study was extremely vigorous and poorly represents what happens in an industrial setup.

4.2 Future work

Nitrogen consumption in synthetic grape must may be different when compared to “real” grape must. Future studies should investigate nitrogen requirements of selected non-*Saccharomyces*

yeasts during pure culture and sequential fermentations performed in “real” grape must. It would also be useful to investigate which single amino acids are preferred by selected non-*Saccharomyces* yeasts by providing individual amino acids as the sole nitrogen sources and monitor the fermentation kinetics accordingly.

Since agitation had a significant effect on fermentation performance as well as aroma compound production, further investigations are needed to look into this interesting observation by repeating the experiment with more strains and at different stirring speeds.

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