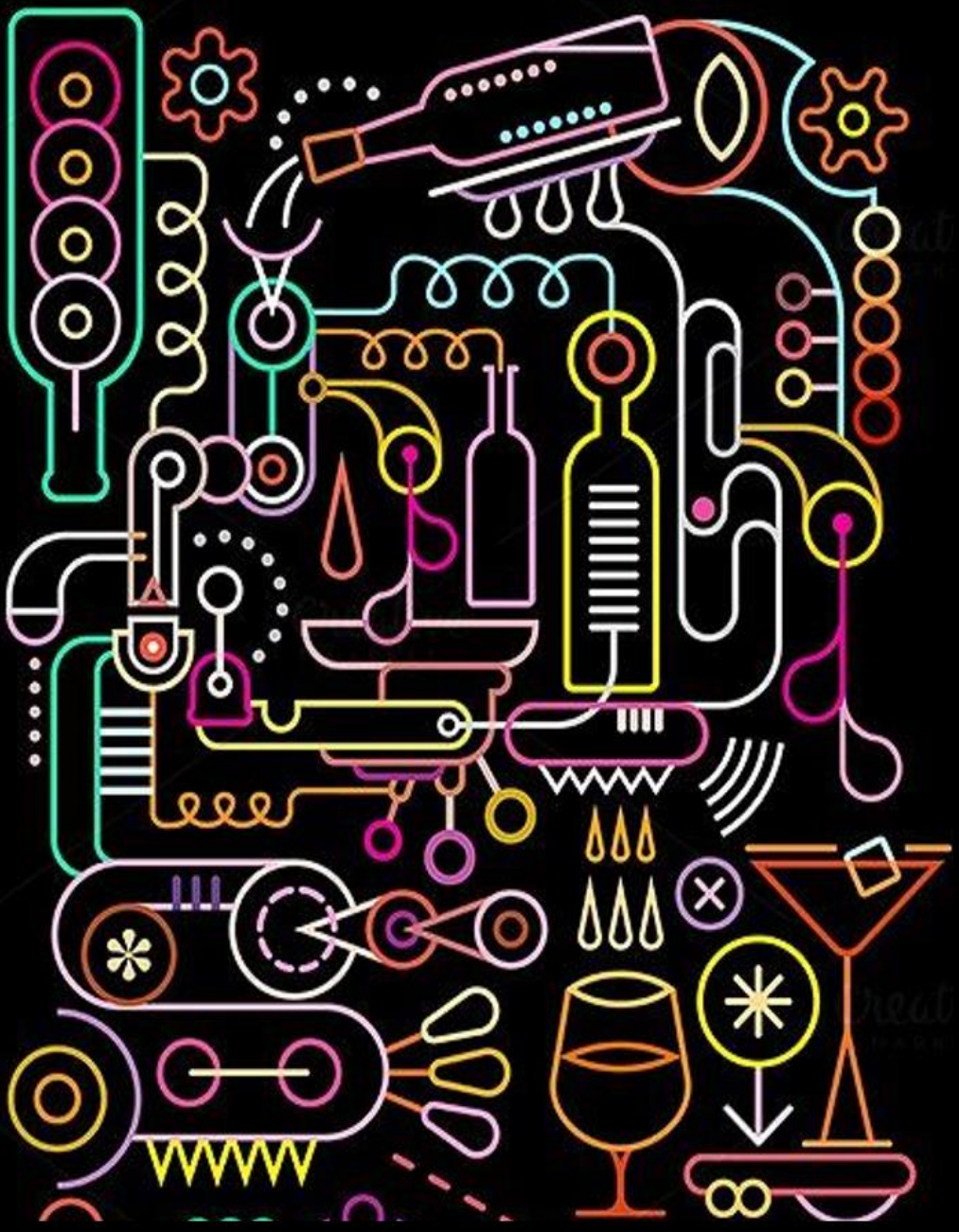
Nitrogen Metabolism of *Saccharomyces* and Non-*Saccharomyces* Wine Strains: Phenotypic and Genomic Characterisation

Ying Su



Nitrogen metabolism of *Saccharomyces* and non-*Saccharomyces* wine strains: phenotypic and genomic characterisation

PhD Thesis

Ying Su

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supervised by

Dr. José Manuel Guillamón Navarro

Dra. Amparo Querol

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Doctorado en Biomedicina y Biotecnología

El Dr. José Manuel Guillamón Navarro y la Dra. Amparo Querol Simón, profesores del Consejo Superior de Investigaciones Científicas (CSIC) en el Departamento de Biotecnología de Alimentos del Instituto de Agroquímica y Tecnología de Alimentos (IATA):

CERTIFICAN

Que Doña. Ying Su, Licenciada en Enología por la Northwest A&F University, ha realizado bajo su dirección el trabajo titulado: "Nitrogen metabolism of *Saccharomyces* and non-*Saccharomyces* wine strains: phenotypic and genomic characterisation", que presenta para optar al grado de Doctor en el programa de Biomedicina y Biotecnología por la Universitat de València. Asimismo, certifican haber dirigido y supervisado tanto los distintos aspectos del trabajo como su redacción.

Y para que así conste a los efectos oportunos, firma el presente certificado en Valencia, a 20 de Julio de 2020.

Fdo Dr. José Manuel Guillamón Navarro Fdo Dra. Amparol Querol Simón

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Introduction

1. Wine fermentation process

1.1 Brief history of viticulture and winemaking

"Il y a plus de philosophie dans une bouteille de vin que dans tous les livres." (A bottle of wine contains more philosophy than all the books in the world.)

-- Louis Pasteur

Wine, the magic liquid which stands together with the history of humankind, is one of the greatest inventions which shed light on our civilization. It is related to almost all the great transitions in human history and inspired the development of language, the arts, and religion (Jagtap et al., 2017). The first drop of wine may come out as a happy accident: the stored grape started to rot and then yeast on the surface of grape fermented the grape juice into wine (Chambers and Pretorius, 2010). Soon people learned to enjoy the taste and the pleasing psychotropic effect of the fermented grape juice. Indeed, the fermented grape juice can be stored for a longer time which solved the problem of preservation.

The first evidence of fermented beverages dates back to 7000 BCE from Jiahu, which was a Neolithic settlement based in the central plain of ancient China (McGovern et al., 2004). It is believed the Caucasus and Mesopotamia region are the provenance of wine (Pretorius, 2000). Grapes were one of the first dominated fruits in this region. Archaeological evidences traced back the origin of wine in Ancient Persia, the Caspian Sea shore (ca. 6000 BCE) and ancient Egypt (ca. 5000 BCE) (Chambers and Pretorius, 2010; Jagtap et al., 2017; Pretorius, 2000). Wine became a part of the ancient Greek diet by around 4000 BCE. By 1500 BCE, the Greeks, the Phoenicians and the Romans colonised southern Europe and northern Africa, and thrived grape

cultivation and winemaking in these regions. By around 100 BCE, the grape variety *Vitis vinifera* was introduced from Ferghana (in modern Uzbekistan) to China, and winemaking become popular around 220 BCE. During the age of discovery, in 1530, the Spanish explorers arrived to the New World and planted *Vitis vinifera* in Mexico, Argentina, Peru and Chile. In 1659, Dutch settlers planted French vine cuttings on the Cape of Good Hope of South Africa. Shortly after, the precious plant arrived at California, and more than a century later, with the arriving of the explorers, *Vitis vinifera* was also planted in Australia and New Zealand (Chambers and Pretorius, 2010; Jagtap et al., 2017; Pretorius, 2000; Pretorius, 2015) (Fig. 1).

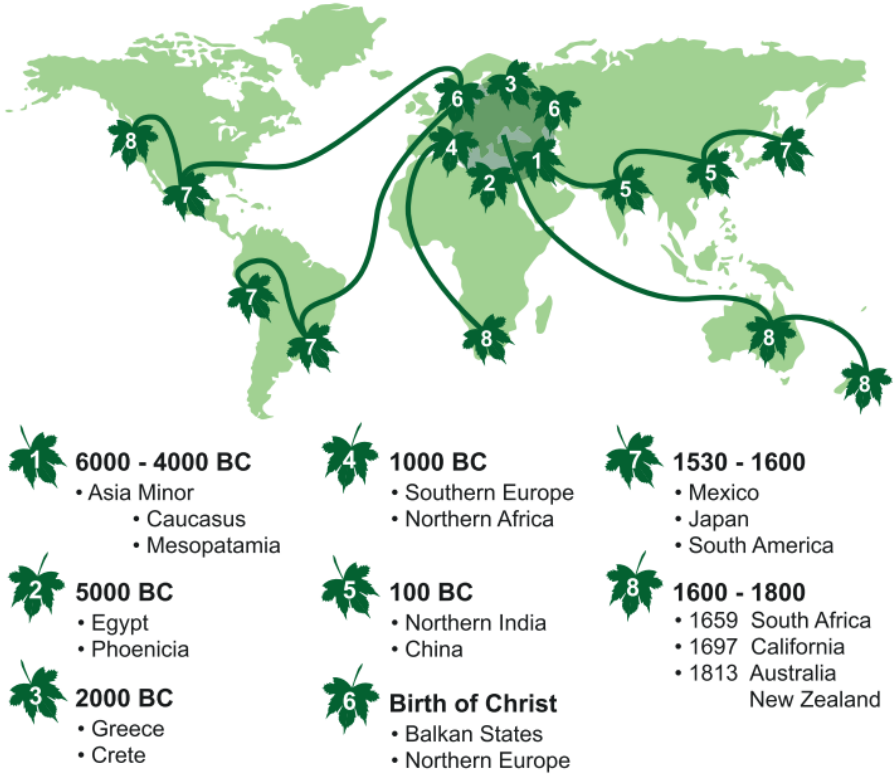

Fig. 1. World distribution of viticulture and winemaking (Jagtap et al., 2017)

1.2 Winemaking process

Depending on the type of wine and the variety of grape, winemaking process varies, but they all share some similar basis. Once harvested from the vineyard, the grapes are sorted, destemmed and crushed. Red wine is made from the red or black grape must, and fermentation is carried out in contact with the grape skins and seeds. On the other hand, for white wine production, maceration is normally avoided or controlled within a very short time. The crushed grapes are pressed to extract the juice and leave behind the skins and seeds and the fermentation of white wine only carried out with grape juice. Alcoholic fermentation is conducted either by the inoculation of yeast into must or may occur naturally with the yeast on grapes which is known as spontaneous fermentation. The fermentation process lasts between one and two weeks. Yeast converts most of the sugars into ethanol and carbon dioxide. After the primary fermentation, a secondary fermentation, malolactic fermentation, is carried out for most of red wine production and some white wine production. Lactic acid bacteria (LAB) are in charge of this process. They transform malic acid to lactic acid which softens the acidic taste of wine. The maturation of wine takes from months to years. Red wine is sometimes stored in oak barrels for maturation. (Fig. 2) The steps for wine making is depending on the goals of the winemaker, they can either be combined or omitted to achieve a certain purpose.

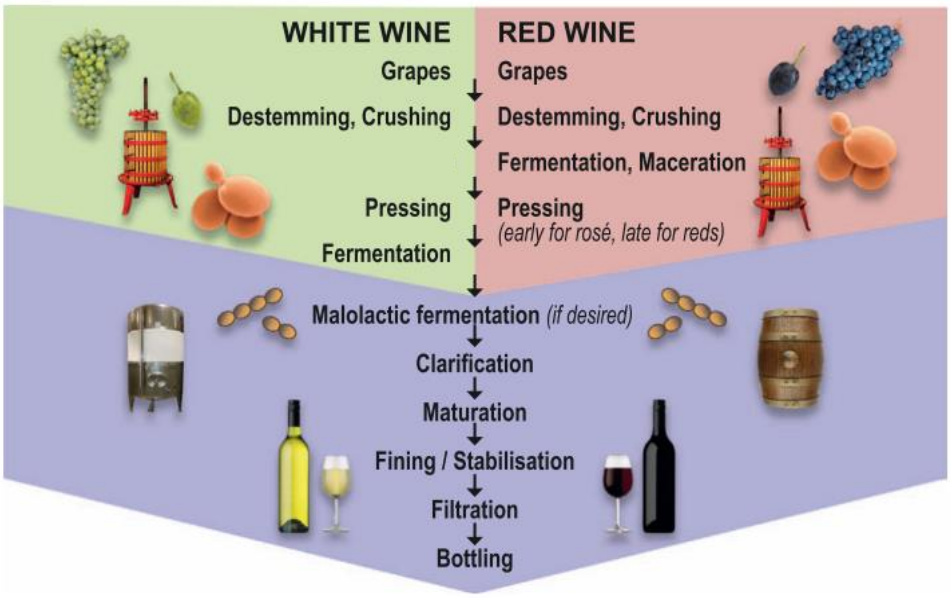

Fig. 2. White wine and red winemaking process. (Adapted from Jagtap et al., 2017)

2. Yeast species used in fermented beverages

2.1 Brief history of yeast research

Yeast research nowadays is prospering and fast-updating. However, it took a long journey to arrive at the recent research achievements. Thanks to the contribution of the great early scientists, we had the chance to explore the yeast realm standing on the shoulders of giants. The story started with Antonie van Leeuwenhoek (1632-1723), a Dutch merchant, who is also known as "the father of microbiology". He is most well-known for his pioneering work in microscopy and his contributions toward the establishment of microbiology as a scientific discipline. Through the handcrafted microscope of van Leeuwenhoek, first yeast cells were observed and the door to the magnificent world of microorganisms is opened. After the

observation of the first yeast cell, Louis Pasteur (1822-1895) proved the catalytic theory of Jöns Berzelius and Justus von Liebig were incorrect and first demonstrated that yeast was responsible for fermentation to produce alcohol from sugar. This important statement made a fundamental contribution to microbiology. Pasteur's research also revealed that the growth of microorganisms was responsible for spoiling beverages, such as beer, wine, and milk. To fight the "diseases" of wine, he invented a process in which liquids were heated to a temperature between 60 and 100 ℃ to kill most bacteria and moulds presented in the liquid. The method became known as pasteurization which still playing an important role nowadays (Barnett, 2000; Pasteur, 1857). It was not until 1883, the first pure culture yeast was isolated by the Danish mycologist and fermentation physiologist Emil Christian Hansen (1842-1909) at Carlsberg Laboratory. The yeast was named *Saccharomyces carlsbergensis*, which is the most important yeast for lager beer brewing until today. In 1890, the German oenologist Hermann Müller-Thurgau achieved the isolation of a pure culture of wine yeast and introduced the concept of inoculating wine ferments with specially selected pure yeast cultures which has become one of the common enological practices in modern winemaking (Dequin et al., 2001; Pretorius et al., 2015). James Watson and Francis Crick discovered the double-helix structure of deoxyribonucleic acid (DNA) in 1951. This helped the later unravelling of the genetic code of all living organisms. Yeast genetic research started during the 1930s and 1940s, with the pioneering work of Øjvind Winge and the work of Carl Lindegren. *Saccharomyces cerevisiae* comprises the first completely finished eukaryotic genome and was sequenced in the early 1996 by an international consortium of researchers from 19 countries working in 94 laboratories using several different sequencing methods and technologies (Goffeau et al., 1996). Now it

comes to today, the advanced technologies of robotics allowed high-throughput researches, which largely speeded up the scientific processes. Exciting discoveries about yeast is happening almost every day from everywhere of the world. Hopefully in the near future, yeast will be fully understood and completely domesticated for the benefit of human beings.

2.2 *Saccharomyces* genus

Saccharomyces genus is closely related to human activities. It is widely used for food and beverage production. *Saccharomyces* genus belongs to Fungi kingdom, *Ascomycota* division, *Saccharomycetes* class, *Saccharomycetales* order, and *Saccharomycetaceae* family. It is a nomadic yeast genus with no clear single habitat or niche that has been proposed (Goddard and Greig, 2015; Bisson, 2019). They have been isolated from different environments and substrates including deciduous tree bark, surrounding soil, tree exudates, fruits, insects and fermented beverages. *Saccharomyces* is from Greek σάκχαρον (sugar) and μύκης (mushroom) and means sugar fungus. Yeasts from *Saccharomyces* genus exhibit magnificent ability to ferment sugars vigorously to produce alcohol under both aerobic and anaerobic conditions (Piškur et al., 2006; Dashko et al., 2014; Guillamón and Barrio, 2017).

All the species included in *Saccharomyces* genus have typical budding shape morphology and possess 16 chromosomes. They are differentiated based on the sequences of their internal transcribed spacer (ITS) and 26S rRNA D1/D2 regions (Naseeb et al., 2017; Schoch et al., 2012; White et al., 1990). The members in *Saccharomyces* genus have been modified various times. According to the latest studies *Saccharomyces* genus including eight species: *S. cerevisiae*, *S. eubayanus*, *S. uvarum*, *S. kudriavzevii*, *S. paradoxus*, *S. mikatae*, *S. arboricolus* and *S. jurei* (Borneman and Pretorius, 2015; Boynton

and Greig, 2014; Hittinger, 2013; Naseeb et al., 2017) (Fig. 3). Moreover, recent studies have reported more species within the genus may exist and still waiting for the isolation from their environment (Legras et al., 2018; Peter et al., 2018).

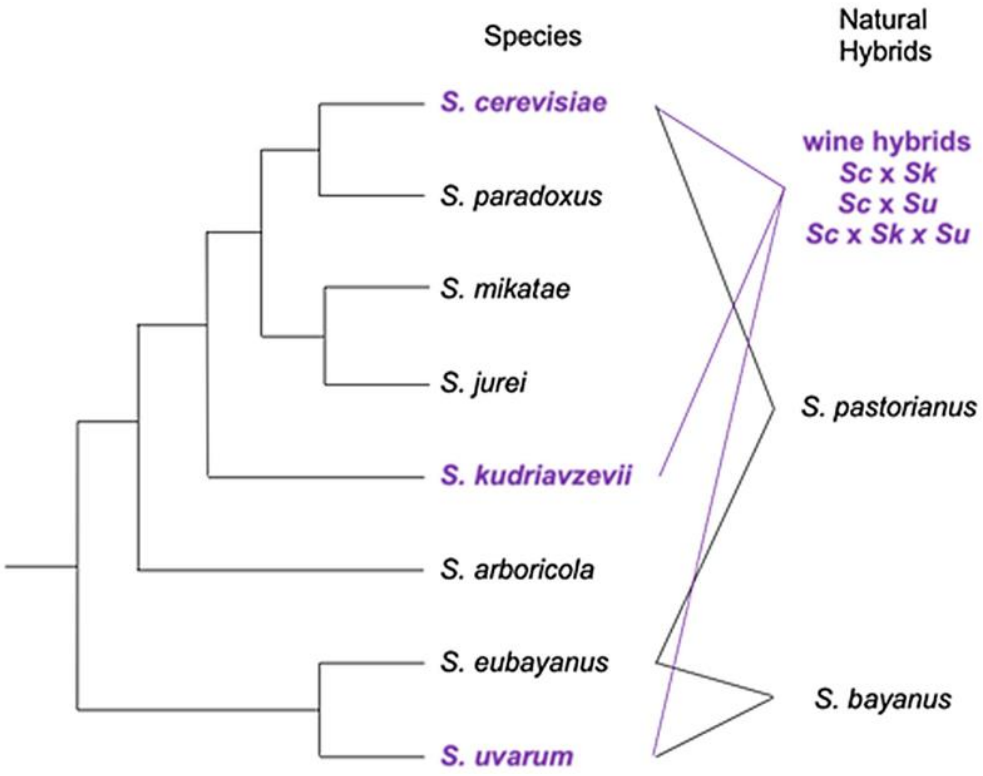

Fig. 3. Phylogeny tree of *Saccharomyces* genes (Vigentini et al. 2019).

Despite nucleotide sequence divergence between species is very high, lacking prezygotic barriers enables different species of *Saccharomyces* to mate and form viable diploids (Morales and Dujon, 2012). In nature, the boundary between these species is fuzzy, and numerous independently formed hybrids have been isolated from industrial fermentations (Boynton and Greig, 2014). Hybrids between *S. cerevisiae* and *S. kudriavzevii* has been isolated from wine, beer and cider (González et al., 2007; Peris et al, 2016; Peris et al., 2018;

Querol et al., 2018); Hybrids between *S. cerevisiae* and *S. uvarum* have been also isolated from wine (González et al., 2006; Le Jeune et al., 2007; Pérez-Torrado et al., 2018; Peris et al., 2018; Querol et al., 2018). The most well-known natural hybrids are *S. cerevisiae* x *S. eubayanus*, which is called *S. pastorianus*. It is the yeast species responsible for lager beer fermentation (Libkind et al. 2011).

2.2.1 *Saccharomyces cerevisiae*

Saccharomyces cerevisiae is arguably one of the most prominent microbial species in human history. It is widely used for the fermentation of bread, wine, beer, cider and other fermented food or beverages. Moreover, it can also be directly used as a functional food and supplement (Moslehi-Jenabian et al, 2010), and as a probiotic animal feed additive (Elghandour et al, 2020). *S. cerevisiae* extract is utilised in some cosmetic products for its moisturizing, wound healing, and cell renewal effects (Gaspar et al., 2008; Kim and Yun, 2006; Péterszegi et al., 2003). Additionally, *S. cerevisiae* is easy to manipulate genetically, and its life cycle is short, which makes it an attractive model organism for exploring gene functions and different mechanisms.

S. cerevisiae mainly reproduce asexually through vegetative multiplication; sexual reproduction by forming ascospores occurs when nutrients are exhausted. The cell division cycle in vegetative multiplication undergoes four stages which are G1 (period preceding DNA synthesis), S (DNA synthesis), G2 (period preceding the mitosis) and M (mitosis and cytokinesis). As soon as the mitosis is complete, the bud as daughter cell separates from the mother cell, as a consequence of this process both mother and daughter cell retain chitinous scar tissue at the point of cytokinesis (Powell et al., 2003). When sexual reproduction is initiated, diploid cells undergo meiosis and form four

haploid spores of two opposite mating types (a and α) encapsulated by an ascus. Meiotic spores are highly resistant to various stresses. When favourable, nutrient-rich conditions are provided, haploid spores germinate and eventually re-establish diploid lines, either by mating with their own mitotic daughter cells after switching mating type (haplo-selfing), by mating with another spore created by the same meiotic event (intra- tetrad mating) or, more rarely, by mating with an unrelated individual (outcrossing) (Knop, 2006) (Fig. 4).

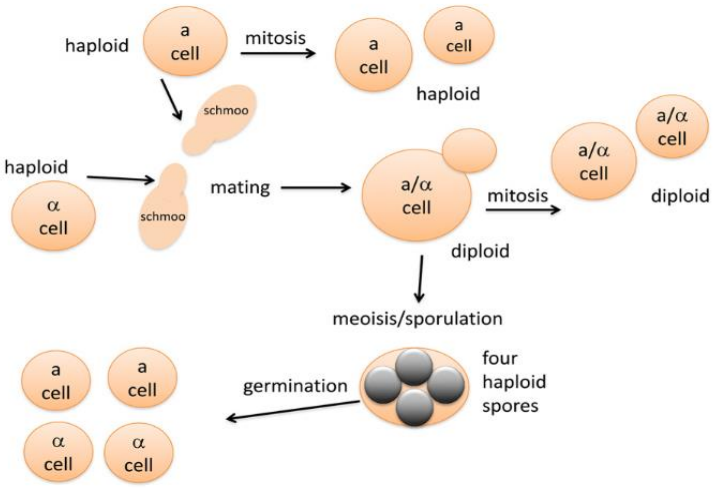

Fig. 4. *S. cerevisiae* life cycle

Saccharomyces cerevisiae is mainly found in fermentation environments related to human activities. They also exist in fruits and insects, in humans as a commensal or pathogen (Angebault et al., 2013; Muller et al., 2011), in soil, on various plants (Wang et al., 2012) and oak trees (Sniegowski et al., 2002; Sampaio and Gonçalves, 2008). Although it has been isolated from different natural environments, there is insufficient evidence concerning the niche or niches that *S. cerevisiae* might be abundant in or adapted to (Goddard and Greig, 2015; Liti, 2015). Although *S. cerevisiae* is the dominant yeast in

spontaneous fermentations, their existence on the skin of grape is not as abundant as we expected. Before maturation, grapes are almost free of *S. cerevisiae* (~0.05%), whereas 25% of ripe damaged grapes harbour such cells (Mortimer et al., 1994; Polsinelli et al., 1996). Goddard and Greig (2015) proposed a nomadic model, which described that *S. cerevisiae* is not adapted to a specific niche, but it is a nomad, able to survive as a generalist at low abundance in a wide range of environments. As *S. cerevisiae* is not airborne, a vector is required to move around. Evidences revealed insects, such as social wasps, play the role as vector and natural reservoir of *S. cerevisiae*. Queens of social wasps overwintering as adults (*Vespa crabro* and *Polistes spp.*) can harbour yeast cells from autumn to spring and transmit them to their progeny (Stefanini et al., 2012). Moreover, the intestine of *Polistes dominula* social wasps provides favourable environmental condition for the mating of *S. cerevisiae* strains (Stefanini et al., 2016).

S. cerevisiae has a relatively small genome containing roughly 6000 protein-encoding genes (Goffeau et al., 1996). These genes distribute along 16 linear chromosomes ranging from 200-2200 Kb in length. Haploid strains contain approximately 12-13 Mb of nuclear DNA. *S. cerevisiae* was the first eukaryote completely sequenced. With the development of next-generation sequencing (NGS) technology, whole-genome sequencing of *S. cerevisiae* strains isolated from different regions in the world and different isolation environment was carried out frequently (Peter et al. 2018; Wang et al., 2012, Legras et al., 2018). The global phylogeny of *S. cerevisiae* strains indicates that strains can be grouped not only by their geographical origin but also by their fermentative process (Fay and Benavides, 2005; Legras et al., 2007; Legras et al., 2018; Liti et al., 2009, Peter et al., 2018) (Fig. 5). The sequencing results largely help the understand of *S. cerevisiae* evolution

history. It has revealed a single "out-of-China" origin for *S. cerevisiae*, followed by several independent domestication events (Peter et al., 2018).

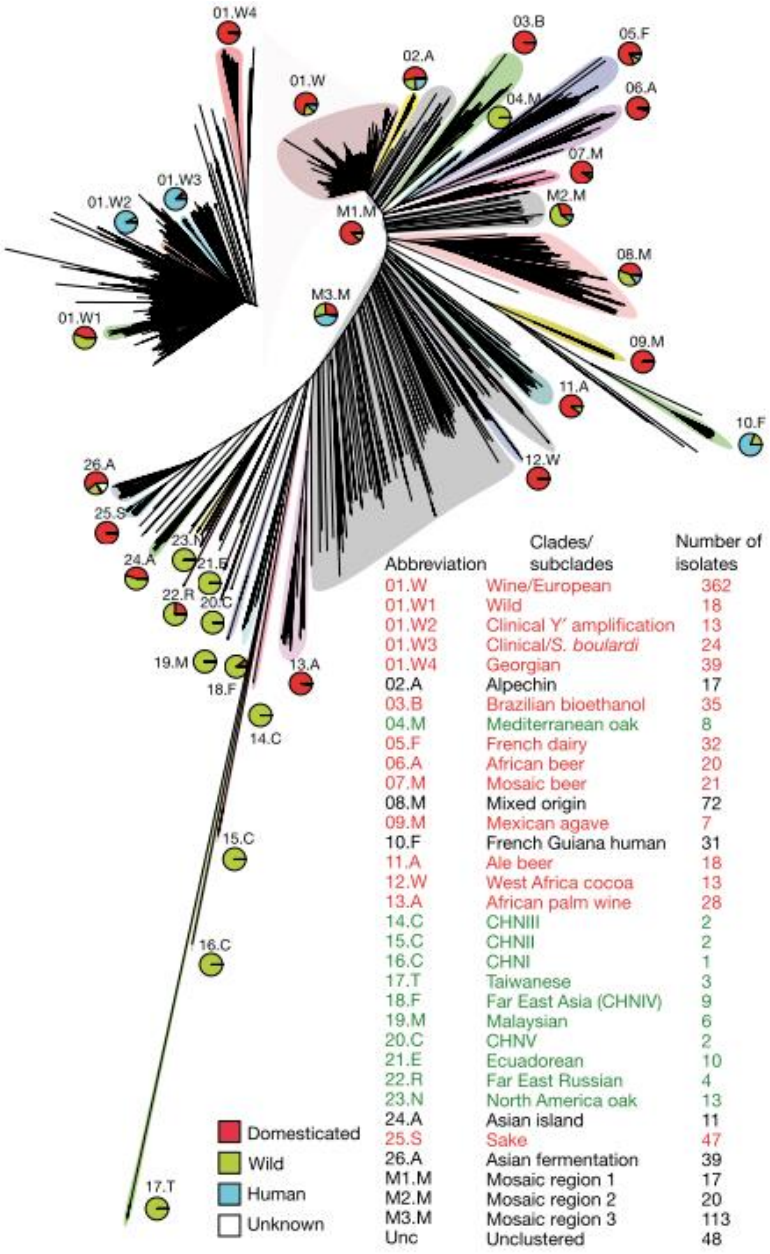

Fig. 5. Neighbour-joining tree built using the biallelic SNPs of 1105 *S. cerevisiae* strain isolated from different origins (Peter et al., 2018)

2.2.2 *Saccharomyces* non *cerevisiae*

Saccharomyces eubayanus

S. eubayanus is a relatively new member in *Saccharomyces* genus. It was not until 2011, the first *S. eubayanus* was isolated and identified by Libkind et al. (2011). In the forest of Northwestern Patagonia, samples collected from environment associated with *Nothofagus* spp. (southern beech), including the bark, the soil and stromata of *Cyttaria hariotii* (an obligate ascomycete parasite of *Nothofagus app*.) first unveiled the existence of *S. eubayanus* (Libkind, 2011). This discovery of the "mystery" non-*cerevisiae* progenitor of *S. pastorianus* is finally settled. After the first isolation, other isolates have also been subsequently obtained in North America (Peris et al., 2014), China (Bing et al., 2014), Patagonia (Rodr ́guez et al., 2014), and New Zealand (Gayevskiy and Goddard, 2016). However, it has only been identified in natural habitats with no isolates that has been obtained from human related fermentation environment. The hybridisation between *S. cerevisiae* and *S. eubayanus* to form *S. pastorianus* can be viewed as a case of unintentional artificial selection. The hybrid is thought to be associated with the emergence of lager beer in Bavaria in the XVth century. Therefore, the isolation of *S. eubayanus* in Europe is expected. However, till now, no isolation has been obtained from Europe. Libkind et al. (2011) hypothesised that early trans-Atlantic traders introduced *S. eubayanus* into the European brewing environment from South America, where it hybridized with *S. cerevisiae*. Alternatively, Bing et al. (2014) suggested that instead of brought from south America, *S. eubayanus* was introduced to Europe from Tibet via the Silk Road since the non-*cerevisiae* progenitor has higher sequence similarity with

Tibetan *S. eubayanus* (99.8%) than with South American *S. eubayanus* (99.4%).

The cryotolerant character of *S. eubayanus* is easy to be identified because the isolation sites are normally at high altitude with low temperature. It is better adapted to growing temperatures at 8-15 ℃, and/or that have a maximum growth temperature in the range 33-35 ℃ (Sampio, 2018). Unlike its well-studied hybrids *S. pastorianus*, about the fermentative characters of *S. eubayanus* is not well-known. The brewing properties of *S. eubayanus* were compared to those of *S. pastorianus*, and it was observed that *S. eubayanus* had better growth performance at low temperatures (10 ℃) and unable to use maltotriose. Nevertheless, as a cryotolerant species *S. eubayanus* performed poorly at 22 ℃ when compared to *S. pastorianus*. The artificially constructed hybrid between *S. eubayanus* and *S. cerevisiae* inherited beneficial brewing properties from both parents and it can ferment faster and produce more ethanol than the parents (Hebly et al., 2015). Origone et al., (2017) studied the stress tolerance ability of *S. eubayanus* under winemaking conditions. The results revealed that in general, *S. eubayanus* is less robust than *S. uvarum* under a combination of different stress conditions including temperature, pH, sugar and SO_2 concentration. However, *S. eubayanus* has better performance than *S. uvarum* in ethanol tolerance when ethanol concentration is below 8%. Ethanol concentration higher than 8% inhibits completely the growth of *S. eubayanus*.

The discovery of *S. eubayanus* not only solved the puzzle of the parents of *S. pastorianus*, but also provided a wild yeast with interesting fermentation abilities. Recently, a beer solely fermented by *S. eubayanus* has been brought to the market (Sampio, 2018).

Saccharomyces uvarum

S. uvarum is a cryotolerant yeast species commonly found in fermented beverages. It was originally described by Martinus Willem Beijerinck in 1894, however it was long considered as a synonym of *S. bayanus* or as one of its two varieties (Vaughan-Martini and Kurtzman, 1985). Pulvirenti et al. (2000) demonstrated that *S. uvarum* is genetically distinct from *S. bayanus*, and finally proposed it as a proper species within the complex *Saccharomyces* sensu stricto (now known as *Saccharomyces* genus). *S. uvarum* occupies a basal position in the phylogeny of *Saccharomyces* genus. It is the sister species of *S. eubayanus*, and *S. bayanus* is a hybrid between *S. uvarum* and *S. eubayanus* (Almeida et al., 2014; Peris et al., 2014; Pérez-Través et al., 2014).

S. uvarum has mainly been isolated from fermentation environments, such as wine, cider, and the traditional Patagonian drink, apple chica (Demuyter et al., 2004; Naumov et al., 2000b, 2001, 2002; Rodríguez et al., 2014). The wines typically fermented by *S. uvarum* are Tokaj (Hungary, Slovakia) (Naumov et al., 2002; Sipiczki et al., 2001), Amarone (Italy) (Torriani et al., 1999), and Txakoli (Spain) (Rementeria et al., 2003); and in France, Sauternes and the whites of northern French vineyards in Burgundy, Champagne, Val de Loire and Alsace (Almeida et al., 2014; Demuyter et al., 2004; Naumov et al., 2000b). Apart from the fermentation environment, *S. uvarum* also occurs on hardwood bark, soil, and insects, and often co-occurs with *S. eubayanus* (Almeida et al., 2014).

In winemaking, *S. uvarum* has a different fermentation profile than that of *S. cerevisiae*. *S. uvarum* has shown higher production of glycerol and lower production of ethanol and acetic acid (Giudici et al., 1995; González Flores et al., 2017; Pérez-Torrado et al., 2016; Rodríguez et al., 2014). Additionally,

S. uvarum has been characterized by its higher capability to release desirable flavor components, such as 2-phenylethanol and 2-phenylethyl acetate (Gamero et al., 2013; Masneuf-Pomarède et al., 2010). All these characters together with the cryotolerant feature make *S. uvarum* a good candidate to fulfil various fermentation requirements.

Saccharomyces kudriavzevii

S. kudriavzevii is another important cryotolerant species within *Saccharomyces* genus. It was first isolated from decaying leaves in Japan (Naumov et al., 2000a). Before obtaining more isolations, natural hybrids of S. *cerevisiae* x *S. kudriavzevii* and triple hybrid *S. cerevisiae* x *S. bayanus* x *S. kudriavzevii* have been isolated in wine fermentation in Europe (Gonzalez et al., 2006). Shortly after, more isolates of *S. kudriavzevii* were obtained from oak trees in Portugal and Spain (Lopes et al., 2010; Sampaio and Gonçalves, 2008).

Apart from the tolerance to low temperature, *S. kudriavzevii* possesses some interesting characters which are highly appreciated in winemaking. In comparison to *S. cerevisiae*, during wine fermentation *S. kudriavzevii* produce higher amount of glycerol and lower ethanol (Gonzalez et al., 2007; Pérez-Torrado et al., 2017; Peris et al., 2016). This not only helps to achieve the goal of making wine of lower ethanol content, but also provide slight sweetness to wine which reduces the astringency, and round, smooth texture which gives fuller wine body (Goold et al., 2017). Moreover, despite they have been isolated only from natural environments far from wineries, *S. kudriavzevii* strains produce larger amounts of higher alcohols and 2-phenylethanol (rose aroma) at low temperatures (Stribny et al., 2015). Given the preferential features of *S. kudriavezevii*, it made an ideal complementary

yeast species to be used in wine fermentation either by collaboration with *S. cerevisiae* or construct interspecific hybrids.

2.3 Non-*Saccharomyces* species related to winemaking

The sugar rich grape juice supports the growth of many different microorganisms. Traditionally, wine fermentation was carried out in a spontaneous way, which allowed yeast existing on grapes or in the ambient to grow and ferment together. During the early stage of spontaneous fermentation, non-*Saccharomyces* yeast strains predominant the grape must. With the increase of ethanol concentration, *S. cerevisiae* gradually outcompete the non-*Saccharomyces* strains and become the dominant yeast species until the end of fermentation (Fleet, 1998). However, the spontaneous fermentation carried out by a complex mixture of microorganisms do not always end up as well as expected. The wine quality was variable, unreliable and sometimes undrinkable. These non-*Saccharomyces* yeasts were originally considered as responsible for the problematic fermentations since they were often isolated from spoiled wine. However, later studies demonstrated that some non-*Saccharomyces* yeast species can benefit wine quality by improving aroma complexity, mouth-feel and showing distinct characters bringing by the indigenous yeast species (Heard, 1999; Soden et al., 2000; Varela et al., 2009). The role of non-*Saccharomyces* yeast species in winemaking was described by Jolly et al. (2013) as a double edged sword. Avoiding the contamination of spoilage yeasts and taking good use of positive yeast species can be appropriate suggestions for the winemaking process.

Despite the sustained presence of certain non-*Saccharomyces* species, the majority disappear gradually during the early stage of vigorous alcoholic fermentation (Fleet et al., 1984; Henick-Kling et al., 1998). The cell death

may be caused by their slow growth, slow fermentation metabolism and inhibition by the combined effects of SO_2, low pH, high ethanol and oxygen deficiency (Heard and Fleet, 1988; Combina et al., 2005). In addition, the population of *S. cerevisiae* in the mixed culture largely influences the existence of other yeast species, which is not only caused by the competition of nutrients but also caused by the cell-cell contact mechanism. For *T. delbrueckii* and *Lachancea thermotolerans*, the cell-cell contact with a high concentration of *S. cerevisiae* inhibits their growth (Nissen et al., 2003).

Non-*Saccharomyces* yeasts are not suitable to carry out wine fermentation as the sole strain. They are often used in cooperation with *S. cerevisiae*. In the recent decade, the utilisation of non-*Saccharomyces* has received growing interests in winemaking industry. Global climate change has deeply influenced the grape composition, resulting in grapes with lower acidity, altered phenolic maturation and tannin content, and increasing sugar content (Jones et al., 2005). The higher sugar concentration in grape must consequently increases alcohol content in wines. Although early harvest can help us to get grapes with ideal sugar content, the phenolic maturity of the grape is often not reached when harvested before the ripeness, which in turn influence the organoleptic complexity of wine. Wines with too high ethanol content are unbalanced and are unpleasant for consumers. It not only increases hotness and bitterness perception but decreases acidity sensation and masks the perception of some important aroma compounds (Ciani et al., 2016; Frost et al., 2015). Non-*Saccharomyces* yeast species are able to carry out fermentation with reduced ethanol yield, for the reason that the metabolic flux distribution is different between *S. cerevisiae* and non-*Saccharomyces* yeast species during fermentation (Ciani et al., 2016). In addition to reduce ethanol concentration, several non-*Saccharomyces* species, particularly *L.*

thermotolerans and *C. zemplinina* are outstanding glycerol producers (Ciani and Ferraro, 1998; Comitini et al., 2011; Soden et al., 2000). Glycerol positively contributes to wine with sweetness, smooth texture and full body. Moreover, some non-*Saccharomyces* strains can decrease volatile acidity and acetic acid concentration (Comitini et al., 2011, Padilla et al., 2016), or increase succinic acid concentration (Ciani and Maccarelli, 1998; Ferraro et al., 2000). Some aroma compounds are present as odourless precursors which need to be hydrolysed by enzymes such as β-glucosidase and β-lyases to produce free volatiles that can improve the aroma complexity. Many studies have observed the increased concentration of the terpenols or thiols in mixed-culture fermentations (Cordero Otero et al., 2003; Garcia et al., 2002; Sadoudi et al., 2012; Seguinot et al., 2020). Some commonly used non-*Saccharomyces* species and their beneficial characters is summarised in Fig. 6.

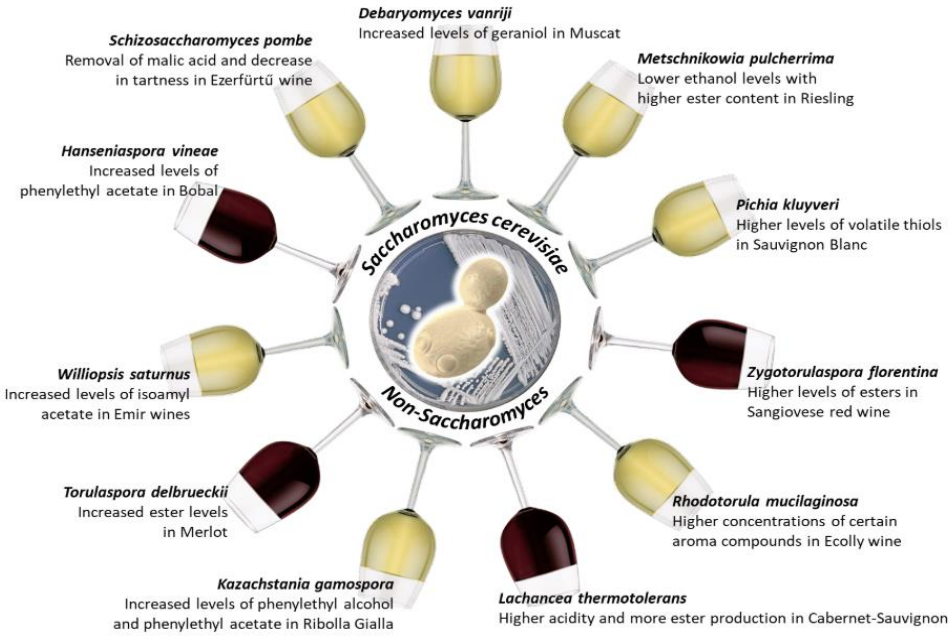

Fig. 6. Examples of the effect of mixed fermentations with non-*Saccharomyces* strains on aroma profile of different wines (van Wyk et al., 2019).

All the preferable features described above together with other beneficial characters such as control of the spoilage wine microflora (Oro et al., 2014), the release of mannoproteins (Domizio et al., 2014) or wine colour stabilization (Morata et al., 2012; Loira et al., 2015; Chen et al., 2018) make non-*Saccharomyces* yeasts a multifunctional toolkit to improve wine quality. Single or mixed non-*Saccharomyces* strains with outstanding fermentation behaviours have been commercialised. The inoculation of selected non-*Saccharomyces* and *S. cerevisiae* strains can help winemaker to meet consumers' requirements.

2.3.1 *Torulaspora delbrueckii*

One of the first commercialised non-*Saccharomyces* yeast was *Torulaspora delbrueckii*. It was formerly classified within the *Saccharomyces* genus (under *S. rosei* or *S. roseus* name). *T. delbrueckii* can be used for both red and white wine making. Comparing to other non-*Saccharomyces* species that can be considered for winemaking, *T. delbrueckii* has outstanding fermentation performance and ethanol tolerance. In pure culture, it can ferment until ethanol concentration reach around 9% (*v*/*v*) (Ciani and Maccarelli, 1998; Benito, 2018), whereas the resistance of most non-*Saccharomyces* yeast to ethanol is no higher than 4% (*v*/*v*) (Benito, 2018). In sequential inoculation with *S. cerevisiae*, due to the nutrients competition and cell-to-cell contact mechanism, the viability of *T. delbrueckii* decrease as soon as *S. cerevisiae* is inoculated (Taillandier et al., 2014). The addition of assimilable nitrogen partially eases the inhibition by *S. cerevisiae*.

Although *T. delbrueckii* has higher tolerance to ethanol, many studies of sequential fermentations between *T. delbrueckii* and *S. cerevisiae* have reported from 0.15 to 0.5% (*v*/*v*) decreases in ethanol production compared to

S. cerevisiae controls (Belda et al., 2017; Cus and Jenko, 2013; Puertas et al., 2017). However, the production of another important metabolite, glycerol, increases in sequential fermentations from 0.1 g/L to approximately 1 g/L (Belda et al, 2017; González-Royo et al., 2015; Medina-Trujillo et al., 2017; Puertas et al., 2017). High concentration of acetic acid (> 0.8 g/L) which gives vinegar like off-flavour to wine is undesirable in fermentations. In *T. delbrueckii* and *S. cerevisiae* sequential fermentation, decreases in acetic acid concentration from 0.13 to 0.27 g/L were observed (Chen et al., 2018; Medina-Trujillo et al., 2017; Taillandier et al., 2014). In pure culture fermentation of *T. delbrueckii* the decrease is more substantial and a reduction of 0.63 g/L was evidenced (Ciani and Maccarelli, 1998).

Sequential inoculation of *T. delbrueckii* and *S. cerevisiae* influence the production of volatile compounds in several aspects. It increases the concentration of some esters such as ethyl propanoate, ethyl isobutanoate, isobutyl acetate and isoamyl acetate (Belda et al., 2017; Renalt et al., 2015). There is a discrepancy in the description of higher alcohol productions, which some studied reported reduced higher alcohols production comparing to *S. cerevisiae* control (Belda et al., 2017; Sadoudi et al., 2012), while other authors observed increases in higher alcohols (Azzolini et al., 2015). The highly strain depended feature may be explained by the complexity and regulation of the Ehrlich pathway, which is responsible for the production of these compounds and varies greatly among strains (Gamero et al., 2016). Some strains of *T. delbrueckii* contribute to a higher synthesis of terpene compounds and volatile thiols (Belda et al., 2017; Cus and Jenko, 2013; Renault et al., 2016; Whitener et al., 2017). Terpene compounds provide floral and fruity aromas in wine and it reflects the varietal character of some terpenic grape varieties. Similarly, thiols are also highly desired aroma

compounds in wine for their pleasant tropical fruit odour. Thiols appear in grapes in their non-volatile precursors. Some *T. delbrueckii* strains possess high β-lyase activity which helps the release of thiols from the odourless precursors (Renault et al., 2016).

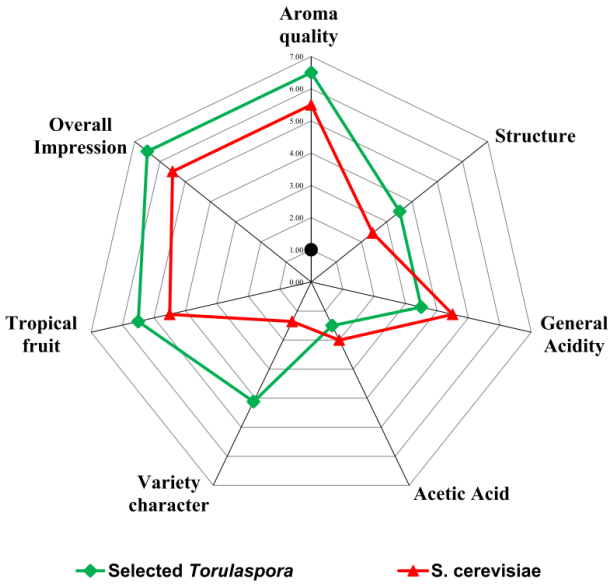

Fig. 7. Influence of *T. delbrueckii* on sensory perception of fermented products compared with fermentation by *S. cerevisiae* (Benito, 2019)

Overall, some *T. delbrueckii* strains are suitable to improve the sensory profile of wine. Fig. 7 shows the summary of the influences on sensory perception by sequential inoculation of *T. delbrueckii* in comparison to *S. cerevisiae* pure culture fermentations. However, several of these beneficial properties are highly strain dependent (Loira et al. 2014; Azzolini et al. 2015; Escribano et al. 2018). Therefore, it is of great significance to select *T. delbrueckii* strains with preferable fermentation characters for winemaking.

2.3.2 *Metschnikowia pulcherrima*

Metschnikowia pulcherrima is a ubiquitous yeast that has been found in fruits (fresh and spoiled), flowers, nectars and tree sap fluxes (Morate et al., 2019). It is also one of the prevalent non-*Saccharomyces* yeast species used for wine fermentation. Unlike *T. delbrueckii*, the fermentative ability of *M. pulcherrima* is relatively low (Contreras et al., 2014). Many strains are able to ferment until reaching 4% (*v*/*v*) of ethanol (Comitini et al., 2011). Strains with ethanol production up to 6-7% (*v*/*v*) have also been observed (Combina et al., 2005). In sequential or mixed cultures with *S. cerevisiae*, viable cells of *M. pulcherrima* decrease rapidly after a few days of fermentation because of the low resistance to the ethanol produced by *S. cerevisiae* (Sadoudi et al., 2012; Wang et al., 2016). This species has also been associated with a reduction in ethanol production in sequential fermentation with *S. cerevisiae* (Contreras et al., 2014; Quirós et al., 2014; Varela et al., 2016). This can be explained by the aerobic respiratory metabolisms of *M. pulcherrima*. Instead of fermentation, *M. pulcherrima* is able to aerobically metabolise more than 40% sugars under suitable aeration conditions. One of the outstanding characters of *M. pulcherrima* is its enzymatic activities. Enzymes such as β-glucosidase and β-lyase can hydrolyse the precursors and release terpenes and thiols which represent the varietal aromas from the grapes (Seguinot et al., 2020; Zott et al., 2011).

M. pulcherrima can be also used as a biological control agent. The antimicrobial activity could be associated with the secretion of pulcherrimic acid, which forms a red pigment named pulcherrimin when it is linked to the iron ions. The production of pulcherrimin depletes the free iron in the medium. In this way, the environment becomes inhospitable to other microorganisms

that require iron for their development (Morata et al., 2019; Sipiczki, 2006; Türkel and Ener, 2009). However, *S. cerevisiae* seems not to be affected by this antimicrobial activity (Oro et al., 2014). The combination of all these characters makes *M. pulcherrima* a perfected candidate to inhibit the growth of spoilage microorganisms and to work together with *S. cerevisiae* to ferment wine with more aroma and less ethanol.

3. Different approaches for yeast strain improvement

Yeasts are a group of organisms with high diversity. No need to mention the differences among species, even strains that are classified as the same species often show a high level of genetic divergence (Steensels et al., 2014). The diversity among yeast strains provide abundant genetic pool for the election of strains to serve different industrial purpose. Despite the immense wealth of natural yeast diversity, the extremely selective and specific conditions of industrial fermentations sometimes require (a combination of) phenotypic traits that might not be commonly encountered in nature. Therefore, artificially improved selected yeast strains to fulfil industrial purpose is necessary. Several ready to use techniques have been developed along the domestication of natural yeasts (Fig. 8). In the following sections, yeast hybridisation, directed evolution, mutagenesis and genomic modification is introduced.

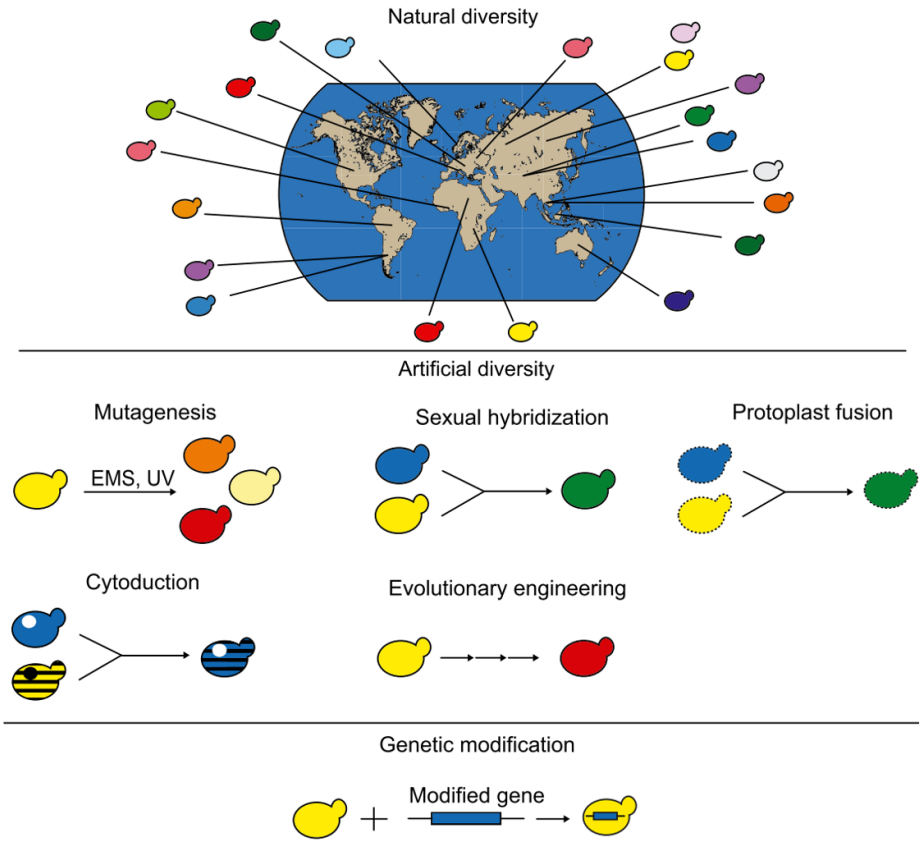

Fig. 8. Overview of strategies to obtain superior industrial yeast strains (Steensels et al., 2014).

3.1 Hybridisation

One of the most prevalent strategies for yeast strain improvement is hybridisation. This technique has been applied by humans for thousands of years in agriculture to choose superior plants from their cultivations. As described in the previous sections, the process of sexual hybridisation naturally occurs in the environment. The modern technologies have paved the way for more targeted and large-scale approaches for yeast breeding. This targeted breeding of yeast strains allows the scientists to generate new strains possessing different characteristics of the selected parents, or to optimise a

single, often complex, trait, by crossing parents selected for the same phenotype. The newly constructed hybrid strains not only obtain the preferable characters from parental strains, but they can also exceed the phenotypic boundaries of the parental strains, which is a phenomenon called heterosis, or hybrid vigour (Lippman and Zamir, 2007). This phenomenon has attracted numerous studies to carry out intraspecific, interspecific, even intergeneric hybridisations. The main approaches used by these studies for sexual hybridisation are direct mating, rare mating, mass mating and genome shuffling. Besides, asexual hybridisation methods like protoplast fusion and cytoduction were also carried out for strains which are not able to sporulate or with low spore viability (Fig. 9).

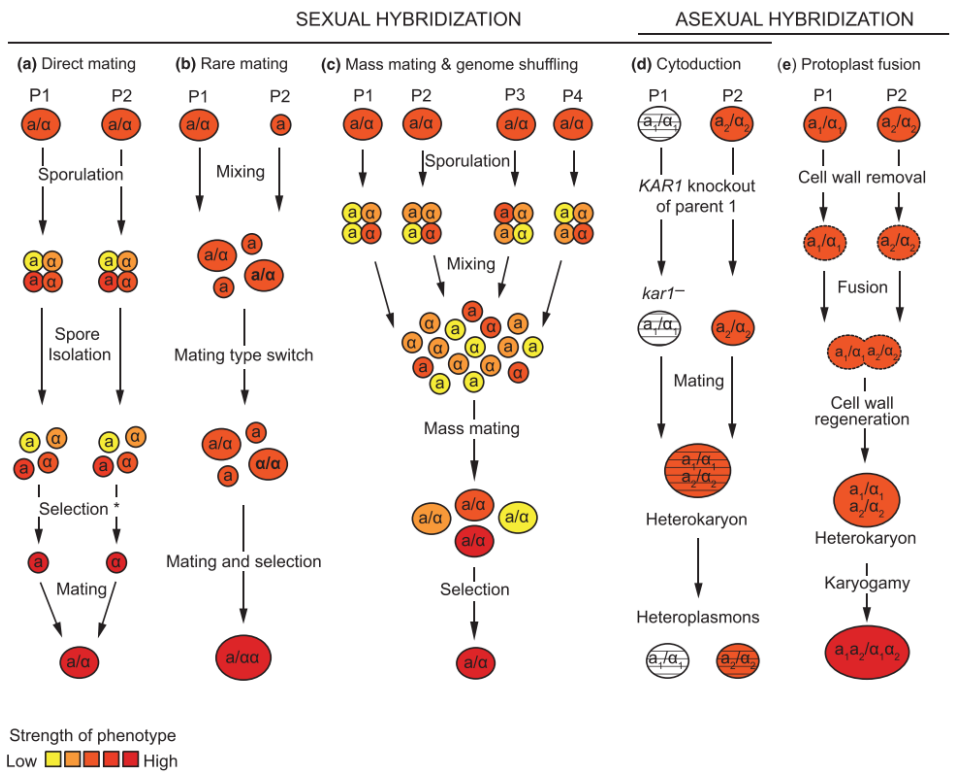

Fig. 9. Overview of different strain improvement techniques using hybridization (Steensels et al., 2014).

Direct mating

Depending on the sexual cycle of the parental strain, direct mating can be classified into three different approaches which are cell-to-cell, spore-to-cell, and spore-to-spore mating. Cell-to-cell mating is carried out simply by mixing two selected stable haploid parents with opposite mating type and subsequent screening for diploid cells, hybrids can be isolated. The main advantage of cell-to-cell mating is that the parental strains are stable haploid cells, therefore they can be carefully phenotyped before mating process, and obtain the hybrid with superior characters. The mating is relative easy to control since no inbreeding can occur. However, since most of industrial related yeast strains are homothallic, this approach is not suitable to be applied. Although the disruption of *HO* gene, which is a gene responsible for mating-type switching, can help to obtain stable heterothallic strains, the resulting hybrids are considered as genetically modified organisms (GMO).

The alternative methods are cell-to-spore or spore-to-spore mating. In these cases, one or both of the parental strains are homothallic, therefore, no stable haploid cells can be obtained for prescreening, and the mating type of the spores are unknown. The mating process is carried out by placing two spores of the strains to be hybridised close to one another on an agar surface by using a micromanipulator. The mating event is monitored, and if the development of zygotes is observed, it is likely a hybrid is successfully constructed. This method is suitable when both parental strains are homothallic or when the hybridisation efficiency of the two parental strains is low, such as interspecific hybridisation experiment (Steensels et al., 2014).

Although the process is time consuming, direct mating has proven to be an effective way to obtain hybrids (Sipiczki, 2008). It has been widely applied

especially for interspecific hybrid construction. The typical examples that many studies have generated hybrids between *S. cerevisiae* and cryotolerant *Saccharomyces* strains such as *S. kudriavezevii*, *S. eubayanus* and *S. uvarum* (Garcia Sanchez et al., 2012; Origone et al., 2018; Pérez-Través et al., 2012). The new hybrids obtain both the outstanding fermentation features of *S. cerevisiae* and the cool tolerance feature of the non-*cerevisiae* parent.

Rare Mating

Some industrial yeasts that have low mating frequencies and low spore viabilities, or that do not mate or sporulate at all. In these cases, rare mating provides an opportunity to construct hybrids without sporulation. The rare mating method depends on the fact that occasional mating-type switching occurs in industrial yeasts, which are normally diploids or of higher ploidy, and may display aneuploidy as well. This results in the occurrence at a low frequency of mating cells, that can then conjugate with a cell with known mating type (Gunge and Nakatomi, 1972). Hybrids isolation requires a strong selection step. Therefore, the parental strains used for hybridisation normally carry selective features such as respiratory-deficiency or auxotrophy (Pretorius, 2000). Consequently, the successfully constructed hybrids are expected to be respiratory proficiency or prototrophy.

Since the frequencies of mating type switch in diploid cells are low, the efficiency of rare mating is correspondingly very poor. However, rare mating has been successfully applied by many studies to construct interspecific hybrids. Recent studies carried out by Pérez-Través et al. (2012), García Ríos et al. (2019), Magalhães et al. (2017) constructed hybrids to improve the cryotolerance of wine or cider yeasts by rare mating. Other studies also used rare mating to generate interspecific hybrids of *S. cerevisiae* and other species

of *Saccharomyces* genus to diversity the flavour profile of wines (Bellon et al., 2011; 2013).

Mass mating

After the ascospores are generated by the homothallic yeast strains, the ascus walls are removed and the spores from different parental strains are mixed and allowed to mate randomly. This technique is called mass mating. Mass mating can significantly increase the chances of successful mating, since a larger population of spores are involved. Therefore, this technique is suitable for constructing hybrids between homothallic strains which show low mating efficiency. Moreover, if the interspecific hybrids carry strong selective markers, mass mating is a convenient technique that can improve the mating efficiency. Mass mating has been applied to generate hybrids with improved characteristics. Recently, Krogerus (2015) has constructed new lager yeast hybrid strains between *S. cerevisiae* and *S. eubayanus* by using mass mating method. The hybrids inherited beneficial properties from both parent strains such as cryotolerance, maltotriose utilisation and strong flocculation, and showed apparent hybrid vigour.

Genome shuffling is a relatively new technique to improve complex phenotypes in yeast strains and it is conceptually related to mass mating (Gong et al., 2009). In a heterogeneous population, different cells may harbour different beneficial mutations. Genome shuffling of these cells can be achieved by applying repeated rounds of genetic recombination and selection to this population (Fig. 9). The aim is to combine all the beneficial characters in the same cell. This method is mainly used in modifications of yeast strains for biofuel production (reviewed by Steensels et al., 2014), and few studies were carried out to improve strain for winemaking.

3.2 Mutagenesis

Mutagenesis is one of the common methods to introduce genetic alterations of yeast which contribute to desired phenotypic characters. The *in vivo* random mutation is induced either by chemical mutagens such as EMS and MNNG or physical mutagens such as UV and ionising radiation. Different mutagens have a different effect on genetic alteration (Table 1). For mutagenesis experiment, since the mutation is random, it is often difficult to predict which kind of mutagen is more suitable to obtain a certain phenotype. Therefore, several rounds of mutagenesis carrying out by using different mutagens are suggested (Rowlands, 1984).

Table 1. Genetic alteration induced by different mutagens (Summarised by Steensels et al., 2014).

	Mutagen	Mode of action	Genetic alteration
Physical	UV	Mitotic crossing over; mitotic gene conversion; pyrimidine dimers; hydroxylated bases; cross-linking DNA strands; reverse mutations	Frameshift mutations, base pair substitutions, transversions
	Ionising radiation	Single- and double-strand breaks in DNA; deamination and dehydroxylated bases	Point mutations
Chemical	EMS	Alkylation	GC-AT transitions
	MNNG	Alkylation, acts close to replication points	Transitions, transversions; clustered mutations

Other factors influencing the result of mutagenesis are mutagenic dose and exposure time. Naturally, a low dose of mutagen will induce less mutations, on the contrary, a high dose can generate mutants with more change, which provide a higher possibility to achieve the desired modifications. However, high dose of mutagen or long exposure time may generate some deleterious mutations, leading to a large fraction of inferior or even unviable cells. Therefore, it is important to choose an optimal dose that gives the largest proportion of beneficial mutants out of all cells that manage to survive. A killing curve that represents the cell viability with increasing dosage to mutagen may help to estimate the proper dosage to be applied. In general, for simple phenotypes that require one or few mutations, the fraction of desired mutants per survivor increases with increasing dose and reaches saturation before the increasing of mutants with deleterious mutations (Crook and Alper, 2012). In the case of complex phenotypes which are influenced by many different genes, the ideal dose and the dose-response curve are more difficult to predict. Industrial strains are normally diploid and only dominant mutations can alter the phenotype. As a method to increase the efficiency of mutagenesis, spores generated directly from the homothallic strains can be used. After the mutagenesis process, the spores will autodiploidise and increases the chance of obtaining the targeted mutation (Romano et al., 1983).

The random mutagenesis method has been applied in wine and brewing industry to generate yeast strains with desired traits. The classical aim of mutagenesis in winemaking is restricted to the elimination of unwanted monogenic traits (Giudici et al., 2015). Some early studies have obtained strains with auxotrophy for a specific amino acid, therefore the production of the corresponding alcohol is reduced, leading to a general improvement of wine flavours (Reviewed by Giudici et al., 2015). More recently, mutants of

a commercial wine strain with reduced H_2S production was obtained by Cordente et al. (2009).

3.3 Directed evolution

Throughout evolution, natural selection promotes the survival of specific organisms at the expense of thousands with trait/s that are not optimal to live in a given environment. Directed evolution also known as "evolutionary engineering" is a method that by mimicking the mutation, recombination and selection processes that occur naturally in evolution to obtain yeast strains that reach specific goals. Directed evolution in yeast is mainly based on the genetic variance among a population of cells and the subsequent selection acting on this variation (Giudici et al., 2005). The cells are grown under continuous selection for the interested characters for many generations. During the process, random mutants may occur and the mutants with a fitness advantage will be enriched. Therefore, an evolved strain with desired traits will be finally obtained. This method can also be used in combination with mutagenesis or sexual hybridisation to increase the efficiency of yeast strain improvement. Since yeast cells have short generation time and are easy to manipulate, directed evolution is a feasible method to achieve yeast strains with improved phenotypes (Buckling et al., 2009; Elena and Lenski, 2003).

Directed evolution can be achieved by several experimental setups such as serial passaging of cells in batch culture, and continuous batch culture like chemostat system. Additionally, the evolution of populations can also be carried out directly in industrial setting where they are to be employed. The most representative example is in beer fermentation, where the brewer's yeast is often harvested and repitched from one batch to another. This process unintentionally selected strains with adjusted phenotypes (Gibson et al, 2007).

In industry fermentation conditions, cells are facing various stresses such as high osmolarity, high levels of ethanol and nutrient deprivation. The selection performed under these harsh conditions allows the isolation of multi-stress tolerant strains. Combining with mutagenesis method, several studies have improved the fermentation capacity of the lager fermentation yeast *S. pastorianus* in high-gravity wort (Blieck et al., 2007; Huuskonen et a., 2010). For winemaking, Cadière et al. (2011) successfully evolved a commercial yeast strain with increased flux through the pentose phosphate pathway by long-term batch culturing the yeast on gluconate as sole carbon source. The evolved strain showed enhanced fermentation rate, decreased formation of acetate and greater production of fermentative aroma (Cadière et al., 2012). Yeast strain with significantly enhanced glycerol production (41% more than the original strain) was obtained by Kutyna et al., (2011) by carrying out adaptive evolution with sulphite at alkaline pH as a selective agent. More recently, Tilloy et al. (2014) conducted evolution based on sequential increases of osmotic/saline stress to divert carbons toward glycerol and away from the production of ethanol. The evolved strain was used for hybridisation and a final strain with lower ethanol and higher glycerol production was obtained.

The development of the new generation sequencing method has made it easier to sequence the genomes of evolved strains. By combining the sequencing result with the expression analysis of evolved clones, it is easier for the scientists to decipher how a specific phenotype is established. This valuable information can in turn guide other methods of strain improvement.

3.4 Genetic modification

Genetic modification is a well-established method used in numerous biological studies for different purposes. Although the technique is widely accepted in different fields, such as pharmaceutical industry, the application of the genetically modified organisms (GMOs) is still strictly controlled in food and beverage industry. However, it can be used as an efficient tool for the study of gene functions which assist yeast strain modification by non-GMO methods. Comparing with non-GMO methods which are described in the previous sessions, genome modification can precisely change the one or several genes in a specific, predetermined manner to get the desired phenotypes without negatively affecting other important characteristics.

Genetic modification often involves the uptake and incorporate foreign extracellular DNA. The transformation protocols have been evolved through many decades from using the intact cells (Hinnen et al., 1978) to the most used method in nowadays improved by Gietz and Woods (2001) and Gietz and Schiestl (2008). This high efficiency method is mediated by LiAc in combination with polyethylene glycol (PEG) and single-stranded carrier DNA. There are two major ways to efficiently express foreign DNA in yeasts, which are using plasmids and fixed integration in the host's genome (or a combination of both) (Steensel, et al., 2014).

Two major ways to efficiently express foreign DNA in yeasts are using plasmids or fixed integration in the host's genome. Different types of plasmids are available which allow variation of the copy number of the introduced DNA fragment. The commonly used yeast episomal plasmids (YEp) allow introduction of 10-40 copies per cell. On the other hand, yeast centromeric plasmids (YCp) allows the 1-2 copies per cell introduction

(Christianson et al., 1992; Clarke and Carbon, 1980; Romanos et al., 1992). Low-copy number YCp vectors carry an origin of replication and a centromere sequence which allows for high segregational stability for the plasmid under selective conditions. However, the segregational stability of multicopy plasmids are limited, which can lead to copy number variation between cells in the same population. Comparing to introducing DNA by plasmids, integrating DNA directly into the yeast genome by homologous recombination is a more stable, robust and repeatable way for the gene expression (David et al., 2015). Vector- or PCR-based generated DNA fragments are generally used for insertion. Homologous recombination is highly efficient in *S. cerevisiae*: small flanking regions of homology (30–45 bp) are sufficient for targeted integration into the yeast genome (Manivasakam et al., 1995). By using the genome integration method, a comprehensive set of gene deletion mutants in *S. cerevisiae* has been obtained which provided valuable tool box for yeast genetics study (Giaever et al., 2002; Winzeler et al.,1999).

For many genetic engineering processes, a selective marker is required for the validation and maintenance of the integrated sequences. The frequently used markers are drug-resistance markers (such as *kan*^{r}, *ble*^{r}) and auxotrophic nutritional markers (such as *URA3*, *ADE2*, *LEU2*). The number of selective marker is limited. When the modification of multiple genes in the same background is required, it is difficult to use the available markers to cover the needs (Delneri et al., 1999). In many cases, the retention of the selective marker is not desired in the genome after the modification, or the same marker is to be used for the modification of another gene, therefore, a marker recycling method was developed to solve the problem. Two good systems for gene recycling are Cre-*LoxP*-mediated recombinase and *delitto perfetto*

(Storici and Resnick, 2003). Cre-*LoxP* system is a powerful and ready-to-use tool for yeast gene knockouts. Many cassettes with different markers were constructed by Güldener et al. 1996 and Gueldener et al., 2000. The marker cassette is flanked by two direct repeats of the 34 bp *LoxP* sequence, which can be converted into a highly efficient gene disruption cassette by the addition of short DNA sequences homologous to the genomic locus of interest using PCR. Once correctly integrated into the genome, the marker can be efficiently rescued by transformation with a plasmid carrying the gene for Cre recombinase under the control of the *GAL1* promoter and induction of Cre expression by shifting the cells to galactose-containing medium. Cre-induced recombination results in loss of the marker cassette, leaving behind a single *LoxP* site at the original integration site (Gueldener et al., 2000). *Delitto perfetto* is another widely used technic for genome alterations. The method is based on the utilisation of counter-selectable reporter (CORE) and the replacement of CORE by a 140-base pair double-stranded molecule that contains 70 bp homologous up- and down-stream of the CORE cassette (Storici et al., 2001). This technique allows seamless modification with no foreign DNA left in the genome.

Unlike the well-studied haploid laboratory strains, industrial yeast strains are normally diploid or polyploidy with a more complex genetic background. Applying the classical PCR-based strategy for the genetic manipulation of industrial strains are normally very laborious, time consuming and sometimes even infeasible (Stovicek et al., 2015). In the recent decade, the development of an alternative genome editing approach, clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) 9 mediated genome editing, can help to solve the problem (DiCarlo et al., 2013; Stovicek et al., 2015). CRISPR systems contain two components: a guide RNA (gRNA)

and a CRISPR-associated endonuclease (Cas protein). The gRNA is comprised of a scaffold sequence necessary for Cas-binding and a user-defined ~20 nucleotide spacer that defines the genomic target to be modified. The 20 nucleotide spacer should be unique compared to the rest of the genome and should be designed to be immediately adjacent to a protospacer adjacent motif (PAM), which serves as a binding signal for Cas9. The Cas9 nuclease is guided to the genomic target DNA by gRNA and then performs the cleavage 3-4 nucleotides upstream of the PAM sequence resulting in a double-strand break (DSB). DSB must be repaired by cells via non-homologous end-joining (NHEJ) or homologous directed recombination (HDR) (Liu et al., 2017, Fig. 10). The NHEJ pathway is error-prone and often causes small nucleotide insertions or deletions at the DSB site, which consequently results in amino acid deletions, insertions, or frameshift mutations leading to premature stop codons within the open reading frame. The function of the gene may lose after the NHEJ repair which fulfil the aim of gene knockout. On the other hand, HDR repair is used for precise modifications of the targeted genomic gene. In HDR pathway, the DSB is repaired by a DNA repair template containing the desired sequence with additional homologous sequence immediately upstream and downstream of the target. The efficiency of HDR is generally low, therefore, a strong verification method should be applied to selected the clones containing the desired edit. DiCarlo et al. (2013) were the first to report a single gene disruption in *S. cerevisiae* haploid strain by HR using the CRISPR/Cas9 system. Thereafter, this method has been used in many studies for the genomic modification of *Saccharomyces* and non-*Saccharomyces* yeast strains (Reviewed by Stovicek et al., 2017). CRISPR method not only facilitates the modification of multiple alleles of the same genes but also

allows the modification of multiple sites of different genes simultaneously by combining several gRNAs. The efficiency of genomic modification is therefore improved dramatically.

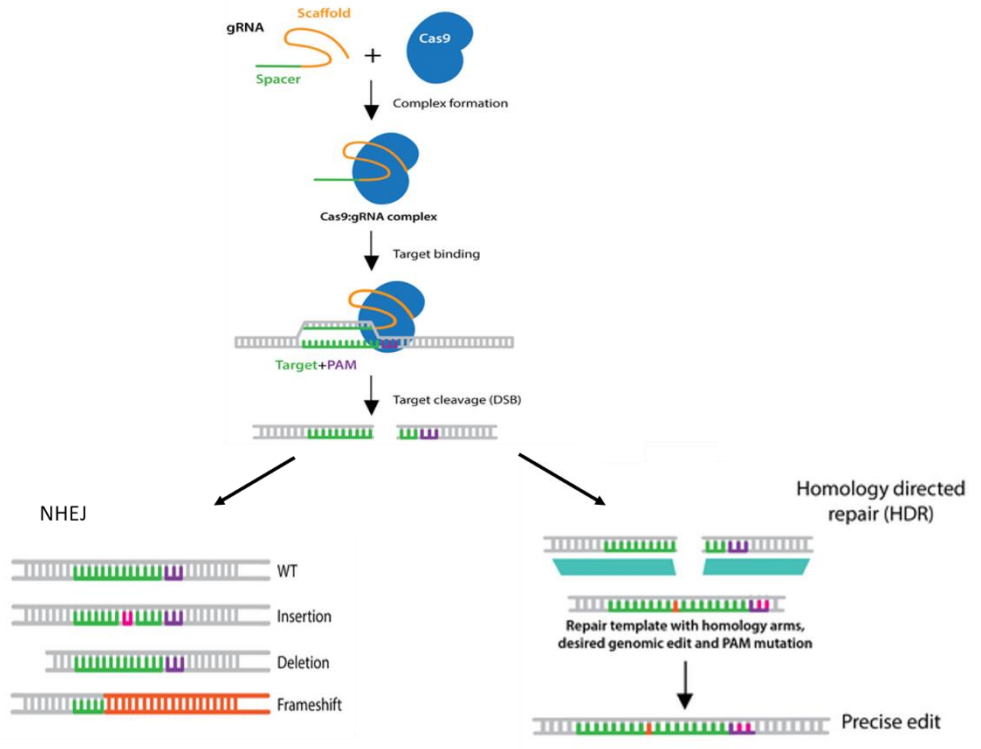

Fig. 10. Engineered CRISPR systems contain two components: a guide RNA (gRNA) and a CRISPR-associated endonuclease (Cas protein). Cas9 is guided to the genomic target DNA (~20 bp) by gRNA. The Cas9 nuclease then cleaves the target DNA resulting in a DSB. DSB must be repaired by the cells via NHEJ or HDR (The figure is adapted from https://www.addgene.org/guides/crispr/).

Another powerful and fascinating technology that can be used for yeast improvement relies on synthetic biology. Synthetic biology is considered as the most exciting 21st Century interdisciplinary science on the scene of, amongst other things, yeast biotechnology and strain development (Jagtap et al., 2017; Pretorius, 2016, 2017, 2018; Steensels et al., 2014). The goal of synthetic biology is the design and construction of new biological parts,

devices and systems, ranging from parts of single genes to completely new organisms (Steensels et al., 2014). In 2009, the first synthetic yeast genome project (Sc. 2.0 project) was launched to redesign and chemically synthesize the entire *Saccharomyces cerevisiae* genome. In 2017, five redesigned yeast chromosomes were completed (Maxen et al., 2017). In 2018, 16 natural chromosomes of *S. cerevisiae* were successfully fused into a single chromosome, similar to those found in prokaryotic cells; the artificial *S. cerevisiae* still has normal cellular functions (Shao et al., 2018). These works break the boundaries between natural and artificial life and shed light on a foreseeable future for tailor designed yeast to fulfil all the customers' needs.

4. Nitrogen metabolism by yeast

4.1 Central nitrogen metabolism by *S. cerevisiae*

Nitrogen is an essential nutrient for yeast. It exists in different forms in the environment. Around 30 distinct nitrogen-containing compounds, including amino acids, ammonium, urea, nitrogen bases, and purine derivatives can be used by yeast and they are named as yeast assimilable nitrogen (YAN). These nitrogen compounds entering yeast cells through different permeases, and subsequently used as building blocks for proteins or catabolised to for the *de novo* synthesis of other essential amino acids. The catabolisation of nitrogen compounds is through either deamination to generate ammonium, or transamination to form glutamate. Glutamate and glutamine are the central cores of nitrogen metabolism in *S. cerevisiae*, and their formation is catalised by different enzymes (Fig. 11). Glutamate dehydrogenase (*GDH1*) combines ammonia with α-ketoglutarate, which is an intermediate of tricarboxylic acid cycle, to form glutamate.

(1) α-Ketoglutarate + NH_4^+ + NADPH + H^+ ↔ glutamate + $NADP^+$

Glutamate can then combine with ammonia in a reaction catalysed by glutamine synthase(*GLN1*) (Magasanik and Kaiser, 2002).

(2) Glutamate + NH_4^+ + ATP → glutamine + ADP + P_i

When glutamate is the sole nitrogen source, glutamate dehydrogenase (*GDH2*) releases ammonia required for the synthesis of glutamine from glutamate (Miller and Magasanik, 1990).

(3) Glutamate + NAD^+ ↔ NH_4^+ + NADH + H^+ + α-ketoglutarate

Glutamine synthetase coded by *GDH2* is essential for the synthesis of glutamine when only glutamate or the precursors of glutamate supplied as nitrogen sources and cells lacking this enzyme require glutamine for growth. In contrast, the function of *GDH1* can be partially compensated by *GLT1*, which codes glutamate synthase.

(4) Glutamine + α-ketoglutarate + NADH + H^+ → 2 glutamate + NAD^+

The combination of this 4 reactions provide glutamate and glutamine for the intracellular nitrogen pool. Glutamate plays a more significant role with around 85% of the total cellular nitrogen is incorporated via the amino nitrogen of glutamate. On the other hand, the amide nitrogen of glutamine provides the remaining 15%.

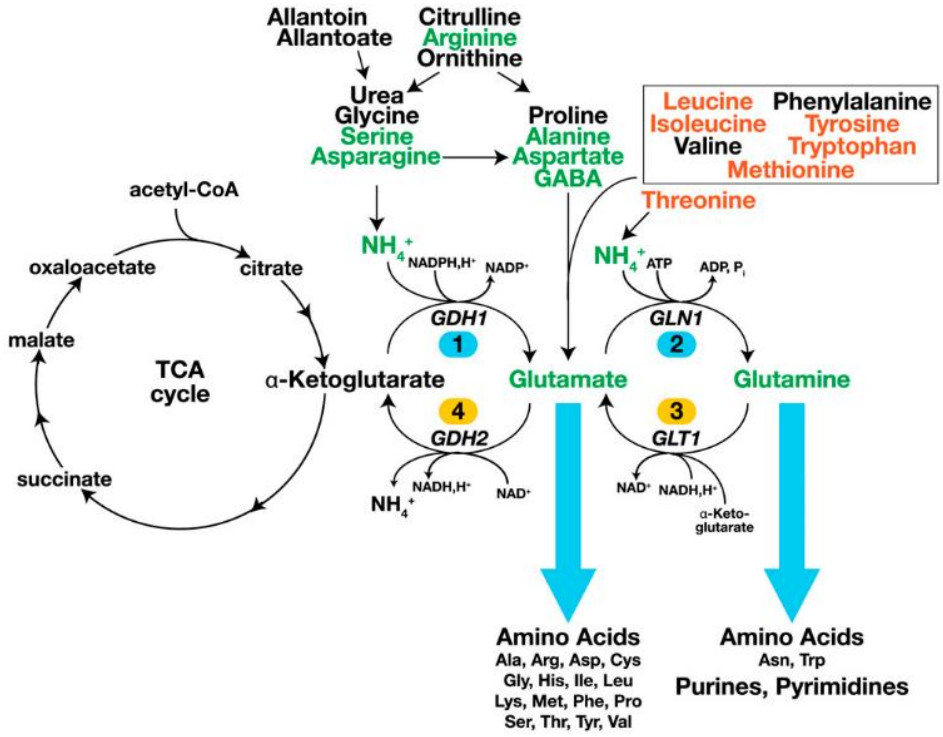

Fig. 11. Schematic diagram of the main pathways of Central Nitrogen Metabolism (CNM) (Ljungdahl and Daignan-Fornier et al., 2012).

4.2 Nitrogen sensing and metabolism regulation

The assimilable nitrogen sources are imported into yeast cells via different specific or non-specific transporters. For the common nitrogen sources existing in the grape must, ammonium is assimilated by three permeases, namely Mep1, Mep2, and Mep3. Among them, Mep2 has the highest affinity for ammonium following by Mep1 and then Mep3. Amino acids are imported through amino acid permeases (AAPs). The AAPs are selective and can transport only one or a group of amino acids, except for Gap1, the general amino acid permease, which allows the transport of all amino acids (Ramos et al., 2016). The major AAPs and their substrates are listed in Table 2. The

AAPs are regulated by several mechanisms. The first is Ssy1p-Ptr3p-Ssy5p (SPS), which is a complex located on plasma membrane with the ability to sense the extracellular amino acids and activates the transcription of amino acid permeases genes (Andréasson and Ljungdahl, 2004; Ljungdahl, 2009). Through the SPS-sensing pathway, extracellular amino acids induce their uptake. In the absence of inducing amino acids, the SPS sensor of extracellular amino acids is present in the plasma membrane in its preactivation conformation, and the transcription of SPS-sensor regulated genes, such as the AAPs, occurs at basal levels. In the presence of extracellular amino acids, the SPS sensor activates the transcription of AAP genes, and AAPs move to the plasma membrane. Consequently, the uptake rate of amino acids increases (Ljungdahl and Daignan-Fornier, 2012) (Fig. 12).

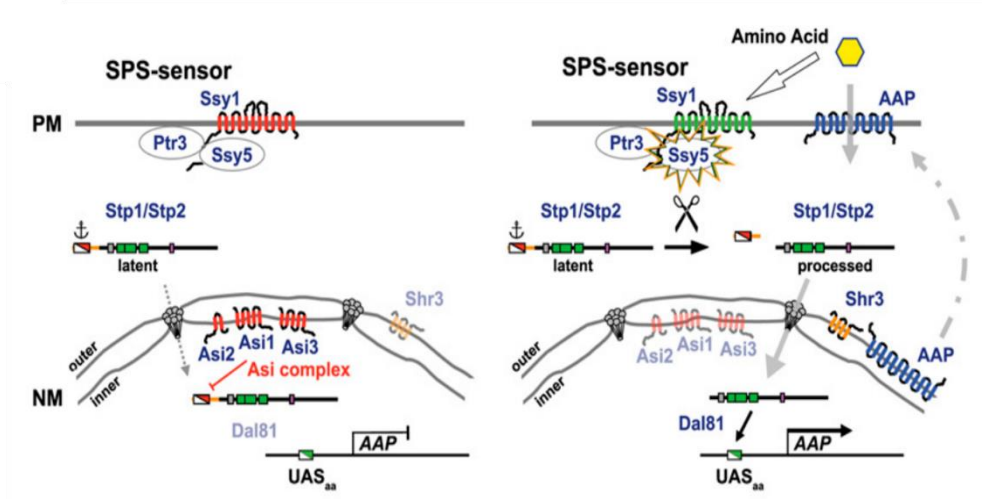

Fig. 12. Schematic diagram of the SPS-sensing pathway of extracellular amino acids (Ljungdahl and Daignan-Fornier et al. 2012).

Table 2. Major amino acid permeases for amino acids uptake

Gene	Description	Substrates
AGP1	Broad-specificity, relatively low-affinity, amino acid permease inducible by most neutral amino acids	L-alanine, L-asparagine, L-cysteine, L-glutamine, L-glycine, L-histidine, L-isoleucine, L-leucine, L-methionine, L-phenylalanine, L-proline, L-serine, L- threonine, L-tryptophan, L-tyrosine, L-valine
BAP2	Broad-specificity amino acid permease inducible by most neutral amino acids	L-cysteine, L-isoleucine, L-leucine, L-methionine, L-phenylalanine, L-tryptophan, L-tyrosine, L-valine, L-alanine,
BAP3	Broad-specificity amino-acid permease inducible by most neutral amino acids	L-alanine, L-cysteine, L-isoleucine, L-leucine, L-methionine, L-phenylalanine, L-threonine, L-tryptophan, L-tyrosine, L-valine
CAN1	Arginine permease	Canavanine, L-arginine
DIP5	Glutamate and aspartate permease able to mediate transport of other amino acids	L-alanine, L-asparagine, L-aspartate, L-glutamate, L-glutamine, L-glycine, L-serine, alpha-aminoadipate
GAP1	General amino acid permease	Canavanine, D-histidine, gamma-aminobutyrate, L-alanine, L-arginine, L-asparagine, L-aspartate, L-citrulline, L-cysteine, L-glutamate, L-glutamine, L-glycine, L-histidine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-ornithine, L-phenylalanine, L-proline, L-serine, L-threonine,

		L-tryptophan, L-tyrosine, L-valine, putrescine, spermidine, spermine, beta-alanine
GNP1	Broad-specificity amino acid permease	L-asparagine, L-cysteine, L-glutamine, L-leucine, L-methionine, L-proline, L-serine, L-threonine
HIP1	Histidine permease	D-histidine, L-histidine, manganese
LYP1	Lysine permease	L-lysine
PUT4	Proline permease	Gamma-aminobutyrate, L-alanine, L-glycine, L-proline, sarcosine
TAT1	Broad-specificity amino acid permease	L-tryptophan, L-tyrosine, L-valine
TAT2	Broad-specificity amino acid permease inducible by most neutral amino acids	L-phenylalanine, L-tryptophan, L-tyrosine

Another regulatory system in *S. cerevisiae* is nitrogen catabolite repression (NCR). NCR system control yeast selectively utilise preferred nitrogen sources, and prevent or reduce the unnecessary transcription of genes encoding enzymes and permeases for the utilisation of poorer nitrogen sources (Magasanik, 1992). It controls the amino acids permeases: *GAP1*, *CAN1*, *PUT4*, *DIP5*, *UGA4* and ammonium permeases: *MEP1*, *MEP2*, *MEP3*.Four transcription factors are involved in regulating the expression of NCR genes, which are two activators, Gln3 and Gat1, and two repressors, Gzf3 and Dal80. In the presence of preferred nitrogen sources, Gat1 and Gln3 are sequestered by Ure2 in the cytoplasm, Dal80/Gzf3 block the UAS_{NTR} binding site. When the preferred nitrogen sources are depleted, Ure2 releases

Gat1 and Gln3 and the complex migrated into the nucleus. Dal80/Gzf3 release the UAS_{NTR} binding site and Gat1/Gln3 complex activates the transcription of NCR controlled genes, which allows the assimilation of non-preferred nitrogen sources (Fig. 13) (Gobert et al., 2019; Ljungdahl and Daignan-Fornier et al., 2012; Zhang et al., 2018).

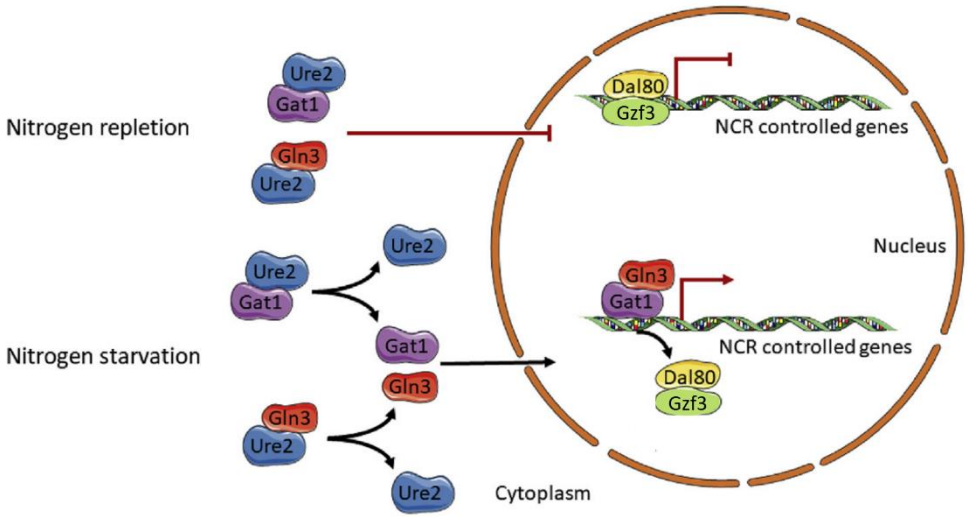

Fig. 13. Schematic diagram of NCR control (Adapted from Gobert et al., 2019).

In *S. cerevisiae*, the NCR system is directly regulated by the target of the rapamycin pathway (TOR) by controlling the Ure2-mediated cytoplasmic retention of Gln3 (Beck and Hall, 1999). TOR is comprised by two complexes, TOR complex 1(TORC1) and TOR complex 2 (TORC2). TORC1 complex is sensitive to the specific inhibitor, rapamycin (Loewith et al., 2002). The presence of rapamycin derepresses the expression of NCR-sensitive genes. TORC1 negatively regulates phosphatase Tap42-Sit4, which in turn influence the extent of Gln3 phosphorylation (Beck and Hall, 1999; Hardwick et al.,

1999; Ljungdahl and Daignan-Fornier et al., 2012). The TOR pathway is responsible for intracellular amino acid sensing (Urban et al., 2007; Zhang et al., 2019). In *S. cerevisiae*, TORC1 is activated and functions in the vacuolar membrane. Although there are many studies dedicated to exploring the TOR pathway, how intracellular levels of nitrogen are sensed by the TORC1 complex and how this pathway responds to preferred and non-preferred amino acid remains unclear (Kessi-Pérez et al., 2020; Zhang et al., 2019).

Under nitrogen starvation conditions the general amino acid control (GAAC) pathway is activated in *S. cerevisiae*. The limiting level of amino acids leads to alterations in the pools of charged tRNAs (Zaborske et al., 2009; 2010). Gcn2 can bind to uncharged tRNAs via its histidyl-tRNA synthetase-like domain. The activated Gcn2 kinase phosphorylates the α-subunit of eIF2, leading to reduced the levels of ternary complex (TC). The diminished levels of TC decrease the efficiency of scanning ribosomes to reinitiate translation, increasing the proportion of ribosomes that reinitiated translation at *GCN4*. Gcn4 activates groups of genes that participate in amino acid biosynthesis, nitrogen utilisation, and signalling through interacting with their promoter regions (Natarajan et al, 2001). Amino acid starvation decreases degradation of Gcn4 by the proteasome and therefore, increases Gcn4 stability (Kornitzer et al., 1994).

4.3 Nitrogen metabolism during wine fermentation

4.3.1 Nitrogen composition in grape must

Grape must contain different types of nitrogen sources. Among them, yeast preferentially uses simple nitrogen sources such as amino acids and ammonium. Besides, some low molecular weight peptides and urea can also

support yeast cells growth, but grape proteins cannot be used as a nitrogen source, since *S. cerevisiae* lacks extracellular proteolytic activity. The concentration and types of nitrogen sources in grape musts are highly variable and may be influenced by a combination of many factors. Viticultural practices such as rate and timing of nitrogen application, trellis style, soil management techniques and even *Botrytis* infection are important determinants of grape nitrogen compounds composition and concentration (Bell and Henschke, 2005; Conradie, 2001; Rodriguez-Lovelle and Gaudillère 2002).

The concentration of total nitrogen increases in grapes and juice during fruit ripping. Specifically, the concentration of total amino acids increases from veraison to harvest. On the other hand, the concentration of ammonium decreases during ripening. Arginine and proline are two of the most abundant amino acids in grapes. Proline, which is not practically used by *S. cerevisiae* during wine fermentation, accumulates rapidly after the veraison. The concentration of arginine, which is an important nitrogen source for yeast, increase quickly from veraison and reaches a maximum, then begins a slow decline during the ripening process (Hernández-Orte et al., 1999, Stines et al., 2000). In the harvested grapes, ammonium accounted on average for 40% of YAN (Bell, 1994). The assimilable amino acids can account for 51–92% of juice YAN at harvest (Bell, 1994; Conradie, 2001). The concentration of different nitrogen sources varies significantly according to grape cultivar, rootstock, site and seasonal conditions and level of maturity. In general, the concentration of ammonium ranges between 5-325 mg N/L (Bely et al., 1991; Butzke, 1998; Henschke and Jiranek, 1993). The concentration of different amino acids in harvested grapes or juice is listed in Table 3.

Table 3. Amino acids concentration ranges in grape berries or juice at harvest. (Reviewed by Bell and Henschke, 2005)

Nitrogen source	Concentration range (mg/L)	Nitrogen source	Concentration range (mg/L)
Alanine	10-227	Leucine	2-160
Arginine	20-2322	Lysine	0.7-45
Asparagine	1-171	Methionine	1-33
Aspartic acid	10-138	Ornithine	0.1-27.2
Citrulline	0.1-83	Phenylalanine	2.8-138
Cysteine	1-8.2	Proline	9-2257
Glutamine	9-4499	Serine	13-330
Glutamic acid	27-454	Threonine	9-284
Glycine	1-20	Tryptophan	0.2-11
Histidine	5-197	Tyrosine	2-33
Isoleucine	1-117	Valine	7-116

In ripe grapes, the majority of nitrogen compounds are located in the skin and pulp. Stine et al. (2000) demonstrated that 19-29% of yeast assimilable amino acids present in grape skin, 61-65% in the pulp, and 10-15% in the seeds (Fig. 14). However, the distribution of nitrogen compounds in each berry part largely depends on the site of the vineyard, viticultural practice, and the cultivar. For example, the pulp of Cabernet Sauvignon contains 1.5 times higher amount of amino acids than that of Riesling berries, although these

two measured cultivars have similar yeast assimilable amino acids (Stine et al., 2000). As large proportion of nitrogen compounds located in the skin and seeds, prolonged skin contact before must pressing, maceration or fermentation with skin makes an important contribution to YAN concentration in grape must (Ribéreau-Gayon, 2000).

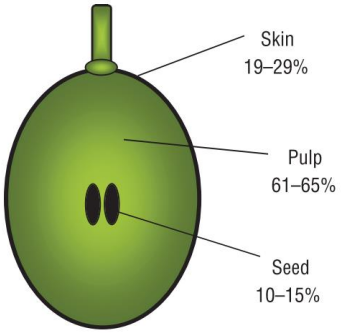

Fig. 14. Distribution of yeast assimilable amino acid N (excluding ammonium and proline N) across the skin, pulp and seeds of Riesling and Cabernet Sauvignon berries (data from Stines et al. 2000) (Bell and Henschke, 2005).

Botrytis is a common infection in vineyard especially when the canopy density is high (Bell and Robson, 1999). Instead of the desirable 'noble rot', the fungus *Botrytis cinerea* might induce 'grey rot', which caused damages on grape berries. Botrytis infected berries displayed a 2- to 7-fold reduction in total amino acid concentration when compared to uninfected berries (Rapp and Versini, 1996). The infection especially reduces the concentration of arginine, which causes nitrogen deficiency in grape musts.

Some post-harvest processes may influence YAN concentration in the must. From the moment of harvest, some grape berry constituents can be subject to degradation by chemical, physical or biological processes, which can potentially impact on the composition and quality of the resultant wine. During mechanical harvesting and transportation of grape berries, the

structure of the berry may be damaged and a variety of degradative processes, such as oxidation and microbial metabolism will be activated consequently. The growth of microorganisms during this period lead to serious losses of grape berry nutrients, which increases the risk of a stuck fermentation. Applying low temperature and inhibitory chemicals, such as sulfites help to reduce the losses of nitrogen compounds during post-harvest processes (Bell and Henschke, 2005).

4.3.2 Nitrogen source quality and sequential utilisations of nitrogen sources

Not all nitrogen sources support yeast growth and fermentation in the same way and they can be classified as preferred (good) or non-preferred (poor) nitrogen sources. The classifications have been defined by several studies by using different methods, either by the efficiency of sole nitrogen source to support growth and fermentation (Henschke and Jiranek, 1993; Jiranek et al., 1995a), by the response of the NCR system (Crépin et al., 2012), or by the response of SPS system (Boer et al., 2006; Ljungdahl, 2009). However, the classification is not absolute. It is highly strain dependent and the results vary from different studies. One of the most representative classifications is based on the research of Godard et al. (2007). Nitrogen sources were grouped based on their regulation mechanisms. With the presence of preferred nitrogen sources, NCR is active, whereas GAAC is inactive. Ammonium, asparagine, glutamine, serine, aspartate, alanine, arginine, and glutamate were considered as preferred nitrogen sources in the study. Oppositely, with the presence of non-preferred nitrogen sources, NCR is derepressed. GAAC, which is associated with nitrogen starvation conditions, is highly active. Leucine, isoleucine, methionine, tyrosine, threonine, and tryptophan were classified as

non-preferred nitrogen sources. Moreover, the catabolisation of the preferred nitrogen sources provides carbon skeletons that are able to be directly integrated into metabolism. They are substrates of transaminases or deaminases that yield pyruvate, oxaloacetate, or α-ketoglutarate. The transaminations of non-preferred nitrogen sources yield carbon skeletons that are converted through Ehrlich pathway to non-catabolisable and toxic fusel oils (Godard et al., 2007; Hazelwood et al., 2008).

As described previously, the nitrogen assimilation by *S. cerevisiae* is regulated by various sensing and metabolism mechanisms. When different nitrogen sources are presented in a complex mixture like in grape must, they assimilated by *S. cerevisiae* at a significantly different rate. Depending on the different assimilation rate during alcoholic fermentation, Crépin et al. (2012) classified the nitrogen sources exist in grape must into three groups, which are prematurely consumed (Lys), early consumed (Asp, Thr, Glu, Leu, His, Met, Ile, Ser, Gln, and Phe), and late consumed (ammonium, Val, Arg, Ala, Trp, and Tyr). Proline is not assimilated by *S. cerevisiae* under anaerobic conditions. The differences in the kinetics of nitrogen consumption are mainly dependent on the regulation and the inherent kinetic properties of the transporters involved in its uptake. The concentration of each nitrogen sources in the medium seems to play a minor or insignificant role in the assimilation order. The permeases associated with the early consumed nitrogen sources are encoded by genes regulated by SPS system (Fig. 15). The early assimilation of these nitrogen sources results in the intracellular accumulation of glutamine and glutamate, which repress the NCR sensitive genes, such as *GAP1*. As a consequence, Ala, Arg, and Gly, which are mainly imported by Gap1, were consumed after the depletion of other nitrogen compounds, during the last part of the growth phase.

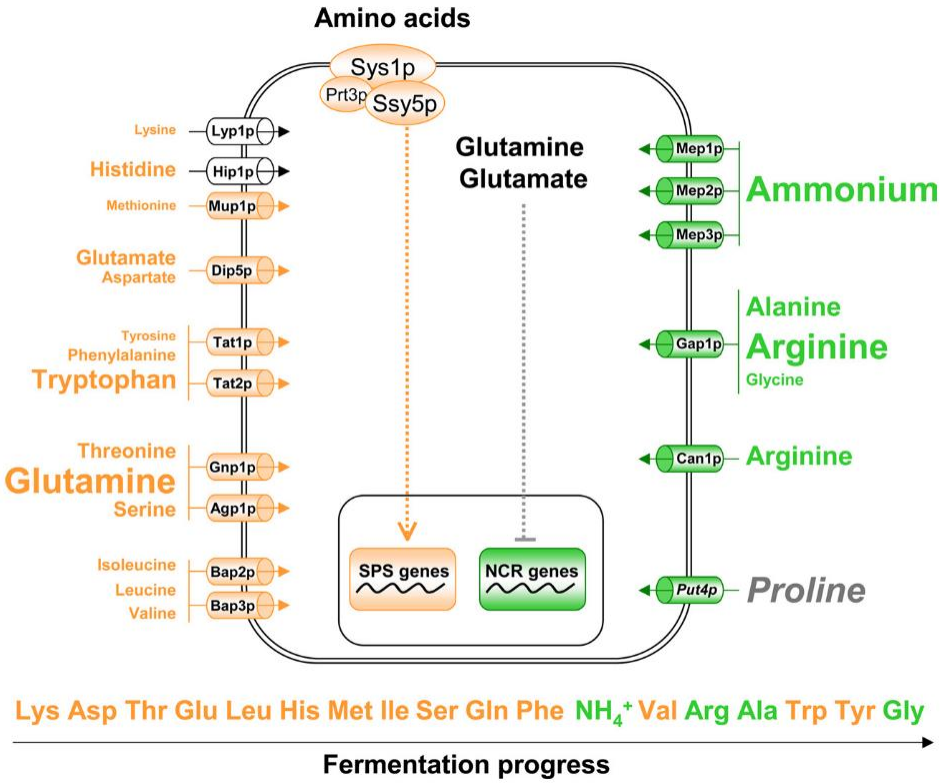

Fig. 15. Major nitrogen source transporters with their regulatory mechanisms. The order of nitrogen source consumption during wine fermentation is indicated at the bottom (Crépin et al., 2012).

4.3.3 Influence of nitrogen on yeast cell growth

When other nutrients are optimal, YAN concentration in the medium largely determines yeast cell population or biomass yield (Henschke and Jiranek, 1993). Nitrogen provides essential building blocks for yeast biosynthesis. The initial YAN concentration is largely influenced by yeast growth in terms of lag phase time, growth rate, and biomass formation. Gutiérrez et al. (2013) and Mendes-Ferreira et al. (2004) studied the influence of nitrogen concentration, either as ammonium or amino acid, on yeast growth. The results demonstrated that nitrogen concentration does not have significant

influences on the specific growth rate, although biomass yield increases with a higher amount of nitrogen. Nevertheless, an excessive amount of nitrogen in the medium may cause nutrient imbalance, and consequently trigger cell death (Tesnière et al., 2013). Apart from nitrogen source concentration, nitrogen source composition also significantly influences yeast cell growth. As described before, *S. cerevisiae* has different preferences for nitrogen sources. Naturally, preferred nitrogen sources support good growth behaviour; non-preferred nitrogen sources support poor growth performance or even no growth. Many studies revealed that a balanced mixture of amino acids and ammonium supports higher growth rates than single nitrogen compounds, even the single compound is highly preferred by yeast. By using a mixture of nitrogen sources, yeasts are able to directly incorporate amino acids into protein, thereby reducing the energy consumption for amino acid *de novo* synthesis (Albers et al., 1996; Beltran et al., 2004; Cooper, 1982; Henschke and Jiranek, 1993; Torija et al., 2003).

4.3.4 Influence of nitrogen on fermentation activity

As for cell growth, the initial YAN concentration is exponentially related to the fermentation rate. An adequate amount of YAN is crucial for a successful fermentation process. Lacking nitrogen may cause sluggish or stuck fermentations, which causes economical losses in the wine industry (Bisson, 1999). Nitrogen deficiency can influence the fermentation rate by two interdependent factors: metabolic activity of each cell and biomass yield (Varela et al., 2004). Under nitrogen starvation conditions, a major decrease in sugar transport activity has been observed. The decreased sugar transport activity leads to an alleviation of the Crabtree effect, with the activation of respiratory genes and decrease in fermentation rate (Mendes-Ferreira et al.,

2007; Salmon, 1989; Salmon et al., 1993). Accordingly, it has been suggested that the transcription activation of genes involved in the tricarboxylic acid cycle and respiration may be associated with a low sugar uptake capacity and/or redox imbalance (Jin et al., 2004). On the other hand, insufficient nitrogen reduces biomass production which in turn reduces the fermentation rate. Varela et al., (2004) compared both factors influencing the fermentation rate and demonstrated that although sugar uptake in nitrogen-deficient cultures is lower, adding biomass to sluggish cultures reduces the fermentation termination time without changing the chemistry of the must.

Various studies have attempted to estimate the minimum concentration of YAN in grape must needed to achieve satisfactory completion of fermentation. Depending on the combination of different factors such as yeast strain (Jiranek et al. 1991, Manginot et al. 1997, 1998, Blateyron and Sablayrolles 2001), initial sugar content, and the different experimental approaches used, estimates range from 70–267 mg/L YAN. In general, a threshold concentration of approximately 140 mg N/L for clarified musts of moderate sugar concentration being considered a practical minimal limit (Agenbach, 1977; Bely et al., 1990, 1991; Mendes-Ferreira et al., 2004). Nitrogen requirement during fermentation is highly yeast strain dependent. Different approaches were applied to explore the nitrogen demands of yeast strains for carrying out risk-free fermentation. Henschke (1991) first demonstrated the difference in fermentation rate among strains by conducting fermentations with low nitrogen concentration (78 mg N/L). Manginot et al. (1998) studied the nitrogen requirement of different yeast strains during the stationary phase and proposed an efficient method which is to measure the amount of nitrogen need to keep a constant rate of CO_2 production during stationary phase. The characterisation of the nitrogen requirement of yeast strains is of great

importance because it provides valuable information for winemakers to choose proper yeast strain depending on the nitrogen status of the must.

4.3.5 Influence of nitrogen on the sensory profile

Nitrogen status in grape must not only affects the yeast's fermentation behaviour but also significantly influences the sensory profile of the final wine product. Nitrogen concentration and composition regulate the metabolic flux through different pathways for the production of various flavour compounds. Moreover, some amino acids are important precursors for higher alcohols and esters.

Ethanol and glycerol

Ethanol and glycerol are two of the major products of wine fermentation. A balanced concentration of ethanol and glycerol largely improves the body and mouthfeel of the wine. When the fermentation is carried out with amino acids as nitrogen sources, little surplus NADH is generated from protein synthesis and growth, resulting in a reduced production of glycerol. However, when ammonium is the sole nitrogen sources, higher *de novo* synthesis of amino acids is required, which results in increased formation of NADH. To reach redox balancing, more glycerol is generated at the expenses of ethanol (Albers et al., 1996).

Carboxylic acids

Nitrogen also impacts the production of carboxylic acids. Succinic acid is one of the main carboxylic acid produced during fermentation. It significantly influences the organoleptic balance by providing acidity and a favourable salt-bitter taste. It was evidenced in some studies that the addition of certain amino acids, such as glutamate, asparagine, proline, glutamine and threonine,

to the medium significantly stimulate the succinate production (Albers et al., 1996; Camarasa et al., 2003). Instead of formed from sugar via the reductive branch of the TCA cycle, succinic acid may be formed by deamination of aspartate and glutamate, which increase the concentration of succinic acid directly. Another representative example is volatile acetic acid. The concentration of acetic acid varies widely from less than 0.2 g/L to more than 2 g/L. A high concentration of acetic acid is undesirable in wine since it gives vinegar-like odour. During wine fermentation, the initial concentration of YAN negatively related to acetic acid formation (Barbosa et al., 2009; Bely et al., 2003; Hernández-Orte et al., 2006). High nitrogen availability in the medium increases the biomass formation, which stimulates NADH production. Therefore, no more NADH is required from the oxidative formation of acetic acid from acetaldehyde (Barbosa et al., 2009).

Higher alcohols

Higher alcohols can be desirable as well as undesirable in wine depending on their concentration. With a concentration below 300 mg/L, higher alcohols provide fruity notes to wine (Rapp and Versini, 1991), however, when the concentration exceeds 400 mg/L, they can present a strong, unpleasant pungent smell and taste (Lambrechts and Pretorius, 2000; Swiegers and Pretorius, 2005). During wine fermentation, higher alcohols can be formed either from central carbon metabolism (CCM) or from catabolisation of amino acids via the Ehrlich pathway (Fig. 16). Through the Ehrlich pathway, degradation of branched-chain amino acids, leucine, isoleucine, and valine provides precursors for the synthesis of branched-chain higher alcohols, isoamyl alcohol, active amyl alcohol, and isobutanol. Moreover, aromatic amino acids, phenylalanine is an important precursor for 2-phenylethanol, which has a pleasant odour of rose. In Ehrlich pathway, amino acids are

transaminated to the corresponding α-ketoacids, which is the key interface between the CCM and the nitrogen metabolism (Crépin et al., 2017; Hazelwood et al., 2008). Then, the α-ketoacids are decarboxylated to aldehydes, following by the reduction of aldehydes to higher alcohols, whereas NADH is oxidised to NAD^+. Additionally, the *de novo* synthesis of valine, leucine and isoleucine share the same pathway of formation of higher alcohols up to the last intermediate, i.e., the appropriate α-keto acid (Boulton et al., 1996).

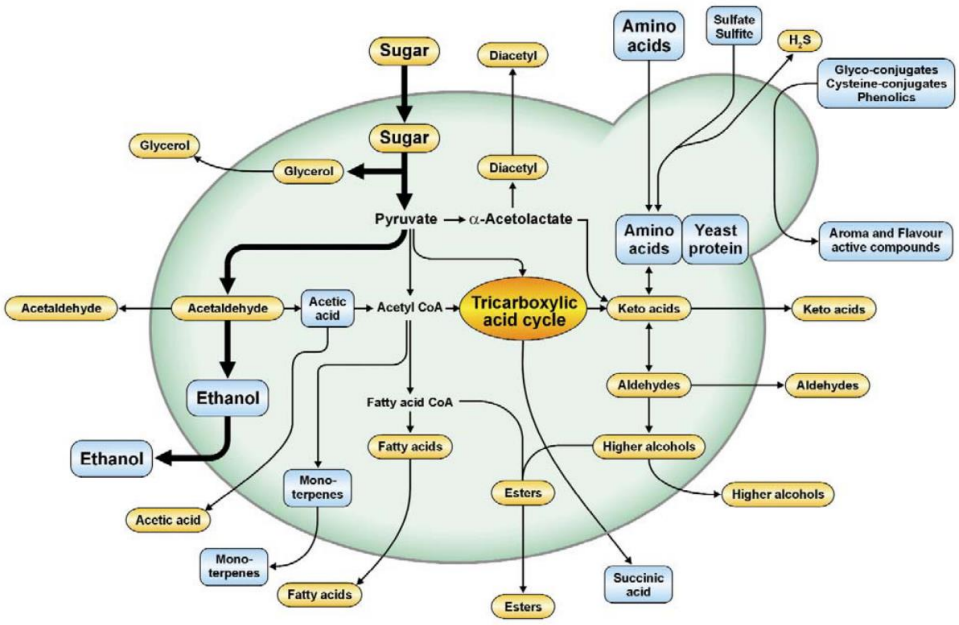

Fig. 16. A schematic representation of derivation and synthesis of flavour-active compounds from sugar and amino acids a by wine yeast (Swiegers et al., 2005).

Although the Ehrlich pathway plays an important role in higher alcohol production, ^{13}C isotopic labelling studies revealed that the majority of volatile compounds originates from the backbones of compounds produced through

the CCM. Only a small fraction of higher alcohols are synthesised using the carbon skeletons of amino acids. This could be explained by the fact that the consumed amino acids are not sufficient to supply the production of the corresponding higher alcohol (Crépin et al., 2017; Rollero et al., 2017). Moreover, increasing the concentration of the corresponding amino acids not necessarily results in greater higher alcohol production. The excretion of higher alcohols tends to occur towards the middle to later stages of fermentation, whereas the bulk of amino acids are consumed early in fermentation, during the yeast growth phase. Rollero et al. (2017) observed that in media containing moderate to high concentration of nitrogen, approximately 70% of the consumed leucine was directly incorporated into biomass instead of producing isoamyl alcohol. In general, when the nitrogen concentration of must is low, a direct relationship between initial nitrogen concentration and the total concentration of higher alcohols exists, whereas at moderate must nitrogen an inverse relationship with higher alcohols prevails (Äyräpää 1971). At high initial must nitrogen, the concentrations of total higher alcohols are at their lowest. This conclusion is verified by Oshita et al. (1995) and Rollero et al. (2017) with the utilisation of isotopically labelled amino acids. When the nitrogen resource is limited, a large amount of α-ketoacids, which are largely synthesised from sugars, is decarboxylated and reduced to higher alcohols, due to the lack of α-amino nitrogen availability from transamination reactions. In the presence of a moderate concentration of amino acids, apart from the a-ketoacids generated from sugar catabolism, the *de novo* synthesis of branched-chain amino acids to fulfil the greater anabolic requirement also results in excess of α-ketoacids. Therefore, a pronounced increase in the flux towards the formation of higher alcohols. Finally, under high nitrogen concentration, more biomass is formed which

increases the anabolic requirement for amino acids. The intracellular a-ketoacids are to a large extent directed towards the synthesis of amino acids at the expense of higher alcohol formation, which results in a decreased flux in the formation of aromas compared with the previous conditions (Fig. 17).

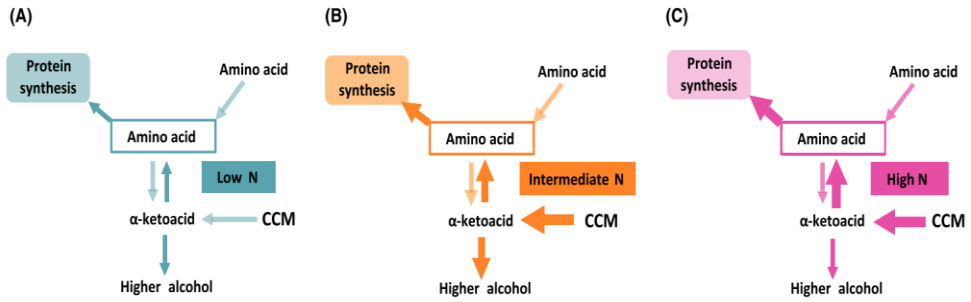

Fig. 17. Distribution of fluxes leading to the formation of higher alcohols on the basis of the initial nitrogen content: (A) low nitrogen concentration, (B) intermediate nitrogen concentration and (C) high nitrogen concentration (Rollero et al., 2017).

Esters

Esters are significant volatile compounds which provide pleasant fruity and floral aromas to wine. Esters in wine are mainly produced by yeast metabolism (through lipid and acetyl-CoA metabolism), but they can also be synthesised by yeast from grape precursors, which is influenced by the grape variety. Esters can be mainly separated into two groups: ethyl esters and acetate esters. Ethyl esters of fatty acids, such as ethyl hexanoate, ethyl octanoate, ethyl decanoate, are derived from the reaction of acyl-CoA compounds with ethanol catalysed by acyl-CoA: ethanol O-acyltransferases. Acetate esters of higher alcohols, such as phenylethyl acetate, isoamyl acetate, are formed by the reactions of acetyl-CoA with higher alcohols, catalysed by alcohol acetyltransferases (Boulton et al., 1996; Bisson and Karpel, 2010).

Nitrogen concentration in the medium affects the formation of esters. Specifically, the synthesis of acetate esters is positively related to nitrogen concentration (Mouret et al., 2014; Rollero et al., 2015; Torrea et al., 2011). This is due to that the synthesis pathway of acetates of higher alcohols is partly linked to nitrogen metabolism via the Ehrlich pathway (Hazelwood et al., 2008). Similarly, the production of ethyl esters, mainly ethyl butanoate, ethyl hexanoate and ethyl octanoate, significantly increase with initial nitrogen concentration (Garde-Cerdán and Ancín-Azpilicueta, 2008; Rollero et al., 2015; Torrea et al., 2011). The nitrogen demand of yeast strains may also influence the synthesis of esters. A high nitrogen demanding strain showed higher production of total esters, when compared to a low nitrogen demanding strain (Torrea et al., 2003).

Monoterpenes

Monoterpenes exhibit floral aroma in wine. They normally exist in grapes as odourless glycoconjugates with only a small proportion present in the free form. During the fermentation, the β-glucosidase from yeasts cleaves the sugar moieties from the glycoconjugates and release the volatile free monoterpenes. Carrau et al. (2005) revealed that some wine strains of *Saccharomyces cerevisiae* are capable of *de novo* monoterpenes production from sugar metabolism although at low concentration. The production of monoterpenes by yeast can be influenced by the YAN and oxygen concentration in the medium. Higher YAN concentration of the medium, which stimulates fermentation rate but not biomass yield, also stimulates monoterpene formation. This may be explained by that the synthesis of monoterpenes involves the conversion of leucine to mevalonic acid. Assimilable nitrogen, as well as oxygen, is known to regulate mevalonic acid and sterol formation, and hence the concentration of intermediates, such as

geranyl pyrophosphate, which can act as a terpene precursor (Vaudano et al. 2004).

Hydrogen sulphide (H_2S)

Hydrogen sulphide is one of the volatile sulphur compounds produced by yeasts during alcoholic fermentation. It imparts a rotten-egg odour, which is considered as off-flavour in wine. H_2S is produced metabolically by yeast from either inorganic sulphur compounds, sulphate and sulphite or organic sulphur compounds, cysteine and glutathione. Many factors influence the production of H_2S, such as the elemental sulphur levels in grapes, the amount of sulphite addition in grape must prior to fermentation, the amount of sulphur-containing organic compounds, vitamin deficiencies, fermentation conditions and yeast strains (Reviewed by Mendes-Ferreira et al., 2011). H_2S production is also associated with a shortage of assimilable nitrogen. As an antioxidant and microbial control agent, sulphite is supplied in grape must to serve antioxidant and microbial control purposes. In the presence of sufficient nitrogen, sulphide is incorporated in the nitrogenous precursors O-acetyl serine and O-acetyl homoserine, which form cysteine and methionine, respectively. Since the concentrations of serine and methionine are usually low in grape must, the biosynthesis of these amino acids is required. Therefore, the accumulated sulphite is derived to the *de novo* synthesis of amino acids instead of H_2S production (Henschke and Jiranek, 1993). However, when nitrogen concentration is limited, amino acids are catabolised to fulfil the intracellular nitrogen pool and the non-preferred sulphur amino acids biosynthesis is minimised. This process leaves no available precursors to combine with the sulphide. Consequently, it diffuses out as the sensorially offensive compound H_2S (Henschke and Jiranek, 1991; Jiranek et al., 1995b; Mendes-Ferreira et al., 2009). It has also been reported that the addition of

diammonium phosphate (DAP) may help with the reduction of H_2S production, however, the addition time is crucial for this process (Mendes-Ferreira et al., 2010).

4.3.6 Nitrogen addition during wine fermentation

As nitrogen deficiency causes sluggish or stopped fermentation, triggers the higher formation of off-flavour compounds such as H_2S and acetic acid, reduces the formation of desirable fruity and floral aroma compounds as esters and monoterpenes, the regulation of initial YAN concentration in the must is essential for a successful fermentation process and good wine quality. A common oenological practice to regulate YAN concentration is the addition of inorganic nitrogen sources mainly DAP into the grape must. More recently, the addition of organic nitrogen sources also has become increasingly popular. Many different commercial products containing combinations of nitrogen sources, inactivated yeast or yeast products are available on the market for winemakers to choose. It is well known that the supplementary of nitrogen sources prevents the sub-optimal fermentations, however, the questions facing by the winemakers are: when is the best timing for nitrogen addition, which kind of nitrogen source is preferable for addition, and how much nitrogen should be supplied. To answer these questions, many studies have been carried out.

Influence of nitrogen addition on fermentation kinetics

Addition at the initial of fermentation of inorganic nitrogen sources such as ammonium chloride, ammonium sulphate and diammonium phosphate (DAP) generally increases fermentation rate and decreases fermentation duration time (Table 4). Moreover, an increase in biomass formation was observed by some studies also (Seguinot et al., 2018). The addition of amino acids does

not have as clear effect as ammonium addition in terms of fermentation time. In fact, some studies observed no impact on fermentation time at all (Garde-Cerdán and Ancín-Azpilicueta, 2008; Hernández-Orte et al., 2004; Martínez-Moreno et al., 2012), while an increase of fermentation rate was observed by Seguinot et al. (2018). The results of different studies all agree that the addition of a combination of ammonium and amino acids promote the fermentation activity by increasing fermentation rate and reducing fermentation time (Arias-Gil et al., 2007; Beltran et al., 2005; Torrea et al., 2011).

When nitrogen was supplied during stationary phase, no significant effect was observed on growth rate. However, the discrepancy exists among different studies on the biomass yield. Half of the related studies showed no significant influence on biomass production (Beltran et al., 2005; Bely et al., 2003; Hernández-Orte et al., 2006), whereas the other half showed increased biomass yield regardless of nitrogen sources (Hernández-Orte et al., 2006; Martínez-Moreno et al., 2014; Seguinot et al., 2018).

Nitrogen addition at the last 1/3 of fermentation was also conducted by Beltran et al. (2005). A reduced fermentation duration was observed, although to a lesser extent comparing to the addition carried out earlier during the fermentation.

Influence of nitrogen addition on volatile compounds formation

The production of volatile compounds during fermentation is closely related to nitrogen concentration in the medium. The influence of timing and type of nitrogen addition on volatile compounds production has been explored by many studies (Arias-Gil et al, 2007; Bely et al., 2003; Beltran et al., 2005; Carrau et al., 2008; Hernándes-Orte et al., 2004; Hernándes-Orte et al., 2006;

Jiménez-Martíet al., 2007; Martínez-Moreno et al., 2014; Miller et al., 2007; Seguinot et al., 2018; Ugliano et al., 2008; Vilanova et al., 2012). However, due to the reasons that the experimental condition in each study are not the same, different yeast strains were used, and the regulation of volatile compounds synthesis is complicated, many discrepancies exists among the results obtained by different studies. Here some general features are summarised (Table 4). The addition of inorganic nitrogen sources such as ammonium chloride, ammonium sulphate and DAP at the beginning of fermentation reduces the higher alcohol production. On the other hand, most studies observed a general increase of the production of esters under this condition (Carrau et al., 2008; Seguinot et al., 2018; Ugliano et al., 2008; Vilanova et al., 2012). Ugliano et al. (2009) demonstrated that DAP addition to Shiraz must induced prolonged formation of H_2S, therefore a higher concentration of H_2S was produced in the final wine.

The addition of amino acids at the beginning of fermentation showed a decrease in higher alcohol production (Hernández-Orte et al., 2004; Seguinot et al., 2018). Some studies also observed an increased production of esters (Garde-Cerdán and Ancín-Azpilicueta, 2008; Seguinot et al., 2018). When a combination of inorganic nitrogen sources and amino acids were supplied into the initial must, a decrease in higher alcohol production and an increase in esters production was detected (Beltran et al., 2005; Torrea et al., 2011). Moreover, the addition of DAP combined with amino acids seems to reduce the H_2S production substantially (Barbosa et al., 2012).

Nitrogen addition during stationary phase seems to reduce higher alcohol production except for using amino acids as the only source (Jiménez-Marti et al., 2007; Seguinot et al., 2018). Supplement of ammonium salts or combination of ammonium and amino acids reduces the formation of esters

(Jiménez-Martí et al., 2007; Martínez-Moreno et al., 2014). The result of amino acids addition is contradictory with one study that observed increase on ester production (Seguinot et al., 2018) whereas another one observed decrease in the formation of esters (Jiménez-Martí et al., 2007).

Table 4. Summary of influences of nitrogen addition on fermentation kinetics and volatile compounds formation

Addition time	N source	Fermentation rate	Fermentation duration	Higher alcohols	Esters
Initial	NH_4^+	↑	↓	↓	↑
	DAP	↑	↓	↓	↑
	AAs	→	→	↑	
	NH_4^++ AAs*	↑	↓	↓	↑
Stationary	NH_4^+	↑	↓	↓	↓
	DAP	↑	↓	↓	↑
	AAs	↑	↓	→	↑
	NH_4^++ AAs	↑	↓	↓	↓

*AAs: amino acids

5. Genetic approaches to explore the differences in nitrogen metabolism among yeast strains

Nitrogen requirements and nitrogen source preferences are highly divergent phenotypes among different yeast strains. Some yeast strains require high amount of extracellular nitrogen sources to conduct proper fermentation

activity. On the other hand, other yeast strains may be better adapted to nitrogen limited environment (Brice et al., 2013; Manginot et al. 1997; 1998). Moreover, the nitrogen source preferences are also strain specific (Gutiérrez et al., 2013). The massive sequencing of 1011 yeast strains has revealed the genetic diversity among *S. cerevisiae* population (Peter et al., 2018). It is important to know which genotype controls the corresponding phenotype diversity in nitrogen metabolism. However, nitrogen metabolism in *S. cerevisiae* from nitrogen sensing to nitrogen catabolism comprise a complicated and sophisticate system that is regulated by several interconnected mechanisms. The relationship between genes and phenotypes is not easy to be revealed straightforwardly. To explore the causative genes that account for the differences in nitrogen metabolism among strains, many attempting has been carried out using various approaches (Table 5).

Beltran et al. (2004) studied the transcriptional profile of the general amino-acid permease (*GAP1*) and the ammonium permeases (*MEP1, 2, 3*) and revealed the existence of NCR mechanism during wine fermentation. Gutierrez et al. (2013) compared several industrial wine strains with different nitrogen utilisation pattern and explored the genetical explanation by a large-scale hemizygosity analysis. One of the most popular and effective method used by studies is the quantitative trait loci (QTL) mapping. It is an approach to map the genetic variation responsible for quantitative traits in *S. cerevisiae*. A population of individuals derived from a cross is genotyped and phenotyped, allowing the statistical correlation between genotype and phenotype to map genes affecting the trait of interest. QTL mapping has been successfully applied for the detection of genes responsible for a certain phenotype, such as high temperature tolerance, sporulation, cell morphology, drug sensitivity, ethanol tolerance, flocculation, wine aroma production, etc

(Brice et al., 2014). A limitation of QTL mapping method is that once the QTL is identified, it is difficult to precisely locate the gene in order to identify the molecular basis. One of the solution approaches is reciprocal hemizygosity analysis (RHA). It can be used to evaluate all genes in a QTL, until the genes with phenotypical influences are identified. The RHA method is based on the construction of two isogenic strains in the hybrid diploid background from both parent strains that differ genetically only in the alleles of one copy of a specific candidate gene. The phenotypes of the two strains demonstrate whether an allele from one genetic background is advantageous over that from the other. Studies using RHA alone or combined with QTL mapping has been carried out to identify the genes responsible the differences in nitrogen metabolism (Brice et al., 2014; Gutiérrez et al., 2013; Molinet et al., 2019). One strategy for applying QTL mapping has been used of recombinant populations derived from wine strains showing opposite phenotypes (high nitrogen demanding and low nitrogen demanding (Brice et al., 2014). Other strategy recombined yeast strains representative of the clean lineages based on the phylogeny (Cubillos et al., 2017). By applying QTL mapping and RHA method, many genes involved in nitrogen sensing, signalling, uptaking and genes related to the TOR pathway were identified (Brice et al., 2014; Cubillos et al., 2017; Kessi-Perez et al., 2016; 2019).

Table 5. Genetic approaches to explore the nitrogen metabolism associated genes involved in the differential nitrogen requirements among strains (Modified from Kessi-Perez et al., 2020).

Method	Genes identified	Phenotype under study	Reference
QTL mapping and microarray	*ABZ1*	Nitrogen consumption & Fermentation kinetics	Ambroset et al., 2011
Riciprocal hemizygosity analysis	*ARO8, ADE5,7, VBA3*	Nitrogen source utilisation	Gutiérrez et al., 2013
QTL mapping and riciprocal hemizygosity analysis	*GCN1, MDS3, ARG81, BIO3*	Nitrogen requirments	Brice et al., 2014
QTL mapping	*AGP1, ASI1, GLT1*	Nitrogen consumption	Jara et al., 2014
QTL mapping	*DAL1, DAL4, RIM15, PUT4*	Growth kinetics	Ibstedt et al., 2015
QTL mapping	*RIM15*	Nitrogen consumption & Fermentation kinetics	Kessi-Perez et al., 2016
Allele specific expression & transcription factor binding	*ASN1*	Nitrogen consumption	Salinas et al., 2016
Allele specific expression	*GDB1*	Fermentation kinetics	Cubillos et al., 2016
QTL mapping and bulk segregant RNA-seq analyses	*ARO1, ALP1, ASI2, CPS1, LYP1, PDC1, RPI1*	Nitrogen consumption	Brice et al., 2018; Cubillos et al., 2017
Comparative transcriptomic analysis	*CAR1, ATF1, DUR1,2, PUT1,* and other genes	Fermentation kinetics	Barbosa et al., 2015

Riciprocal hemizygosity analysis	*GTR1, TOR2, SIT4, SAP185, EAP1, NPR1, SCH9*	Nitrogen consumption	Molinet et al., 2019
Gene deletion collection study	*UBC13, MMS2, UBP7, UBI4, BRO1, TPK1, EAR1, MRP17, MFA2, MVB12, MFA2, AAT2*	Fermentation kinetics	Peter et al., 2018
QTL mapping	*KAE1*	Fermentation kinetics	Kessi-Perez et al., 2019

As listed in Table 5, a lot of effort has been devoted to explore the genetic basis of different nitrogen metabolism related phenotypes. However, they are carried out under different experimental conditions, different strains were used and the focused phenotypes were diverse, therefore, no general conclusion can be drawn so far. Future exploration is still needed with more strains involved in a better controlled way. With the rapid development of tools and services in system biology, a combination of methods from genomics, proteomics, metabolomics and bioinformatics may provide the ultimate answer for what is behind the phenotype differences in nitrogen metabolism in *S. cerevisiae*.

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Background & objectives

Wine is one of the most popular alcoholic beverages. Every year, more than 0.2 billion hectolitres of wine is consumed all over the world (data source: OIV http://www.oiv.int/). However, wine production nowadays is facing a lot of challenges. Among them, nitrogen deficiency in grape must is an important issue which largely influences the quality of wine. Nitrogen deficiency causes sluggish or stuck fermentations which can lead to economic losses. A common solution to deal with this problem is nitrogen addition. However, it is difficult to control and over dose of nitrogen may have negative influence on wine fermentation such as the microbial instability and toxic compound formation. Nitrogen requirements and nitrogen source preferences are highly yeast strain dependent. Therefore, exploring the nitrogen requirement of different wine related yeasts will provide valuable information for the wine makers to choose the most suitable yeast strain depending on the grape must nitrogen content.

With the purpose of studying nitrogen metabolism of different yeast species, I started my phD in the Systems Biology in Yeast of Biotechnological Interest (SBYBI) group located at the Institute of Agrochemistry and Food Technology (IATA) of the Spanish Scientific Research Council (CSIC), Valencia (https://www.iata.csic.es/es). The project was supported by Lallemand which is one of the leading yeast producers in the world. The combination of scientific research and industry interests form a perfect collaboration which results in many ready-to-use information benefiting for industrial production. The research group is very experienced in wine-related yeast research. Many previous work of the group focusing on yeast nitrogen metabolism has built a solid background in this research field. The aim of my thesis is to strengthen and deepen the knowledge we have obtained about *S. cerevisiae* and exploring the unknown field of non-*cerevisiae* and non-

Saccharomyces wine yeasts (Fig. 18). A comprehensive overview of nitrogen metabolism by wine related yeast species can be obtained from the result of the thesis.

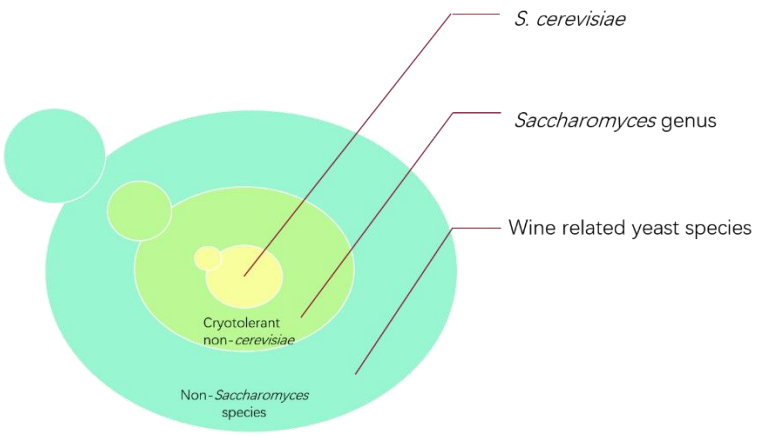

Fig. 18. Flow of thesis research. The work started with central core yeast species for wine making, *S. cerevisiae*. Then cryotolenrant species in *Saccharomyces* genus were studied. At the end, the research was expanded to non-*Saccharomyces* species.

For wine fermentation, *S. cerevisiae* has its prominent position in the realm with its importance being extensively described. Nowadays, the inoculation of commercialised *S. cerevisiae* yeast for wine fermentation is a common oenological practice. The experienced wine makers choose the yeast strains thoughtfully to match the potential of the grape must and the type of wines. Nitrogen concentration is one of the most important features of grape must to be determined before fermentation. To choose the most suitable yeast strains which are able to carry out problem-free fermentations in a certain nitrogen condition, it is crucial to understand the nitrogen requirement of the yeast strain. Therefore, the first objective of my thesis is to explore the nitrogen requirement differences of commercial *S. cerevisiae* strains both

phenotypically and genetically.

Fermentations carried out at lower temperature (10-15ºC) is becoming a popular practice especially for white and rosé wine fermentations. Low temperature fermentation increase not only the retention but also the production of some volatile compounds which improve the sensory profile of wine. However, low temperature is not suitable for the growth of *S. cerevisiae*. The cold environment increases the lag phase, reduces the growth rate, and even leads to sluggish and stuck fermentations. Luckily, there are some cryotolerant species in *Saccharomyces* genus can help to solve the problem. *S. uvarum*, *S. eubayanus* and *S. kudriavzevii* are known for their outstanding cold tolerance during fermentation. However, the nitrogen requirements of these species is unclear, especially during low temperature fermentations. As part of the i-link project "Bases fisiológicas y moleculares de la utilización de nitrógeno durante la fermentación alcohólica", led by José M. Guillamón and in collaboration with the Santiago de Chile University (Chile) and with the Comahue National University (Argentina), the second objective of my thesis was to study the fermentation behaviour of *Saccharomyces* non-*cerevisiae* yeast species under different nitrogen concentrations and temperatures. This work was done in collaboration of Dr. Christian Lopes and his group from Instituto de Investigación y Desarrollo en Ingeniería de Procesos, Biotecnología y Energías Alternativas (PROBIEN), (Neuquén, Argentina). The cryotolerant strains used in the study were isolated by them from the Patagonia region.

Although the cryotolerant yeast species possess many beneficial characteristics for fermentations at low temperature, they have lower tolerance to ethanol and sometimes are not able to finish the fermentation when used as pure culture. Artificial interspecific hybridisation within

Saccharomyces genus is a well-established method to combine the desired traits of both parental strains and gain hybrid vigour. The hybrid construction between *S. cerevisiae* and cryotolerant species perfectly combined the outstanding fermentation capability of *S. cerevisiae* and the cold tolerant character of these non-*cerevisiae* strains. Furthermore, using the selected low nitrogen demanding strains for hybridisation may help to obtain hybrids with both low nitrogen requirement and cold tolerance. This comprises the third objective of the thesis which is to construct interspecific hybrids between *S. cerevisiae* and selected low nitrogen demanding cryotolerant strains and study the fermentation capacity of the hybrids with different nitrogen concentration.

Traditionally, wine was made by spontaneous fermentation, in which a mixture of different microorganisms were involved. The wine quality was often difficult to control, and, in many cases, the wine ended up completely undrinkable. For a long period of time, yeasts other than *S. cerevisiae* were considered as spoilage microorganisms for wine making and they were controlled by inoculation of *S. cerevisiae* at the beginning of fermentation. However, the current trend in oenology has brought some of these "old players" back to the field. One of the reasons that non-*Saccharomyces* yeast strains have gained popularity is their distinctive patterns of aroma production. It diversifies the characteristics of wine and improves wine quality. These non-*Saccharomyces* strains are used in combination with *S. cerevisiae* in forms of mixed-culture or sequential fermentations. Their implementation in the fermentation change the nutrient condition for *S. cerevisiae*, and may cause competition between yeast strains. Therefore, it is of great importance to know the nitrogen requirements and nitrogen source preferences of the non-*Saccharomyces* strains. To explore the nitrogen requirements of non-*Saccharomyces* strains, studies were carried out in collaboration with Dr.

Carole Camarasa and Pauline Seguinot from Institut national de recherche pour l'agriculture, l'alimentation et l'environnement (INRAE), Montpellier, under the support of Lallemand. As the fourth objective of the thesis, two commonly used and commercialised yeast species *T. delbrueckii* and *M. pulcherrima* were studied for their nitrogen source preference and nitrogen assimilation pattern during fermentation.

Unlike the well-studied model organism *S. cerevisiae*, non-*Saccharomyces* species remain as a "mystery land" waiting to be explored. There are many questions relating to nitrogen metabolism still to be answered. One of the most important questions is how nitrogen sources are metabolised and redistributed after entering the cell. To get the answer of this question, I went to Montpellier for a short stay in Dr. Carole Camarasa's group. The group has solid experience on studying yeast nitrogen sources metabolism by quantitative isotope labelling-based analysis. There I carried out isotope labelling experiment by using ^{15}N- and ^{13}C- labelled nitrogen sources, to explore the flux distribution in *M. pulcherrima* and *T. delbrueckii*.

Objectives:

1. Phenotypically determine the nitrogen requirement of commercial *S. cerevisiae* strains and explore the decisive genetic differences on nitrogen requirement (Chapter 1)
   - Determine the growth and fermentation characters of a set of *S. cerevisiae* strains in minimised scale under different nitrogen concentrations.
   - Select high and low nitrogen demanding strains and verify the nitrogen requirement by large scale fermentations

- Determine the competition capability, biomass yield, and nitrogen requirement of high and low nitrogen demanding strains during fermentation of synthetic must with different nitrogen concentration.
- Explore the possible genetic basis influencing the differences on nitrogen requirements.

2. Explore growth, fermentative behaviour and competition capacity of cryotolerant *Saccharomyces* species in different nitrogen conditions (Chapter 2)
   - Determine growth parameters of cryotolerant strains in function of nitrogen concentration.
   - Determine the fermentation capacity of different stains in 3 nitrogen concentrations.
   - Evaluate the competition capacity between *S. cerevisiae* and non-*cerevisiae* strains during fermentation at different temperature and nitrogen conditions.
3. Construct interspecific hybrid among different *Saccharomyces* species and evaluate the fermentative characters (Chapter 3)
   - Construct hybrids between *S. cerevisiae* and non-*cerevisiae* strains
   - Evaluate the fermentation capability of hybrid strains at different temperature and nitrogen concentration.
   - Compare the differences in metabolites and volatile compounds production between hybrids strains and parental strains.
   - Determine the nitrogen sources assimilation order during fermentations at different conditions.
4. Explore nitrogen sources preferences of non-*Saccharomyces* yeasts to sustain growth and fermentation under winemaking conditions (Chapter 4)

- Determine the growth parameters of non-*Saccharomyces* strains with sole nitrogen source.
- Study the fermentation capacity of each strain with sole nitrogen source.
- Explore nitrogen source assimilation order during fermentation.

5. Explore the partitioning of nitrogen sources in the metabolic network of non-*Saccharomyces* strains by quantitative ^{15}N- and ^{13}C-isotope labelling-based analysis (Chapter 5)
   - Study the redistribution of major nitrogen sources through central carbon metabolism pathway by using ^{15}N-labelled ammonium, arginine and glutamine.
   - Determine the flux distribution through Ehrlich pathway by using ^{13}C-labelled valine and leucine.

Chapter 1

Phenotypic and genomic differences among *S. cerevisiae* strains in nitrogen requirements during wine fermentations

Ying Su, Laura Gutiérrez Macías, José María Heras, Amparo Querol and José Manuel Guillamón

Food microbiology (Submitted)

Abstract

Nitrogen requirements by *S. cerevisiae* during wine fermentation are highly strain-dependent. Different approaches were applied to explore the nitrogen requirements of 28 wine yeast strains. Based on the growth and fermentation behaviour displayed at different nitrogen concentrations, high and low nitrogen-demanding strains were selected and further verified by competition fermentation. Biomass production with increasing nitrogen concentrations in the exponential fermentation phase was analysed by chemostat cultures. Low nitrogen-demanding (LND) strains produced a larger amount of biomass in nitrogen-limited synthetic grape musts, whereas high nitrogen-demanding (HND) strains achieved a bigger biomass yield when the YAN concentration was above 100 mg/L. Constant rate fermentation was carried out with both strains to determine the amount of nitrogen required to maintain the highest fermentation rate. Large differences appeared in the analysis of the genomes of low and high-nitrogen demanding strains showed for heterozygosity and the amino acid substitutions between orthologous proteins, with nitrogen recycling system genes showing the widest amino acid divergences. The CRISPR/Cas9-mediated genome modification method was used to validate the involvement of *GCN1* in the yeast strain nitrogen needs. However, the allele swapping of gene *GCN1* from low nitrogen-demanding strains to high nitrogen-demanding strains did not significantly influence the fermentation rate.

Keywords

S. cerevisiae, nitrogen requirement, genetic basis, biomass production, competition experiments, CRISPR/Cas9

1. Introduction

In modern oenology, a common practice followed to conduct wine fermentation is inoculating commercial wine yeast to grape must to ensure wine quality. Specific yeast strains are selected by experienced oenologists to serve difference fermentation purposes. Some of the most substantial features to be considered in yeast selection are their fermentation ability, aroma production, stress tolerance and nutrition requirements. The climate of each vintage, viticulture management and degree of ripeness largely determine the quality of grapes and, subsequently, wine quality (Henschke and Jiranek, 1993; Alexandre and Charpentier, 1998; Bell and Henschke, 2005). The right yeast strains can help oenologists to compensate any defects and enhance the merits of grape must. Nitrogen is one of the most important nutrients for yeast, and its availability in grape must is a key parameter for the wine fermentation progress. Grape-must contains a wide variety of nitrogen sources, including ammonium, amino acids, and small peptides (Kevvai et al., 2016). The nitrogen sources that can be utilised by yeast to support growth and fermentation are defined as yeast assimilable nitrogen (YAN). A sufficient amount of YAN is crucial for the fermentation process to be successful. It regulates yeast biomass formation which, in turn, impacts fermentation kinetics and fermentation duration (Bell and Henschke, 2005; Mart ńez-Moreno et al., 2012; Mendes-Ferreira et al., 2004; Varela et al., 2004). Previous studies reveal that a minimum of 140 mg/L of YAN is required for yeast to complete alcoholic fermentation (Bell and Henschke, 2005; Bely et al., 1990; Butzke, 1998). Deficient YAN in grape-must may cause stuck or stopped fermentation, which can diminish wine quality or lead to wine becoming completely spoiled (Bisson, 1999; Bell and Henschke, 2005). Moreover, many nitrogen sources provide important precursors for aroma

compounds. Therefore, the availability and quality of nitrogen sources directly determine wine quality (Barbosa et al., 2009, 2012; Bell and Henschke, 2005; Seguinot et al., 2018). Winemakers regulate the grape-must nitrogen concentration by adding external nitrogen sources, such as di-ammonium phosphate. However, this practice is not easy to control, and may lead to microbial instability and an undesirable organoleptic profile in the final product (Ough and Amerine, 1988). Consequently, it is relevant to understand the nitrogen requirement of yeast strains in order to choose a suitable candidate to match the nitrogen condition of grape must.

Despite belonging to the same species, the ability of different *S. cerevisiae* strains to carry out fermentations at different nitrogen concentrations is highly strain-dependent (Bely et al., 1990; Gutiérrez et al., 2012; Su et al., 2019a). Some yeast strains are able to conduct fermentations with a limited amount of YAN, whereas others may need more to accomplish the process. Many studies have been carried out to determine yeast nitrogen requirements by analysing their growth and fermentation characters at different nitrogen concentrations (García-Ríos et al., 2013; Gutiérrez et al., 2012, 2013). Competition fermentations under nitrogen-limited (60 mg/L YAN), intermediate (140 or 180 mg/L YAN) and excessive (300 mg/L YAN) conditions were performed by García-Ríos et al. (2015) and Su et al. (2019) to compare the nitrogen demands of two yeast strains. Manginot et al. (1997; 1998) firstly described a creative nitrogen requirement analysis method, which involves maintaining a stable fermentation rate by adding nitrogen in the stationary fermentation phase, and the amount of nitrogen supplied during fermentation was used as a criterion to quantify a certain yeast strain's nitrogen requirement. This method was later applied by Brice et al. (2014a) to assess the nitrogen requirements of different *S. cerevisiae* wine yeasts.

In *S. cerevisiae*, nitrogen metabolism is regulated by a series of interconnected processes, such as the Ssy1-Ptr3-Ssy5 (SPS) signalling sensor system (Forsberg and Ljungdahl, 2001; Ljungdahl and Daignan-Fornier, 2012), the target of rapamycin (TOR) regulatory pathway (Conrad et al., 2014), nitrogen catabolite repression (NCR) (Hofman-Bang, 1999; Beltran et al. 2004), the general amino acid control (GAAC) pathway (Hinnebusch and Natarajan, 2002; Hinnebusch, 2005), and other related regulatory mechanisms. These pathways form a complicated and sophisticated system (Reviewed by Zhang et al., 2018), which sets challenges to understand the underlying genetic basis of nitrogen requirement differences. Brice et al. (2013) compared various physiological traits between high and low nitrogen-demanding strains and conducted a transcriptomic analysis. Their results revealed an overexpression of stress genes in high nitrogen requirement strains and a higher expression of biosynthetic genes in low nitrogen requirement strains. Quantitative trait loci (QTL) and RNA-seq analyses were carried out to identify the genetic variants between strains with different nitrogen requirements (Brice et al. 2014; Cubillos et al. 2017; Jara et al., 2014). Depending on allelic variations, several nitrogen sensing and metabolising genes, namely *GCN1*, *ARG81*, *MDS3* (Brice et al., 2014) and *CPS1*, *LYP1*, *ALP1* and *ASI2* (Cubillos et al., 2017), were identified, and their function in the difference of nitrogen requirements was verified. The confluence of these genes may compose molecular mechanisms that influence nitrogen utilisation by yeast.

In the present study, we comprehensively determined the nitrogen requirements of a set of industrial wine yeast strains by applying different approaches. The growth and fermentation characters of yeast strains were determined under three nitrogen conditions. The strains of high and low

nitrogen requirements were selected and their phenotypic features were further confirmed by competition fermentation, chemostat fermentation and constant rate fermentation. The genome sequences of two representative high and low nitrogen demanding strains were obtained. The genetic differences between strains were analysed and the gene function was studied by the CRISPR/Cas9-mediated genome editing method. New insights are introduced to understand yeast nitrogen requirements both phenotypically and genetically.

2. Material and Methods

2.1 Strains and media

In our study, 28 industrial wine yeast strains provided by Lallemand Inc. (Canada) were used. The strain codes and their corresponding commercial names are showed in Table 1. The oenological features of these strains can be obtained from the company's website (http://www.lallemandwine.com).

Table 1. Strains used in this study

Code Name	Commercial Name
P1	Lalvin®ICVD254
P2	Uvaferm®WAM
P3	Lalvin®ICVD80
P4	Lalvin® Rhone2056
P5	Lalvin®ICVGRE
P6	Lalvin®EC1118
P7	Lalvin®ICVD47
P8	Uvaferm®CEG
P9	Lalvin®Rhone2323
P10	Uvaferm®BC
P11	Uvaferm®VRB
P12	Uvaferm®43

P13	CrossEvolution®
P14	Lalvin®71B
P15	Lalvin®BM45
P16	Enoferm®M1
P17	Enoferm®M2
P18	Uvaferm®BDX
P19	Uvaferm®CM
P20	Lalvin®ICVD21
P21	Lalvin®Rhone2226
P22	Lalvin®CY3079
P23	*
P24	*
P25	*
P26	*
P27	*
P28	T73

*The commercial names are not available for these strains, since they are still under development.

The derivative strains of P12, P13, P17, P20 and P26 were constructed by introducing geneticin resistance gene *KanMX4*. One copy of the open reading frame (ORF) of gene *GAL1* was replaced with deletion cassette *KanMX4* by the short flanking homology (SFH) method. Plasmid pUG6 (Güldener et al, 1996) was used as a template to obtain this deletion cassette. Yeast transformation was carried out by the lithium acetate method (Gietz and Woods, 2002). Transformants were selected by resistance to geneticin and later confirmed by PCR. Primers used for gene deletion and transformants verification is listed in Table 5 in Annex I.

Yeast propagation was carried out firstly in yeast extract peptone dextrose (YPD) medium, which contains 20 g/L glucose, 20 g/L peptone and 10 g/L yeast extract. It was then transferred to SD media (2% glucose, 0.017% yeast nitrogen base) with 230.8 mg/L NH_4Cl (corresponding to 60 mg/L YAN) to

eliminate the influence of YPD nitrogen-rich media. YPD with 200 mg/L G418 disulphate salt (Formedium, UK) was used as geneticin resistance selective medium.

Synthetic grape must (SM) was used for the growth and fermentation experiments. The recipe was modified based on Bely et al. (1990), which contains 100 g/L glucose, 100 g/L fructose, 5 g/L malic acid, 0.5 g/L citric acid and 3 g/L tartaric acid. Minerals and vitamins were supplied at the same concentration as those described by Su et al. (2019a). Nitrogen sources were supplied as a mixture of 40% ammonium chloride and 60% amino acids. The composition of amino acids in the 1-litre stock was 1.5 g L-tyrosine, 13.4 g L-tryptophan, 2.5 g L-isoleucine, 3.4 g L-aspartic acid, 9.2 g L- glutamic acid, 28.3 g L-arginine, 3.7 g L-leucine, 5.8 g L-threonine, 1.4 g glycine, 38.4 g L-glutamine, 11.2 g L-alanine, 3.4 g L-valine, 2.4 g L-methionine, 2.9 g L-phenylalanine, 6 g L-serine, 2.6 g L-histidine, 1.3 g L-lysine, 1.5 g L-cysteine and 46.1 g L-proline, which corresponded to 22.15 g/L of YAN. The nitrogen source concentration in SM was adjusted according to the requirements of different experiments.

2.2 Determination of growth parameter at different nitrogen concentrations

The growth parameters of the various strains were determined at three different nitrogen concentrations: 60, 140, and 300 mg/L YAN. Growth characters were determined by the microtiter plate screening method described by Warringer and Blomberg (2003) and Su et al. (2019). 96 well-microtiter plates were used. Each well was filled with 250 μL of synthetic must and inoculated with 10^6 yeast cells/mL. Growth curves were monitored by recording the increase in optical density (OD) at wavelength 600 nm.

Microtiter plates were incubated in a SPECTROstar Nano® microplate reader (BGM Labtech, Offenburg, Germany) at 28 ℃ with 500 rpm orbital shaking. The optical density of each well was measured every 30 min until the growth reached the stationary phase. All the nitrogen conditions were assayed in triplicate. The growth characters of each strain at different nitrogen conditions can be calculated by directly fitting OD measurements *versus* time to the Gompertz equation proposed by Zwietering et al. (1990), which has the following expression:

$$y = D * exp\ \{-exp[((\mu_{max} * e)/D) * (\lambda - t) + 1]\}$$

where y= ln (OD_t/OD_0), OD_0 is the initial OD and OD_t is the OD at time t; D = ln (OD_∞/OD_0) is the OD value reached with OD_∞ as the asymptotic maximum, μ_{max} is the maximum specific growth rate (h^{-1}) and λ is the lag phase period (h). Phenotypic data were fitted to the Gompertz model by non-linear least-squares fitting using the Gauss-Newton algorithm as implemented in the nls function in the R statistical software, v.3.0.

2.3 Determination of fermentation characters at different nitrogen concentrations

The fermentation characters at the different nitrogen concentrations were screened by microscale fermentation. Fermentations were carried out in 15 mL tubes with 10 mL of synthetic must. The screw caps of tubes were loosened to allow CO_2 to release. SM was modified with three different nitrogen concentrations: 60, 140 and 300 mg/L of YAN. These values correspond to nitrogen limited, standard and excessive conditions (Bely et al., 1990). Around 2 x 10^6 cells/mL were inoculated. Fermentations were followed by CO_2 production, which can be represented by the weight loss of fermentation tubes. The tubes filled with 10 mL SM without inoculation were

used as the controls for evaporation weight loss (EWL). The weight of fermentation tubes was determined around every 12 h. Fermentations were considered completed when tube weight stopped lowering. Fermentation curves can be obtained by plotting weight loss against time. Data were fitted to the Gompertz model as described in 2.2. The maximum fermentation rates (V_{max}) were obtained by applying a similar equation to the calculation with μ_{max}, but by changing only the OD value to the weight loss value. The fermentation experiment was carried out in triplicate.

2.4 Laboratory-scale fermentation

Fermentations were carried out in 100 mL bottles with 80 mL of SM at two nitrogen concentrations (60 and 300 mg/L YAN) at 28 ℃ with continuous orbital shaking at 120 rpm. SM was inoculated with 2×10^6 cells/mL. Fermentation kinetics was followed by measuring the density of SM with a portable densitometer (Densito 30PX, Mettler Toledo, Switzerland). Samples were centrifuged to remove yeast cells before density measurements. Fermentation was considered finished when density reached 998 g/L (Gutiérrez et al., 2012).

2.5 Competition fermentation

In order to determine the competitiveness of the different yeast strains at certain nitrogen concentrations, one-to-one competition fermentation was carried out between different pairs of *S. cerevisiae* strains. Fermentations were performed in 80 mL SM as described in 2.3. In all, 2×10^6 cells/mL were inoculated with 50% of each strain. One of the strains in the competition pairs was labelled with geneticin (G418) resistance marker *KanMX4*. The yeast cell population was determined by plating on YPD and counting colony-forming

units (CFU). The percentage of each strain throughout fermentation was monitored by replica plating from YPD to YPD-G418.

2.5 Chemostat fermentation and dry weight measurements

Chemostat cultures were carried out to determine the influence of nitrogen concentration on yeast biomass production. Continuous cultures were performed at 28 ℃ in a 0.5 L reactor (MiniBio, Applikon Biotechnology) with a working volume of 0.35 L. The operation parameters in the bioreactor were temperature 28 ℃, pH 3.3 and 300 rpm stirring. The dilution rate (D) was set at 0.2 h^{-1}, which corresponds to the exponential growth phase. pH at 3.3 was controlled by the automatic addition of 2 M NaOH and 2×10^6 cells/mL yeast were inoculated. Prior to starting the continuous culture, cells were allowed to grow in a batch culture under the same condition as the continuous culture in SM with 60 mg/L of YAN to achieve enough biomass in a batch phase. The continuous culture started when the batch culture entered the stationary phase. Steady states were sampled only after all the continuous cultures had been running for at least five residence times and the biomass values were constant. The continuous cultures were fed with the SM of the high YAN concentration. Every time the steady state was achieved, the introduced SM was changed to an SM with a higher YAN concentration. The continuous culture was started by introducing SM with 60 mg/L of YAN before being increased to 80, 100, 140, 160, 180, and finally to 200 mg/L of YAN. For the biomass dry weight analysis, 5 ml of samples were collected in a pre-weighed tube and centrifuged at 4000 rpm for 5 minutes to remove the supernatant. The cell pellet was washed 3 times with distilled water and dried in a 70 ℃ oven for 2 days until constant weight.

2.6 Constant rate fermentation by nitrogen addition

Nitrogen requirements during fermentations were also determined by constant rate fermentation as described by Manginot et al. (1997, 1998). Batch fermentations were carried out at 28 ℃ in a 0.5 L bioreactor (MiniBio, Applikon, the Netherlands) with a working volume of 0.35 L. 2×10^6 cells/mL were inoculated in SM at the initial nitrogen concentration of 140 mg/L of YAN. The CO_2 production rate was monitored by a gas monitor (Multi-Gas Monitors INNOVA 1316, LumaSense Technologies) throughout fermentation. When the CO_2 production rate reached the peak value, the automatic addition of nitrogen compounds (40% NH_4Cl and 60% amino acids) was activated to maintain the CO_2 production rate at the maximum value until the residual sugar become limiting. Residual nitrogen was determined by HPLC as described by Su et al. (2019b). The amount of nitrogen added to fermentation throughout this process was considered to be the nitrogen required to maintain a constant fermentation rate.

2.7 Genome sequencing and analysis of two *S. cerevisiae* strains.

Two of the selected strains, P17 and P20, were sequenced by Illumina HiSeq with 150 bp paired-ends reads. Trimmomatic v.0.33 (Bolger, Lohse, and Usadel 2014) was used to remove adapters, while Sickle (version 1.33, available at https://github.com/najoshi/sickle) was run to read trimming by quality. *De novo* genome assemblies were obtained using SPAdes v.3.1.0 (Bankevich et al. 2012). Annotation was done by transferring the annotation from the *S. cerevisiae* S288c reference strain (R64-2-1) to our assemblies using RATT (Otto et al. 2011). Then a *de novo* gene prediction with Augustus (Stanke and Waack 2003) was performed. Finally, both annotations were merged and manually corrected.

Phylogeny reconstruction was performed using the genome sequencing data from 90 representative strains of different *S. cerevisiae* lineages (Peter et al., 2018). In all, 1732 orthologous genes were translated into proteins, aligned with MAFFT v.7.2 (Katoh and Standley, 2013) and backtranslated into codons. The concatenated codon alignment was carried out for maximum-likelihood phylogeny reconstruction using RAxML v.8.2 (Stamatakis, 2014) with 100 rapid bootstraps and the GTRGAMMA model.

To perform a chromosome copy number variation analysis, reads were mapped against the reference assembly of *S. cerevisiae* S288C (version R64-2-1) using bowtie2 v2.3.0 (Langmead and Salzberg, 2012). The position read depth was computed using an in-house Python script of a sliding-window method in which the mean mapping reads was calculated and represented in 10 kb windows along each chromosome.

A variant calling analysis was performed using FreeBayes v1.2. (Garrison and Marth 2012) with default parameters. Low quality SNPs were removed with vcftools (Danecek et al. 2011).

To analyse the differences between genomes of P17 and P20 at the coding sequence level, pair-wise amino acid substitutions were calculated using aligned pairs of orthologous proteins between P17 and P20. In this way, a total number of 6035 orthologs were found and analysed using an in-house Python script.

2.8 CRISPR/Cas9-mediated allele swapping

CRISPR/Cas9-mediated genomic editing was performed to explore the genetic basis for the differences in nitrogen requirement. CRISPR/Cas9 plasmid pWS174 (Addgene plasmid # 90961; http://n2t.net/addgene:90961;

RRID: Addgene 90961) was used in our study. This plasmid contains *NatR* as a yeast marker, *KanR* as an *E. coli* marker and GFP, which is replaced with the designed guide RNA (gRNA). The 20 bp gRNA target was designed by following the instructions described in Benchling (https://www.benchling.com). A *Bsm*BI restriction site (GACT overhang at the 5' and GTTT at the 3', and an additional TT included between the 5' overhang and the gRNA sequence) was added to the target sequence. The oligonucleotides were assembled into pWS174 as described by Shaw et al., (2019). They were firsly phosphorylated by T4 PNK, annealed and then assembled to the vector in a 10 µL *Bsm*BI golden gate assembly using T4 DNA ligase (Thermo Fisher Scientific). The reaction mix was transformed into *E. coli* and plated on LB medium with kanamycin. In the successfully constructed plasmids, GFP should be replaced with gRNA. Therefore, the *E. coli* colonies without florescent were selected, and plasmids were extracted and verified by PCR and sequencing. The gRNA sequences and primers are shown in Table 5 in Annex I. To accomplish a precise genome modification, the homology directed repair (HRD) method was applied for double strand break (DSB) repair. Donor DNA was transformed into yeast cells together with CRISPR plasmids. Yeast transformation was carried out by the lithium acetate method (Gietz and Woods, 2002). For each transformation, around 100-200 ng of CRISPR plasmid and more than 2 µg of donor DNA were used as illustrated by Shaw et al. (2019).

Two successive CRISPR events were carried out with the gene *GCN1* of P17 to achieve allele swapping as described by Biot-Pelletier et al. (2016). In the first step, the CRISPR-Cas9 system introduced DSB in *GCN1* and the *KanMX4* cassette obtained from the pUG6 plasmid (Güldener et al, 1996) with 40 bp homology arms of the target was used as donor DNA to repair

DSB. 628 bp of the target gene were replaced with *KanMX4*. Transformants were selected by double resistance of G418 and nourseothricin. Primers up- and down-stream of the *KanMX4* was designed and the insertion was verified by sequencing. In the second step, the new gRNA that targeted the inserted *KanMX4* was designed. CRISPR plasmid introduced the DSB in the *KanMX4* gene, and part of *GCN1* gene including the mutation sites (762 bp) from P20 was used as the donor DNA for DSB repairing. Transformants were selected by replicate plate from YPD-clonNAT to YPD-G418. The transformants without resistance to G418 were further confirmed by sequencing. The newly constructed strain was named P17m.

Laboratory-scale fermentations (80 mL, 28 ℃) were carried out with SM of 60 mg/L of YAN to check the fermentative ability of P17 modified with the P20-*GCN1* allele (P17m) in a nitrogen-limited environment. P17 and P20 were used as the control strains. Fermentations were monitored by measuring density throughout the fermentation process and were considered completed when density went below 998 g/L.

2.9 Statistical analysis

All the growth and fermentation experiments were carried out in triplicate. Parameters of fermentation kinetics was calculated by fitting the density data to the 4-parameter logistic model. The time needed to consume 5% (T5), 50% (T50) and 100% sugars (T100) was extracted from the smoothed data. A one-way analysis of variance (ANOVA) was conducted with version 7.0 of the Statistica software package. The statistical level of significance was set at $p \leqslant 0.05$ with a Tukey HSD test.

3. Results

3.1 Growth performance of the different strains in SM at three concentrations

The 28 commercial wine yeasts showed different growth performances in SM with 60, 140 and 300 mg/L of YAN (Fig. 1). As expected, the strains grown in SM with 60 and 300 mg/L of YAN (SM60 and SM300) obtained the lowest and highest maximum growth rate, respectively. Surprisingly, the SM with 140 mg/L of YAN (SM140) did not promote significantly higher μ_{max} than SM60, which suggests that higher nitrogen concentrations are needed to speed up cell duplication. The Area Under the Curve (AUC; Fig. 2) was also calculated, which represents the three main growth parameters: lag phase, μ_{max} and maximum population (yield). The AUC values showed clear differences in all three nitrogen conditions, and SM140 had the highest value and SM60 had the lowest one. Thus the highest nitrogen content (SM300) promoted the highest growth rate, but prolonged the lag phase (Fig. 2E).

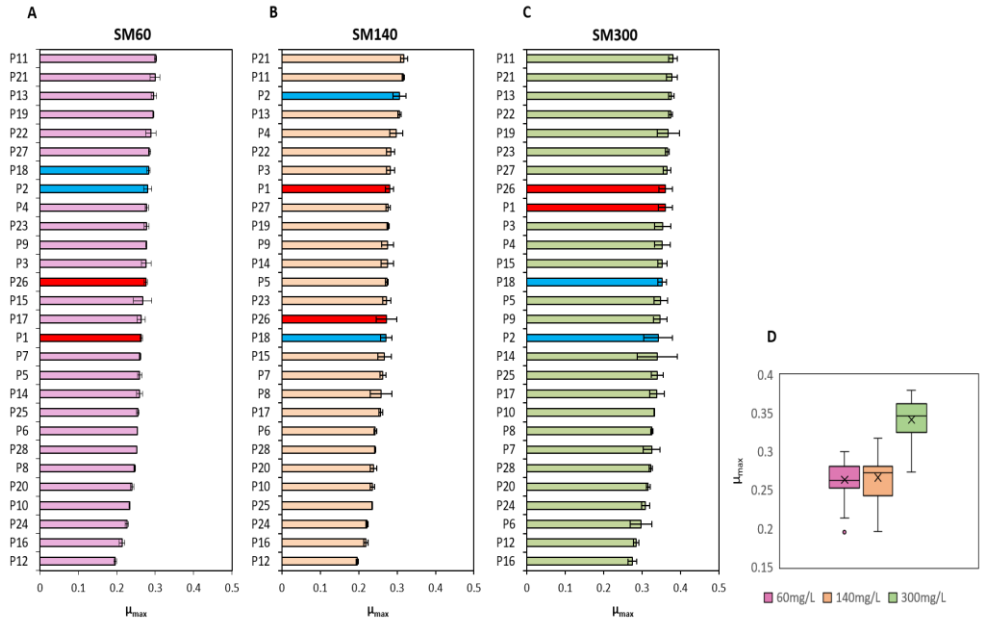

Fig. 1. Maximum growth rate (μ_{max}) of 28 strains in SM60 (**A**), SM140 (**B**) and SM300 (**C**). Box plot summaries the μ_{max} of all the strains at three nitrogen concentrations (**D**).

On the Y-axis of Fig. 1, strains were ranked from high to low by their μ_{max} under each growth condition. Strains P11, P13 and P21 showed the best growth performance for all three conditions. On the contrary, strains P12, P16 and P24 were among the worst ones regardless of the nitrogen concentration. Most strains maintained their ranking position within three nitrogen concentrations. Some representative exceptions were P1 and P26, which the μ_{max} showed lower ranking at a low nitrogen concentration, but their μ_{max} substantially increased with SM300 and their ranking consequently went up on the Y-axis. Conversely, the ranking position of P2 and P18 dropped with increased nitrogen concentrations. This indicates that a higher nitrogen concentration significantly influences the growth performance of these strains. P1 and P26 may need a higher amount of nitrogen to obtain better growth,

whereas the strains P2 and P18 seemed to grow better than other tested strains when nitrogen concentration is limited.

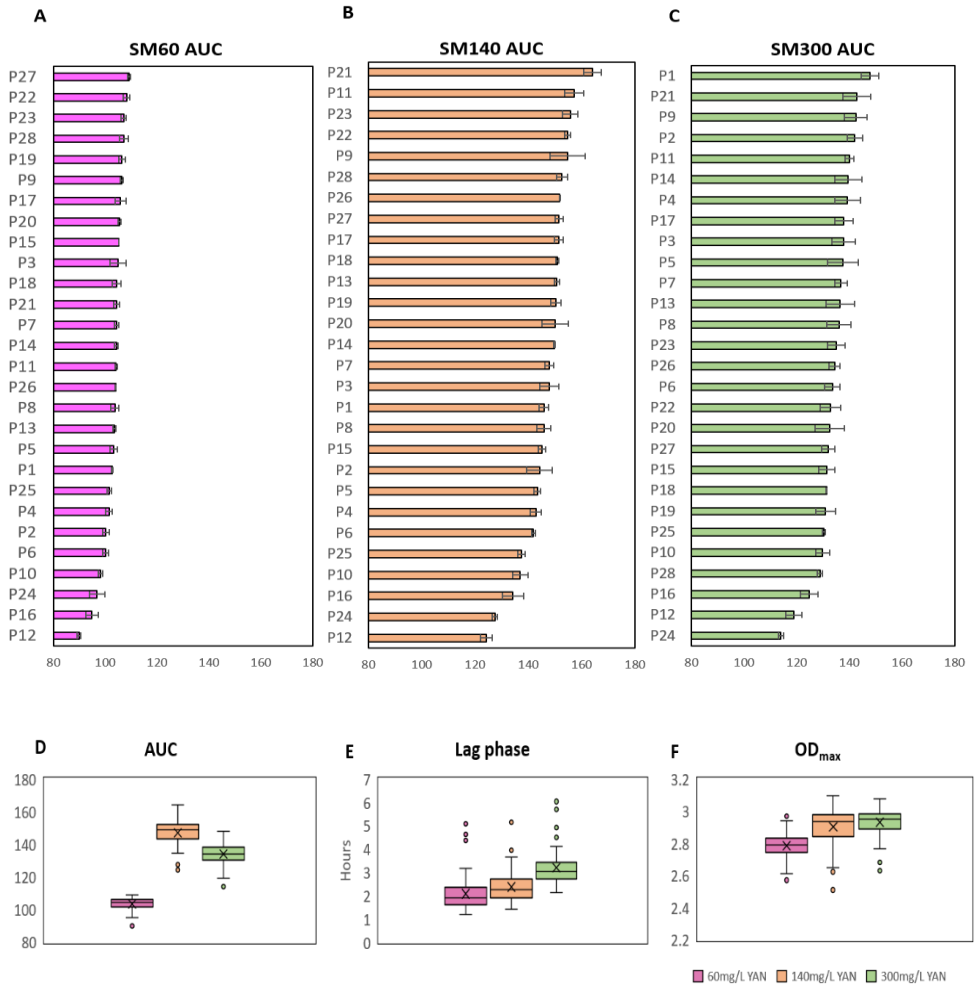

Fig. 2. Ranking of the AUC value of the different strains with SM60 (**A**), SM140 (**B**) and SM300 (**C**). Box plot summarises AUC (**D**), Lag phase (**E**) and OD_{max} (**F**) of all the strains at three nitrogen concentrations.

3.2 Fermentation performance of different strains at three nitrogen concentrations

Similar to the growth performances, the same wine yeast strains showed distinct fermentation behaviours with SM60, SM140 and SM300. An overlook of the maximum fermentation rate clearly demonstrated that a higher nitrogen concentration in the SM resulted in higher fermentation rates (Fig. 3D). The maximum fermentation rate of each strain was ranked high to low on the Y-axis (Fig. 3A-C). Different ranking pictures were observed for each nitrogen concentration. Unlike that observed for the growth rate, the ranking positions of many strains changed at three nitrogen concentrations, which indicated the significant influence that nitrogen availability had on the fermentation rates. Several representative strains were selected. Strains P9, P11, P13 and P20 showed good fermentation performance with limited nitrogen. However, when carrying out the fermentations with higher nitrogen concentrations, fermentation rates were not as promoted as other strains, which resulted in lower rankings. Conversely, strains P12, P17 and P26 showed poor fermentation performance with SM60, but their fermentation rates dramatically increased with higher nitrogen concentrations, therefore, ranked higher on the Y-axis. In our study, strains P9, P11, P13, and P20 were considered as low nitrogen-demanding strains (LND) for their outstanding fermentation performance with low nitrogen concentration, and their average to poor performance with a high nitrogen concentration; *vice versa*, strains P12, P17 and P26 were considered as high nitrogen-demanding strains (HND) as their fermentation performance clearly enhanced only by a high nitrogen concentration.

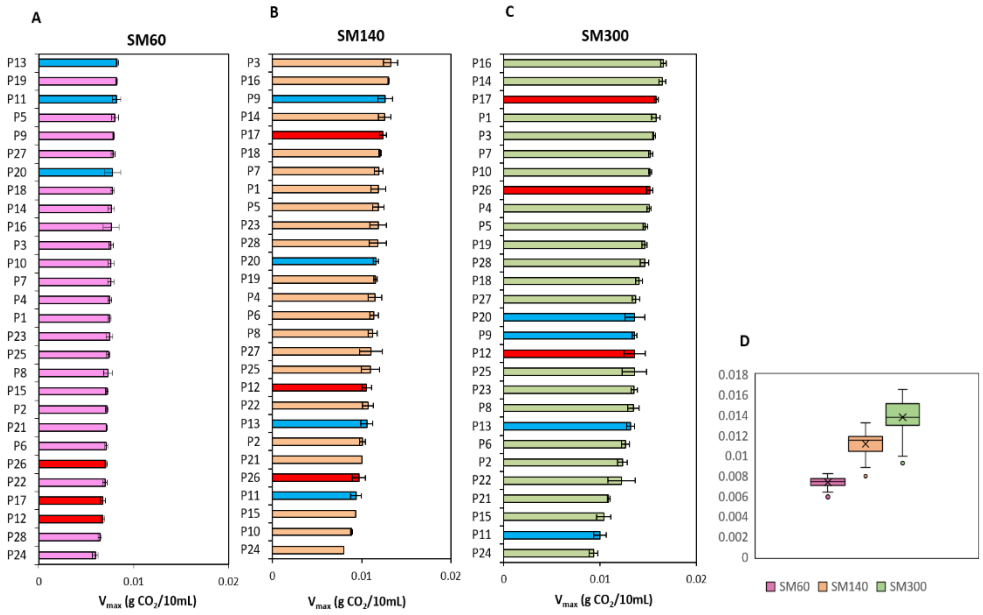

Fig. 3. Maximum fermentation rate (V_{max}) of 29 strains in SM60, SM140 and SM300 (**A**-**C**). The selected high nitrogen-demanding strains are marked in red, and the low nitrogen-demanding strains are marked in blue. **D.** Box plot summaries the V_{max} of all the strains at three nitrogen concentrations.

3.3 Laboratory-scale fermentation to confirm the selected strains

The fermentation performances of seven selected high or low nitrogen-demanding strains were further evaluated by carrying out microvinification in 80 mL of SM with 60, 140 and 300 mg/L of YAN. Fermentation kinetics were determined more precisely by measuring density reduction. Strains P13 and P20, which were selected as low nitrogen-demanding strains, finished fermentations (T100) in a significantly shorter time in SM60 than the strains selected as high nitrogen-demanding P17 and P26 (Fig. 4), with SM300, P13, P17, P20 and P26 finished fermentation almost at the same time. Strains P9 and P12 did not show the same features as observed in the fermentations done with 10 mL of SM. Depending on the results obtained with the 10 mL

fermentation, P12 was classified as high nitrogen-demanding. However, in 80 mL of SM, it was the first strain to finish the fermentation with SM60, and the last strain to finish the fermentation with SM300, which correspond to the features of a low nitrogen-demanding strain. Strain P9 required a longer time to finish fermentations at all three nitrogen concentrations. Strain P11 displayed an average behaviour for all three conditions. According to our results, candidates were narrowed down to P12, P13 and P20 as LND strains, and to P17 and P26 as HND strains.

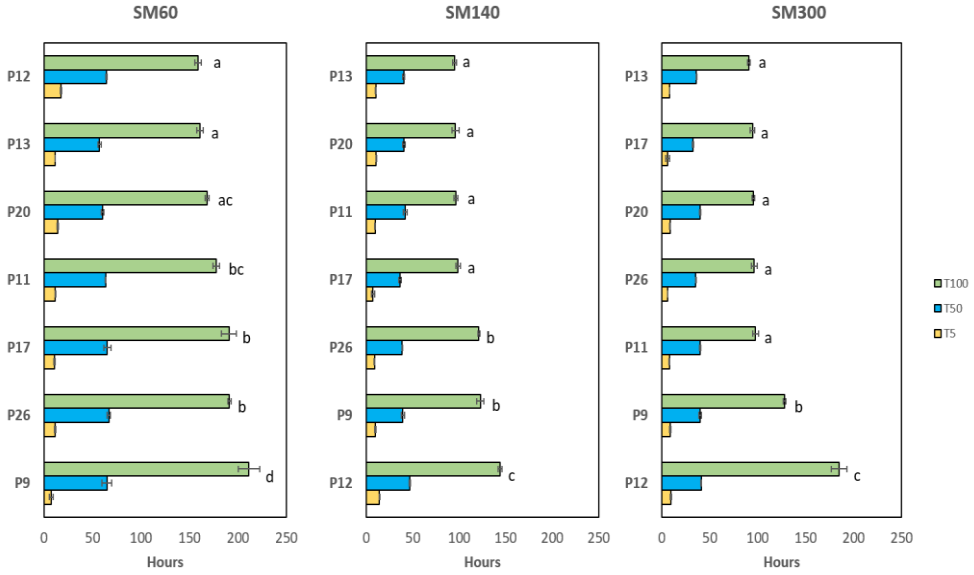

Fig. 4. Time (hours) required to consume the 5% (T5), 50% (T50) and100% (T100) of the sugar content in SM60, SM140 and SM300.

3.4 Competition fermentation

Competition fermentations were carried out with pairs of strains under the nitrogen-limited (SM60) and excess nitrogen (SM300) conditions. Five competition pairs were analysed: P17(HND) vs. P20(LND), P17(HND) vs.

P12(LND), P17(HND) vs. P13(LND), P26(HND) vs. P20(LND) and P26(HND) vs. P13(LND). The percentage of each strain was monitored during fermentation (Fig. 5). In four of the five analysed pairs, one strain always outcompeted the another one regardless of the nitrogen concentration in SM: P13(LND) outcompeted P17(HND); P26(HND) outcompeted P12(LND); P20(LND) outcompeted P26(HND); P17 (HND) outcompeted P12 (LND). These results suggest that the outcome of the competition between these pair of strains was not dependent on nitrogen concentration. Conversely, pair P17(HND) vs. P20(LND) exhibited different competition behaviours. When fermentation was carried out with SM60, the percentage of P20 gradually increased from 50% to 62% at the end of fermentation, whereas the percentage of P17 dropped to around 37%. P17 showed a growth advantage with SM300, and its percentage increased to 64% at the end of fermentation, which left only 36% of P20 in fermentation. Nitrogen concentration clearly determined the dominant population in fermentation. This competition pair perfectly matched our assumption, and further confirmed that P17 was LND and P20 was HND.

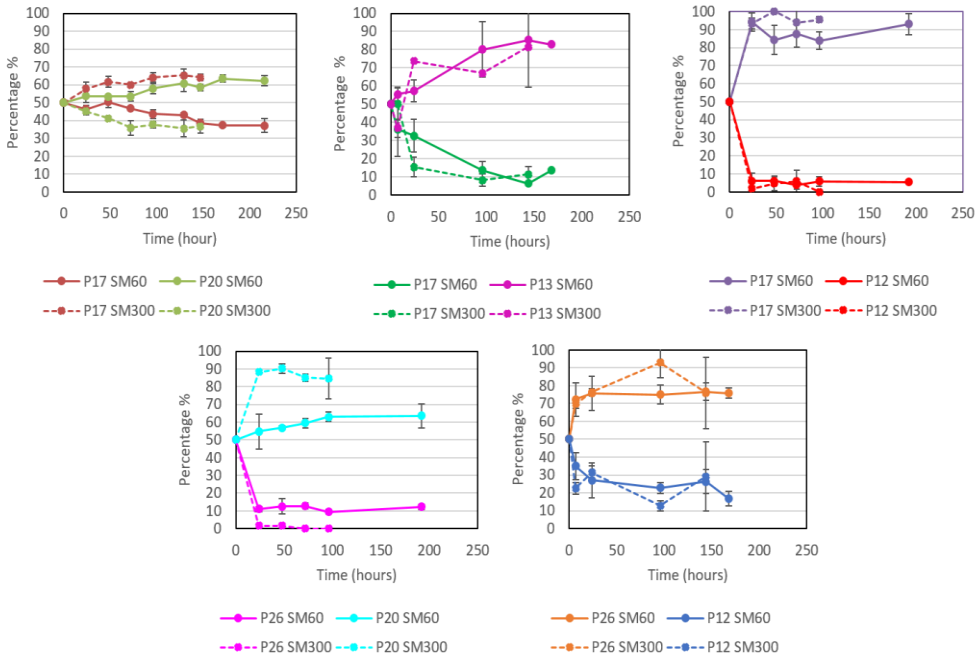

Fig. 5. The percentage of each strain during fermentation within each competition pair of strains HND and LHD in SM60 and SM300.

3.5 Influence of nitrogen concentration on biomass production

Nitrogen availability significantly influences yeast biomass production. A continuous culture with an increased nitrogen concentration was carried out to determine the influence of nitrogen concentration on biomass production in the exponential fermentation stage. The selected HND P17 and LND P20 strains were evaluated. Overall for both strains, biomass production increased with nitrogen concentration until YAN reached 140 mg/L (Fig. 6A). Then the biomass remained stable with a nitrogen concentration higher than 140 mg/L. Interestingly when SM with 60 and 80 mg/L of YAN was supplied to the continuous culture, the LND strain P20 produced more biomass than P17. When YAN exceeded 100 mg/L, the biomass production by HND strain P17 rapidly increased and was significantly higher than that of P20. When the

nitrogen concentration continued to increase to 140 mg/L of YAN, both strains reached their maximum carrying capacity. SM with more than 140 mg/L of YAN triggered flocculation formation with P17, which consequently resulted in a lower unstable biomass production. The biomass formation by P20 remained stable with SM from 140 to 200 mg/L of YAN. By way of conclusion, when the nitrogen concentration was limited (SM60 and SM80), the LND strain produced more biomass than the HND strain. The HND strain started to produce more biomass than LND with SM100. Biomass formation stopped increasing when the nitrogen concentration reached 140 mg/L, which can be considered the nitrogen-limiting concentration to reach the maximum biomass.

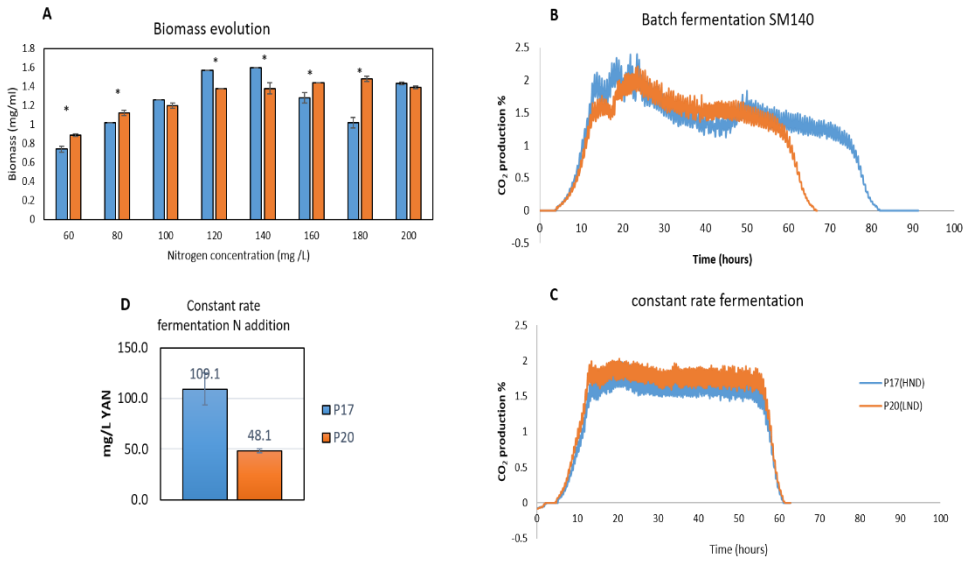

Fig. 6. **A**. Biomass production of P17 (HND) and P20 (LND) in a chemostat with increasing nitrogen concentrations. *indicates a significant difference between two strains (HSD Tukey test $p<0.05$). **B**. CO_2 production during batch fermentation of strain P17 (HND) and P20 (LND). **C**. CO_2 production of P17 (HND) and P20 (LND) during constant rate fermentation as consequence of nitrogen addition. **D.** Amount of nitrogen added during constant rate fermentation.

3.6 Determining yeast nitrogen requirement by constant rate fermentation

Constant rate fermentation was carried out to evaluate the amount of nitrogen required by yeast strains to maintain the fermentation rate (CO_2 production) at the same level throughout fermentation. In a normal batch fermentation of SM140, P20 required some 15 h less than P17 to complete fermentation (Fig. 6B). When the fermentation rate was controlled and remained constant by supplying nitrogen to SM, the two strains finished fermentation almost at the same time (Fig. 6C). This indicates that the availability of nitrogen sources could be the limiting factor for the differences found in the fermentation times between two strains. Nitrogen sources were added in the form of a mixture containing amino acids and ammonium. The result showed that P17 required twice as much nitrogen as P20 to maintain a constant fermentation rate (109.1 *vs*. 48.1 mg/L YAN; Fig. 6D).

3.7 Genome sequencing and comparison between LND and HND strains

The genomes of P17 and P20 were sequenced and analysed, and 6161 genes were annotated for P17, as were 6145 genes for P20. The sequencing data are available at NCBI database under bioproject accession number PRJNA625869. The phylogenetic analysis using representative genomes of the different described *S. cerevisiae* lineages (Peter et al., 2018) (Table S1) showed that strains P17 and P20 were grouped into the Wine/European clade (Fig. 7).

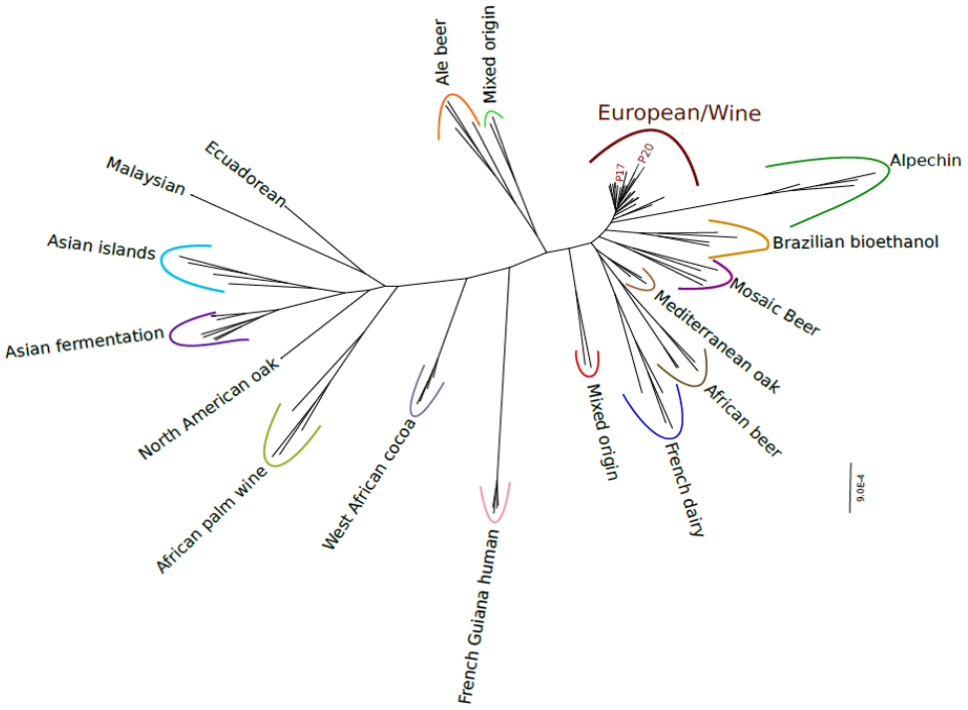

Fig. 7. Phylogenetic tree with 90 strains from different *S. cerevisiae* lineages.

The chromosome copy number analysis did not show any signal of aneuploidy in either of the two strains. Interestingly, the variant calling analysis evidenced that P17 was completely homozygous along the genome, but P20 presented higher heterozygosity levels with some patterns of loss of heterozygosity in specific chromosome regions (Fig. 8). Position read depth is shown in Fig. 9. Another major difference between the two strains was that strain P20 had a region of 35 genes in chromosome XV duplicated. The genes contained in this region are shown in Table S2. Neither gene ontology (GO) nor pathway enrichment was found for this set of genes.

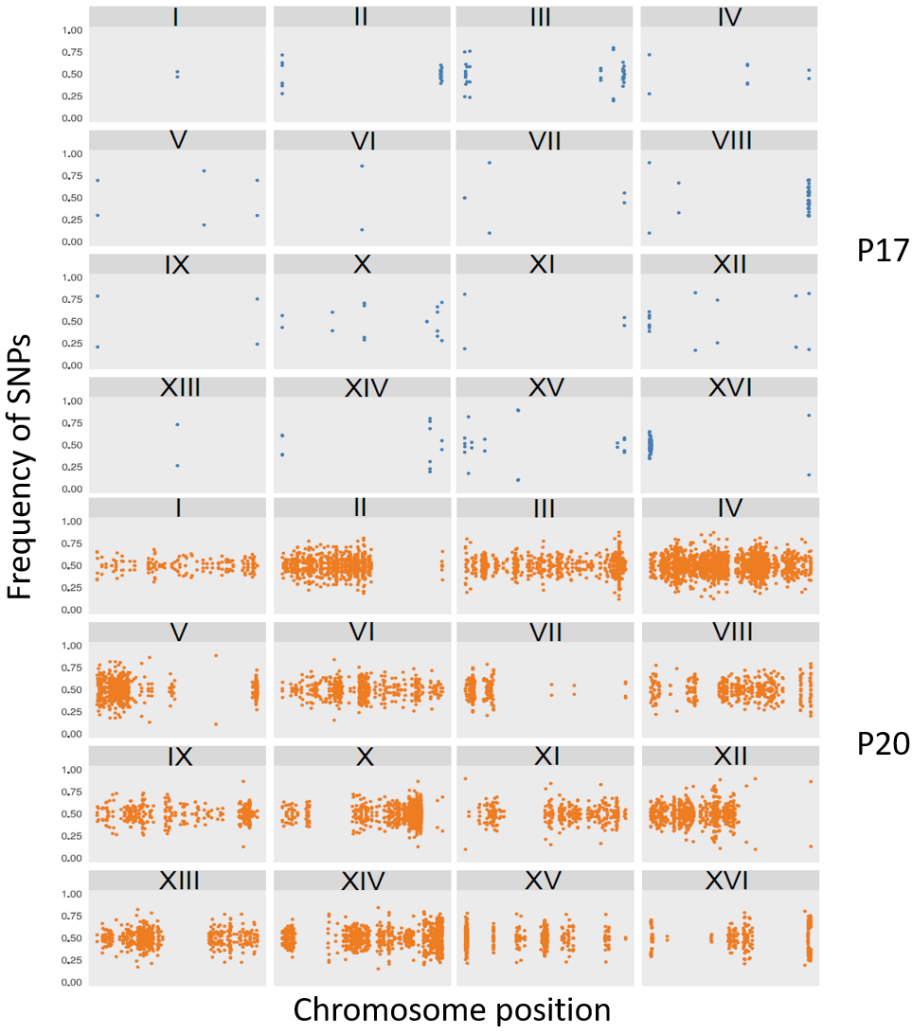

Fig. 8. Herozygotic positions along chromosomes P17 (blue) and P20 (orange).

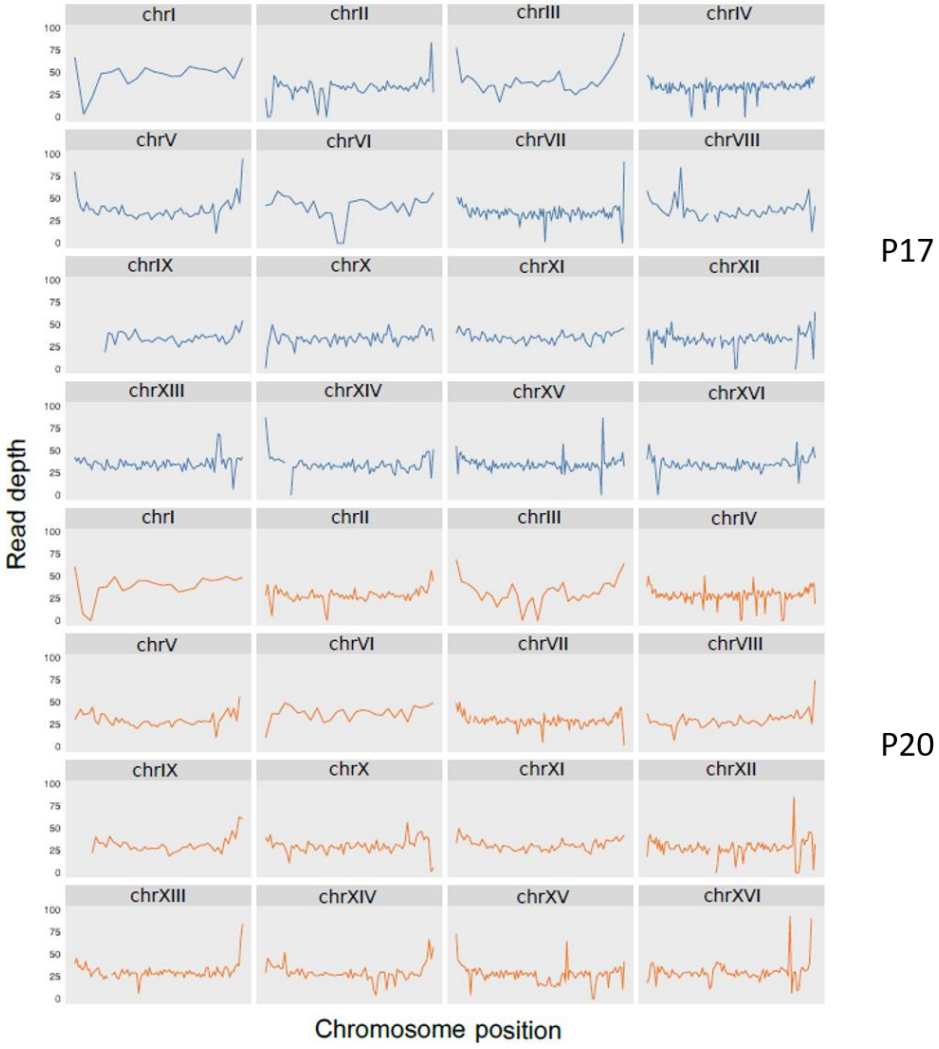

Fig. 9. Position read depth along P17 (blue) and P20 (orange) chromosomes.

The proportions of amino acid substitutions were calculated between the pair-wise orthologous proteins from the annotation of genomes P17 and P20 (Table S3). Higher values indicate a higher proportion of amino acid positions in which amino acid residues differ compared to the orthologous aligned proteins of P17 and P20. The genes encoding the proteins with the highest values were: YLR255C, a protein of unknown function, but one that is

conserved across *S. cerevisiae* strains; *VPS61*, a dubious ORF whose deletion causes a vacuolar protein sorting defect (Fisk et al., 2006); *EHD3*, a 3-hydroxyisobutyryl-CoA hydrolase whose deletion affects fluid-phase endocytosis (Wiederkehr et al., 2001) and is related to the proteome of *S. cerevisiae* mitochondria (Sickmann et al., 2003).

3.8 CRISPR/Cas9-mediated allele swapping and the gene function analysis

Of all the genes showing amino acid divergence, *GCN1* was identified as our gene of interest. This gene has been previously connected to nitrogen metabolism regulation (Brice et al., 2014) and we considered that it was worth evaluating the different alleles in both strains. This gene is a positive regulator of the Gcn2p kinase and forms a complex with Gcn20p (Garcia-Barrio, et al., 2000; Kubota et al., 2001). It is involved in nitrogen sensing and signalling in response to nitrogen availability (Hinnebusch, 2005). Four non-synonymous mutations were found between *GCN1* of P17 and P20 (Fig. 10A). Allele swapping was performed to investigate the influence of mutations on nitrogen requirement. As *GCN1* is a relatively large gene of 8kb, it is difficult to make modifications with the entire gene. Therefore, a 762-bp piece, including three mutation points at amino acid positions 377, 556 and 585 in P17, was replaced with the allele of P20 by the CRISPR/Cas9-mediated genomic editing method. Fermentations were carried out with P17, P20 and modified strain P17m in SM60. SM60 is nitrogen-limited medium and our previous data showed large differences in fermentation performance in both strains under this condition. However, the result demonstrated that the fermentation behaviour of P17m was similar to that of P17 (Fig. 10B). No significant differences in T5, T50 and T100 were observed between P17 and P17m, unlike those between P17m and P20 (Fig. 10C). The introduction of the SNPs of the allele showing a

better fitness (P20) did not improve the fermentation performance of the strain that was more affected by nitrogen limitation (P17).

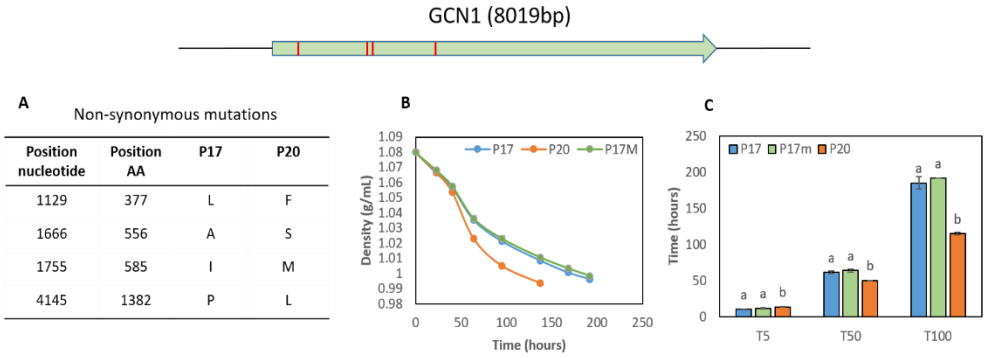

Fig. 10. **A.** Mutation positions of *GCN1* between P17 and P20. **B.** Fermentation kinetics in SM60 with modified strain P17m. **C.** T5, T50 and T100 of fermentation in SM60. Different letters above bars indicate significant differences (HSD; Tukey test $p<0.05$).

4. Discussion

Nitrogen is one of the most important nutrients for yeast that has a substantial impact on wine fermentation process. The nitrogen requirement of yeast is highly strain-dependent. Understanding the nitrogen demand of yeast strains is of significant importance for a successful winemaking process. A set of *S. cerevisiae* wine strains was herein assessed for their growth and fermentation activities in SMs with different nitrogen concentrations. HND and LND strains were selected and the corresponding genetic evidence was explored.

Our first aim was to assess the impact of nitrogen concentration on yeast growth and fermentation activity in a set of 28 *S. cerevisiae* industrial wine yeast strains. In growth behaviour terms, we focused on the influence of nitrogen on the maximum growth rate (μ_{max}) and the AUC. Surprisingly both parameters did not show any direct correlation with nitrogen concentration.

The maximum growth rate increased only with the highest nitrogen concentration, whereas the highest AUC value was obtained for SM140. In a similar study, Gutiérrez et al. (2012) reported that the nitrogen-limiting concentration required to reach the μ_{max} value was much lower than the nitrogen concentration to obtain the maximum area. This perfectly matches our results when SM300 was excluded (this high nitrogen concentration was not tested in Gutiérrez et al. (2012)). In any case, this indicates that SM140 did increase the final cell population and the lag phase time was relatively shorter, but the concentration was not high enough to improve the maximum growth rate. SM300 promoted the maximum growth rate, but the highest nitrogen concentration may have some inhibiting effect for yeast to start growing because a much longer lag phase was observed (Fig. S1). As expected, we observed a positive correlation between μ_{max} and OD_{max} and a negative correlation between OD_{max} and the lag phase time regardless of nitrogen concentration (Fig. 11). However, both the positive and negative correlations were stronger for SM140 than for SM300. Ibstedt et al. (2014), who exhaustively evaluated the lag, rate and efficiency of *S. cerevisiae* in a wide variety of nitrogen-limited environments, concluded that growth components were well correlated under nitrogen restriction and pointed out a concerted evolution of yeast nitrogen use.

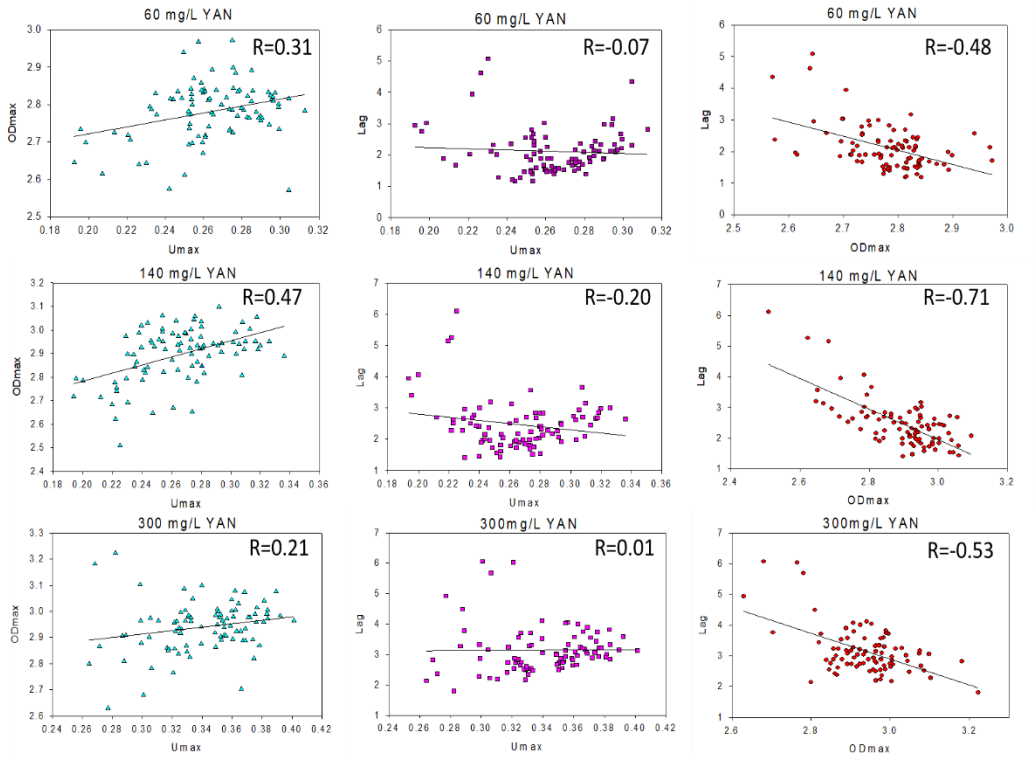

Fig. 11. Pearson correlation analysis between the maximum growth rate (μ_{max}), the maximum biomass yield (OD_{max}) and the lag phase time (Lag) for each nitrogen concentration. Linear regression (black line) is displayed. The correlation coefficient (R) is also shown.

When we compared the growth and fermentation performance among strains at the different nitrogen concentrations, we observed that nitrogen concentration clearly determined the fermentation fitness of most strains, as seen from the changing ranking orders under different conditions. These changes in strain ranking were not so evident for growth performance. The yeast fermentation rate can be influenced by either glycolytic flux per cell or the yeast population. Several previous studies have concluded that in nitrogen-deficient fermentation, the fermentation rate is driven by yeast cell population rather than by specific flux per cell (Albertin et al., 2011; Valera et al., 2004). However, Gutiérrez et al. (2012) provided evidence for the non-

correlation between biomass production and nitrogen requirement. Similarly, Barbosa et al. (2014) compared growth and fermentation performance at two different concentrations, and concluded that the highest fermentation rates were observed under high nitrogen conditions due to the higher final yeast cell biomass. However, at the same nitrogen concentration when comparing yeast strains, the same correlation between the yeast cell final biomass and better fermentative performance was not established.

The first screening based on miniaturised systems for determining the growth and fermentation activity of the different strains allowed us to select several strains to be categorised as HND and LND. Pair-wise combinations of strains (LND vs. HND) were used to ferment SMs with a limiting (SM60) and non-limiting (SM300) nitrogen concentration to determine the competitiveness of each strain according to nitrogen availability. During the fermentation process many species and strains are competing for the resources and the absolute numerical presence, and persistence over time, of these species/strains are affected by parameters such as temperature, sulphur dioxide (SO_2), pH, osmotic pressure, oxygen and nutrient availability (Bagheri et al., 2020; Garcia-Rios et al., 2014; Pérez-Torrado et al., 2017; Su et al., 2019). In our experiment of competitions, only the fitness of one pair of strains was clearly determined by nitrogen concentration. In the other strain combinations, other abiotic or biotic factors had to determine the capacity to outcompete other strains. So the fermentation tests provided us with valuable information to select the best candidates as LND and HND strains.

After selecting the most promising candidates with different nitrogen requirements, we assessed the influence of increasing nitrogen concentration on biomass production and fermentation rate in a more controlled and accurate system. For biomass production, we used different continuous

cultures with increasing nitrogen concentrations in the feed medium, which ensured a strict control of the fermentation conditions (Vázquez-Lima et al., 2014). The results clearly showed that the LND strain was more efficient in producing biomass at low nitrogen concentrations whereas it was the other way around at a high nitrogen concentration. We also determined the measurement of the nitrogen supply required to maintain the fermentation rate constant in the stationary phase (constant rate fermentation method). This method has been previously proposed to quantify the nitrogen needs of yeast strains (Julien et al. 2000; Manginot et al. 1997; Manginotetal. 1998). As expected, strain HND needed the addition of 2-fold nitrogen to keep a constant fermentation rate. When combining the results of biomass production and fermentation rate in SM140, we concluded that the HND strain yielded a higher biomass, but needed more available nitrogen to keep a constant fermentation rate in the stationary phase. As previously mentioned, these data revealed a possible decoupling between the biomass yield and the fermentation rate.

Many questions are undisclosed about what determines the ability of strains to carry out fermentation in low nitrogen-containing media and the molecular mechanisms underlying these differences. Some authors conclude that these differences in nitrogen demand correlate with the nitrogen uptake rate (Gutiérrez et al., 2012; Jara et al., 2014), nitrogen sensing (Brice et al., 2014; Tesnière et al., 2015; Kessi-Pérez et al., 2020) or the nitrogen recycling system, through autophagy and the ubiquitin–proteasome-associated system (Piggott et al., 2011; Walker et al., 2014). To explore the genetic background that supports the differences in nitrogen requirements, the selected LND and HND strains were whole-genome sequenced and analysed. One of the striking results disclosed that P17 is completely homozygous as most cases industrial

strains are heterozygous (Gallone et al., 2018; Peter et al., 2018). Industrial strains tend to display higher levels heterozygosity due to their facultative asexual reproduction, while homozygous strains are efficient sporulators (Magwene et al., 2011). At times of nutritional stress, such as carbon or nitrogen starvation, diploid yeasts cells tend to undergo the sporulation process. Cells were observed during fermentation to check whether the sporulation of P17 at a low nitrogen concentration was the reason for a slower fermentation speed. However, the result did not support this hypothesis because no strain sporulated during the fermentation of SM60 (data not shown). It is not clear whether the homozygosity of P17 had any impact on its nitrogen requirement. However, it shed light on some possible future explorations.

As many of the genes showed different amino acid position between P17 and P20, determining which genes were responsible for nitrogen requirement differences was complicated. Regarding the genes with the highest amino acid divergences, most were related to the nitrogen recycling system, which underscores the importance of autophagy and the proteasome system for yeast fitness under wine fermentation conditions. However, Brice et al. (2014) carried out the QTL analysis with a population of F2 segregants generated from a cross between one HND and one LND, to find that most genes were involved in sensing and signalling nitrogen. These authors pointed out that *GCN1* was an allele with a direct effect on the fermentation rate. *GCN1* is known to be a positive regulator of general amino acid control (GAAC), and one required for *GCN2* activity, which triggers the preferential translation of the *GCN4* transcription factor which, in turn, controls the expression of the genes encoding the proteins involved in amino acid biosynthesis (Hinnebusch, 2005). In our study, four non-synonymous differences between P17 and P20

were found in *GCN1*. However, these SNPs differed from the mutation positions described by Brice et al. (2014). In their study, the *GCN1* allele from the LND strain showed a negative effect on the fermentation rate. However, the allele swapping between P20 and P17 did not significantly influence fermentation activities under the nitrogen-limited condition. As illustrated in many studies and reviews (Forsberg and Ljungdahl, 2001; Ljungdahl and Daignan-Fornier, 2012; Hofman-Bang, 1999; Beltran et al. 2004; Hinnebusch and Natarajan, 2002; Hinnebusch, 2005; Zhang et al., 2018), nitrogen metabolism in *S. cerevisiae* is regulated by several complicated and sophisticated systems. Thus the nitrogen requirement of different yeast strains can be expected to result from the collaboration of a group or many groups of genes. A further in-depth analysis with more LND and HND strains should be conducted to decipher the general genetic pattern that determines yeast nitrogen requirements.

We would like to stress that this modification of *GCN1* was conducted by the novel genome editing technique CRISPR/Cas9, which is less time-consuming and its transformation efficiency is better than the classical PCR-based gene deletion and replacement method, mainly for industrial diploid strains. This technique has been applied to *S. cerevisiae* to serve different industrial purposes (reviewed by Stovicek et al., 2017). In our study, the two-step modification by CRISPR/Cas9 (Biot-Pelletier et al., 2016) was carried out to help the selection of successful transformants easier. As the *GCN1* gene size was relatively large (8019 bp), only one part of the gene, which contained three mutation positions, was swapped between two strains.

5. Conclusions

This study combined different methods to phenotypically explore the nitrogen requirement of a set of commercial *S. cerevisiae* strains. LND and HND strains were selected based on the alteration in growth and fermentation behaviour at different nitrogen concentrations. Their nitrogen requirements were further verified by competition fermentation, biomass production and constant rate fermentation at different nitrogen concentrations. The genome information of these two strains revealed large differences in heterozygosity terms and in the amino acid substitutions between orthologous proteins, which stresses the importance of the cell recycling system under nitrogen restriction conditions. The CRISPR/Cas9 genomic engineering technique was implemented to investigate the genetic basis of nitrogen requirement differences. However, the allele swapping of the *GCN1* gene from the LND strain to the HND strain did not show any significant impact on fermentation behaviour in nitrogen-limited fermentation. Our study provides a guideline for the comprehensive analysis of nitrogen requirements by yeast during fermentation. Further genetic analyses are still required to gain a comprehensive understanding of this complex yeast feature.

Supplementary material

The additional files can be downloaded from:

https://drive.google.com/drive/folders/189OHVwIBEyzQexl9A9gYpM0UNme7_cw9?usp=sharing

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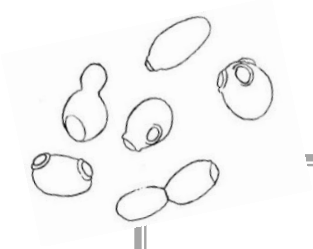

Chapter 2

Fermentative behaviour and competition capacity of cryotolerant *Saccharomyces* species in different nitrogen conditions

Ying Su, Andrea Cecilia Origone, María Eugenia Rodríguez, Amparo Querol, José Manuel Guillamón and Christian Ariel Lopes

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Abstract

The selection of yeasts with low nitrogen requirement is a current need in winemaking. In this work, we analysed nitrogen requirements of strains belonging to the cryotolerant species *S. uvarum*, *S. eubayanus* and *S. kudriavzevii*, in order to evaluate their potential for conducting the fermentation of low nitrogen content grape musts. Our result demonstrated that *S. eubayanus* is the species less influenced by the increasing nitrogen concentrations in both growth and fermentation conditions. Strains showing the best behaviours, *S. eubayanus* NPCC 1285 and *S. uvarum* NPCC 1317, were selected to be tested in mixed cultures with *S. cerevisiae* T73 at different temperatures (12 ℃, 20 ℃ and 28 ℃) in synthetic grape must with different nitrogen concentrations (60, 140 and 300 mg/L YAN). The cryotolerant strains dominated the fermentations carried out at 12 ℃ while *S. cerevisiae* prevailed at 28 ℃ independently from the nitrogen concentration. At intermediate temperature, 20 ℃, *S. eubayanus* mono and mixed cultures showed the best fermentative behaviour especially with low and intermediate nitrogen concentration. In summary, cryotolerant *Saccharomyces* species, particularly *S. eubayanus*, could be interesting tools to avoid fermentations stucks caused by low nitrogen content in grape musts.

Keywords: *Saccharomyces eubayanus*, *Saccharomyces uvarum*, *Saccharomyces kudriavzevii*, nitrogen, wine.

1. Introduction

Nitrogen is a key nutrient during wine fermentation, affecting both fermentation kinetics and the formation of wine aroma. It is the major limiting nutrient for growth under oenological conditions. Although alternative nitrogen sources, such as oligopeptides, amides, biogenic amines and nucleic acids, can be found and might constitute a substantial nitrogen resource in grape juice (Ough et al., 1991; Henschke and Jiranek, 1993; Perry et al., 1994; Marsit et al., 2015), yeast assimilable nitrogen (YAN) is mainly composed of ammonium and amino acids (Henschke and Jiranek, 1993). Different factors including grape variety, geographical origin, climate conditions and some technological processes affect the YAN content in musts and thus the fermentation kinetics (Butzke, 1998; Dubois et al., 1996; Nicolini et al., 2004). Previous works have determined that, in general, a minimum of 140 mg/L of YAN is required for yeast to complete alcoholic fermentation (Bell and Henschke, 2005; Bely et al., 1990; Butzke, 1998). Nevertheless, it strongly depends on the yeast species developing during the fermentation process.

Saccharomyces cerevisiae is the main yeast species used in the wine industry. Its favoured characteristics such as high fermentation efficiency, high ethanol tolerance and consistency of wine quality help *S. cerevisiae* to maintain its dominant position (Mas et al., 2016). However, the context of the worldwide Oenology has altered with the climate change, which affects the grape composition and ends up with grape musts with low nitrogen and high sugar concentrations (van Leeuwen et al., 2016). This situation gives the wine industry a big challenge to meet consumers' preference for wines with lower alcohol and fruitier aromas. One of the oenological practices applied by winemakers is to use lower fermentation temperatures, as far as 10–12 ℃ to preserve aroma compounds in wines (Beltran et al., 2006; Alonso del Real et

al., 2017). However, low-temperature fermentation produced similar metabolic and transcriptional effects to those obtained in nitrogen-limited fermentations (Beltran et al., 2006; Pizarro et al., 2008). Both conditions decreased biomass yield and fermentation rate during wine fermentation. This is not unexpected, because low temperature produces a rigidification of the plasma membrane that impairs the activity of some permeases, reducing the transport of nutrients (Beltran et al., 2006). Thus, low-temperature influences the quantity and the quality of yeast nitrogen requirements. In summary, the lack of nitrogen, the high sugar concentration, and the low fermentation temperature have made a very complicated harsh condition for yeasts and often causes the main fermentative problems.

In this context, strains belonging to cryotolerant *Saccharomyces* species, showing good adaptation at low temperature, lower nitrogen requirements and lower ethanol yields, may play an important role in wine fermentations. *Saccharomyces uvarum*, *Saccharomyces kudriavzevii* and *Saccharomyces eubayanus* have been identified as natural cryotolerant species in the genus. In particular, *S. uvarum* has been isolated from both natural habitats (Almeida et al., 2014; Bing et al., 2014; Gayevskiy and Goddard, 2016; Rodr ́guez et al., 2014) and fermentation environments including wines, ciders and apple chichas (for a review see Rodr ́guez et al., 2016). The fermentation profile of *S. uvarum* is different from that shown by *S. cerevisiae*. This species has a shorter lag phase than *S. cerevisiae* at low fermentation temperature (around 13 ℃). Comparing with *S. cerevisiae*, it also produces higher amounts of glycerol and lower amounts of ethanol (Castellari et al., 1994; Masneuf et at., 2010), and it generates a differential aromatic profile, particularly characterised by a higher production of 2-phenylethanol which gives a very pleasant rose-like floral odour (Bertolini et al., 1996; Masneuf et al., 2010;

Origone et al., 2018). These different traits support the great potential of *S. uvarum* being widely used in wine industry at low temperature fermentation. The other two cryotolerant species, *S. kudriavzevii* and *S. eubayanus*, have only been found in natural habitats, although it has been demonstrated that they contribute to ferment beverages through their presence as part of the genome of chimeric strains with other *Saccharomyces* species, predominantly *S. cerevisiae* (González et al., 2006; González et al., 2007; Libkind et al., 2011).

Many studies have been carried out to explore the nitrogen demanding character of *S. cerevisiae* both phenotypically and genotypically (García-Ríos et al., 2014; Gutiérrez et al., 2012, 2013, 2016). However, there are few studies about the nitrogen requirement of the cryotolerant species of the genus. It has only been described that *S. uvarum* strains have lower nitrogen requirement than *S. cerevisiae* (Masneuf et al., 2010), which suggests the putative competitiveness of this species during specific wine fermentations.

In our study, we phenotypically analysed the combined effect of temperature and nitrogen availability on the fermentation behaviour of cryotolerant yeast strains. Firstly, Nitrogen requirements of a set of *S. uvarum* and *S. eubayanus* strains recently isolated from Patagonia as well as *S. kudriavzevii* strains from natural habitats in Spain were studied. Secondly, Competition assays have also been evaluated by selected cryotolerant strains against *S. cerevisiae* in different conditions of temperature and nitrogen content in order to find out the optimal condition for the better implantation of these cryotolerant species.

2. Materials and methods

2.1 Strains and media used in the study

Thirty-two strains belonging to *S. uvarum*, *S. eubayanus* and *S. kudriavzevii* species, isolated from natural (Lopes et al., 2010; Rodr ́guez et al., 2014) and fermentative (Rodr ́guez et al., 2017) environments were used in this work. *S. uvarum* BMV58 and CECT12600 and *S. cerevisiae* T73 were also used for comparative purpose.

Yeast extract peptone dextrose (YPD) medium (2% glucose, 2% peptone, 1% yeast extract) was used for yeast propagation. A synthetic grape must (SM) is similar to natural grape must, but with a defined composition. It was used in our study to determine yeast growth and fermentation characteristics. The SM was prepared as described by Riou et al. (1997), but with some modifications. The SM contains 200 g/L reducing sugar (100 g/L glucose and 100 g/L fructose), malic acid 5 g/L, citric acid 0.5 g/L and tartaric acid 3 g/L, KH_2PO_4 0.75 g/L, K_2SO_4 0.5 g/L, $MgSO_4$ 0.25 g/L, $CaCl_2$ 0.16 g/L, NaCl 0.2 g/L, trace elements ($MnSO_4$ 4 mg/L, $ZnSO_4$ 4 mg/L, $CuSO_4$ 1 mg/L, KI 1 mg/L, $CoCl_2$ 0.4 mg/L, H_3BO_3 1 mg/L and $(NH_4)_6Mo_7O_{24}$ 1 mg/L), vitamins (myo-inositol 20 mg/L, calcium pantothenate 1.5 mg/L, nicotinic acid 2 mg/L, thiamine hydrochloride 0.25 mg/L, pyridoxine hydrochloride 0.25 mg/L and biotine 0.003 mg/L). The composition of nitrogen source in the SM is 40% ammonium and 60% amino acids, as described in Beltran et al. (2004). The composition of amino acids in 1 liter amino acid stock is L-tyrosine 1.5 g, L-tryptophan 13.4 g, L-isoleucine 2.5 g, aspartic acid 3.4 g, glutamic acid 9.2 g, L-arginine 28.3 g, L-leucine 3.7 g, L-threonine 5.8 g, glycine 1.4 g, L-glutamine 38.4 g, L-alanine 11.2 g, L-valine 3.4 g, L-methionine 2.4 g, L-phenylalanine 2.9 g, L-serine 6 g, L-histidine 2.6 g, L-lysine 1.3 g, L-cysteine 1.5 g and L-proline 46.1 g, which corresponded to 13.75 g/L assimilable nitrogen. The final pH of the SM was adjusted to 3.3 with sodium hydroxide.

The SM was sterilised by filtration through 0.22 μm pore size membrane filter (Thermo scientific).

Fermentation medium was inoculated with a yeast overnight preculture to reach an OD600nm of approximately 0.1. Preliminary experiments have been carried out to relate cell counts, by using Neubauer chamber (Brand GMBH, Germany), with OD value. The result showed that, for the different species used in our study, OD600nm=0.1 approximately equals to 1×10^6 cells/mL (data not shown).

2.2 Strain screening based on nitrogen requirement

2.2.1 Growth character screening

S. uvarum, *S. eubayanus* and *S. kudriavzevii* strains were screened by both growth and fermentation character under different nitrogen concentrations. The growth character was determined by microtiter plate screening method. 96 well microtiter plates were used. Each well was filled with 250 μL of synthetic must of different nitrogen concentrations and with 10^6 yeast cells/mL. Growth curves were monitored by recording the increase of optical density (OD) at wavelength 600 nm. The microtiter plates were incubated in SPECTROstar Nano® microplate reader (BGM Labtech, Offenburg, Germany) at 25 ℃ with 500 rpm orbital shaking. The optical density of each well was measured every 30 minutes until the growth reached the stationary phase. The growth characters of 32 strains were screened under 12 nitrogen concentrations (20, 40, 60, 80, 100, 120, 140, 160, 180, 200, 220, 300 mg/L YAN). All the nitrogen conditions were assayed by triplicate. The growth character of each strain under different nitrogen condition can be calculated by directly fitting OD measurements versus time to the Gompertz equation proposed by Zwietering et al. (1990), which has the following expression:

$$y = D * exp\ \{-exp[((\mu_{max} * e)/D) * (\lambda - t) + 1]\}$$

Where y= ln (OD_t/OD_0), OD_0 is the initial OD and OD_t is the OD at time t; D = ln (OD_∞/OD_0) is the OD value reached with OD_∞ as the asymptotic maximum, μ_{max} is the maximum specific growth rate (h^{-1}), and λ is the lag phase period (h). R code (statistical software R, v.3.0) was used to facilitate calculating μ_{max} and λ with Gompertz equation.

2.2.2 Fermentation character screening

The fermentation character was screened by microscale fermentation method. The fermentations were carried out in 15 mL tubes with 10 mL synthetic must. The screw caps of the tubes were not completely tightened in order to allow CO_2 release. Synthetic must was modified with three different nitrogen concentrations which were 60, 140 and 300 mg/L YAN. These values correspond to nitrogen limited, standard and excessive conditions (Bely et al., 1990). The fermentations were followed by CO_2 production which can be represented by the weight loss of the fermentation tubes. Three tubes filled with 10 mL SM without inoculation are used as triplicate controls for the evaporation weight loss (EWL). The fermentation tubes were weighted around every 12 hours. The weight loss (WL) can be simply calculated as: $WL= W_0 – W_t – EWL$. The fermentations were considered as finished when WL stops to increase. Lag phase time (λ) and maximum fermentation rate (V_{max}) were calculated by applying the weight loss data into Gompertz model as described in 2.2.1.

2.3 Competition fermentation

In order to determine the influence of nitrogen concentration and fermentation temperature on the yeast population in a mixed culture, competition

fermentation was carried out between *S. cerevisiae* strain T73 and one of the non-*cerevisiae* strains. The strains used for fermentation were first propagated in liquid YPD media overnight and then transferred to SD media (2% glucose, 0.017% yeast nitrogen base) with 230.8 mg/L NH_4Cl (corresponding to 60 mg/L YAN) in order to eliminate the influence of YPD nitrogen-rich media.

Fermentations were carried out in 100 mL bottles with 80 mL synthetic must of three nitrogen concentrations (60, 140 and 300 mg/L YAN). All the fermentations were inoculated with 2 $x10^6$ cells/mL. In the case of mix-culture fermentations, $1x10^6$ cells/mL of each strain was inoculated. In order to observe the influence of temperature on competition capacity of each strain, the fermentations were carried out at three temperatures: 12, 20 and 28 ℃, representing low, medium and high temperature respectively. The fermentation process is monitored by measuring the density of the media (g/L), after a slight centrifugation at 5.000 rpm for 5 min for cell removal and using a portable densitometer (Mettler Toledo, USA). The fermentation was considered as finished when the density reaches 998 g/L (Gutiérrez et al., 2012). Mono-culture fermentations were also carried out as references.

2.4 Strain population dynamics in competition fermentation

As *S. uvarum* and *S. eubayanus* are less heat tolerant than *S. cerevisiae*, we confirmed the incapability of *S. uvarum* and *S. eubayanus* to grow on at 37 ℃, whereas they perfectly grow at 30 ℃ in YPD (data not shown). On the other hand, the colony forming ability of *S. cerevisiae* strains is the same at 30 ℃ and 37 ℃. The percentage of strains belonging to *S. cerevisiae* or to non-*cerevisiae* can be simply determined by plating the sample on two YPD plates and incubated at 30 ℃ and 37 ℃. CFUs were counted after 2 days of incubation. The CFU number on the 37 ℃ plate represents the population of

S. cerevisiae in the mixed culture and the CFU difference between the two plates represents the population of non-*cerevisiae* strain in the mixed culture.

At the end of the competition experiment, fermentation samples were taken and analysed for the main wine chemical parameters including glucose, fructose, ethanol, glycerol and main organic acids. The samples were first centrifuged and diluted 3 times with deionized water, then filtrated through 0.22 mm pore size nylon filters (Micron Analitica, Spain). The analysis was carried out on a UHPLC (Thermo Scientific, ultimate 3000) equipped with a refraction index detector and a UV-visible detector (Thermo Scientific). The mobile phase used was 1.5 mM H_2SO_4 with a flux of 0.6 mL/min and a column temperature of 45 ℃. The metabolites were separated by a HyperREZ XP Carbohydrate H+ 8 mm column (Thermo Scientific) and their concentration was calculated by using external standards.

2.5 Statistical analysis

All the experiments were carried out at least in triplicate. Physiological data were analysed by the Sigma Plot 13.0 software and the results are expressed as mean and standard deviation. Significance was determined by analysis of variance (ANOVA) using the Statistica, version 7.0, software package. The statistical level of significance was set at a P value of ≤ 0.05 with a Tukey HSD test. Multi-factorial ANOVA based on the percentage of *S. cerevisiae* in the mixed culture fermentation was also carried out (Statistica, version 7.0) in order to analyse the significance of the influences of temperature (12, 20 and 28 ℃), nitrogen concentration (60, 300 mg/L YAN), and the type of mixed culture (*S. cerevisiae* + *S. eubayanus*, *S. cerevisiae* + *S. uvarum*). The data group of fermentation carried out at 20 ℃ with 140 mg/L YAN was not included in the multi-factorial ANOVA, since it is the unique group with 140

mg/L YAN. Phenotypic data were fitted to the modified Gompertz model by non-linear least-squares fitting using the Gauss-Newton algorithm as implemented in the function in the R statistical software, v.3.0. Hierarchical clustering used in heatmap plot was performed by MeV MultiExperiment Viewer with Euclidean distance metrics and group clustering was based on group averages (average linkage).

3 Results

3.1 Nitrogen requirements of *S. eubayanus*, *S. uvarum* and *S. kudriavzevii* in pure cultures

Nitrogen requirements of a set of 32 non-conventional cryotolerant *Saccharomyces* yeast strains including *S. uvarum* (17 different strains), *S. eubayanus* (13 different strains) and *S. kudriavzevii* (2 different strains) were evaluated in synthetic grape must at 25 ℃. During the first stage, yeast growth was evaluated by OD increases in microtiter plates containing synthetic must with different total nitrogen concentrations (20, 40, 60, 80, 100, 120, 140, 160, 180, 200, 220 and 300 mg/L YAN). Growth curves were fitted to modified Gompertz equations for all yeast strains and conditions (a total of 1260 growth curves, including the three reference strains). After calculating by Gompertz equation, μ_{max} (maximum growth rate) and λ (lag phase time) values were successfully obtained for all analysed strains and conditions and their absolute values are exhibited in Supplementary Material Tables 1 & 2. Lag phase time is a mainly a strain dependent value. Increasing of nitrogen concentration does not have significant differences on λ of each strain. In order to easily detecting different nitrogen requirement among analysed species and strains, a heatmap analysis was carried out with μ_{max} data (Fig. 1). For this analysis, the μ_{max} value obtained for each strain was normalised by mean value of all strains at each particular nitrogen concentration. Values

lower and higher than the average are indicated in green and red respectively in the heatmap (Fig. 1). This heatmap is hierarchically clustered into four clusters. The first cluster is made up of the strains with the highest μ_{max}, which mostly belong to *S. eubayanus*, with the exception of two *S. uvarum* strains (NPCC1317 and NPCC1323), which were isolated from apple chicha. The second cluster is formed by a group of strains with higher or similar growth rate than the average value of all strains (red to black colour). These strains were mostly isolated from *A. araucana* tree (Wild), which is a gymnosperm endemic of the lower slopes of the Chilean and Argentinian south-central Andes, typically above 1000 m of altitude (Rodr ǵuez et al, 2014). The third group was represented by strains mostly with lower μ_{max} than the average (black to green colour). This cluster is mainly made up of *S. uvarum* isolated from wild and fermentative environments (chicha and cider) and, curiously, the three wine strains, two *S. uvarum* references BMV58 and CECT12600, and the control *S. cerevisiae* T73, are included in this third cluster, showing similar interspecific growth features. The fourth group includes two strains of *S. kudriavzevii*, showing the lowest growth rate of all the tested strains.

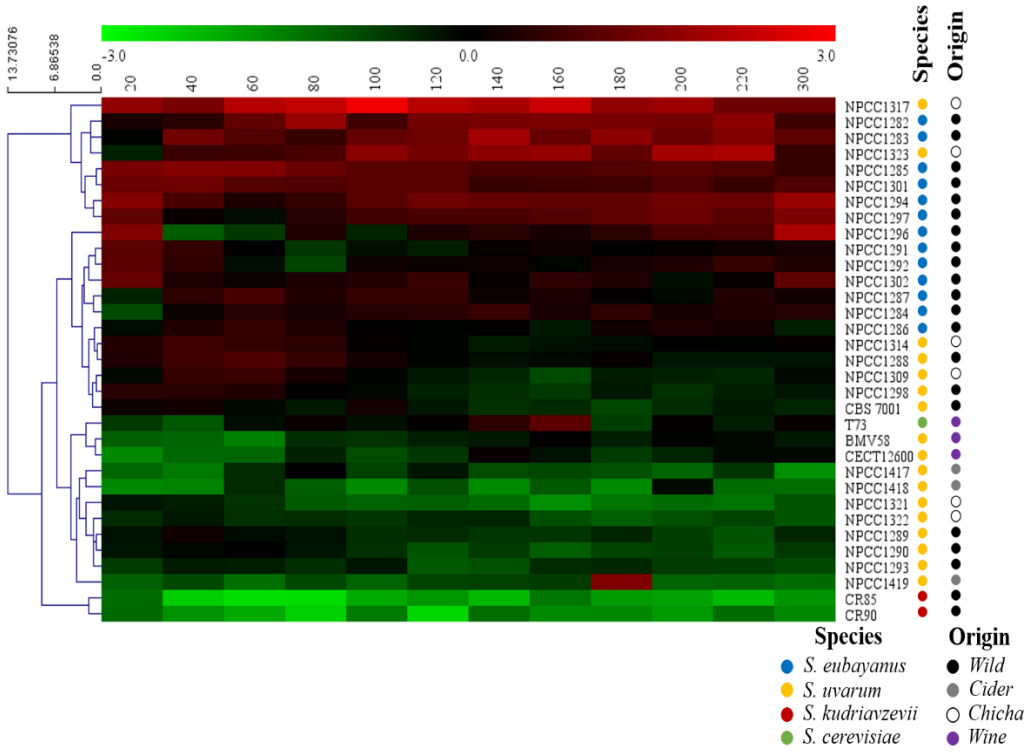


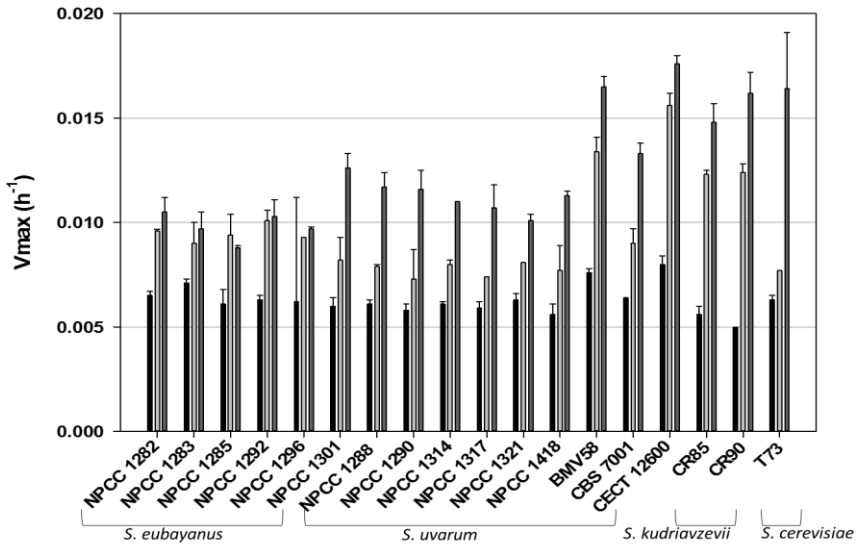
Fig. 1. Heatmap representation of μ_{max} values of the analysed strains at increasing nitrogen concentrations. Each line corresponds to a strain (indicated at the right of the figure) and each column to a particular nitrogen concentration (indicated at the top of the figure). The colour key bar at the top indicates μ_{max} value relative to the average for each particular nitrogen concentration: values higher than the average are in red and values lower than the average are in green. Hierarchical clustering is showed on the left. Colour dots on the right of the figure indicate both species identity and origin of the strains. The statistical significance is shown in supplementary material table 1.

In order to evaluate if the behaviour evidenced in yeast growth is also observed in the fermentation performance of the yeasts, we selected strains showing high, low and intermediate nitrogen requirements to be evaluated in fermentations with low (60 mg/L YAN), high (300 mg/L YAN) and intermediate (140 mg/L YAN) nitrogen concentrations. The strains with high

nitrogen requirements were selected among those showing low μ_{max} values at low nitrogen concentrations and high μ_{max} values at high nitrogen concentrations according to the normalised data from Fig. 1 (*S. eubayanus* NPCC 1296, 1301 and *S. uvarum* NPCC 1321, 1418). Conversely, the strains with low nitrogen requirements were selected among those showing high μ_{max} values at low nitrogen concentrations and also high (or average) μ_{max} values at high nitrogen concentrations (*S. eubayanus* NPCC 1282, 1283, 1285 and *S. uvarum* NPCC 1288, 1314, 1317). *S. eubayanus* strain NPCC 1292 and *S. uvarum* strain NPCC 1290, showing intermediate nitrogen requirements, were also evaluated. For comparative purposes, we additionally included the two *S. kudriavzevii* strains CR85 and CR90, the *S. uvarum* reference strains BMV58, CBS 7001 and CECT 12600 and the *S. cerevisiae* reference strain T73.

Weight loss data due to CO_2 release were calculated by Gompertz equation and parameters V_{max} (representing the maximum rate of CO_2 production) and λ_{CO2} (representing the time required to start the fermentation) were obtained for each strain at three nitrogen conditions: 60, 140 and 300 mg/L (Fig. 2 and Supplementary Material Table 3). Interestingly, contrary to the expected, the strains selected for their high or low nitrogen requirements showed similar fermentative behaviour in each nitrogen concentration. Generally, the increasing nitrogen concentration enhanced the V_{max}, but with different intensity in the various group of strains. *S. eubayanus* strains were less affected by the increasing nitrogen concentration, showing similar values in V_{max} obtained at both 140 mg/L and 300 mg/L. The V_{max} of S. uvarum Patagonian strains (NPCC strains) improved greatly along with increasing nitrogen concentration, especially high value was observed at 300mg/L. The greatest influence of nitrogen concentration on V_{max} was observed for the

wine *S. uvarum* and *S. kudriavzevii* strains, which showed around twice V_{max} values at high nitrogen concentrations compared with that observed at 60 mg/L YAN (Fig. 2A).



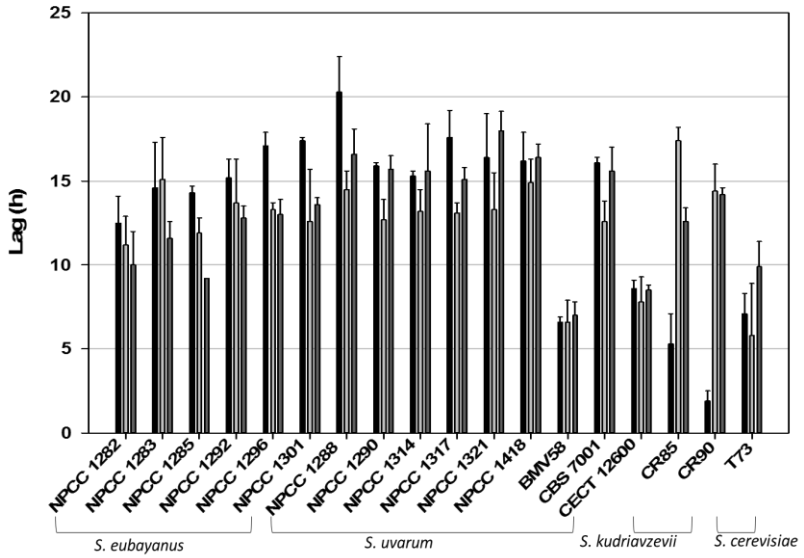


Fig. 2. (A) Maximum fermentation velocity and (B) lag stage of strains from several origins at fermentation performed at different YAN concentrations (■ 60mg/L, ■ 140 mg/L and ■ 300 mg/L). The statistical significance is shown in supplementary material table 3.

Regarding the time needed to start fermentation (Fig. 2B; λ_{CO2}), *S. eubayanus* and *S. uvarum* mostly shortened this lag period as nitrogen concentration increased. Conversely, the two *S. kudriavzevii* strains showed a negative correlation between nitrogen concentration and the time needed for starting fermentation, which showed higher λ_{CO2} values at intermediate and high nitrogen concentrations. Finally, the shortest λ_{CO2} was observed in the wine strains *S. uvarum* BMV58, CECT12600 and *S. cerevisiae* T73, regardless the nitrogen concentration. This is of great significance for the reason that, it evidences that regardless of the species or the nitrogen concentration, the isolating origin of the strains is the most influencing factor for a good adaptation to the growth medium.

3.2 Competition tests *S. cerevisiae* vs. *S. eubayanus*/*S. uvarum*

For their industrial application potential, one *S. eubayanus* strain (NPCC 1285) and one *S. uvarum* strain (NPCC 1317), showing the best performance at low nitrogen concentrations (especially evident in growth assays) were selected to perform mixed culture fermentation with *S. cerevisiae* reference strain T73, in order to evaluate their competition capacity in synthetic grape musts with different nitrogen concentrations. We evaluated, in the first stage, the competition at both low and high nitrogen content (60 mg/L and 300 mg/L YAN). Moreover, due to the well-known differential temperature preference of the yeasts involved in the competitions, two different fermentation temperatures (28 ℃ and 12 ℃) were evaluated for the mixed cultures. For

comparative purposes, monoculture fermentations were also carried out with the three strains involved in the competitions.

Our result showed that both fermentation temperature and nitrogen concentrations have significant influences on the fermentation kinetics and on the competitiveness of the strains. At 28 ℃, most of the fermentations were completed with both nitrogen concentrations (only *S. eubayanus* monoculture was unable to end up fermentation). However, the low YAN concentration yielded sluggish fermentations (Fig. 3A-B). Conversely, at 12 ℃, the combination low temperature and low YAN resulted in unfinished fermentations for all the tested monocultures or mixed cultures (Fig. 3C). At low temperature but high YAN concentration, *S. uvarum* and *S. eubayanus* monocultures showed the highest fermentation activity and the shortest fermentation times (Fig. 3D). The higher fermentation activity for *S. cerevisiae* at 28 ℃ and for *S. eubayanus*/*S. uvarum* at 12 ℃ generally correlated with higher CFU counts (Fig. 4). Regardless YAN concentration, the cryotolerant species showed greater final population size at 12 ℃. Conversely, only *S. eubayanus* produced more cells than *S. cerevisiae* at 28 ℃ and low YAN.

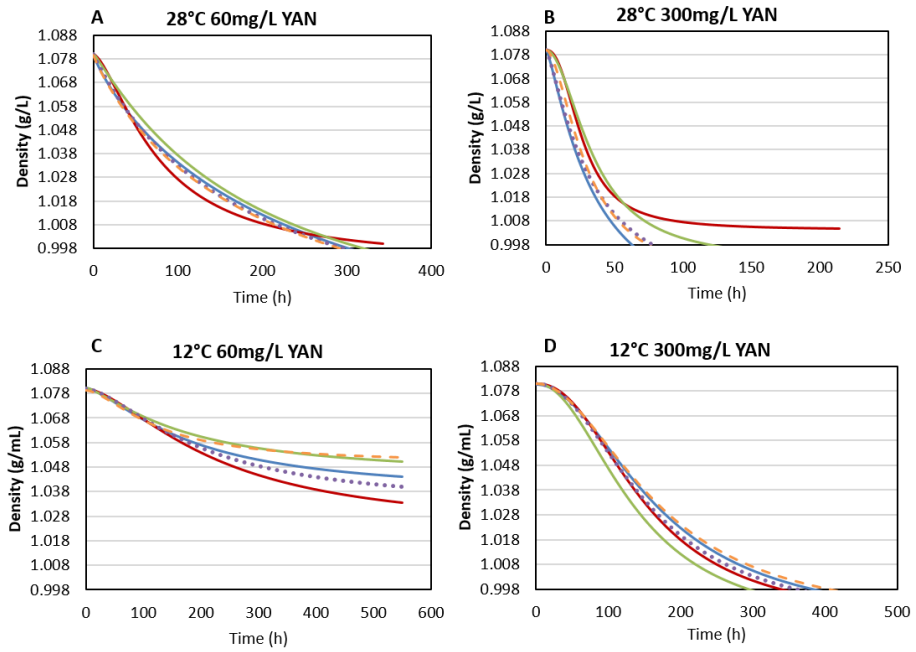


Fig. 3. Fermentation kinetics at 28 ℃ 60 mg/L YAN (A), 28 ℃ 300 mg/L YAN (B), 12 ℃ 60 mg/L YAN (C) and 12 ℃ 300 mg/L YAN (D)

— *S. cerevisiae*, — *S. eubayanus*, — *S. uvarum*, ···*S. eubayanus* + *S. cerevisiae*, — — *S. uvarum* + *S. cerevisiae*.

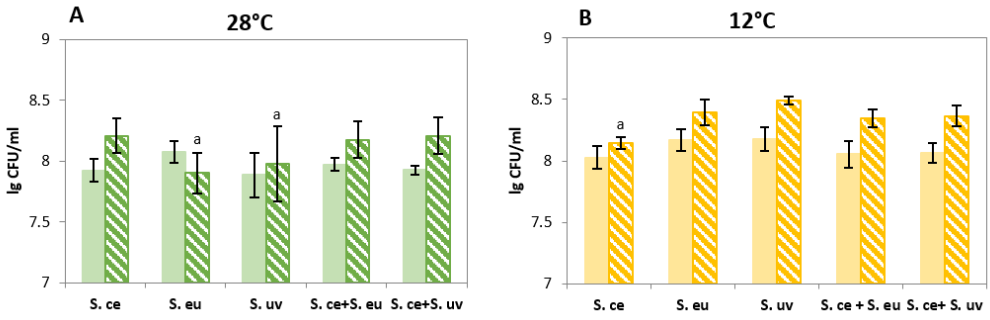


Fig. 4. Maximum population during stationary phase of fermentation at 28 ℃ (A) and 12 ℃ (B). Light green (28 ℃) and light yellow (12 ℃) bars represent 60mg/L YAN. Dark green (28 ℃) and dark yellow (12 ℃) bars with diagonal lines represent 300mg/L YAN. One-way

ANOVA was performed within each nitrogen concentration. Letters on top of the bars indicate significant difference ($p<0.05$).

In terms of strain composition in mixed culture fermentations, temperature of fermentation determined the competitiveness of the different strains and nitrogen did not have any impact on their implantation. At 28 ℃, *S. cerevisiae* completely dominated the two mixed-culture fermentations, with implantation percentages higher than 60% in all samples only after 6 h of fermentation (Fig. 5A-B). Conversely, at 12 ℃, the cryotolerant yeasts *S. eubayanus* and *S. uvarum* dominated the mixed fermentations. Nevertheless, this implantation was not absolute, with percentages of *S. cerevisiae* around 10-30% at the end of the fermentations (Fig. 5C-D).

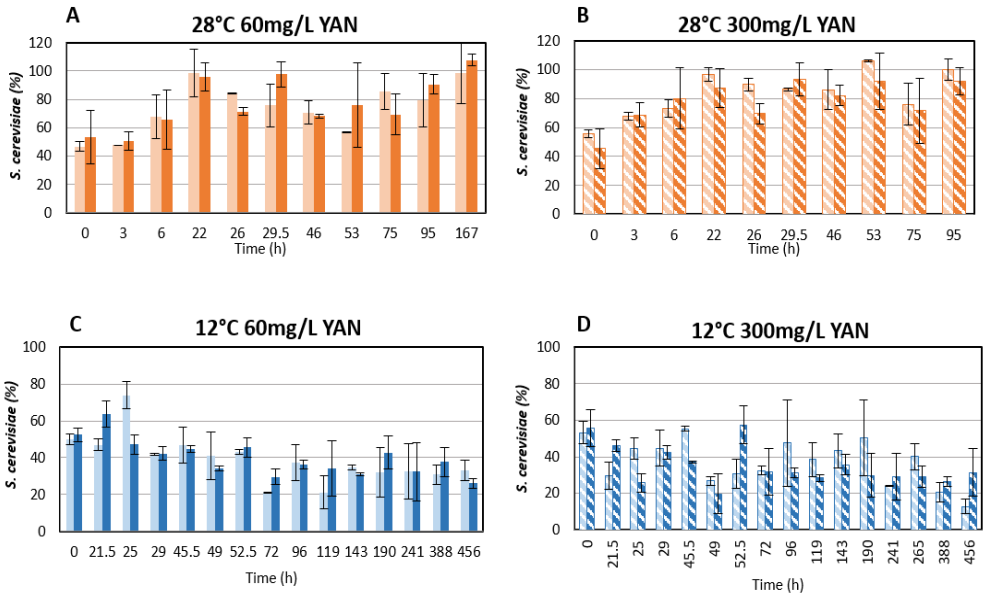


Fig. 5. *S. cerevisiae* growth evolution in mixed culture fermentations at 28 ℃ 60 mg/L YAN (A), 28 ℃ 300 mg/L YAN (B), 12 ℃ 60 mg/L YAN (C) and 12 ℃ 300 mg/L YAN (D). Bars in light colour represent *S. eubayanus* + *S. cerevisiae* and bars in dark colour represent *S.*

uvarum + *S. cerevisiae*. Bars with diagonal lines show the value of 300 mg/L YAN. Bars without patterns show the value of 60 mg/L YAN.

As 12 ℃ is an extreme low fermentation temperature to conduct fermentation in the wineries, we decided to test the selective effect of a milder temperature for yeast development. Thus, we subsequently evaluated the same mono and mixed cultures at 20 ℃. In this case, we also considered to include, additionally, an intermediate nitrogen concentration of 140 mg/L. In this assay, since the influence of the temperature has been reduced, a strong effect of nitrogen concentration on fermentation capacity, and hence, in the competition capacity of the yeast species involved in the mixed cultures was observed (Fig. 6). The implantation percentages of *S. cerevisiae* at 300 mg/L YAN were around 60-90%, whereas the same at 60 mg/L YAN was only 40-50%, independently from the analysed mixed culture (*S. cerevisiae* + *S. uvarum* or *S. cerevisiae* + *S. eubayanus*). Moreover, at this high nitrogen concentration, the imposition of *S. cerevisiae* is stronger competing with *S. eubayanus* than with *S. uvarum*. Interestingly, with 140 mg/L YAN, the implantation of *S. cerevisiae* kept around 50-60% throughout the fermentation (Fig. 6A). This is of great importance, since under this condition, most of the fermentations were able to finish (Fig. 6B), and the implantation of the cryotolerant strains remain considerable. Furthermore, the monoculture of *S. eubayanus* also showed the fastest fermentation activity. As at 12 ℃, none of the strains finished the nitrogen-limited fermentations (60 mg/L YAN) and the cryotolerant strains exhibited higher CFU counts than *S. cerevisiae* monocultures, regardless of the nitrogen concentration (Fig. 6B-C).

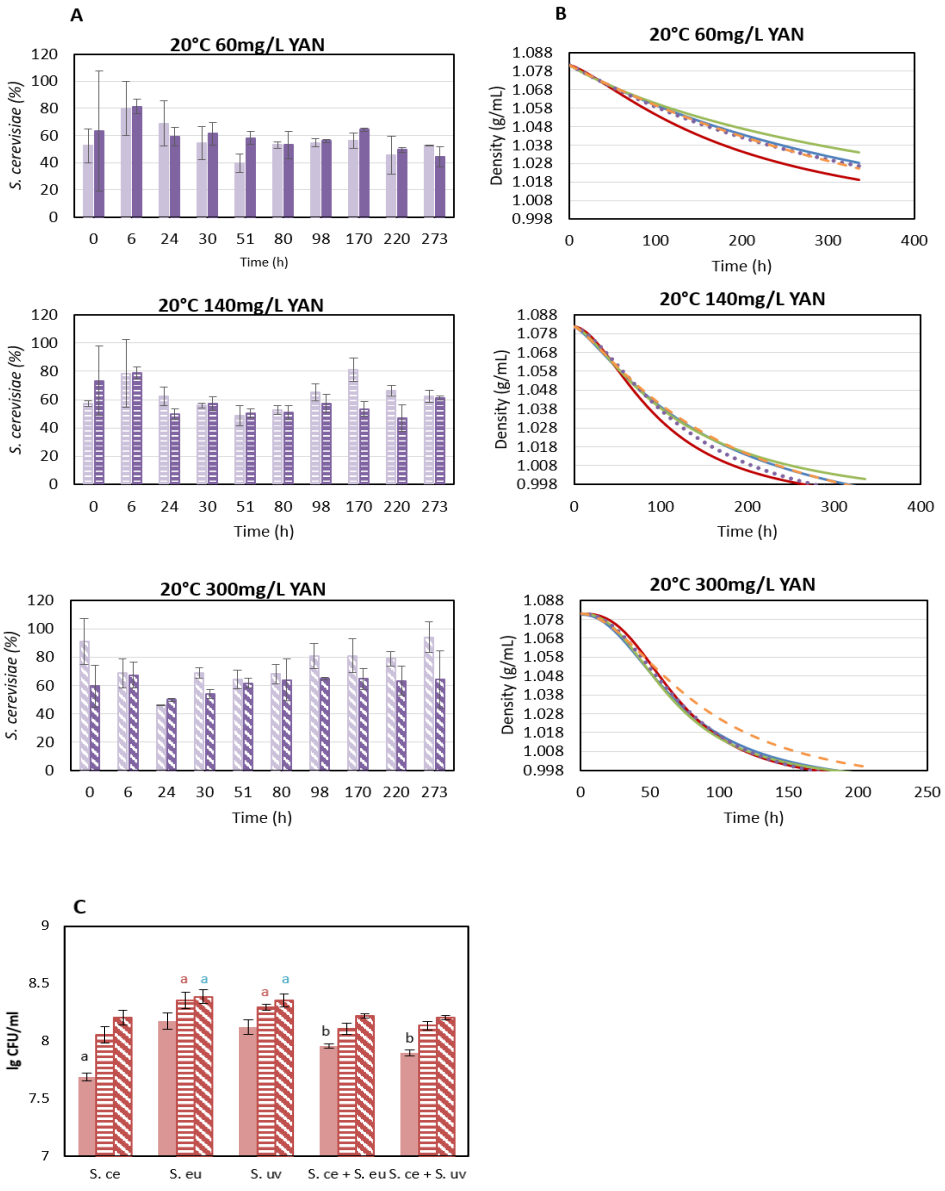


Fig. 6. (A) *S. cerevisiae* growth evolution in mixed culture fermentations. Light purple bars represent *S. eubayanus* + *S. cerevisiae* and dark purple bars represent *S. uvarum* + *S. cerevisiae*. 60, 140 and 300 mg/L of YAN are represented by bars without patterns, bars with horizontal lines, and bars with diagonal lines, respectively. (B) Fermentation kinetics at 20 ℃: — *S. cerevisiae*, — *S. eubayanus*, — *S. uvarum*, ▪▪▪ *S. eubayanus* + *S. cerevisiae*, — — *S. uvarum* + *S. cerevisiae*. (C) Maximum population during stationary phase of fermentation.

Pink bars without pattern represent 60mg/L YAN. Red bars with horizontal lines represent 140mg/L YAN and red bars with diagonal lines represent 300mg/L YAN. One-way ANOVA was performed within each nitrogen concentration. Letters on top of the bars indicate significant difference ($p<0.05$).

Based on the percentage of *S. cerevisiae* at the end of mixed culture fermentation, a multi-factorial ANOVA was performed (Table 1). In the multi-factorial ANOVA, the effects of three parameters have been compared: temperature (12 ℃, 20 ℃ and 28 ℃), nitrogen (60mg/L and 300mg/L) and the cryotolerant strain used in the mixed culture (*S.cerevisiae*+*S. eubayanus* and *S. cerevisiae*+*S. uvarum*). According to the results, temperature (T), nitrogen concentration (N) and the combination of T and N had significant and strong influences on the competitiveness of *S. cerevisiae* in the mixed culture; regardless of the cryotolerant species used in combination with *S. cerevisiae*. When combining all the three parameters together, the impact is also significant with P value lower than 0.05.

Table 1. F-values and P-values for percentage of *S. cerevisiae* at the end of mix-culture fermentation obtained by a multi-factorial ANOVA

	Sources of variation						
	temp (T)	**N concen-tration (N)**	**Mix-culture[a](M)**	**T*N**	**T*M**	**N*M**	**T*N*M**
F value	386.80	9.97	0.10	31.76	3.26	0.18	6.02
P value[b]	$p < 0.001$	$p < 0.01$	0.76	$p < 0.001$	0.076	0.68	$p < 0.05$
DF	2	1	1	2	2	1	2

a. There are two mix-culture type which are *S. eubayanus* + *S. cerevisiae* and *S. uvarum* + *S. cereivisiae*

b. The data is considered as significant when p value is less than 0.05.

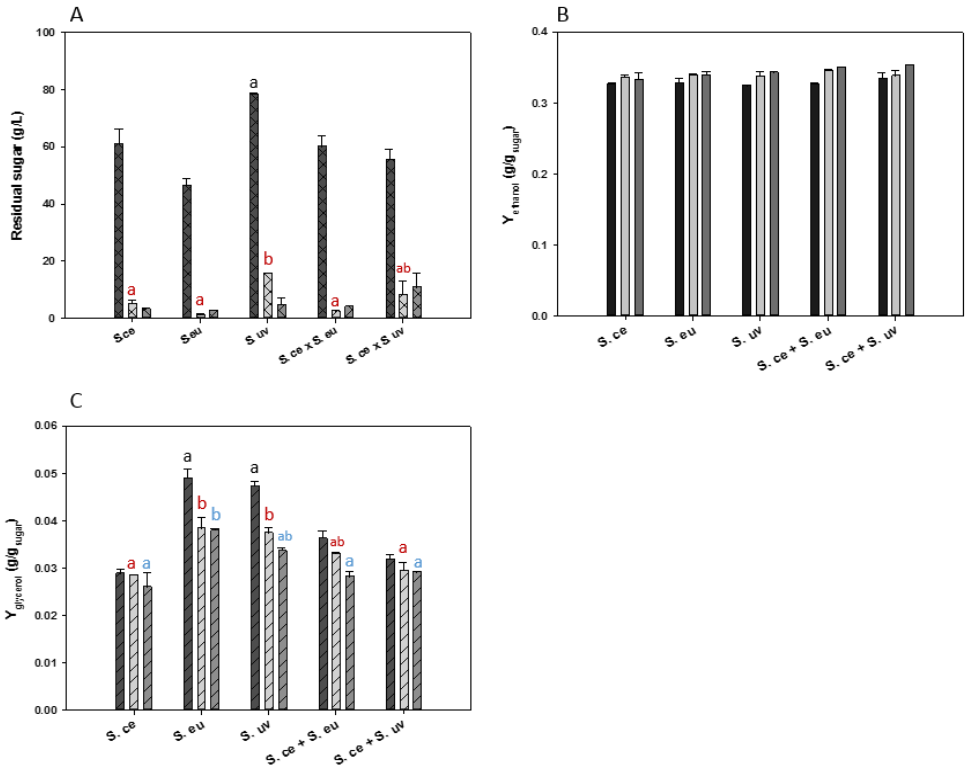


Fig. 7. Chemical characterisation of products obtained after fermentation at 20 ℃ of synthetic musts containing different nitrogen concentrations (60, 140 and 300 mg/L). ■ 60mg/L, ■ 140 mg/L and ■ 300 mg/L (A) Residual sugar glucose + fructose. (B) Ethanol yield (C) Glycerol yield. One-way ANOVA was performed within each nitrogen concentration. Letters on top of the bars indicate significant difference ($p<0.05$).

The analysis of main chemical parameters at the end of fermentations has been carried out for fermentations at 20 ℃. As mentioned above, no culture was able to complete the fermentation at low nitrogen concentration (60 mg/L), with the presence of high values of residual sugars in the final wines (Fig. 7A). Since not all the fermentations were completed, the production of ethanol and glycerol is shown as yield produced per gram of sugar (glucose + fructose) consumed (Fig. 7B-C). No significant differences were observed

in ethanol yield among different nitrogen concentrations and different strains. On the contrary, *S. eubayanus* and *S. uvarum* strains have much higher glycerol yield than *S. cerevisiae*, especially with low nitrogen concentration 60 mg/L YAN. Nitrogen concentration does not have big influence on glycerol yield by *S. cerevisiae*.

4 Discussion

4.1 Growth and fermentative performance are variable according to species and life history of yeasts in monocultures

It has been described that the nitrogen requirement during wine fermentation is a strain-specific feature (Gutiérrez et al., 2012). We demonstrated, for the first time, the existence of a differential behaviour in nitrogen requirement among strains of the cryotolerant species *S. uvarum*, *S. eubayanus* and *S. kudriavzevii*. The effect of different nitrogen concentrations was evidenced in both yeast growth and fermentation rate of the strains. However, a good growth performance was not necessarily associated with a good fermentation performance of a particular yeast, i.e. *S. eubayanus* or *S. uvarum* strains, with the highest and the lowest μ_{max} growth values, showed no differences in their maximum fermentation rate evaluated by CO_2 release. Gutiérrez et al. (2012) and Kemsawasd et al. (2015) have also reported this lack of correlation between growth and fermentation behaviour. In the study of Gutiérrez et al. (2012), the nitrogen requirements of four commercial *S. cerevisiae* wine strains were analysed under growth and fermentation conditions. One of the strains, which showed the poorest growth capacity, had the best fermentation performance. Kemsawasd et al. (2015) investigated the influence of different nitrogen sources on growth and fermentation activity of *S. cerevisiae* and four wine-related non-*Saccharomyces* yeast species (*Lachancea thermotolerans*, *Metschnikowia pulcherrima*, *Hanseniaspora uvarum* and *Torulaspora*

delbrueckii), and they have also concluded that a good growth capacity does not always result in a good fermentation behaviour. Therefore, the two parameters, growth and fermentation performance, must be evaluated in order to understand the yeast performance during alcoholic fermentation.

Interestingly, most of the strains identified as *S. eubayanus* showed higher μ_{max} values in microplate assays than those belonging to *S. uvarum* species, for all different nitrogen concentrations tested. Even *S. uvarum* strains isolated from the same natural habitats as *S. eubayanus* (*A. araucana* trees) showed lower μ_{max} values, similar to those observed for the *S. uvarum* strains isolated from fermentative environments. This observation evidences a clear species-specific behaviour, potentially associated with a particular ecological strategy. The species-specific effect of nitrogen on both growth rate and fermentation performance was also reported by Kemsawasd et al. (2015) for a set of non-*Saccharomyces* species including *Lachancea thermotolerans* and *Metschnikowia pulcherrima*. The authors suggested that some of the differences between the responses of non-*Saccharomyces* species and *S. cerevisiae* could be related to genetic differences shaped by human activity (domestication) of *S. cerevisiae*. Spor et al. (2009) also observed that populations harboured different strategies depending on their ecological niches. These authors found that forest and laboratory strains reach a large carrying capacity (population size) and a small cell size in fermentation, but they have a low reproduction rate in respiration and produce lower quantities of ethanol, suggesting that they store cell resources rather than secreting secondary products. Contrarily, the industrial strains of this species reproduce slowly, reach a small carrying capacity but have a big cell size in fermentation and a high reproduction rate in respiration with higher glucose consumption rates. These two contrasted behaviours were metaphorically defined by the

authors (Spor et al., 2009) as ‘‘ant’’ and ‘‘grasshopper’’ strategies of resource utilisation. Similar results were obtained in this work when compared with different strains of *Saccharomyces* non-*cerevisiae* species isolated from diverse habitats. We observed that independently from the nitrogen concentration, both the highest μ_{max} in microplates assays and the highest CFU numbers in competition experiments were observed for *S. eubayanus* strains isolated from natural habitats. It is interesting to note that for the specific case of *S. uvarum* species, including strains from natural habitats and fermentative environments, previous phylogenetic analyses have evidenced the existence of at least two different populations with low genetic flux among the strains of this species isolated from natural habitats in Patagonia named Southamerica-A and Southamerica-B/Holartic (Almeida et al., 2014). On the other hand, all strains isolated from fermented beverages in the same region belonged to the Southamerica-A/Holartic subpopulation (Almeida et al., 2014; Rodr ́guez et al., 2017). This data also supports the fact that the origin, more than the phylogenetics relationships, are involved in the physiological response of the yeast to the analysed culture conditions. In previous work carried out in our laboratory with a set of *S. uvarum* and *S. eubayanus* strains including those used in this work, we also evidenced clear physiological differences between strains from natural and fermentative habitats (Gonz ́alez Flores et al., 2017; Origone et al., 2017).

The effect of nitrogen content on both the growth and fermentative capacity of the evaluated yeasts is a complex issue. Although several works have evaluated the chemical composition of grape must in different winemaking areas, the lack of information about the same in natural habitats makes the evaluation of this phenomenon difficult. Strikingly, the strongest effect of increasing nitrogen concentrations on the maximum fermentative rate was

observed for strains associated with industrial fermentations (*S. cerevisiae* T73, *S. uvarum* BMV58 and CECT 12600) as well as for strains strictly associated with natural habitats (S. *kudriavzevii* CR85 and CR90). This was observed for pure culture fermentations with 60, 140 and 300 mg/L YAN. However, the same assay carried out with *S. eubayanus*, the other species associated only with natural habitats, demonstrated only a slight difference in this parameter at increasing nitrogen concentrations. These results suggest that both the yeast species and their ecological life-history are involved in the response strategy of yeasts to this environmental factor. This phenomenon became relevant for the development of mixed cultures for winemaking as it has been demonstrated to enhance the organoleptic complexity of wines. This fact, added to the tendency to produce wines at low temperature as well as to produce more aromatic wines, makes the study of *Saccharomyces* non-*cerevisiae* cryotolerant yeast strains an interesting topic for research, especially in mixed cultures with *S. cerevisiae*. The competition ability of several *S. cerevisiae* strains under different nitrogen concentration has been evaluated (Garc á-R ós et al., 2014; Lemos Junior et al., 2017; Vendramini et al., 2017); however, little is known about the competition ability between *S. cerevisiae* versus other species from the *Saccharomyces* genus.

4.2 Low temperature and low nitrogen concentration favour *Saccharomyces* non-*cerevisiae* implantation in mixed cultures

The implantation capacity of two *Saccharomyces* non-*cerevisiae* strains (*S. eubayanus* NPCC 1285 from natural habitats and *S. uvarum* NPCC 1317 from apple chicha), which were selected according to their performance in fermentations with low nitrogen concentrations, was evaluated in mixed cultures with the wine commercial *S. cerevisiae* strain T73 at different temperatures.

Our results indicate that, as expected, increased temperature improves the advantage of *S. cerevisiae*, whereas, conversely, lowered temperature reduces its competitiveness, favouring cryotolerant strains, independently from the nitrogen concentration. This phenomenon was first reported by Arroyo-López et al. (2011) and Salvadó et al. (2011) for competition experiments between different yeast strains, and recently evidenced by Alonso del Real et al. (2017) for competitions between one strain of *S. eubayanus*, *S. uvarum* or *S. kudriavzevii* and *S. cerevisiae*. In our work, we also evidenced that temperature is the most important parameter governing the implantation of both *S. eubayanus* and *S. uvarum* in mixed cultures with *S. cerevisiae*. However, we observed a better competition capacity of our strain *S. eubayanus* NPCC 1285 against *S. cerevisiae* T73 at 20 ℃. This fact was particularly observed with an intermediate nitrogen concentration (<140 mg/L). This could be due to the fact that the strains used in competition fermentation were selected because of their low nitrogen requirement, according to the assays previously discussed.

Several other factors can be related to the competitive capacity of a yeast strain including competition for space and for other specific nutrients, oxygen affinity, production of antagonist compounds as killer toxins or other diffusible molecules, among others (Fleet, 2003). In the same way, Pérez-Torrado et al. (2017) demonstrated that both cell-to-cell contact and differential sulphite production and resistance are important factors that determine the dominance of one *S. cerevisiae* strain over another. Additionally, Cheraiti et al. (2005) observed that acetaldehyde production and redox interactions are involved in competitions between *S. cerevisiae* and *S. uvarum*.

A particular strategy that seems to adopt *S. eubayanus*, and, to a lesser extent, *S. uvarum*, is a significantly higher CFU number than in *S. cerevisiae* at lower temperatures. This phenomenon was observed in most of the analysed conditions in monocultures (both microplates and competition assays). These differences were more evident at lower nitrogen concentrations, i.e. *S. cerevisiae* evidenced an increase in its population size directly proportional to the nitrogen content in the medium while this response was not in the same magnitude for *S. eubayanus*. Natural environments are usually nutrient poor; in this context, yeast strains able to grow in the presence of low nitrogen concentrations are selected, and this could be the situation for *S. eubayanus*. This species seems not to be able to respond efficiently to increasing nitrogen concentrations. Contrarily, it has been argued that *S. cerevisiae* does not show adaptations to any particular habitat, but rather an ability to survive in a wide range of conditions (Goddard and Greig, 2015) which is consistent with its life history of nomadic generalist that inhabits diverse niches (Jouhten et al., 2016; Liti et al., 2009). Interestingly, it has also been demonstrated that one genomic region that shows strong differentiation between the oak and wine European populations of *S. cerevisiae* correspond to a gene coding for a transporter for oligopeptides that can act as nitrogen sources in wines (Marsit et al., 2015). Additionally, Treu et al. (2014) evidenced that gene sequences involved in nitrogen metabolism are more variable among *S. cerevisiae* strains from vineyard than in industrial strains, suggesting a different aptitude in nitrogen uptake and management. Altogether, these data indicate that nitrogen metabolism is strongly dependent on the origin of the yeast strains in both *S. cerevisiae* and *Saccharomyces* non-*cerevisiae* yeasts. The exploitation of these differential metabolic behaviours could be of interest for the development of mixed cultures for rational winemaking.

5. Conclusions

The results obtained in this work evidenced for the first time that both nitrogen content in must and fermentation temperature are important factors involved in the competition of *S. eubayanus* and *S. uvarum* in mixed culture fermentations with *S. cerevisiae*. Moreover, *S. eubayanus* in mono and mixed culture with *S. cerevisiae* showed the best fermentation performance in synthetic must, at low nitrogen concentration and low fermentation temperature. The employment of this kind of mixed cultures can become a strategy for winemakers to overcome the problem of nitrogen-limited musts.

Supplementary material

The additional files can be downloaded from:
https://www.sciencedirect.com/science/article/abs/pii/S0168160518304859

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Chapter 3

Interspecific hybridisation among diverse *Saccharomyces* species: a combined biotechnological solution for low-temperature and nitrogen-limited wine fermentations

Ying Su, Amparo Gamero, María Eugenia Rodríguez, Christian Ariel Lopes, Amparo Querol and José Manuel Guillamón

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Abstract

Lack of the prezygotic barrier in the *Saccharomyces* genus facilitates the construction of artificial interspecific hybrids among different *Saccharomyces* species. Hybrids that maintain the interesting features of parental strains have been applied in industry for many beneficial purposes. Two of the most important problems faced by wine makers is nitrogen deficiency in grape must and low-temperature fermentation. In our study, hybrids were constructed by using selected low nitrogen-demanding cryotolerant *S. eubayanus*, *S. uvarum* strains and *S. cerevisiae*. The fermentation capacity of the hybrid strains was tested under four conditions by combining two temperatures, 12 ℃ and 28 ℃, and two nitrogen concentrations, 60 mg/L and 300 mg/L. The hybrid strains obtained combined characters of both parental strains and conferred better fermentation rates under low-temperature or low-nitrogen conditions. The hybrid strains also produced larger amounts of acetate esters and higher alcohols, which increase aroma intensity and complexity in wine. Nitrogen sources were more rapidly consumed by the hybrid strains, which allows greater competition ability under nitrogen-deficiency conditions. Therefore, the interspecific hybridisation between low nitrogen-demanding cryotolerant strains and *S. cerevisiae* is a potential solution for low-temperature or low-nitrogen fermentations.

Keywords: *S. cerevisiae*, *S. eubayanus*, *S. uvarum*, hybridisation, nitrogen requirement, temperature

1. Introduction

Nitrogen is one of the substantial nutrients for yeasts that regulates biomass formation and fermentation activity, and provides important precursors for wine aroma-related compounds, such as higher alcohols, esters and volatile fatty acids (Bely et al., 1990; Bisson, 1991; Swiegers et al., 2005). Lack of nitrogen is one of the main causes of sluggish or stuck wine fermentation and H_2S production (Bell and Henschke, 2005; Giudici and Kunkee, 1994). While nitrogen plays a significant role in fermentation processes and sensory wine characters, yeast assimilable nitrogen (YAN) deficiency in grape must has been well-identified in many wine regions in Europe and elsewhere in the world (Butzke, 1998; Hagen et al., 2008; Henschke and Jiranek, 1993; Nicolini et al., 2004). Many studies have revealed that at least 140 mg/L YAN is needed to achieve complete wine dryness (Bely et al., 1990, Mendes-Ferreira et al., 2004). Technically speaking, a higher YAN concentration is needed to match a higher sugar concentration in grape must. Nitrogen addition to grape must can be one solution, but it is often difficult to handle. Excessive nitrogen additions may lead to the presence of non-assimilated residual nitrogen at the end of fermentation, which leads to microbial instability and ethyl carbamate accumulation in wine (Ough and Amerine, 1988). Therefore, it is of much interest to develop yeast strains with lower nitrogen requirements to complete fermentations.

The *Saccharomyces* genus has been used as an ideal paradigm for hybridisations. In spite of the fact that eight identified species (*S. cerevisiae*,

S. paradoxus, S. mikatae, S. kudriavzevii, S. arboricola, S. uvarum, S. eubayanus and *S. jurei*) are highly divergent in nucleotide sequences, they barely show any prezygotic barriers, which enables them to mate and form viable diploids (Morales and Dujon, 2012). Natural hybrids have been isolated from different fermentation processes. The most well-known lager beer yeast *S. pastorianus* is a natural hybrid between *S. cerevisiae* and *S. eubayanus* (Gibson and Liti, 2015). Besides, *S. cerevisiae* x *S. uvarum*, *S. cerevisiae* x *S. kudriavzevii*, and even triple hybrid *S. cerevisiae* x *S. kudriavzevii* x *S. uvarum*, have been isolated from wine, cider and beer (Antunovics et al., 2005; González et al., 2006; Krogerus et al., 2018; Le Jeune et al., 2007; Lopandic et al., 2007; Masneuf et al., 1998). Interspecific hybridisation could be an evolution strategy for better adaptation to the environment or could also be a result of domestication. Apart from natural hybrids, artificial hybridisation can be used as a powerful tool for developing yeast strains with desirable merits for industrial uses. Many hybrid strains have been constructed in laboratories to combine beneficial attributes, such as stress tolerance, aroma production, fermentation rate, sugar utilisation, etc (reviewed by Bisson, 2017; Lopandic, 2018; Sipiczki, 2018; Sipiczki, 2019; Steensels et al., 2014). These artificial hybrids, together with natural interspecific hybrids, not only possess the characters of both parents, but also demonstrate superior performance in many cases (García Ríos et al., 2019; Krogerus et al., 2017; Pérez Través et al., 2015; Sipiczki, 2008). Some of them have been successfully used in the fermentation-based food and beverage industry. One example is a hybrid developed in our laboratory

between *S. cerevisiae* and commercial strain *S. uvarum* Velluto BMV 58 (Lallemand Inc.) to improve the ethanol tolerance of *S. uvarum*, which has been commercialised as Velluto Evolution (Lallemand Inc.).

Fermentation at lower temperature is becoming increasingly popular because volatile compounds can be better preserved and it, therefore, provides wine with fruitier and fresher notes (Molina et al., 2007). However, cold temperature is one of the major stresses for *S. cerevisiae*, which largely influences the fermentation rate. *S. eubayanus* and *S. uvarum* are two of the most applied species for artificial hybridisation. Their well-known cryophilic nature favours the requirement of low-temperature fermentation. *S. uvarum* strains have been isolated as the dominant strain from different fermentation environments, including wine, cider and apple chicha (reviewed by Rodriguez et al., 2016). This species, together with *S. cerevisiae*, is able to tolerate different stress conditions and allows wine fermentations to finish (Alonso del Real et al., 2017). Physiologically, *S. uvarum* produces less acetic acids and ethanol, but more glycerol than *S. cerevisiae* (Castelleri et al., 1994; Masneuf-Pomarède et al., 2010). In particular, this species produces more 2-phenylethanol and 2-phenylethyl acetate, which confer wine a pleasant rose-like odour (González Flores et al., 2017; Masneuf-Pomarède et al.; 2010; Stribny et al., 2015). *S. eubayanus* was first isolated and identified by Libkind et al. (2011) from Patagonia. After its identification, it has been isolated in various locations in North America (Peris et al., 2014), China (Bing et al., 2014) and New Zealand (Gayevskiy and Goddard, 2016), and also from other substrates and locations in Patagonia (Rodríguez et al., 2014). However, no

isolate has yet been obtained from Europe. Keen research interest has been shown in *S. eubayanus* after its identification. Nowadays, the beer fermented only by *S. eubayanus* can be found on the market.

Several studies have previously constructed hybrids between *S. cerevisiae* and *S. eubayanus*, *S. uvarum* aiming to improve the cryophilic character of *S. cerevisiae* (Diderich et al., 2018; Garc á-R ós et al., 2019; Hebly et al., 2015; Kishimoto, 1994; Magalh ães et al., 2017; Origone et al., 2018; Rainieri et al, 1998; Zamboneli et al, 1997). However, so far, hybridization has not been used to improve the nitrogen requirements of a wine yeast. In our previous study (Su et al., 2019), we compared the nitrogen requirements of the *S. eubayanus* and *S. uvarum* Patagonian strains to *S. cerevisiae* by carrying out both fermentation and growth experiments with different nitrogen concentrations and several temperatures. Our results revealed that both species, especially the *S. eubayanus* strains, have relatively lower nitrogen requirements than the *S. cerevisiae* control strain. Therefore, the combination of two interesting oenological features, cold tolerance and low-nitrogen requirements, in *S. eubayanus* and *S. uvarum* makes these two species ideal candidates for hybridising with *S. cerevisiae*. Thus, the objective of our study was to improve yeast fermentation activity at a low temperature and a limited nitrogen concentration by constructing hybrids between *S. cerevisiae* and the low nitrogen-demanding cryotolerant strains of *S. eubayanus* and *S. uvarum*. In fact, a low-temperature and nitrogen-limited fermentation is a very challenging situation for wine yeasts. Both conditions produce similar metabolic and transcriptional effects, decreasing biomass yield and

fermentation rate during wine fermentation (Beltran et al., 2006; Pizarro et al., 2008)

Different strategies to construct artificial hybrids, including protoplast fusion, rare mating, spore to spore mating, mass mating, etc have been successful applied (reviewed by Morales and Dujon, 2012; Sipiczki, 2008; Steensels et al., 2014). In this study, we applied the direct cell to cell mating method which is simply mixing cell cultures of two selected stable haploid parents. This approach is not used regularly to develop novel yeast hybrids due to the homothallic nature of most industrial yeast strains, making them unsuited for this approach. However, homothallic strains would be amenable to this approach after genetically disrupting the *HO* endonuclease gene, a gene responsible for mating-type switching (Katz Ezov et al., 2010), making these strains fit for cell-to-cell mating experiments (Steensels et al., 2014). Despite this requires a genetic transformation, which implies that the resulting hybrid is classified as a GMO, we used this direct and quick hybridization method. We aimed at this stage to get a proof of concept that hybridisation of *S. cerevisiae* with *S. eubayanus* and *S. uvarum* was a feasible method to get strains with good fermentation performance at low temperature and nitrogen-limited conditions. The constructed hybrids were evaluated by conducting fermentations under four different conditions by combining two temperatures and two nitrogen concentrations. Fermentation metabolite and volatile compounds production by different yeast strains was determined. Nitrogen assimilation during fermentation was monitored throughout the fermentation process.

2. Materials and Methods

2.1 Strains and media

S. eubayanus (*Se*) strain NPCC1285 was isolated from *A. araucana* seeds and *S. uvarum* (*Su*) strain NPCC1317 was obtained from fermented apple chichi, both from the Patagonia region in Argentina. These strains were identified as low nitrogen-demanding strains in our previous work (Su et al., 2019) and were used as cryotolerant parental strains. Lalvin T73 (Lallemand Inc., Montreal, Canada) was used as the *S. cerevisiae* (*Sc*) parental strain.

Yeast extract peptone dextrose (YPD) medium, which contains 20 g/L glucose, 20 g/L peptone and 10 g/L yeast extract, was used for yeast propagation.

Fermentations were carried out with synthetic grape must (SM) which was prepared as described by Riou et al. (1997) with some modifications. This medium contains 200 g/L of sugar as a 50% glucose and 50% fructose mix. The concentrations of organic acids were the following: 5 g/L malic acid, 0.5 g/L citric acid and 3 g/L tartaric acid. Minerals and vitamins were supplied at the same concentration as those described by Su et al. (2019). The composition of nitrogen sources in the SM was 40% of ammonium chloride and 60% of amino acids. The composition of amino acids in the 1L stock was 1.5 g L-tyrosine, 13.4 g L-tryptophan, 2.5 g L-isoleucine, 3.4 g aspartic acid, 9.2 g glutamic acid, 28.3 g L-arginine, 3.7 g L-leucine, 5.8 g L-threonine, 1.4 g glycine, 38.4 g L-glutamine, 11.2 g L-alanine, 3.4 g L-valine, 2.4 g L-methionine, 2.9 g L-phenylalanine, 6 g L-serine, 2.6 g L-histidine, 1.3 g L-

lysine, 1.5 g L-cysteine and 46.1 g L-proline, which corresponded to 13.75 g/L of Yeast Assimilable Nitrogen (YAN is the sum of the amino acid and ammonium concentrations, except proline). The final pH of the SM was adjusted to 3.3 with sodium hydroxide. Synthetic musts were sterilised by filtration through 0.22 μm pore-sized membrane filters (Thermo scientific, MA, USA).

2.2 Hybrids construction

Firstly, we generated stable haploid versions of the diploid parental strains by deleting one copy of the *HO* gene using the dominant drug resistance markers nourseothricin and hygromycin (Goldstein and McCusker 1999). The *HO* deletion was performed by PCR-mediated gene replacement (short flanking homology method). The deletion cassette was obtained by PCR using the pAG25 plasmid that contains nourseothricin resistance and the pAG32 plasmid that contains hygromycin resistance. Namely, one copy of the *HO* gene of T73 was replaced with *NatMX6* and the *HO* genes of *S. eubayanus* and *S. uvarum* were replaced with *HphMX6*. The primers used for gene deletion are listed in Supplementary Table 1. Transformation was performed by the lithium acetate method (Gietz and Schiestl, 2008). Transformants were selected by resistance to nourseothricin or hygromycin, and correct deletion cassette integration was confirmed by diagnostic PCR using the primers upstream and downstream of the deleted region (Table 5 in Annex I).

The successfully transformed diploid parental strains (heterozygous for *HO*) were sporulated on acetate medium (1% potassium acetate, 2% agar) for 5-7

days. Asci were digested with 0.5 mg/mL zymolyase at 37 ℃ for 30 minutes. Tetrads were dissected using a micromanipulator (MSM 400; Singer Instruments, Watchet, UK) on YPD agar plates (2% Bacto peptone, 1% yeast extract, 2% glucose, and 2% agar). Viable spore clones were then streaked onto YPD with nourseothricin or hygromycin agar plates for Δ*ho* spore clone selection. The haploid cells with nourseothricin or hygromycin resistance were again verified by diagnostic PCR, as mentioned above.

The Δ*ho* haploid strains from both parental with opposite mating types were crossed in complete media (YPD) and grown overnight. Patches were replica-plated on YPD plates containing both hygromycin and nourseothricin to select diploid hybrids. These hybrids were then confirmed by the *Msp*I restriction pattern of the *KEL2* gene (Pérez et al., 2015). Eleven hybrids of each combination (*Sc*x*Su* and *Sc*x*Se*) were tested for growth capacity at 60, 140 and 300 mg/L YAN, and no significant differences among the different hybrids were observed (data not shown).

2.3 Fermentation activity analysis

Fermentations were carried out in 100-mL bottles with 80 mL of the SM at two nitrogen concentrations (60 and 300 mg/L YAN) at 12 ℃ or 28 ℃. The SM was inoculated with both pure and mix cultures to a population size of $2x10^6$ cells/mL. The mix-culture fermentations were inoculated with 50% of each of the two tested strains. Fermentation kinetics was followed by measuring the density reduction of the SM. Fermentation was considered finished when density went below 998 g/L (Gutiérrez et al., 2012).

Unlike *S. cerevisiae*, *S. uvarum* and *S. eubayanus* strains are less heat-tolerant and are unable to form colonies on YPD plates at 37 ℃. Therefore, in the mix-culture fermentations, the percentage of *S. cerevisiae* was determined simply by plating the sample on two YPD plates and incubating at 30 ℃ and 37 ℃. Colony-forming units (CFU) were counted after 2 days of incubation. The CFU number on the 37 ℃ plates represents the *S. cerevisiae* population and the CFU difference between the two plates represents the population of the non-*cerevisiae* strains in the mixed culture (Su et al., 2019).

2.4 HPLC analysis for residual sugars and fermentation metabolites

At the end of the fermentations, samples were taken and analysed for the main wine chemical parameters, including glucose, fructose, ethanol, glycerol, succinic acid and acetic acid. Samples were first centrifuged to remove yeast cells and the supernatants were diluted 3 times with deionised water to then be filtered through 0.22 μm pore-sized nylon filters (Phenomenex, CA, USA). HPLC, equipped with a refraction index detector and a UV-Visible detector, was used for the analysis. The employed mobile phase was 1.5 mM H_2SO_4 with a flux of 0.6 mL/min. Metabolites were separated by a HyperREZ XP Carbohydrate H+ 8 mm column with a column temperature of 45 ℃ (Thermo Scientific, MA, USA).

2.5 HPLC analysis for residual amino acids

The analysis of the residual amino acids was carried out in a ultimate 3000® UPLC (Thermo Scientific, MA, USA) equipped with a UV-visible detector (Thermo Scientific, MA, USA). The HPLC analysis method is based on

Gómez-Alonso et al. (2007), but with some modifications. The 400 µL samples were derivatised with 12 µL diethylethoxymethylenemalonate (DEEMM), together with 300 µL of methanol. The reactions were performed in screw-cap test tubes in an ultrasonic bath for 30 minutes, followed by heating at 80 ℃ for 2 h to degrade any excess DEEMM. After derivatisation, samples were filtrated through 0.22 µm nylon syringe filters (Phenomenex, CA, USA). Chromatographic separation was performed with an Accucore® C18 LC column (Thermo Scientific, MA, USA). The binary gradient was applied (phase A: 25 mM acetate buffer, pH 6.0, and phase B: acetonitrile) at a flow rate of 1.2 mL/min and a column temperature of 30 ℃. The gradient is shown in Table 4 in Annex I.

2.6 GC analysis for volatile compounds

Higher alcohols and esters were analysed by the headspace solid-phase microextraction (HS-SPME) technique using a 100 µm poly-dimethylsiloxane (PDMS) fibre (Supelco, Sigma-Aldrich, Madrid, Spain). The extraction method was the same as that described by Stribny et al. (2016). 2-heptanone (0.005%) was added as an internal standard. A TRACE GC Ultra® gas chromatograph (Thermo Scientific, MA, USA) with a flame ionization detector (FID) was used, equipped with an HP-INNOWax 30 m × 0.25 mm capillary column coated with a 0.25 µm layer of cross-linked polyethylene glycol (Agilent Technologies, CA, USA). The oven temperature programme was: 5 min at 35 ℃, 2 ℃/min to 150 ℃, 20 ℃/min to 250 ℃ and 2 min at 250 ℃. The detector temperature remained constant at 300 ℃.

Chromatographs were analysed by the Chrom Quest programme. Volatile compounds were identified by the retention time for the reference compounds. Quantification of volatile compounds was determined using the calibration graphs of the corresponding standard volatile compounds.

2.7 Statistical analysis

All the fermentations were carried out in triplicate. Fermentation kinetics was calculated by fitting the density data to the 4-parameter logistic model. The time needed to consume all the sugars (T100) was extracted from the smoothed data. One-way analysis of variance (ANOVA) was conducted with version 7.0 of the Statistica software package. The statistical level of significance was set at $p \leq 0.05$ with a Tukey HSD test. For the nitrogen uptake order analysis, the area under the curve (AUC) value was calculated by using the R statistical software v. 3.0 with the "growth curver" package (Sprouffske, 2018). A heatmap was plotted by Mev MultiExperiment Viewer, and hierarchical clustering was based on Euclidean distance metrics. A principal component analysis (PCA) was performed using the software package LatentiX 2.12 (http://www.latentix.com). Data were autoscale-normalised.

3. Results

3.1 Fermentation kinetics and cell composition in the mix-culture fermentation

Fermentations were carried out at four different conditions with a combination of two temperatures, 12 ℃ and 28 ℃, and two nitrogen

concentrations, 60 mg/L and 300 mg/L YAN. The strains used in these fermentations were parental strains *S. cerevisiae* (*Sc*), *S. eubayanus* (*Se*) and *S. uvarum* (*Su*) and two hybrid strains, *Sc*x*Se* and *Sc*x*Su*. For comparison purposes, the mix-culture fermentations between *S. cerevisiae* and the cryotolerant parental strains (*Sc*+*Se* and *Sc*+*Su*) were also carried out. The results indicated that 12 ℃ and 60 mg/L YAN formed an extremely harsh condition and none of the tested strains or combinations of strains were able to complete fermentations within 500 h. However, the lowest residual sugar concentration was detected in the *Su* fermentation, which was more than 3-fold lower than the *Sc* fermentation (21 *vs.* 75 g/L; Supplementary Table 3). For the other fermentation conditions, the time needed by the different strains to end fermentations (T100) are shown in Fig. 1 (A-C). As expected, *Sc* displayed such better performance and spent less time to finish fermentations than *Se* and *Su*, mainly under the optimum condition for *S. cerevisiae* (28 ℃ and 300 mg/L YAN). The cryotolerant strains carried out the fermentations more slowly. They had difficulties to deplete all the sugars at 28 ℃ and 60 mg/L YAN and more than 10 g/L fructose remained in the must (Supplementary Table 3). On the contrary, the hybrid strains displayed outstanding fermentation performance, and even exceeded their *Sc* parental strain by completing fermentations in shortest time under all the conditions. The fermentation time of the mix-culture fermentations was somewhere between that of the *Sc* and cryotolerant strains. In order to determine the competitiveness of each strain in the mix-culture fermentations, the percentage of each inoculated strain was also monitored (Fig. 1D). Our results

showed that temperature strongly influenced the imposition of *Sc* on *Se* and *Su*. *Sc* was less competitive than the cryotolerant strains at 12 ℃. Regardless of nitrogen content, at the end of these low temperature fermentations, the percentage of *Sc* represented only 20-30% of the total population. In contrast at 28 ℃, the percentage of *Sc* was around 60%-80%. A high nitrogen concentration also favoured the implementation of *Sc*, but the influence was not as determinant as temperature.

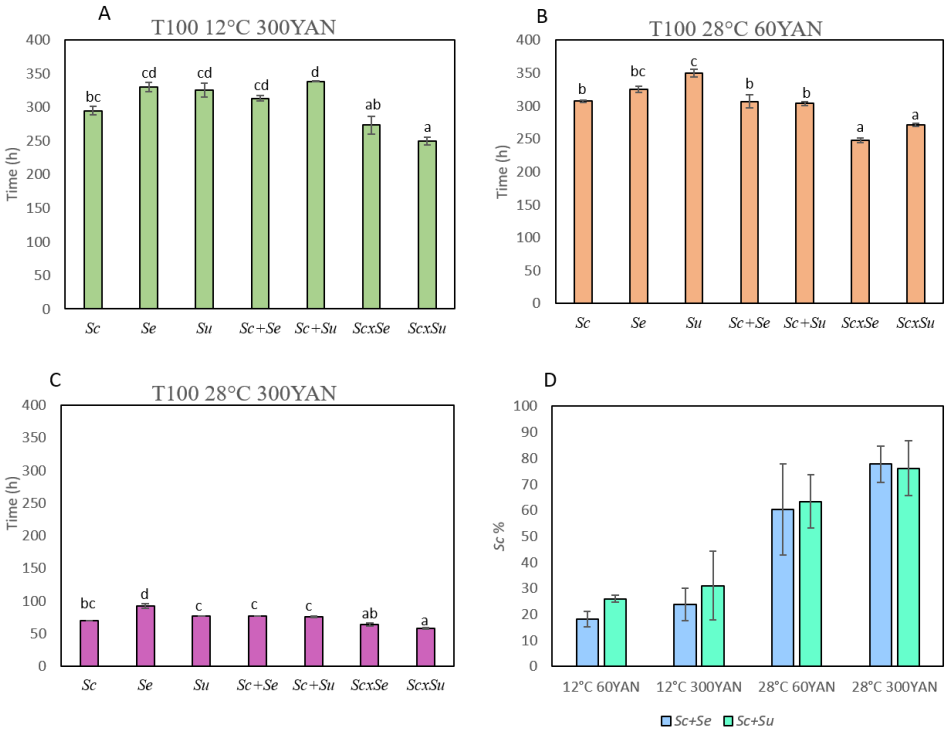


Fig. 1. **A-C**. Time (hours) required to consume the 100% (T100) of the sugar content in the synthetic must with 12 ℃ 300 mg/L YAN, 28 ℃ 60 mg/L YAN, and 28 ℃ 300 mg/L YAN. The letters at the top of bars indicate the significant difference groups (HSD Tukey test $p \leq 0.05$). **D**. Percentage of *S. cerevisiae* in the mix culture at the end of fermentations. Blue

bars represent the mix culture of *S. cerevisiae* and *S. eubayanus*; green bars represent the mix culture of *S. cerevisiae* and *S. uvarum*.

3.2 Metabolites production by different fermentations

At the end of the fermentations, major metabolites were analysed by HPLC (Supplementary Table 3). The yields of ethanol, glycerol, and organic acids by sugar consumption were calculated. No significant differences were observed for the ethanol yields in most of the fermentation conditions (Fig. 2, Supplementary Table 4). However, significant differences were observed for the glycerol yields for the various tested conditions. Generally speaking, *Sc* was a low glycerol producer, whereas the cryotolerant strains gave the highest glycerol yields for all the conditions. Interestingly, under the low-nitrogen condition at 28 ℃, the hybrid strains achieved significantly higher glycerol production than their *Sc* parental strain (more than 2 g/L higher), but no significant differences were found in the fermentations done with 300 mg/L YAN. Acetic acid synthesis also strongly depended on yeast strains. The hybrid strains produced significantly smaller amounts of acetic acid than *Sc*, especially when nitrogen was limited in the SM. *Su* was the lowest acetic acid producer under all the conditions. Another metabolite whose concentration seemed to be nitrogen-dependent was succinic acid. A negative correlation was found between nitrogen concentration and succinic acid production, with higher yields for all the strains when nitrogen was limited.

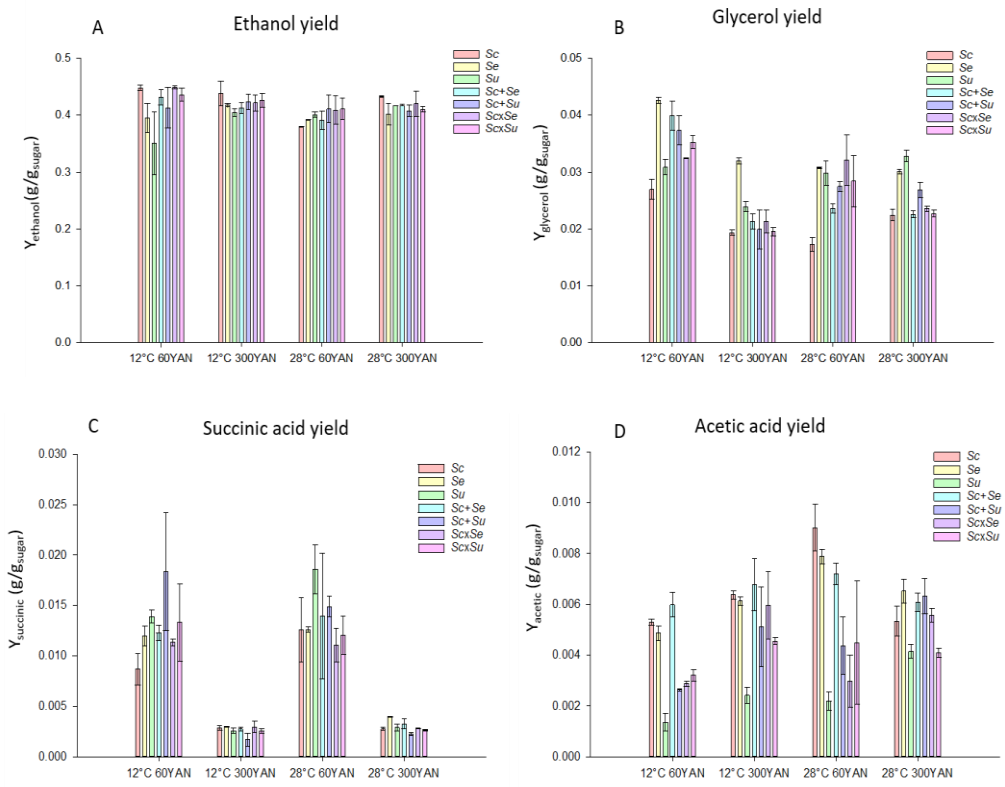


Fig. 2. Yield of major metabolites ethanol, glycerol, succinic acid and acetic acid, at the end of fermentations. Different strains are represented by different coloured bars.

3.3 Quantification of volatile compounds

Volatile compounds production was considerably influenced by fermentation temperature, nitrogen concentration and yeast strains (Supplementary Table 5). The concentrations of eleven volatile compounds were determined, and they were classified as ethyl esters (ethyl hexanoate, ethyl octanoate, ethyl decanoate, diethyl succinate), acetate esters (ethyl acetate, isoamyl acetate, 2-phenylethyl acetate), and higher alcohols (isobutanol, isoamyl alcohol, benzyl

alcohol, 2-phenylethanol). The unfinished fermentations at 12 ℃ and 60 mg/L of YAN were not included in the comparisons. Fig. 3 shows the distribution of these three groups of aroma compounds from all the fermentations under the same condition. Regardless of the inoculated strain, the fermentations carried out with limited nitrogen demonstrated slightly higher acetate esters and ethyl esters production, but these differences were not statistically significant. Higher alcohols production was strongly influenced by fermentation conditions. A much larger amount of higher alcohols was produced when fermentations were carried out with 60 mg/L YAN, with double the concentration of that in the fermentations run with 300 mg/L YAN. A PCA was carried out with the aroma data of all the fermentations (Fig. 4). The first two principal components accounted for 65% of total variance. The topography distribution of the PCA showed that the environmental factors (nitrogen and temperature) more strongly impacted aroma production than the inoculated strain because all the strains were grouped according to fermentation conditions. The only exceptions were strains *Se* and *Su* at 28 ℃/300 mg/L YAN, which grouped with the samples at low temperature, which could be interpreted as a consequence of their cryotolerant feature. The fermentations performed with the higher YAN concentration grouped mainly for the higher production of some ethyl esters, isoamyl acetate, and benzyl alcohol, while the fermentations performed with the lower YAN concentration stood out for higher alcohols production.

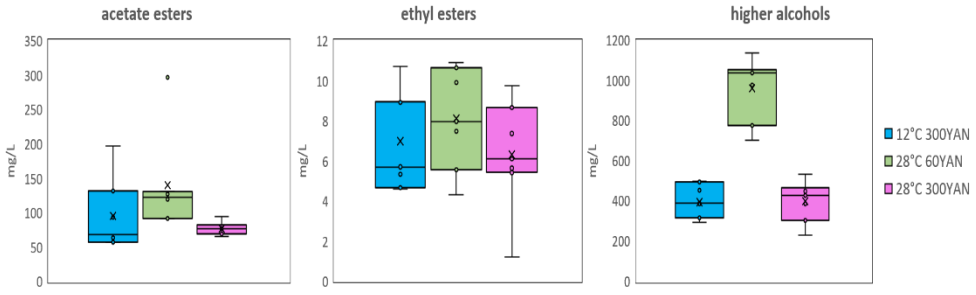


Fig. 3. Box plots represent the production distribution of the acetate esters, ethyl esters and higher alcohols of three fermentation conditions.

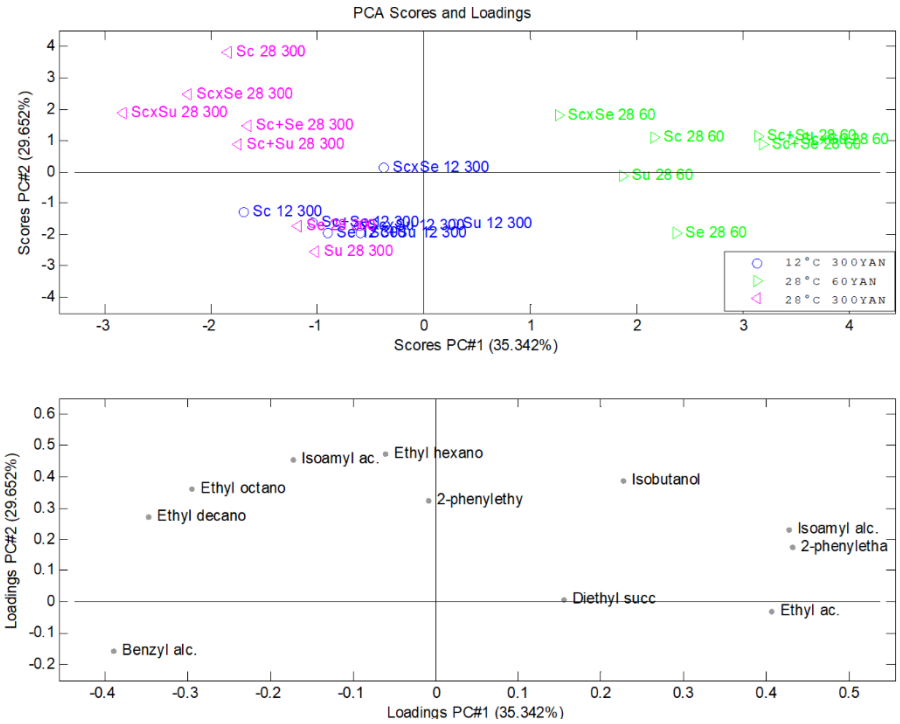


Fig. 4. The PCA plot of the volatile compounds produced by different strains under three fermentation conditions. Scores represent fermentations. Loadings denote different volatile compounds.

Se produced the largest amount of acetate esters (mostly ethyl acetate) at 28 ℃ and *Su* exhibited the same behaviour at 12 ℃ (Fig. 5; Supplementary Table 5). Conversely, *Sc* and hybrids yielded larger amounts of ethyl esters, mainly diethyl succinate, than the cryotolerant parental strains at low temperature or under low-nitrogen conditions. Significant differences in ester production were observed by the two hybrids: *Sc*x*Se* produced a higher level of ethyl esters than *Sc*x*Su*, regardless of the fermentation conditions. Regarding the higher alcohols, *Sc* and the hybrid strains were the best producers at 28 ℃. At 12 ℃, hybrids also maintained good higher alcohols production, whereas the synthesis of *Sc* significantly lowered. The production of 2-phenylethyl acetate and 2-phenylethanol was higher for both the cryotolerant and hybrid strains, and occurrence was more obvious for the fermentations conducted at low temperature or at a limited YAN concentration (Supplementary Table 5). The mix-culture fermentations generally gave intermediate production values between the pure culture of *Sc* and the pure cultures of the cryotolerant strains, but behaviour was more irregular because it depended mostly on the degree of imposition of each inoculated strain (Fig. 1D).

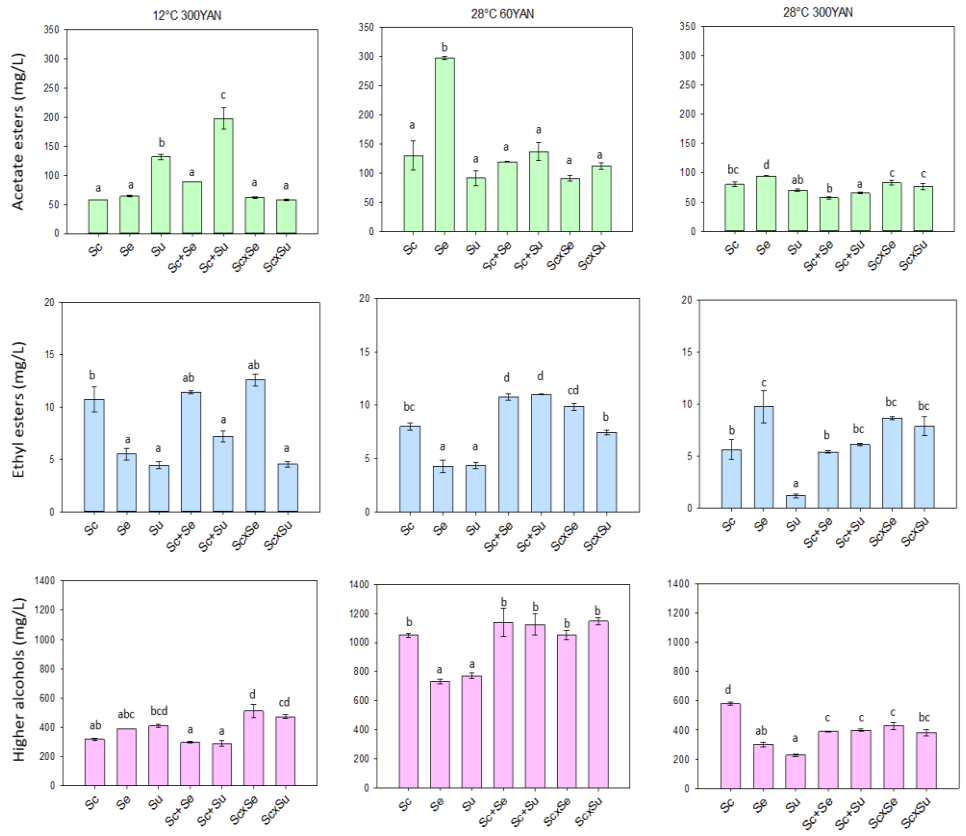


Fig. 5. Production (mg/L) of acetate esters, ethyl esters and higher alcohols by different strains under three fermentation conditions.

3.4 Nitrogen source uptake order by hybrid strains under different conditions

Nitrogen source preference and nitrogen source assimilation order shed light on the nitrogen metabolism regulation by certain yeast species. In order to explore nitrogen uptake order by hybrids and their parental strains, the residual nitrogen in the must was analysed by HPLC during fermentation. The percentage of nitrogen consumption was plotted against time as the nitrogen

consumption curve. The area under the curve (AUC) provided comprehensive information about the time to start consumption, the consumption rate and the maximum consumption percentage. A high AUC value indicates a more rapid complete consumption of certain nitrogen sources. Conversely, a low AUC value denotes slow or incomplete consumption (individual consumption curves are found in Supplementary Figs. 1-4). Although the fermentations run at 12 ℃ with 60 mg/L YAN did not finish, the nitrogen consumption under this condition was complete. Therefore, this condition was included for the analysis. For an overview of nitrogen consumption efficiency, the AUC value of the total YAN consumption was calculated for the different strains and conditions (Fig. 6). The nitrogen uptake rate of the parental strains was clearly influenced by fermentation temperature. *Sc* showed the quickest nitrogen consumption efficiency at a high temperature, while *Se* and *Su* more rapidly consumed total nitrogen at 12 ℃. These differences were more pronounced with 300 mg/L YAN because all the strains quickly exhausted total nitrogen under the 60 mg/L YAN conditions. It is noteworthy that the hybrid strains were more similar to the fittest parent at each temperature.

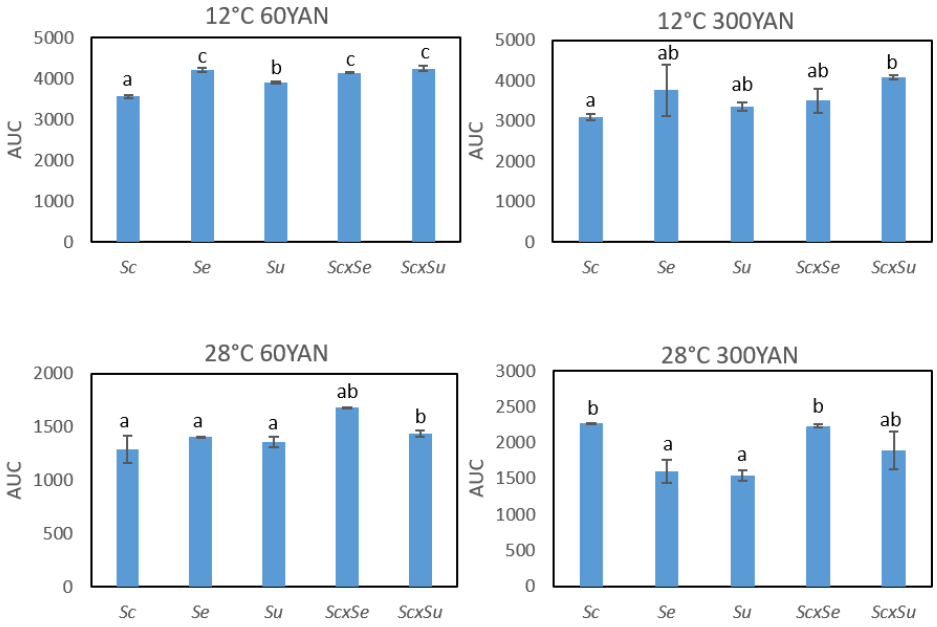


Fig. 6. The AUC value of total nitrogen consumption for the different strains under each fermentation condition. AUC provides the summary information of the maximum consumption percentage of YAN during the time-lapse analysed.

The AUC values of 20 individual nitrogen sources for each fermentation condition were also calculated and ranked from high to low to indicate the uptake order. Ranking orders were represented on a heatmap with hierarchical clustering (Fig. 7). Three major groups (A-C) were obtained that, as with aroma production (PCA; Fig. 4), were determined mainly by the fermentation conditions. Once again, the exception came from the parental *Se* and *Su* at 28 ℃ and 300 mg/L YAN, which were grouped in cluster C (12 ℃ and 300 mg/L of YAN). Another interesting exception was the parental *Sc* and the two hybrids at 28 ℃ and 300 mg/L of YAN, which were not included in any of

these major groups and represented the three conditions with a shorter fermentation time.

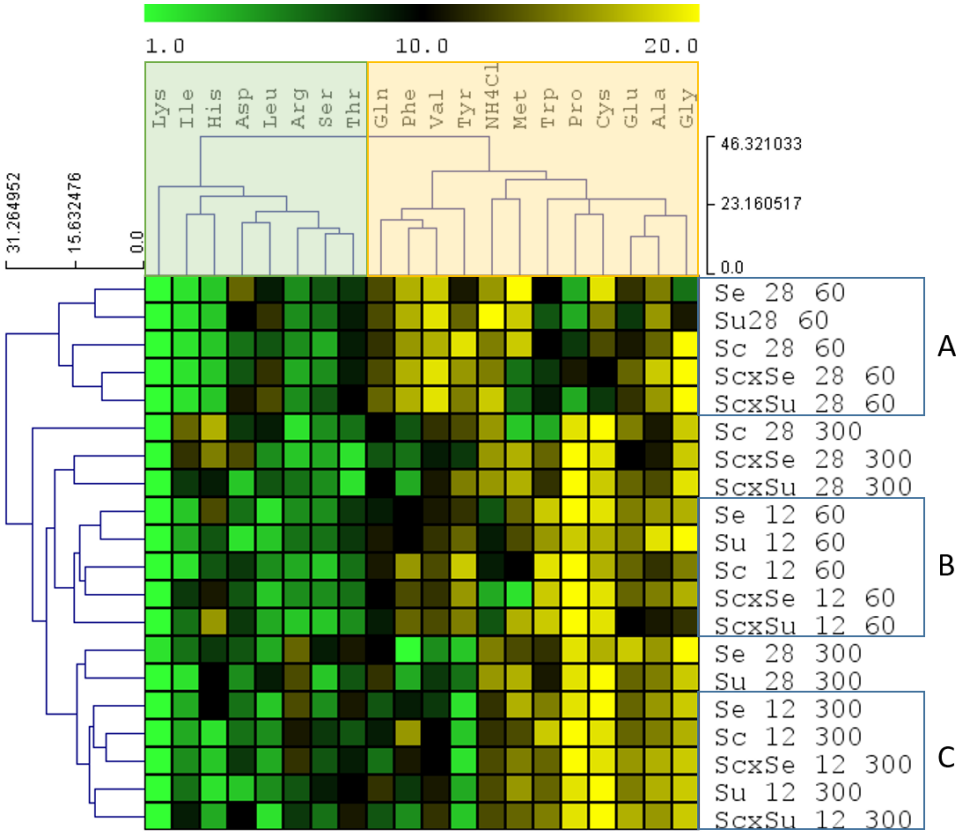


Fig. 7. The heatmap of the consumption order of different nitrogen sources with a hierarchical cluster calculated by Euclidean distance. The colour scheme from green to yellow indicates the consumption order from the 1st to the 20th.

Two main groups were obtained when this hierarchical clustering was applied to the individual 19 amino acids and ammonium chloride (Fig. 7). Regardless of the fermentation conditions, the green cluster gathered the most rapidly consumed amino acids (the greenest squares) and the yellow cluster grouped

all the amino acids of a delayed uptake (the yellowest squares). Black squares represent the amino acids of intermediate consumption. A more detailed consumption order for each individual nitrogen source for the different strains and fermentation conditions is showed in Fig. 8. As reported for other *S. cerevisiae* strains, Lys was practically the first amino acid to be consumed by all the strains and fermentation conditions, followed by other amino acids that were early consumed, e.g. Ile, His, Asp, Leu, Arg, Ser and Thr. Lastly, Pro, Cys, Glu, Ala and Gly were among the last consumed amino acids. From this individual nitrogen source consumption overview, some strain-specificities are worth mentioning. The biggest differences among strains were detected at 28 ℃ and 300 mg/L YAN as total nitrogen was quickly exhausted in the nitrogen-limiting fermentations. *Sc* was the fastest nitrogen-consuming strain that depleted all the nitrogen sources within 30 h, except for proline and cysteine (Fig. 3). The hybrid strains showed similar consumption profiles to *Sc*, and almost all the sources were depleted. Conversely, *Se* and *Su* consumed nitrogen more slowly than *Sc*, with many residual nitrogen sources after 30 h of fermentation. As expected, both nitrogen concentration and fermentation temperature clearly conditioned the uptake profile of the amino acids in the different strains. The cryotolerant strains consumed Arg more quickly in the nitrogen-limiting fermentations than in 300 mg/L of YAN. The Trp consumption performed by all the strains at 12 ℃ was much slower than that at 28 ℃. *Su* consumed Asp more quickly than the other strains at 12 ℃. One remarkable finding was the preference Phe consumption by the cryotolerant

and hybrid strains. In all the strains, Tyr was very rapidly consumed at 12 ℃ with 300 mg/L YAN (Fig. 8).

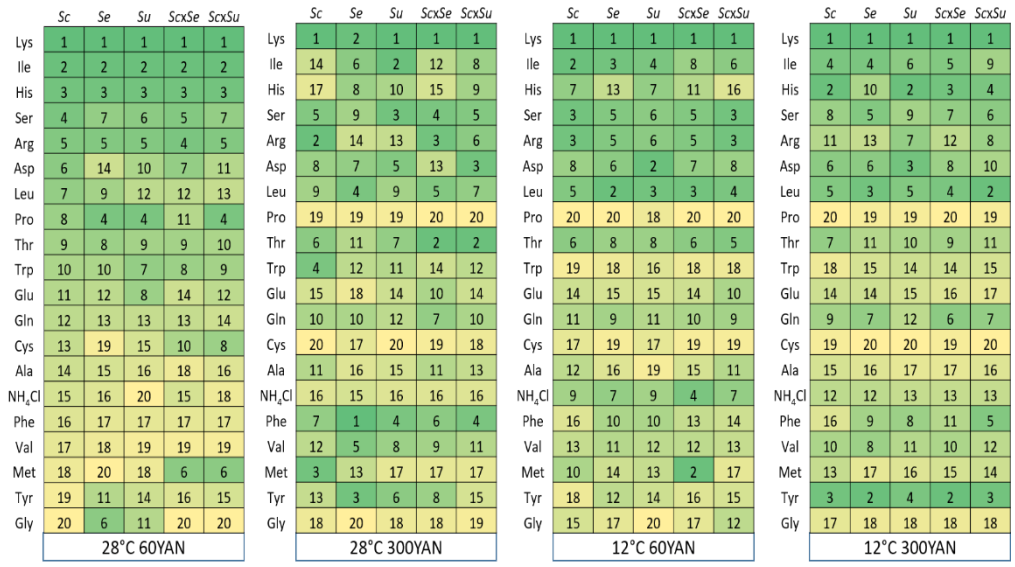


Fig. 8. The uptake order of different nitrogen sources under four fermentation conditions. The ranking is based on AUC values. A lower ranking number represents early consumption; a higher ranking number denotes late consumption. The colour scheme from yellow to green represents the ranking for low to high, respectively.

4. Discussion

4.1 Advantage of interspecific hybridisation for yeast strain adaptation to different fermentation environments

Interspecific hybridisation in the *Saccharomyces* genus was successfully applied for yeast strain improvement. Strains were crossed to obtain robust hybrids that carried different favourable traits of parental strains. The hybrid

strains between *S. cerevisiae*, and the selected low nitrogen-demanding cryotolerant strains of *S. eubayanus* and *S. uvarum* (Su et al., 2019), were constructed herein. These hybrids showed good adaptation to different fermentation conditions and outcompeted their parental strains in fermentation rate terms. The phenomenon in which hybrids possessed phenotypic superiority over parental strains has been previously reported and is known as heterosis or hybrid vigour (Lippman and Zamir 2007; reviewed by Steensels et al., 2014). The hybrid process provides a heterozygous advantage to buffer against deleterious recessive alleles and provides genetic plasticity to adapt to variable environmental conditions. Apart from hybridisation, the co- or sequential inoculation of non-*Saccharomyces* or *Saccharomyces* non-*cerevisiae* strains has also been suggested as an oenological practice with multiple purposes, such as increasing aroma complexity, lowering ethanol content and increasing glycerol in wine (Alonso del Real et al., 2017; Jolly et al., 2014; Padilla et al., 2017; Zhang et al., 2018). For comparison purposes, mix-culture fermentations were also included herein. As in our previous work (Su et al., 2019), we observed that the population of different strains was strongly influenced by both fermentation temperature and nitrogen concentration. Low temperature favoured the growth of *S. eubayanus* and *S. uvarum*, while higher temperature gave rise to *S. cerevisiae* dominating in fermentation, especially at a high nitrogen concentration. Generally speaking, the mix-culture fermentation behaviour was similar to that of the strains with a higher carrying capacity. The intermediate metabolites production level between both strains in the mix-

culture fermentation was also observed. Although the mix-culture fermentation improved the fermentation rate under suboptimum conditions, they were not as efficient as the hybrid strains. For applications in industry, the instability of the co- or sequential inoculation could be more difficult to manage, and wine quality could be hard to predict. Therefore, interspecific hybridisation could be a better alternative to improve yeast fermentation capacity.

4.2 Influence of fermentation conditions on yeast metabolism

Fermentation conditions have greatly impact yeast metabolism. The combination of two temperatures and two YAN concentrations was applied in the present study to explore the behaviour of the hybrid strains. The factorial ANOVA illustrated that the fermentation temperature, the initial nitrogen concentration, strains and the combination of different factors significantly impacted most of the metabolites herein analysed (Supplementary Table 6). Specifically, nitrogen availability strongly influenced succinic acid production. A more than 2-fold larger amount of succinic acid was produced when fermentations were carried out at a lower nitrogen concentration. Rollero et al. (2015) analysed the combined effects of nitrogen, lipids and temperature on fermentation metabolites production. Correspondingly, they also pointed out that succinic acid production was negatively regulated by the initial nitrogen concentration. The possible involved mechanism was explained by Camarasa et al. (2003): when nitrogen is limited in media, less glutamate is produced from α-ketoglutarate. Hence

succinic acid synthesis acts as a safety valve (Rollero et al., 2015) that directs the accumulated α-ketoglutarate pool to the TCA cycle. Thus nitrogen-limited fermentations could potentially contribute to biological acidification, a very desirable process, if we take into account the current drop in titratable acidity, or the increase in pH, in grape musts and wines as a consequence of climate change (Jones et al., 2005). Acetic acid yield is a substantial wine quality factor because it confers off-flavour. Our results demonstrated that the hybrid strains produced significantly less acetic acid than *Sc*, especially when nitrogen was limited in the SM. This feature could be inherited from cryotolerant parental strains as *S. uvarum* and *S. eubayanus* produced lower acetic acid levels. In general, acetic acid production was quite high in our study, which could be due to the absence of lipids in our SM. As previously described (Beltran et al.,2008; Rollero et al., 2015), acetic acid production is largely influenced by lipid concentration, and more acetic acid is released by yeast in the SM than in natural grape must due to the difference in lipid concentration (Beltran et al. 2008).

Volatile compounds production was also strongly impacted by the fermentation conditions. The largest amount of volatile compounds was generally produced when fermentations were carried out at 28 ℃ with 60 mg/L YAN. Previously, several authors (Beltran et al., 2005; Carrau et al., 2008; Rollero et al., 2015) have demonstrated the negative relation between nitrogen concentration and higher alcohols production. The larger amount of higher alcohols synthesis under nitrogen-limited conditions could be due mainly to the *de novo* synthesis of branched-chain amino acids (BCAAs)

through the anabolic pathway (reviewed by Stewart, 2017). In our study, higher production took place only for higher alcohols, but also for ethyl esters and acetate esters at low nitrogen concentrations. Interestingly, ethyl acetate and diethyl succinate were two compounds with the biggest contribution for the concentration of acetate esters and ethyl esters, respectively. The production of these two compounds was higher when nitrogen was limited and matched the very large succinic and acetic acids productions. Without considering ethyl acetate and diethyl succinate, a positive correlation between acetate esters and ethyl esters production and nitrogen concentration was observed (Fig. 9), which agrees with the conclusion drawn by several previous studies (Mouret et al., 2014; Rollero et al., 2015). Low-temperature fermentation has normally been applied to better conserve aromas (Beltran et al., 2002; Gamero et al., 2010; Molina et al., 2007; Torija et al., 2003). However, Mouret et al. (2014) mentioned the possible overestimated influence of temperature on ester synthesis. Our results illustrate that the initial nitrogen concentration, instead of temperature, seems to more strongly impact volatile compounds formation. Nevertheless, as the fermentations performed at 12 ℃ and 60 mg/L YAN did not finish, this evidence may not be sufficient to reach this conclusion. Comparing with *S. cerevisiae*, the cryotolerant parental strains produced larger amounts of acetate esters and smaller quantities of ethyl esters and higher alcohols. Among the acetate esters, a bigger amount of ethyl acetate was detected, which will confer wines a solvent-like off-flavour. For this reason, the *S. eubayanus* and *S. uvarum* strains used in our study may not be perfect strains for pure culture

fermentations in sensory character terms. However, the hybrid strains, mainly *Sc*x*Se*, produced bigger amounts of ethyl esters and higher alcohols. These compounds are highly desirable in wine because they are the main donors of fruity and floral aromas. Moreover, the higher production of both 2-phenylethyl acetate and 2-phenylethanol by the hybrid strains provided the wine a pleasant rose-like odour, which enhanced aroma complexity.

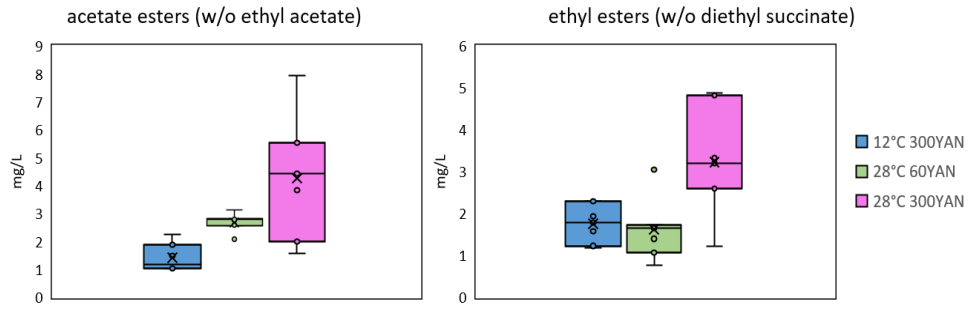


Fig. 9. The production distribution of acetate esters without ethyl acetate, and ethyl esters without diethyl succinate by different strains under three fermentation conditions.

4.3 Nitrogen requirement and sequential utilisation by hybrid strains

In our previous work (Su et al., 2019), competition fermentations were carried out between *S. cerevisiae* and the cryotolerant strains at low nitrogen concentrations. The result revealed that the selected cryotolerant strains outcompeted *S. cerevisiae* at low nitrogen concentrations, with fermentation temperature no higher than 20 ℃. However, the cryotolerant strains displayed poor fermentation capacity at 28 ℃. The hybrid strains demonstrated outstanding fermentation behaviour at 28 ℃ with only 60 mg/L YAN, while the cryotolerant parental strains did not manage to deplete all the fermentable

sugars under the same condition. During fermentations, nitrogen sources were consumed more rapidly by the hybrid strains than by the parental strains, which indicates better ability to compete in the uptake of key nutrients. Under the nitrogen-limiting condition, this strategy could ensure an optimum population size.

For *S. cerevisiae*, the nitrogen source uptake order is regulated mainly by nitrogen catabolite repression (NCR) and the Ssy1p-Ptr3p-Ssy5 (SPS) mechanism. As *S. eubayanus* and *S. uvarum* are phylogenetically closely related to *S. cerevisiae*, an alike regulation system is also expected. Similar nitrogen source uptake order has been observed among *S. cerevisiae*, cryotolerant strains and hybrid strains. In our study, fermentations were conducted under the same condition, and grouped together on the heatmap of nitrogen uptake order. Therefore, instead of yeast strains, the fermentation conditions more substantially influenced the nitrogen source uptake order. Notably, phenylalanine was consumed relatively more quickly by the *S. eubayanus*, *S. uvarum* and hybrid strains, which corresponded to higher productions of 2-phenylethanol and 2-phenylethyl acetate by these strains via the Ehrlich pathway.

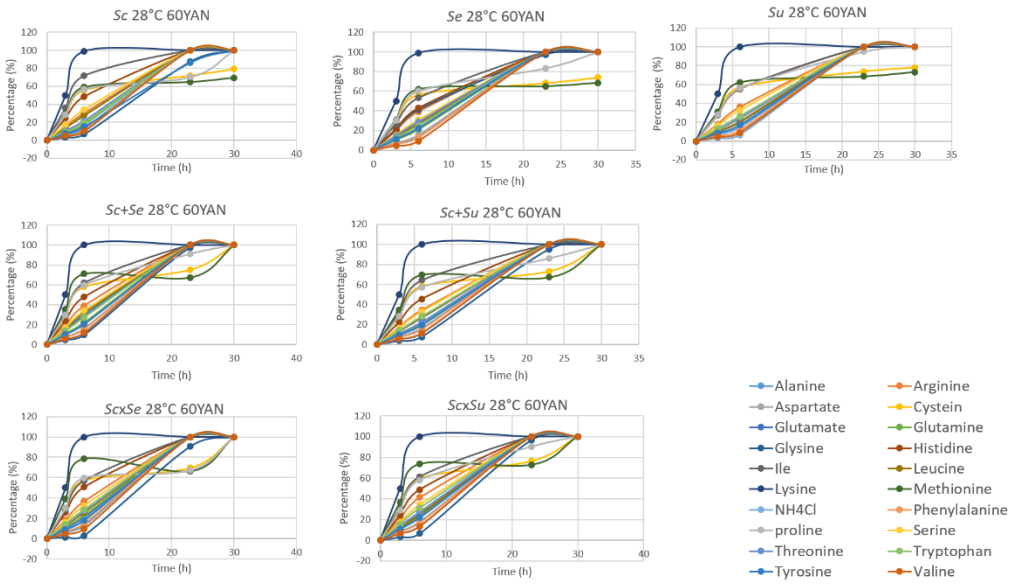
5. Conclusions

For the first time, this work used selected low nitrogen-demanding cryotolerant strains for hybrid construction with *S. cerevisiae*. The obtained hybrid strains showed good adaptation to low-temperature or low-nitrogen fermentation conditions and better fermentation performance than the

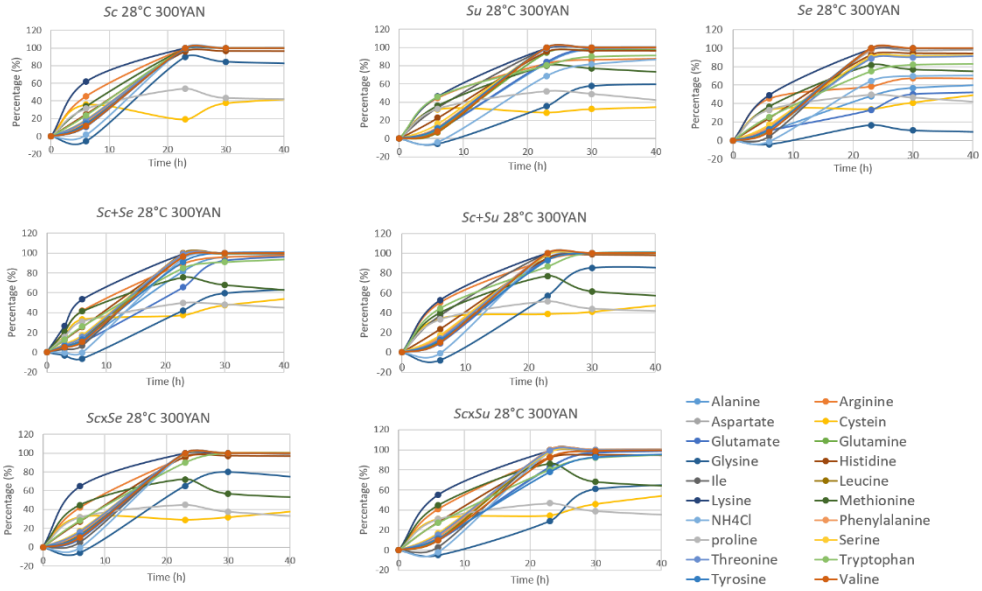
parental strains. A high fermentation speed not only saves time and energy for wineries, but also prevents contamination by undesirable microorganisms during fermentation. Hybrid strains are very promising for conducting fermentations under stressful conditions. We are well aware that our constructed strains are genetically modified and unable to be directly used in industry. However, as a result of these preliminary insights, we are now constructing non-GMO strains by applying natural auxotrophy and temperature as selection markers, as previously reported by Magalhães et al. (2017). Due to the inherent instability of interspecific yeast hybrids, the constructed hybrids will be subjected to a genomic stabilisation process after several generations of vegetative propagation. This process of stabilisation can be exploited for obtaining segregants (evolved hybrids) that display a high range of phenotypes (Sipiczki, 2018).

Supplementary material

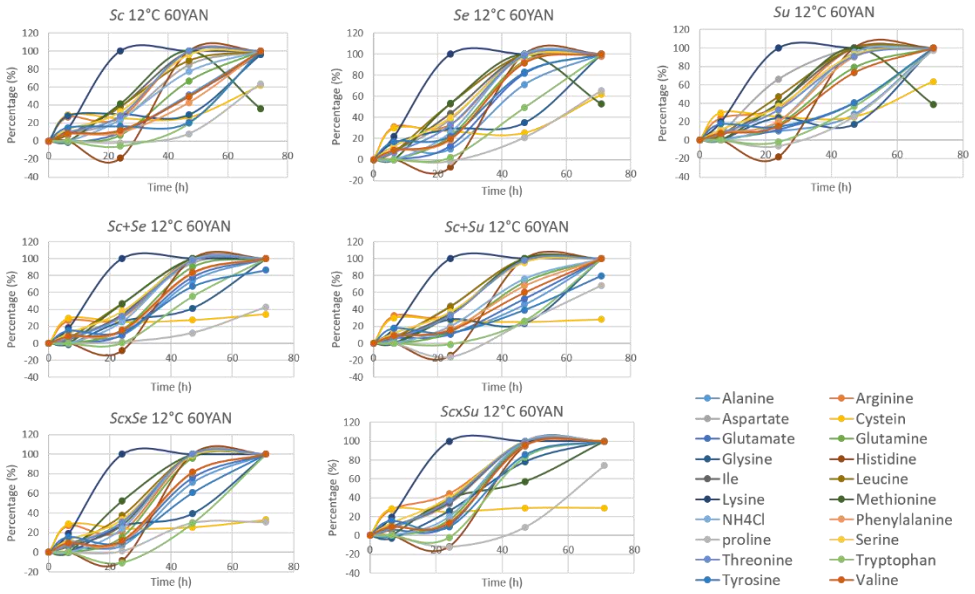
Other supplementary data to this article can be found online at 10.1016/j.ijfoodmicro.2019.108331



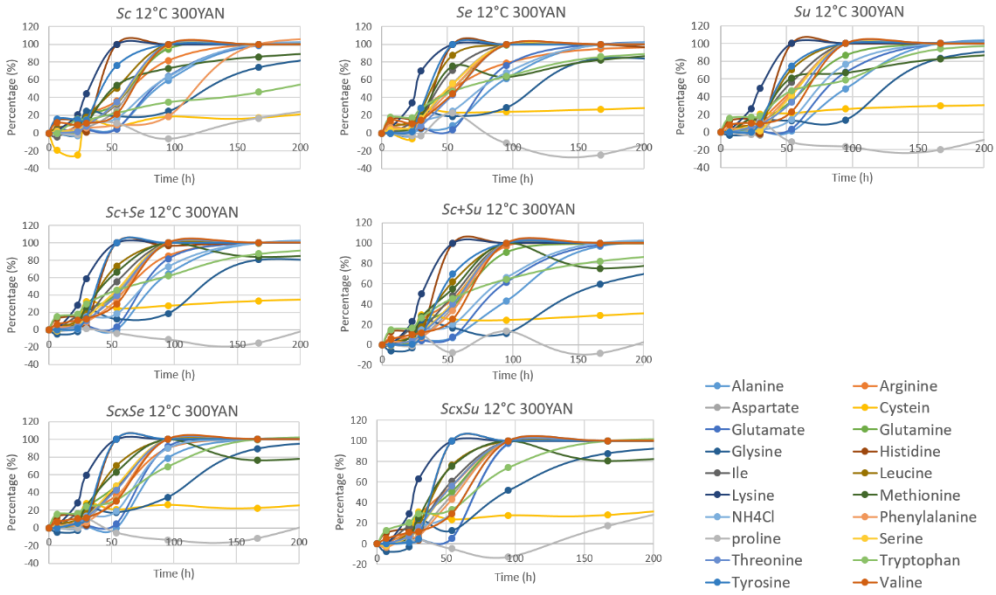
Supplementary Fig. 1. The nitrogen consumption curves of the fermentations carried out at 28 ℃ and 60 mg/L YAN.



Supplementary Fig. 2. The nitrogen consumption curves of the fermentations carried out at 28 ℃ and 300 mg/L YAN.



Supplementary Fig. 3. The nitrogen consumption curves of the fermentations carried out at 12 ℃ and 60 mg/L YAN.



Supplementary Fig. 4. The nitrogen consumption curves of fermentations carried out at 12 ℃ and 300 mg/L YAN.

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Chapter 4

Nitrogen sources preferences of non-*Saccharomyces* yeasts to sustain growth and fermentation under winemaking conditions

Ying Su, Pauline Seguinot, Isabelle Sanchez, Anne Ortiz-Julien, José Maria Heras, Amparo Querol, Carole Camarasa and José Manuel Guillamón

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Abstract

Wine-related non-*Saccharomyces* yeasts are becoming more widely used in oenological practice for their ability to confer wine a more complex satisfying aroma, but their metabolism remains unknown. Our study explored the nitrogen utilisation profile of three popular non-*Saccharomyces* species: *Torulaspora delbrueckii*, *Metschnikowia pulcherrima* and *Metschnikowia fructicola*. The nitrogen source preferences to support growth and fermentation as well as the uptake order of different nitrogen sources during wine fermentation were investigated. While *T. delbrueckii* and *S. cerevisiae* strains shared the same nitrogen source preferences, *Metschnikowia* sp. displayed a lower capacity to efficiently use the preferred nitrogen compounds, but were able to assimilate a wider range of amino acids. During alcoholic fermentation, the non-*Saccharomyces* strains consumed different nitrogen sources in a similar order as *S. cerevisiae*, but not as quickly. Furthermore, when all the nitrogen sources were supplied in the same amount, their assimilation order was similarly affected for both *S. cerevisiae* and non-*Saccharomyces* strains. Under this condition, the rate of nitrogen source consumption of the non-*Saccharomyces* strains and *S. cerevisiae* were comparable. Overall, this study expands our understanding about the preferences and consumption rates of individual nitrogen sources by the investigated non-*Saccharomyces* yeasts in a wine environment. This knowledge provides useful information for a more efficient exploitation of non-*Saccharomyces* strains that improves the management of the wine fermentation.

Keywords: *Torulaspora delbrueckii*, *Metschnikowia pulcherrima*, *Metschnikowia fructicola*, nitrogen preference, nitrogen uptake

1. Introduction

In the past 50 years, the inoculation of selected *Saccharomyces cerevisiae* strains has become increasingly prevalent in winemaking. It is an excellent way to control alcoholic fermentation by ensuring the complete exhaustion of sugars and avoiding the formation of undesirable off-flavours. During wine fermentation, the predominant non-*Saccharomyces* yeasts in grape juice are rapidly outcompeted by *S. cerevisiae* because they poorly adapt to increasing ethanol concentrations and to low levels of dissolved oxygen (Bauer and Pretorius, 2000; Boulton et al., 1995, Bisson, 1999). Some of these non-conventional yeasts have attracted more attention as they positively modify the wine chemical composition and, consequently, improve wine flavour and bouquet. (Padilla et al., 2016). However, as a result of their low ethanol tolerance, these species are unable to achieve complete sugar consumption. Consequently, in order to take advantage of their phenotypic specificities while avoiding stuck or sluggish fermentations, they are usually combined in industry with *S. cerevisiae* in sequential or co-inoculation. In this context, both the growth and the metabolic activity of non-*Saccharomyces* yeasts may impact *S. cerevisiae* performance in mixed culture fermentation mainly due to their nutrient consumption (Medina et al., 2012; Taillandier et al., 2014).

Nitrogen availability in grape must is a key parameter for the wine fermentation progress as it acts at many levels. Firstly, nitrogen is an important nutrient for sustaining yeast growth and, for the vast majority of fermentations, it is the limiting factor for biomass production (Bell and Henschke, 2005; Martínez-Moreno et al., 2012; Mendes-Ferreira et al., 2004; Varela et al., 2004). Nitrogen also plays a key role in fermentation kinetics and fermentation duration because insufficient nitrogen source availability in must can lead to stuck or sluggish fermentation (Bely et al., 1990; Mendes-

Ferreira et al., 2004). Lastly, the catabolism of nitrogen sources, together with central carbon metabolism, is involved in the synthesis of fermentative aroma precursors in wine yeasts (Barbosa et al., 2012; Bell and Henschke, 2005).

Grape musts contain a wide range of yeast assimilable nitrogen (YAN) sources, including not only amino acids and ammonium, but also urea, citrulline, ornithine and small peptides (Kevvai et al., 2016). The utilisation of these compounds during wine fermentation has been extensively described in *S. cerevisiae*. Nitrogen source quality, in terms of the capacity to support yeast growth and fermentation activity, has been evaluated in this species (Cooper, 1982; Fairbairn et al., 2017; Godard et al., 2007; Gutiérrez et al., 2013), with major differences between poor nitrogen sources, such as tryptophan, tyrosine, threonine or methionine, and rich nitrogen sources like ammonium, glutamine or asparagine. When provided as a mixture during fermentation, some nitrogen compounds are taken up early by yeasts, such as lysine, aspartate or threonine, while others (arginine, glycine, tryptophan, etc.) are consumed later (Jiranek et al., 1995; Crepin et al., 2012; Rollero et al., 2018). Indeed, many processes required for the uptake and growth in lower quality nitrogen sources are suppressed in the presence of a higher quality nitrogen source (Godard et al. 2007). Nitrogen sources are sequentially assimilated through various transporters that are regulated by two main systems: nitrogen catabolite repression (NCR) and the Ssy1p-Ptr3p-Ssy5 (SPS) system (Crépin et al., 2012; Godard et al., 2007; Ljungdahl and Daignan-Fornier, 2012). Consistently with phenotypic changes, the nitrogen source available in the medium determines the transcription level of almost 10% of all the genes in *S. cerevisiae* (Godard et al., 2007). The different regulation pathways triggered by various nitrogen sources can explain this transcriptional reprogramming.

Unlike *S. cerevisiae*, very little is known about the physiology and behaviour of non-*Saccharomyces* yeasts during wine fermentation, and their consumption profiles and preferences for amino acids have been poorly explored. In previous studies, Kemsawasd et al. (2015) studied the influence of pure nitrogen sources on the growth, glucose consumption and ethanol production of four wine-related non-*Saccharomyces* yeast species, and revealed that the effect of nitrogen sources on yeast growth and fermentation performance is species-specific. Furthermore, Gobert et al. (2017) and Rollero et al. (2018) investigated the nitrogen source uptake order by wine-related non-*Saccharomyces* strains while fermenting natural and synthetic grape musts, respectively. Both studies highlighted the importance of monitoring nutrient concentration during sequential fermentations with *S. cerevisiae* to avoid stuck or sluggish fermentations. Consequently, a comprehensive analysis of the efficiency of amino acids, used as the sole nitrogen source, to sustain the growth and fermentation performances of non-*Saccharomyces* species can be regarded as valuable research. This information would be essential for implementing the sequential or co-fermentation of these species with *S. cerevisiae*.

For this purpose, four commercially available non-*Saccharomyces* strains, which belong to the species *Torulaspora delbrueckii*, *Metschnikowia fructicola* and *Metschnikowia pulcherrima* were chosen to explore their nitrogen source management under fermentation and growth conditions. This study generally highlights the differences between the *S. cerevisiae* and non-*Saccharomyces* species in single nitrogen source preferences and the assimilation order of each compound in mixed nitrogen sources. Furthermore, the lack of relationship between the sequence of consumption of amino acids and their capacity to support growth are highlighted, regardless of species.

2. Material and Methods

2.1 Strains and media

All the yeast strains herein used were provided by Lallemand Inc. (France). These strains are *S. cerevisiae* Lalvin QA23® (SC), *Torulaspora delbrueckii* Biodiva® (TD), *Metschnikowia pulcherrima* Flavia® (MP), and *Metschnikowia fructicola* Gaia® (MFa) and LYCC7706 (MFb).

Yeast extract peptone dextrose (YPD) medium (20 g/L glucose, 20 g/L peptone, 10 g/L yeast extract) and SD medium (20 g/L glucose, 0.17 g/L yeast nitrogen base with 0.23 g/L NH_4Cl) were used for yeast propagation.

A synthetic grape must (SM) base without nitrogen was prepared based on the recipe of Bely et al. (1990) with some modifications. This medium contains 200 g/L of sugar as a mixture of 50% glucose, 50% fructose and 5 mg/L of phytosterols. The concentrations of organic acids, minerals and vitamins were the same as those described by Su et al. (2019). The concentration of the different nitrogen sources was adjusted to specific levels, and detailed concentrations are explained in the following sections. When small volume (growth experiment) was needed, synthetic must was sterilised by filtration through 0.22 μm pore-size membrane filters (Thermo scientific). When large volume was needed (fermentation experiment), synthetic must was sterilised by steam pasteurisation for 15 min.

2.2 Determination of the growth rate, lag phase length and biomass yield in different nitrogen sources

The growth of the four non-*Saccharomyces* strains and one *S. cerevisiae* control strain in the different sole nitrogen sources was determined by the 96-well microtiter plate screening method, as described by Warringer and Blomberg (2003), with some modifications. In all, 27 individual nitrogen

sources, including amino acids and ammonium, were used at two concentrations: 140 mg/L and 200 mg/L of YAN (Table 1). As a control fermentation, a mixed nitrogen source was used, which was made up of a mixture of 70% of amino acids and 30% of ammonium chloride (Table 2).

To assess the growth parameters, yeast cells were first pre-cultured in YPD medium overnight before being transferred to SD medium, which contains only 60 mg/L YAN, to be cultivated for 24 h to eliminate the influence of the nitrogen-rich YPD medium. Cells were washed and inoculated into SM with different nitrogen sources at a density of 10^6 yeast cells/mL. 250 µL of inoculated SM were dispensed to each microtiter plate well. Each condition was assayed in triplicate. Microtiter plates were incubated in a SPECTROstar Nano® microplate reader (BGM Labtech, Offenburg, Germany) at 25 ℃ with 500 rpm orbital shaking. Growth curves were monitored by recording the increase in optical density (OD) at wavelength 600 nm every 30 minutes. The growth parameters (lag phase λ, maximal specific growth rate μ_{max}) were deduced from the growth curve by directly fitting OD measurements *versus* time to the Gompertz equation proposed by Zwietering et al. (1990), which takes the following expression:

$$y = D * exp\{-exp[((\mu max * e)/D) * (\lambda - t) + 1]\}$$

where y= ln (OD_t/OD_0), OD_0 is the initial OD and OD_t is the OD at time t; D = ln (OD_∞/OD_0) is the OD value reached with OD_∞ as the asymptotic maximum. R script (statistical software R, v.3.0) was used to facilitate the calculations of μ_{max} and λ with the Gompertz equation.

Table 1. Concentration of each individual nitrogen sources in synthetic must

	Assimilable nitrogen	Molecular mass (g/mol)	Concentration (g/L) for 200 mg/L of YAN	Concentration (g/L) for 140 mg/L of YAN
Ala	1	89.1	1.273	0.891
NH_4Cl	1	53.5	0.764	0.535
Arg	3	174.2	0.830	0.581
Asp	1	133.1	1.901	1.331
Cys	1	121.2	1.731	1.212
Glu	1	147.1	2.102	1.471
Gln	2	146.1	1.044	0.731
Gly	1	75.1	1.072	0.751
His	1	155.2	2.217	1.552
Ile	1	131.2	1.874	1.312
Leu	1	131.2	1.874	1.312
Lys	2	146.2	1.044	0.731
Met	1	149.2	2.132	1.492
Phe	1	165.2	2.360	1.652
Pro	1	115.1	1.645	1.151
Ser	1	105.1	1.501	1.051
Thr	1	119.1	1.702	1.191
Trp	1	204.2	2.918	2.042
Tyr	1	181.2	2.588	1.812
Val	1	117.2	1.674	1.172
Ade	1	135.1	1.930	1.351
Cyt	1	111.1	1.587	1.111
GABA	1	103.1	1.473	1.031
Orn	2	168.6	1.204	0.843
Asn	2	132.1	0.944	0.661
Cit	2	175.2	1.251	0.876
Urea	2	60.1	0.429	0.300

Table 2. Concentration of each individual nitrogen source in synthetic must MS140, MS300 and MS300E.

	MS140		MS300		MS300E	
	Concentration (g/L)	YAN (g/L)	Concentration (g/L)	YAN (g/L)	Concentration (g/L)	YAN (g/L)
Ala	0.048	0.008	0.103	0.016	0.095	0.015
Arg	0.123	0.030	0.264	0.064	0.062	0.015
Asp	0.015	0.002	0.031	0.003	0.143	0.015
Cys	0.004	0.000	0.009	0.001	0.130	0.015
Glu	0.040	0.004	0.085	0.008	0.158	0.015
Gln	0.166	0.032	0.357	0.068	0.078	0.015
Gly	0.006	0.001	0.013	0.002	0.080	0.015
His	0.011	0.001	0.023	0.002	0.166	0.015
Ile	0.011	0.001	0.023	0.002	0.141	0.015
Leu	0.016	0.002	0.034	0.004	0.141	0.015
Lys	0.006	0.001	0.012	0.002	0.078	0.015
Met	0.010	0.001	0.022	0.002	0.160	0.015
Phe	0.013	0.001	0.027	0.002	0.177	0.015
Pro	0.202	0.000	0.432	0.000	0.123	0.000
Ser	0.026	0.003	0.055	0.007	0.113	0.015
Thr	0.025	0.003	0.054	0.006	0.128	0.015
Trp	0.059	0.008	0.127	0.017	0.109	0.015
Tyr	0.006	0.000	0.013	0.001	0.194	0.015
Val	0.015	0.002	0.031	0.004	0.126	0.015
NH_4Cl	0.151	0.040	0.324	0.085	0.057	0.015

2.3 Fermentation kinetic experiment

Fermentations were conducted with synthetic must containing sole nitrogen sources with four non-*Saccharomyces* strains and one *S. cerevisiae* control strain. Two nitrogen concentrations, 140 mg/L and 200 mg/L, were used, and the detailed concentration of each nitrogen source is shown in Table 1. Fermentations were carried out in 15 mL glass tubes closed with a filter tip to allow the release of CO_2. Tubes were kept at 24 ℃ with continuous magnetic

stirring (260 rpm). Fermentations were followed by monitoring released CO_2 by weighing tubes every 4 h. The experiment was carried out in duplicate. This measure was automatically taken by a robotic device (Bloem et al., 2018).

2.4 Nitrogen source uptake order during alcoholic fermentation

Alcoholic fermentations were carried out at 25 ℃ with a mixture of nitrogen sources to disclose the uptake order of the different nitrogen sources during fermentation. Firstly, fermentations were conducted with SM140 and SM300 (synthetic must of 140 and 300 mg/L YAN), whose composition is described in Table 2. Out of the two *M. fructicola* strains, only MFa (Gaia) was used for assessing the nitrogen uptake order. Samples were taken every 4 h until 48 h of fermentation. Fermentations were also conducted with SM300E in which an equal amount of each nitrogen sources (in terms of YAN) was supplied. For the SM300E fermentations, the *T. delbrueckii* and the *M. pulcherrima* strains, together with the control *S. cerevisiae*, were included in the experiment. Samples were taken every 4 h until 24 h. Yeast cells were removed from samples by centrifugation. The residual nitrogen in samples was analysed by UHPLC equipped with a UV detector (Thermo Scientific, MA, USA). The HPLC analysis method was based on Gómez-Alonso et al. (2007), but with some modifications. Samples were derivatised by diethylethoxymethylenemalonate (DEEMM). An accucore® C18 (Thermo scientific) LC column was used to separate amino acids.

2.5 Statistical analysis

All the experiments were carried out at least in duplicate. To study the growth on different media, heatmaps were plotted by Mev MultiExperiment Viewer, hierarchical clustering was performed with Euclidean distance metrics and group clustering was based on group averages (average linkage). The area

under the curve (AUC) was calculated by the R statistical software, v. 3.0., with the growth curver package (Sprouffske, 2018).

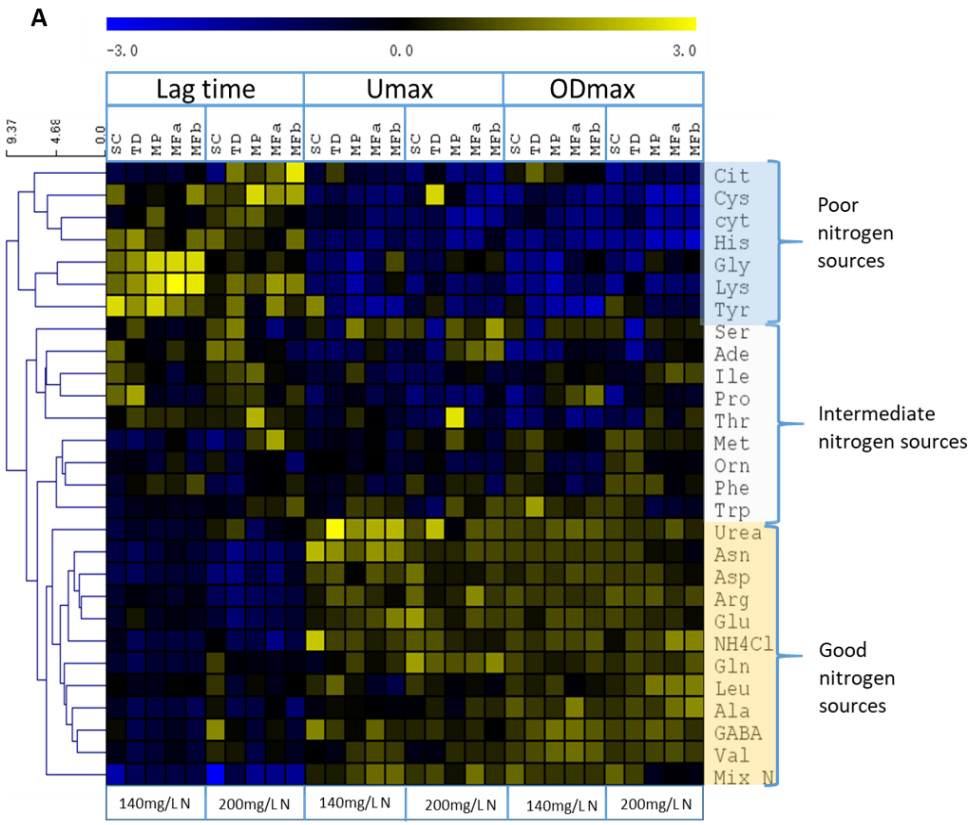
The CO_2 production curves were smoothed on the same time vector using the cellGrowth package, version 1.22.0 (Gagneur and Neudecker, 2012). These data were clustered by employing the Euclidean distance between CO_2 points at the same time, and by the Ward aggregation method. The number of clusters was set at seven. A principal component analysis (PCA) was carried out with the FactoMineR package (Le et al., 2008).

3. Results

3.1 Influence of the single nitrogen source on the yeast growth rate, lag phase length and biomass yield

The lag phase, growth rate (μ_{max}) and maximum OD (OD_{max}) of four non-*Saccharomyces* and one *S. cerevisiae* strains were compared during growth in 27 different single nitrogen sources (140 or 200 mg/L YAN). The heatmap, which summarises the comparison of the growth parameters depending on the nitrogen sources, the concentrations and strains (Fig. 1A), revealed that the herein used nitrogen sources hierarchically clustered into three categories, namely good, intermediate and poor nitrogen sources. Generally, the good nitrogen sources supported a relatively shorter lag phase time, a higher maximum growth rate and a higher maximum OD. Conversely, the poor nitrogen sources only supported weak, or even no, strain growth. NH_4Cl, glutamate, glutamine, aspartate, asparagine, GABA, arginine, alanine, leucine and valine were classified as good nitrogen sources for both *S. cerevisiae*, as previously reported (Godard et al., 2007; Gutiérrez et al., 2013), and the non-*Saccharomyces* species: *Torulaspora delbrueckii*, *Metschnikowia pulcherrima* and *Metschnikowia fructicola*. Similarly,

cysteine, cytosine, citrulline, lysine, glycine, tyrosine and histidine poorly sustained the growth of all the strains. Surprisingly according to our results, proline, which has always been considered an unassimilable nitrogen source for *S. cerevisiae* strains under anaerobic conditions, was clustered in the intermediate nitrogen sources because the non-*Saccharomyces* strains, especially the *Metschnikowia* strains, efficiently used proline for growth.



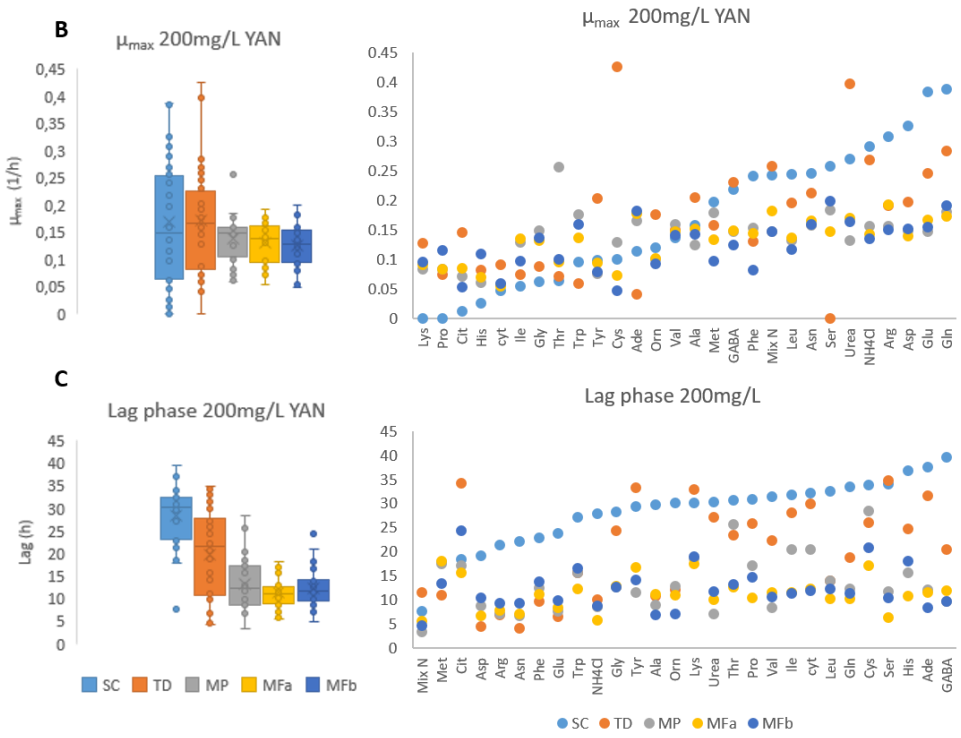


Fig. 1. A. Growth parameters of the different strains grown in synthetic must with only one nitrogen source. Each row on the heatmap represents the sole nitrogen source used for growth. Three growth parameters, lag phase time, maximum growth rate (μ_{max}) and maximum OD (OD_{max}), and two nitrogen concentrations, 140 mg/L and 200 mg/L, are compared on the heatmap. For each growth parameter, data were normalised by the mean value of all the nitrogen sources within the same strain and at the same nitrogen concentration. Hierarchical clustering was done based on Euclidean distance. The colour scheme from blue to yellow represents the normalised value from low to high. Clustering generally separates the nitrogen sources into three clades, which represent good nitrogen sources, intermediate nitrogen sources and poor nitrogen sources. **B.** Maximum growth rate and **C.** lag phase of the different strains when grown on synthetic must with 200 mg/L of the sole nitrogen sources. Box plots demonstrate the distribution of all the nitrogen sources for each strain. Dot plots show the value of each sole nitrogen source by specific strains. SC: *S. cerevisiae*; TD: *T. delbrueckii*; MP: *M. pulcherrima*; MFa: *M. fructicola* (Gaia); MFb: *M. fructicola* (LYCC7706).

A further analysis of the dataset revealed marked differences in the growth rate (Fig. 1B) and the lag phase time (Fig. 1C), depending on both the nature of the nitrogen source and the yeast species. The *Metschnikowia* strains displayed poorer growth performance for most nitrogen sources and a narrower variance in their growth characteristics according to the nitrogen source. However, the *Metschnikowia* strains exhibited greater growth capacity than *S. cerevisiae* in some nitrogen sources, such as isoleucine, glycine, threonine, histidine, citrulline and proline. The *Metschnikowia* and *T. delbrueckii* strains were able to use lysine as a sole nitrogen source, whereas *S. cerevisiae* was unable to metabolise it. On the contrary, *S. cerevisiae* grew much faster than the *Metschnikowia* strains in aspartate, asparagine, glutamine, glutamate, GABA, arginine and urea. *T. delbrueckii* took an intermediate position with higher μ_{max} than the *Metschnikowia* strains, but a lower μ_{max} than *S. cerevisiae* in the richer nitrogen sources. To summarise these specific growth traits, it was concluded that *S. cerevisiae* had a higher μ_{max} than the non-*Saccharomyces* strains when grown on good nitrogen sources, while the non-*Saccharomyces* strains displayed better growth performances than *S. cerevisiae* on the poorest nitrogen sources (proline, cysteine, lysine and histidine).

Interestingly enough, although the non-*Saccharomyces* strains, especially the *Metschnikowia* strains, showed less growth capabilities than *S. cerevisiae*, the non-*Saccharomyces* strains displayed approximately a 10-hour shorter lag phase than *S. cerevisiae* for more than half the single nitrogen sources. Only under the mix-N condition did the lag phase time of *S. cerevisiae* substantially lower and reach a similar value to the non-*Saccharomyces* strains.

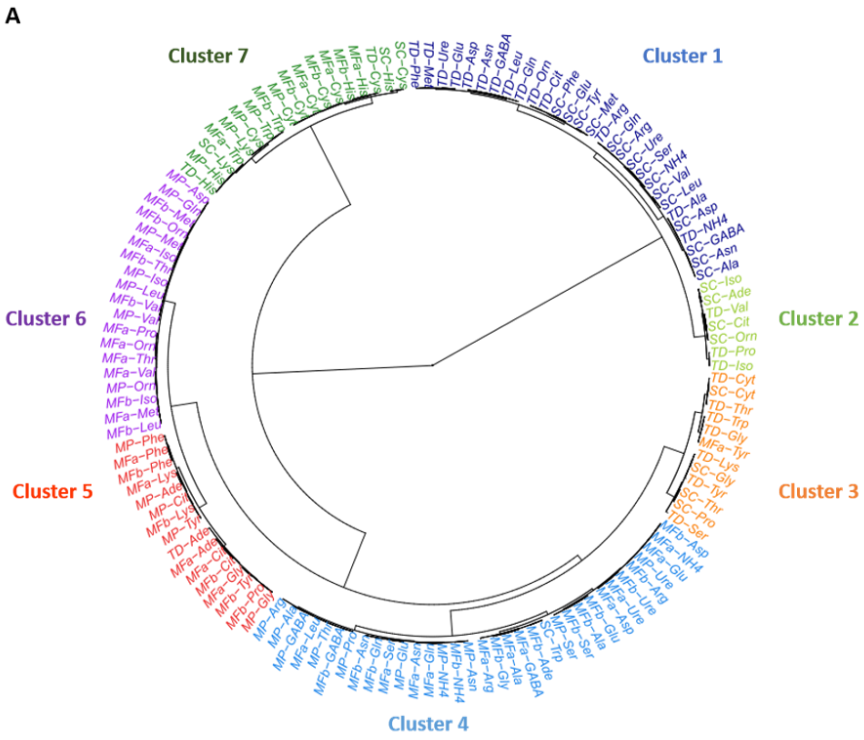
Two YAN concentrations (140 mg/L and 200 mg/L), which correspond to the nitrogen levels commonly found in natural grape must, were assayed for each

pure nitrogen source in our study. No substantial differences were observed for either the lag phase time or the maximum growth rate between both concentrations for most nitrogen sources (Suppl. Fig. 1-2), except for the poorer nitrogen sources (lysine, glycine, histidine, proline and tyrosine). The increased concentration of these poorer nitrogen sources yielded an increase in μ_{max} and a reduction in the lag phase. Finally, as expected, a higher maximum OD value was obtained with 200 mg/L YAN than with 140 mg/L YAN for almost all the nitrogen sources (Suppl. Fig. 3).

3.2 Capability of nitrogen sources to sustain fermentation

In parallel, four fermentation parameters were assessed: the fermentation lag phase, the maximal fermentation rate, the time to reach this maximum and the ability of strains to complete fermentation. A wide range of fermentation profiles was obtained from the five strains (Fig. 2). By applying Ward's agglomerative hierarchical clustering procedure to compare the profiles at 200 mg/L YAN, seven representative clusters of specific behaviours were differentiated (Fig. 2). Clusters 1, 2 and 3 collected most of the *S. cerevisiae* and *T. delbrueckii* fermentations, characterised by high fermentation rates from 0.55 g/L/h to 0.99 g/L/h, which allowed fermentation to be completed in less than 350 h. These three clusters were differentiated by lag phase length, which was shorter in Cluster 1 (a mean lag phase of 18.8 h), intermediate for Cluster 2 (mean value of 44.5 h), but much longer in Cluster 3 (a mean lag phase of 80.5 h). Clusters 4, 5 and 6 grouped the fermentations in which sugars were not exhausted. All these conditions corresponded to the cultures carried out with *Metschnikowia* sp., apart from the *S. cerevisiae* and *T. delbrueckii* fermentations on tryptophan and adenine, respectively. Under these conditions, fermentation activity was moderate, and a sharp drop in the fermentation rate occurred after approximately 50 h of fermentation. The

average duration of the lag phases was shorter than in Clusters 1-3, and varied from 16.8 h to 29.3 h. This reduction in the fermentation lag phases agrees with the above-mentioned shorter growth lag phases for *Metschnikowia* sp. Finally, Cluster 7 grouped the conditions that were unable to support efficient fermentation, in which the lag phase was long and the fermentation rate was very slow. Histidine and cysteine were the only identified amino acids as being unable to support fermentation for all four species.



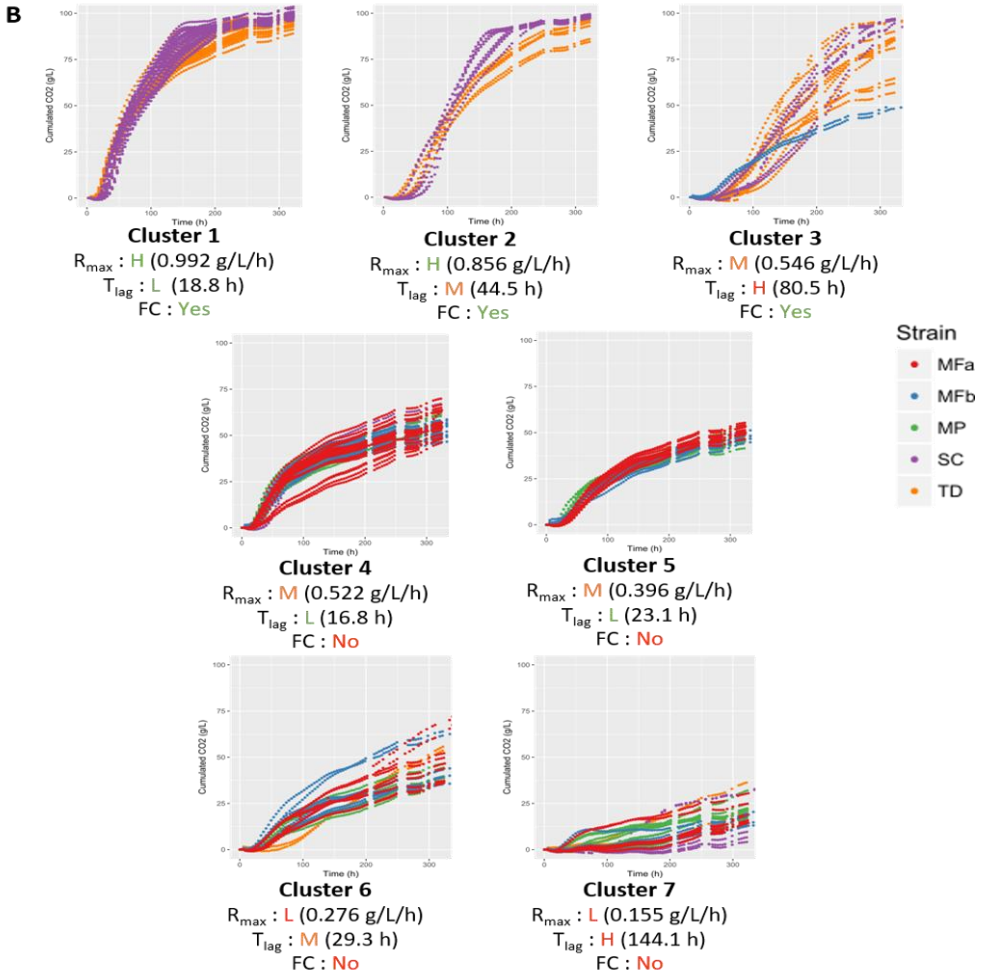


Fig. 2. Clustering of the fermentation profiles obtained on 27 media containing only one N source with the five studied strains. The means of the fermentation parameters were calculated for each cluster. **R_{max}:** Maximum fermentation rate (g CO_2/L/h). **T_{lag}**: lag phase time (h) **FC:** fermentation completion. **H:** high, **M:** medium, **L:** low.

To further investigate the effect of the different nitrogen sources on the fermentation performance of the four yeast species, three quantitative parameters, which describe fermentation kinetics (lag phase duration, maximal fermentation rate and time to reach this maximum; Fig. 3) were compared by a PCA analysis. The first principal component explained 76%

of the variance in the dataset on the basis of lag phase duration and the time to reach the maximal fermentation rate. A group of individuals (blue circle), which corresponded mainly to the fermentations done with cysteine and histidine as the nitrogen source, for which the four species' activity was very limited, was clearly distinguished from the other conditions according to these two parameters. A clear differentiation was also observed on the second axis between the fermentation parameters obtained with *T. delbrueckii* and *S. cerevisiae* (light green circle) on the one hand, and with *Metschnikowia sp.* on the other hand (yellow circle). The fermentations performed by the *Metschnikowia* strains had a low R_{max}, but a shorter lag phase. *T. delbrueckii* and *S. cerevisiae* achieved better performances with higher fermentation rates. These strains were also characterised by a wider variety of profiles depending on the amino acid, with differentiation based mainly on the fermentation rate (Fig. 3). For example, *T. delbrueckii* and *S. cerevisiae* both had a low fermentation rate on cytosine and glycine. However, *S. cerevisiae* obtained a high fermentation rate for adenine, whereas *T. delbrueckii* proved less effective on this medium. Overall, most of the amino acids that efficiently supported fermentation were comparable for the five strains (Table 3). These amino acids included alanine, aspartate, asparagine, GABA, glutamate, ammonium and urea.

Furthermore, even if *T. delbrueckii* and *S. cerevisiae* similarly responded to the majority of amino acids, some differences can be highlighted between these species (Suppl. Fig. 4-5, Table 3). In general, *S. cerevisiae* had higher fermentation rates than *T. delbrueckii*, especially on adenine, glycine, tryptophan, tyrosine, valine and serine. Conversely, *T. delbrueckii* fermented more efficiently than *S. cerevisiae* on lysine, and exhibited a shorter lag phase and a higher fermentation rate on citrulline and ornithine. No significant

differences in fermentation performances were observed between *M. pulcherrima* and *M. fructicola*, apart from the more efficient fermentation on lysine for *M. fructicola*.

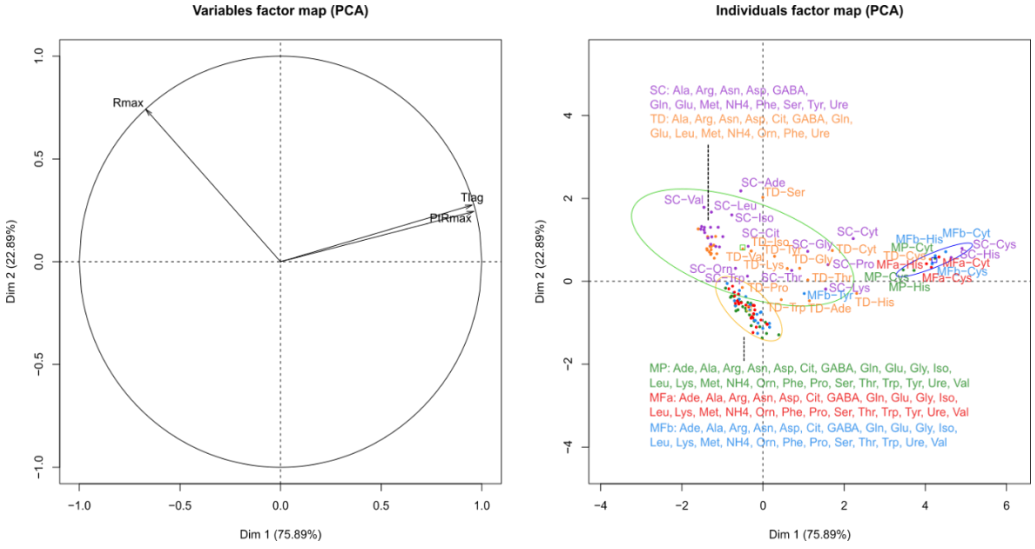


Fig. 3. Principal component analysis of the fermentation parameters obtained with the five strains in the 27 media. **Rmax:** Maximum fermentation rate; **Tlag:** lag phase time; **PtRmax:** time to reach Rmax. Labels indicate the strain and the nitrogen source available in the medium. Points are coloured depending on the strain; SC: purple, TD: orange, MP: green, MFa: red and MFb: blue. Labels of overlapping points are shifted over or under the points following the dashed lines. The conditions form three groups, highlighted by the light green, yellow and dark blue confidence ellipses (confidence level of 0.95).

Finally, the nitrogen concentration (140 or 200 mg/L of YAN) did not impact the classification of nitrogen sources. Higher maximal fermentation rates were obtained for higher nitrogen concentrations, except for the fermentation of *T. delbrueckii* on adenine, and the fermentation of *S. cerevisiae* on cysteine and histidine (Suppl. Fig. 4). Lag phases were longer on the medium containing the lower nitrogen concentration (140 mg/L of YAN), especially for the non-*Saccharomyces* yeasts (Suppl. Fig. 5).

Table 3. Classification of nitrogen sources depending on their ability to sustain fermentation.

	T. delbrueckii		*Metschnikowia sp.*		*S. cerevisiae*	
++	Ala	Urea	Ala	NH_4	Ala	Met
	Arg	Leu	Arg	Ser	Arg	NH_4
	Asn	Met	Asn	Urea	Asn	Phe
	Asp	NH_4	Asp		Asp	Ser
	Cit	Orn	GABA		GABA	Tyr
	GABA	Phe	Gln		Gln	Urea
	Gln		Glu		Glu	Val
	Glu		Leu		Leu	
+	Cyt	Ile	Ade	Orn	Cyt	Trp
	Gly	Pro	Cit	Pro	Gly	
	Lys		Gly	Thr	Pro	
	Ser		Lys	Val	Thr	
	Thr		Phe	Trp	Ade	
	Trp		Tyr		Cit	
	Tyr		Ile		Ile	
	Val		Met		Orn	
-	Ade		Cys		Cys	
	Cys		Cyt		His	
	His		His		Lys	

3.3 Correlation between the growth and fermentation activity of each strain by using a single nitrogen source

The correlation between maximum growth rate μ_{max} and maximum fermentation rate R_{max} by using different nitrogen sources at 200 mg/L YAN was calculated (Fig. 4). A generally good correlation was observed between both parameters. However, this correlation was better for the *Metschnikowia* strains than for *S. cerevisiae* and *T. delbrueckii*. For the last two strains, the two parameters were well correlated when the μ_{max} values were under 0.1 h^{-1}. Only for the amino acids that supported poor growth, the fermentation capacity was limited by biomass (a linear correlation between μ_{max} comprised between 0 and 0.1 h^{-1}). When using the richer nitrogen sources, *S. cerevisiae* and *T. delbrueckii* showed higher growth rates (μ_{max} higher than ≈0.1), but

R_{max} reached a constant value around 0.95 g/L/h for *T. delbrueckii* and 1.1 g/L/h for *S. cerevisiae*. As the maximum growth rates were lower than 0.1 h^{-1} for the *Metschnikowia* strains with all the nitrogen sources, a linear correlation was observed between R_{max} and μ_{max} for these strains.

For all the strains, some specific nitrogen sources did not show the aforementioned correlation between μ_{max} and R_{max}. They were mainly poor nitrogen sources like Cys, His and Ade, which supported neither good growth nor high fermentation speed. Special cases were observed for T. *delbrueckii*, with Cys sustaining high μ_{max}, but very low R_{max}, or Ser showing low μ_{max}, but high R_{max}.

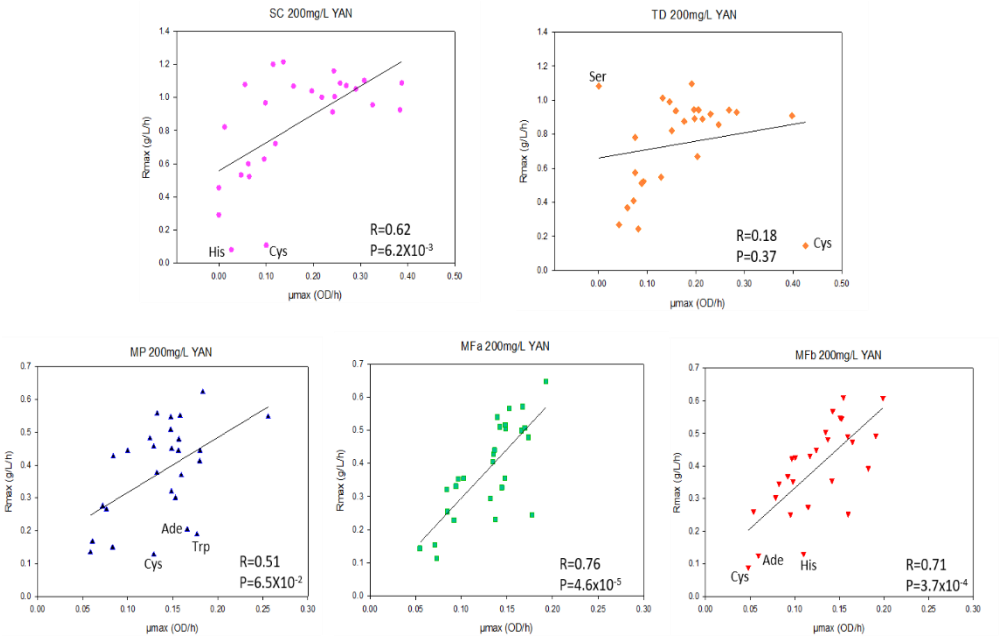


Fig. 4. Pearson's correlation analysis between the maximum growth rate (μ_{max}) and the maximum fermentation rate (R_{max}) for each strain. Linear regression (black line) is displayed. The squared Pearson correlation coefficient(R) and the *p*-value (P) are provided in this figure.

3.4 Nitrogen sources utilisation efficiency by different strains during alcoholic fermentation.

To explore the sequential utilisation of different nitrogen sources by each yeast strain during synthetic must fermentation, fermentations were carried out with SM140 and SM300, which respectively corresponded to intermediate and high nitrogen availability conditions (Table 2). As the behaviour of two *Metschnikowia fructicola* strains was similar, only one of them (MFa Gaia) was included. To gain a clearer view of the nitrogen consumption profiles in each strain and for each fermentation condition, the AUC of each individual nitrogen source was calculated, which represents information about the time to start consumption, the maximum consumption rate and the maximum consumption percentage (Fig. 5A). Thus the nitrogen sources with higher AUC values could be considered an earlier and more complete consumed compound. Conversely, a low AUC value represented slow and partial nitrogen source consumption. The box plots in Fig. 5B-C represent a general distribution of the AUC values of 19 nitrogen sources for the different strains in SM140 and SM300 for 24 h of fermentation. These plots clearly show a quicker and better utilisation efficiency of most nitrogen sources by *S. cerevisiae* and *T. delbrueckii* than by the *Metschnikowia* strains. These AUC values were ranked to represent the nitrogen sources uptake order by each strain (Fig. 6A-B). Each species showed a specific order of assimilation of the nitrogen substrates. However, nitrogen concentration barely influenced this order of assimilation. This result illustrated that, without changing the proportion of the different nitrogen sources, increasing the nitrogen concentration did not significantly influence the nitrogen source uptake order. The representation of the AUC values on a heatmap hierarchically clustered the nitrogen sources into four categories (Fig. 6D).

Firstly, nitrogen sources lysine, leucine, isoleucine, histidine, methionine, glutamate, glutamine, and valine were the most rapidly consumed by all the studied strains. Of these, lysine was the first nitrogen source to be consumed in most cases. Of the early consumed nitrogen sources, differences appeared in the order of uptake in distinct strains. Remarkably, Ile, Gln and Val were more quickly depleted by the *Metschnikowia* strains, whereas His and Glu were poorly or slowly consumed by *T. delbrueckii*. It is noteworthy that all these early consumed amino acids were transported to yeast cells through SPS regulated permeases. The second group of nitrogen sources consumed at a moderate speed was made up of Tyr, NH_4, Arg, Ala, Phe, Thr, Ser, and Asp. Once again, specific differences were observed among the studied strains for this group of amino acids. *T. delbrueckii* consumed Asp and Ser more quickly than the other strains. *S. cerevisiae* and *T. delbrueckii* also showed a higher uptake speed for Phe than the *Metschnikowia* strains. The third group was formed by Gly and Trp, which were the last amino acids to be consumed, despite the fact that the *Metschnikowia* strains consumed Trp earlier than other amino acids. Finally, Pro was clustered alone and represented the nitrogen source that was practically not consumed under any condition (only partial consumption by *T. delbrueckii* in SM140 was detected).

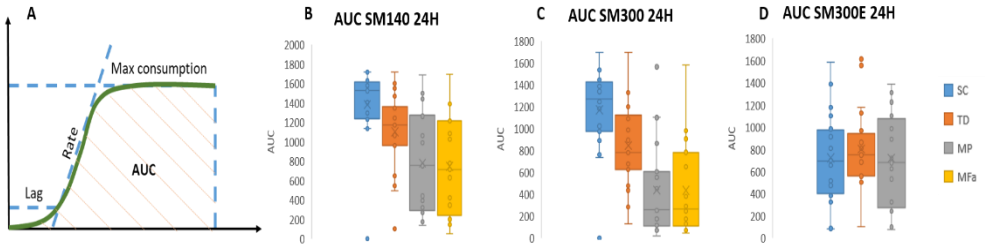


Fig. 5. A. AUC provides the summary information of the lag phase, consumption rate and maximum consumption percentage. AUC values of the different strains with **B.** SM140, **C.** SM300 and **D.** SM300E.

The individual consumption of each nitrogen source is shown in Suppl. Fig. 6 and 7. All the nitrogen sources were completely depleted by the *S. cerevisiae* strain within the first 24 h of both fermentations, except for proline, which was not consumed at all within the studied time frame. With the *T. delbrueckii* strain, most nitrogen sources were consumed within the first 48 h during both fermentations, with some exceptions. Trp and Gly were partially consumed in SM300 (60% and 30%, respectively) and Pro was partially consumed (48%) in SM140, but was not consumed at all in SM300 within the studied time frame. For both *Metschnikowia* strains, the depletion of different nitrogen sources was clearly slower. In SM300, only lysine, the two branched-chain amino acids (BCAA) leucine and isoleucine and glutamine were almost fully consumed (more than 90%). *M. pulcherrima* consumed more than 90% of valine, the third BCAA, but only 50% of this amino acid was depleted by *M. fructicola*. This could indicate a specific preference of *M. pulcherrima* for this amino acid compared to *M. fructicola*, but more strains should be studied to confirm this hypothesis. For both *Metschnikowia* strains, glutamate, glutamine, histidine and methionine were moderately utilised, with consumption exceeding 50% in 48 h. Proline and glycine were not consumed within the first 48 h.

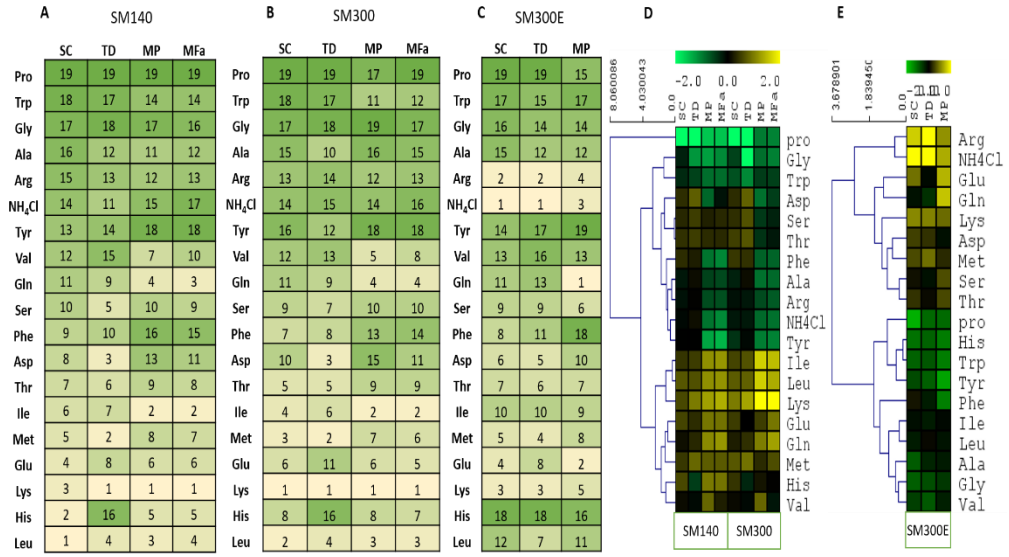


Fig. 6. Ranking of the nitrogen source uptake order of **A.** SM140, **B.** SM300 and **C.** SM300E. **D.** Heatmap of the AUC values of SM140 and SM300. **E.** Heatmap of the AUC values of SM300E. The nitrogen sources with a low ranking number indicate that they were quickly consumed during alcoholic fermentation; Conversely, the nitrogen sources with a high ranking number indicate that they were slowly consumed or were not assimilated within the first 48 h of fermentation. The colour key from green to yellow represents the AUC value from low to high. Data were normalised by the mean value of each strain. Hierarchical clustering was done by Euclidean distance metrics.

3.5 Influence of the initial concentration on the nitrogen source uptake order

To assess the influence of the initial concentration of the different nitrogen sources on the uptake order, fermentations were carried out by the strains of *S. cerevisiae*, *T. delbrueckii* and *M. pulcherrima* on a synthetic grape must with an equal amount (in YAN terms) of each nitrogen source and a final YAN concentration of 300 mg/L (SM300E; Table 2). Strangely enough, the equal proportion of each nitrogen source in the final YAN substantially changed the general uptake profile compared to SM300. By comparing the

distribution of the AUC values (Fig. 5C-D), the first point to be underlined is that, unlike the results of the SM300 fermentations, no major differences were observed in the general nitrogen source consumption efficiency for the three strains. The analysis of the distribution of the consumed nitrogen sources showed a delayed uptake of some nitrogen sources by *S. cerevisiae* (Suppl. Fig. 8) because several amino acids had only been partially consumed after 24 h, but they had all been completely depleted in SM300 after 20 h (except Pro). Interestingly, these amino acids poorly sustained yeast growth and fermentation (Trp, Gly and His), whereas SM300E seemed to favour the uptake of many amino acids for the *M. pulcherrima* and *T. delbrueckii* strains, with more amino acids completely depleted after 24 h (Suppl. Fig. 8).

The SM300E AUC values were also represented on a heatmap (Fig. 6E). To better compare them with SM140 and SM300, the uptake order is shown in Fig. 6C. NH_4Cl and Arg were the fastest nitrogen sources to be consumed by the *S. cerevisiae* and *T. delbrueckii* strains in SM300E, while these nitrogen sources were ranked from position 13 to 15 in SM300 (Fig. 6B). We should bear in mind that these compounds are the most abundant nitrogen sources in SM300 and their concentrations sharply drop in SM300E. Although both NH_4Cl and Arg were also rapidly depleted by the *M. pulcherrima* strain, Gln and Glu were preferentially consumed by it. Another major difference between SM300E and SM300 was observed for Leu and Ile as their consumption was significantly delayed in the SM300E fermentations by the three strains. Valine was more quickly exhausted by the *M. pulcherrima* strain when nitrogen sources were provided in similar amounts. All these BCAAs were provided in bigger quantities in SM300E. Lys was also one of the quickest amino acids to be consumed in the three strains, but took longer than NH_4Cl and Arg depletion. For proline, all the strains showed the assimilation

of this amino acid in SM300E (*T. delbrueckii* even completely depleted it), but it was not assimilated in SM300.

4. Discussion

Mixed culture fermentations of the non-*Saccharomyces* yeasts and *Saccharomyces cerevisiae* involve different synergic or antagonistic interactions between species and strains, including competition for nutrients, especially nitrogen sources, which play a significant role in winemaking (Rollero et al., 2018). Consequently, understanding the nitrogen resource management by non-*Saccharomyces* yeasts is essential to modulate the implementation of different species in sequential inoculations. This study provides a comprehensive overview of the nitrogen assimilation of four non-*Saccharomyces* industrial strains by comparing their nitrogen source preferences in terms of not only growth and fermentation performances, but also of their consumption sequence of these nitrogen sources. During cultures with good nitrogen source, *T. delbrueckii* displayed high growth and fermentation rate, similar to the behaviour of *S. cerevisiae*. This could be due to the close phylogenetic relationship between those two species (Kurtzman and Robnett, 2003). Both these species displayed a high degree of specialisation in their nitrogen preferences, with wide variability in the characteristic parameters of their capacity to grow and complete wine fermentation depending on the nitrogen source. Based on their efficiency to support growth, as measured by the generation time, amino acids and ammonium have been previously classified as “preferred”, “intermediate” and “non-preferred” nitrogen sources for lab and wine *S. cerevisiae* strains (Cooper et al., 1982, Godard et al., 2007; Guti érrez et al., 2013). Interestingly, the growth characteristics of both commercial *S. cerevisiae* and *T. delbrueckii* strains are consistent with this classification, apart from some specific amino

acids likely reflecting differences in the genetic background between both species. For example, *T. delbrueckii* exhibited substantial growth in a lysine medium, whereas *S. cerevisiae,* in which the key enzymes involved in the lysine degradation pathways have not yet been identified (Bhattacharjee, 1992; Neshich et al., 2012), was unable to grow when using this amino acid as the sole nitrogen source. On the contrary, glutamine, glutamate and aspartate, which are key elements of the central core of nitrogen metabolism, supported *S. cerevisiae* growth more efficiently than that of *T. delbrueckii*.

Previous studies (Prior et al, 2019; Barbosa et al, 2018) have revealed the poor fermentation activity of the *Metschnikowia* strains in synthetic and natural grape musts. In the present study, the phenotypic traits of the *Metschnikowia* strains also stood out from those of the other two species as they were unable to complete wine fermentation, regardless of the nature of the nitrogen source, and they generally exhibited comparatively low growth and fermentation rates. Compared to the *T. delbrueckii* and *S. cerevisiae* species, the characteristics of the *Metschnikowia* strains were less influenced by the nature of nitrogen sources, even though these compounds had been differentiated between preferred and non-preferred nitrogen sources. Consequently, their fitness during growth and fermentation on poor nitrogen sources, including lysine, histidine and threonine, was better than that of *S. cerevisiae*. Notably, *Metschnikowia* strains are able to use proline more efficiently than *S. cerevisiae*. The presence of dissolved oxygen during microplate cultivations may favour proline consumption. However, the growth conditions were the same for both non-*Saccharomyces* and *S. cerevisiae*. Thus, our results indicate that proline might be differently metabolised by these species. This observation also indicates that the nitrogen quality plays a more relevant role in controlling the growth of the *S. cerevisiae*

and *T. delbrueckii* yeasts than that of the *Metschnikowia* strains. Finally, the *Metschnikowia* strains were characterised by a shorter lag phase compared to the *T. delbrueckii* and *S. cerevisiae* species for most nitrogen sources. This shorter lag phase, a fermentation trait during the wine fermentation process to which very little attention has been paid (Novo et al., 2003), confers the *M. pulcherrima* and *M. fructicola* species a great competitive advantage for establishing in grape juice and starting fermentation, despite the rise in ethanol and oxygen depletion favouring the progressive imposition of *S. cerevisiae*. Our previous observations indicated that, in mixed culture fermentations, increasing the abundance of some non-preferred nitrogen sources could favour the implementation of *Metschnikowia* strains. These considerations may be particularly relevant for winemaking as the metabolic activities of the non-*Saccharomyces* species, including *M. pulcherrima* and *M. fructicola,* have been reported to impart a positive impact on the sensory quality of wines (Ruiz et al., 2018).

The literature reported that good growth fitness is not necessarily associated with the good fermentation performance of a particular yeast (Crépin et al., 2012; Gutiérrez et al., 2013; Kemsawasd et al., 2015; Prior et al., 2019; Su et al., 2019). In our study, very good correlations between these two parameters were observed for the *Metschnikowia* strains in most individual nitrogen sources. However, this correlation only remained for the nitrogen sources with μ_{max} values below 0.1 h^{-1} in the case of the *S. cerevisiae* and *T. delbruecki* strains. In these strains, the most preferred nitrogen sources clearly improved the growth rate, but this growth did not impact the fermentation rate. When fermentations were carried out in good nitrogen sources, YAN concentration may become the main limiting factor for the yeast carrying capacity, which in turn limited the maximum fermentation rate.

The four commercial wine species consumed the available nitrogen in synthetic must with an assimilation sequence that was generally conserved between strains. The differentiation between the early (including Leu, Met, Ile, Thr, Glu, His) and late (ammonium, Ala, Arg and Tyr) consumed amino acids, reported for a collection of 14 *S. cerevisiae* strains (Crépin et al., 2012), *P. kluyveri*, *T. delbrueckii*, *M. pulcherrima* and *L. thermotolerans* (Prior et al., 2019), also applied to all the yeasts that were herein characterised. In *S. cerevisiae*, this nitrogen source ranking is explained mainly by two molecular mechanisms: the Ssy1p-Ptr3p-Ssy5 (SPS) system, which regulates the permeases involved in transporting early assimilated amino acids, and the nitrogen catabolite repression (NCR) system, which controls the uptake of late consumed nitrogen compounds (Beltran et al., 2004; Godard et al., 2007; Ljungdahl, 2009; Crépin et al., 2012). However, some exceptions to this general pattern were evidenced in the non-*Saccharomyces* strains, which are worth pointing out in the winemaking context as they could result in the limited availability of a specific nitrogen compound to support *S. cerevisiae* growth in mixed or sequential fermentations. In particular, aspartate and serine were consumed earlier by *T. delbrueckii* than other strains, while isoleucine, valine, glutamine and tryptophan were exhausted more quickly during fermentation with the *Metschnikowia* species. Given the similarity of the nitrogen source uptake profiles between the *S. cerevisiae* and non-*Saccharomyces* strains, we hypothesised that similar regulatory mechanisms like NCR and SPS could also be present in the *T. delbrueckii* and *Metschnikowia* strains. Lleixà et al. (2018) recently demonstrated that the NCR mechanism also operated in *Hanseniaspora vineae*. Furthermore, changes in the concentration of nitrogen sources also affected the consumption sequence of N-containing molecules differentially between the

S. cerevisiae and non-*Saccharomyces* strains. Compared to the concentrations usually found in natural grape must (used in SM, Bely et al., 1990), when all the nitrogen sources were supplied at the same concentration, *S. cerevisiae* slowed down the uptake of many nitrogen sources, whereas the non-*Saccharomyces* strains more efficiently took up these compounds, with the consequent reduced fitness advantage of *S. cerevisiae* over other species. This observation likely reflects the adaptation of *S. cerevisiae* wine strains to grape must environments in order to efficiently use a scarce nitrogen resource. It also indicates that the competitiveness of the different species during co- or sequential fermentations can be modulated by modifying nitrogen source compositions and concentrations. The uptake of arginine and ammonium was most significantly affected by their concentration in SM. The delayed consumption of these nitrogen sources under wine fermentation conditions can be explained by their high concentration in grape juice, combined with a repressed uptake by NCR. Nonetheless, the uptake of some nitrogen sources was not affected by their availability in the medium, like lysine, which was early consumed in both media, or tryptophan and proline, which were exhausted later regardless of the nitrogen resource composition. With lysine, this behaviour could be related to the kinetic properties of major lysine transporter Lyp1p, which allows the fast and efficient uptake of this compound (Bianchi et al., 2016). In a population of *S. cerevisiae* wine strains, a positive correlation has been reported between the growth efficiency and uptake rate of nitrogen provided as a mixture of ammonium and amino acids (Gutiérrez et al., 2012). This can be extended to the species employed herein with the strains displaying the least growth capacity (*Metschnikowia* strains) with the lowest nitrogen consumption rate, and *vice versa*. However, no correlation was found between the uptake order of the different nitrogen

sources and their capacity to support growth and fermentation activity as some of the most rapidly consumed nitrogen sources poorly sustained yeast growth. Conversely, some late consumed sources supported good growth and fermentation capacity. The most paradigmatic example is the early consumption of lysine, although this amino acid poorly supports the growth of the non-*Saccharomyces* strains or is unable to sustain *S. cerevisiae* growth. Conversely, alanine was consumed late, but is considered a preferred nitrogen source because it efficiently sustains the growth and fermentation of wine yeast species. The differences between these two classifications can be explained by the fact that the sequence of assimilation of nitrogen sources is controlled by the regulation of the permeases involved in transporting these molecules, while different mechanisms underlie the efficiency of nutrients to sustain growth. Hence the carbon derivatives resulting from the catabolism of most preferred nitrogen sources produces C-compounds that are directly assimilable by the central carbon metabolism, while the transamination of less efficient sources leads to the formation of keto-acids, which are converted into higher alcohols (Godard et al., 2007; Gutiérrez et al., 2013).

5. Conclusions

Nitrogen source preference and the metabolism of wine-related non-*Saccharomyces* species are very important for the wine industry. This work thoroughly studied the nitrogen preferences and its utilisation profile of a *T. delbrueckii* and three *Metschnikowia* commercial strains. Regardless of the nitrogen source, clear differences were observed between the *T. delbrueckii* and *Metschnikowia* strains. The *T. delbrueckii* strain showed a similar growth character and fermentation performance to *S. cerevisiae*, whereas the *Metschnikowia* strains exhibited a much lower growth rate and less fermentation activity. However, the *Metschnikowia* strains were able to grow

much better than *S. cerevisiae* in the so-called poor nitrogen sources. This result, together with a shorter lag phase than *S. cerevisiae*, can confer these species a great competitive advantage for starting fermentation. In addition, the initial proportion of nitrogen sources in the medium has important influence on their assimilation order, especially for arginine and ammonium, and consequently on the fitness of the species. The non-*Saccharomyces* strains had a similar nitrogen source uptake profile with *S. cerevisiae*, which indicates that similar nitrogen regulation systems may also exist in the non-*Saccharomyces* species during alcoholic fermentation. Overall, these results point out that an appropriate management of the nitrogen nutrition during mixed fermentation is required for an optimal use of the non-*Saccharomyces* species during winemaking.

Supplementary material

Supplementary data to this article can be found online at 10.1016/j.fm.2019.103287

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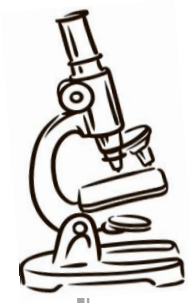

Chapter 5

Isotopic tracers unveil distinct fates for nitrogen sources during wine fermentation with two non-*Saccharomyces* strains

Ying Su, Pauline Seguinot, Audrey Bloem, Anne Ortiz-Julien, José María Heras, José Manuel Guillamón, Carole Camarasa

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Abstract

Non-*Saccharomyces* yeast strains have become increasingly prevalent in the food industry, particularly in winemaking, because of their properties of interest both in biological control and in complexifying flavour profiles in end-products. However, unleashing the full potential of these species would require solid knowledge of their physiology and metabolism, which is, however, very limited to date. In this study, a quantitative analysis using 15N-labelled NH_4Cl, arginine, and glutamine, and ^{13}C-labelled leucine and valine revealed the specificities of the nitrogen metabolism pattern of two non-*Saccharomyces* species, *Torulaspora delbrueckii* and *Metschnikowia pulcherrima*. In *T. delbrueckii*, consumed nitrogen sources were mainly directed towards the de novo synthesis of proteinogenic amino acids, at the expense of volatile compounds production. This redistribution pattern was in line with the high biomass-producer phenotype of this species. Conversely, in *M. pulcherrima*, which displayed weaker growth capacities, a larger proportion of consumed amino acids was catabolised for the production of higher alcohols through the Ehrlich pathway. Overall, this comprehensive overview of nitrogen redistribution in *T. delbrueckii* and *M. pulcherrima* provides valuable information for a better management of co- or sequential fermentation combining these species with *Saccharomyces cerevisiae*.

Keywords: ^{15}N- and ^{13}C-isotope labelling, quantitative analysis of metabolism, nitrogen resource management, *T. delbrueckii*, *M. pulcherrima*

1. Introduction

Traditionally, wine fermentation is a process in which sugars are converted into ethanol through interactions between microorganisms, mainly yeast species, also imparting pleasant notes to wines (Fleet, 2003). However, some spoilage microorganisms may cause problematical wine fermentations. In 1883, Emil Christian Hansen successfully isolated the first pure yeast culture (Hansen, 1883), which started a new era for yeast research and utilisation. In modern oenology, inoculation of pure cultures of *Saccharomyces cerevisiae* is widely used as an efficient way to prevent the growth of spoilage non-*Saccharomyces* species, thus ensuring the completion of fermentation and a stable wine quality (Bisson, 1999; Bauer and Pretorius, 2000; Fleet, 2001). Nevertheless, in the last decade, more attention has been paid to non-*Saccharomyces* yeasts for their technological properties of interest in winemaking. Two of the most studied and commercialised of these species are *Torulaspora delbrueckii* and *Metschnikowia pulcherrima* (Belda et al., 2017), used to reduce the production of ethanol and volatile acids while, on the contrary, increasing glycerol concentration and wine aromatic complexity (for a review see Jolly et al., 2013, and references therein). However, due to their low tolerance to ethanol or SO_2, non-*Saccharomyces* yeasts are unable to consume all the sugars available in grape juices, and thus often used together with *S. cerevisiae* in co- or sequential fermentations to ensure complete wine fermentation. The main consequence of these combined inoculation procedures is that competition for nutrients, mainly nitrogen resources, may occur between the two species involved, resulting in fermentation issues. Furthermore, it has been widely reported that the quantity and quality of nitrogen sources in grape must directly affect the formation of volatile compounds and determine wine organoleptic profile (Seguinot et al,

2020). Therefore, understanding nitrogen metabolism during fermentation is of utmost significance.

In *S. cerevisiae,* many studies have been carried out to draw a comprehensive picture of both the mechanisms responsible for the import and use of nitrogen sources during winemaking, and their regulation. Nitrogen is imported into cells by different transporters, controlled by three regulation systems: the Nitrogen Catabolic Repression (NCR), the SPS sensors and the General Amino acids Control (GAAC) (Hinnebusch, 2005; Ljungdahl and Daignan-Fornier, 2012; Magasanik and Kaiser, 2002). The combination of these different regulation mechanisms explains the order in which nitrogen sources are consumed in *S. cerevisiae* (Crépin et al., 2012). Once inside the cells, amino acids can be either directly incorporated into proteinogenic amino acids or degraded. A recent study using labelled amino acids showed that the amount of amino acids directly incorporated into biomass is low, except for arginine, histidine and lysine (Crépin et al., 2017). The largest part of amino acids is catabolised to release ketoacids and nitrogen as ammonium or glutamate. Ketoacids can then be used for the *de novo* synthesis of amino acids, catabolised towards the central carbon metabolism (CCM) or towards the production of aroma compounds through the Ehrlich pathway. Ammonium and glutamate are redirected towards the core nitrogen metabolism composed of reversible reactions between ammonium, glutamate and glutamine, and provide nitrogen necessary for the *de novo* synthesis of amino acids (Magasanik and Kaiser, 2002). The carbon skeleton required to achieve this *de novo* synthesis can originate from the degradation of consumed amino acids or be provided through CCM. Thus, carbon and nitrogen metabolisms are interconnected. The fluxes through these metabolisms are dependent of the amount of available nitrogen that affects

the proportion of amino acids directly incorporated into the biomass, CCM contribution and finally aroma production (Rollero et al., 2017).

Currently, only little information is available about these non-*Saccharomyces* yeasts compared to *S. cerevisiae*. Their metabolism, especially nitrogen metabolism, has been addressed, determining the preferred and non-preferred nitrogen sources of several strains and pointing out specific features compared to *S. cerevisiae* (Gobert et al., 2017; Kemsawasd et al., 2015; Su et al., 2020). In addition, underlying mechanisms have been investigated, either at the level of gene expression regulation in *Hanseniaspora vinae* (Lleixà et al. 2016) or in terms of fluxes partitioning in the metabolic network, explaining some specific metabolic traits of *Kluyveromyces marxianus* (Rollero et al., 2019). However, these works each focused on a single species, and cannot be extrapolated to other yeasts, due to the important variability between non-*Saccharomyces* strains regarding nitrogen resource management (Rollero et al., 2018). A better understanding of nitrogen metabolism in non-*Saccharomyces* species would help using them at their best potentiality during fermentation and optimise their co-culture with *S. cerevisiae*.

In this context, the aim of this study is to explore nitrogen resources management by the two non-*Saccharomyces* commercial yeasts most used in winemaking, viz. *Metschnikowia pulcherrima* Flavia and *Torulaspora delbrueckii* Biodiva, and thus gain information on nitrogen sources fate. These strains have shown metabolic specificities compared to *S. cerevisiae*, especially for nitrogen preferences and volatile compounds production (Azzolini et al., 2015; González-Royo et al., 2015; Varela et al., 2017; Su et al., 2020; Seguinot et al., 2020) that should be further explored. To this end, we applied a quantitative metabolic analysis approach using ^{15}N- or ^{13}C-

labelled nitrogen sources to trace their partitioning through the metabolic network during alcoholic fermentation. Through the isotopic enrichment data, we identified the metabolic origin of both proteinogenic amino acids and volatile compounds, through the quantitative determination of nitrogen sources use. Our results provide essential information for a deeper understanding of nitrogen metabolism by non-*Saccharomyces* species and new insights for ultimately a better management of yeast nitrogen nutrition in co- or mixed-culture fermentation.

2. Materials and methods

2.1 Yeast strains and media

The two non-*Saccharomyces* strains, *Torulaspora delbrueckii* (Biodiva, Lallemand) and *Metschnikowia pulcherrima* (Flavia, Lallemand) used in the study were propagated on Yeast extract peptone dextrose (YPD) medium (2% glucose, 2% peptone, 1% yeast extract). Fermentations were carried out with a synthetic must (SM) that is similar to grape juice but with a defined composition, with modifications as in Bely et al. (1990). For nitrogen source labelling experiment, the synthetic must contained 100 g/L glucose, 100 g/L fructose and a mixture of 40% ammonium chloride and 60% amino acids as nitrogen sources (300 mg/L YAN). For glucose labelling experiment, the SM contained 100 g/L glucose as sole carbon source and ammonium chloride (300 mg/L YAN) as the nitrogen source. The concentration of organic acids, minerals and vitamins in the SM were the same as those described by Su et al. (2019). SM was sterilised by filtration through 0.22 μm pore-size membrane filters (Labbox, Spain).

The labelled nitrogen sources, ^{15}N ammonium chloride (99%), $^{15}N_2$ L-glutamine (98%), U-$^{15}N_4$ L-arginine (98%), $^{13}C_5$ L-valine (97-98%), $^{13}C_6$ L-

leucine (97-99%), and labelled $^{13}C_6$ glucose (99%) were obtained from Euriso-top (Cambridge Isotope Laboratories).

2.2 Fermentations and sampling

For nitrogen source labelling experiment, fermentations were carried out in duplicate in 250 ml SM with one nitrogen compound labelled at a time. Samples were taken at four different stages (1/4, 1/2, 3/4, and maximum cell population, referred as $N_{1/4}$, $N_{1/2}$, $N_{3/4}$, and EN). Since *Torulaspora delbrueckii* and *Metschnikowia pulcherrima* have different fermentation rates, different sampling points were applied, i.e. 16 h, 20 h, 24 h and 40 h for *T. delbrueckii* and 12 h, 18 h, 24 h and 40 h for *M. pulcherrima*.

2.3 Quantification of consumed and proteinogenic amino acids

Biomass weight was determined by filtering 10 mL well-mixed fermentation cultures through a pre-weighed 0.45 μm nitrocellulose filter. Filters were washed twice with 50 mL deionised water and dried at 105°C for 48 h before weighing.

The protein concentration of the biomass was quantified using a bicinchoninic acid (BCA) protein assay kit (Sigma-Aldrich). Proteins were extracted from 1-2 mg biomass by incubation with 50% (*v*/*v*) dimethyl sulfoxide (DMSO) for 1 h at 105°C and their concentration determined following manufacturer's instructions.

In order to determine the protein composition in amino acids, cell pellets were incubated overnight in 10% (*v*/*v*) trichloracetic acid at -20°C for protein precipitation. Proteins were then hydrolysed in 6 N HCl at 105°C for 24 h. Amino acid concentrations were determined with a specific amino acid analyser (Biochrom 30, Biochrom) combining ion-exchange chromatography and spectrophotometric detection after ninhydrin revelation. The percentage

of each amino acid in proteins was calculated by dividing the concentration of each amino acid by the total amount of amino acids in the protein extract.

The residual nitrogen compounds in the SM were analysed by UHPLC equipped with a UV detector (Thermo Scientific, MA, USA) based on Gómez-Alonso et al. (2007), with some modifications. Samples were derivatised with diethylethoxymethylenemalonate (DEEMM). An accucore® C18 (Thermo scientific) LC column was used to separate amino acids, with acetonitrile and acetate buffer as mobiles phases, with the working gradient as described in Su et al. (2019) (Table 1). Since 10 fermentations were carried out for each strain, we obtained the residual nitrogen data in 10 replicates.

Table 1. Eluent gradient for the HPLC determination of amino acids.

Time (min)	**0.0**	**3.0**	**5.0**	**11.0**	**12.5**	**14.0**	**18.0**	**21.0**	**23.0**	**25.0**	**26.0**
Phase A (%)	95.0	94.0	92.0	90.0	88.0	82.0	80.0	70.0	60.0	25.0	20.0
Phase B (%)	5.0	6.0	8.0	10.0	12.0	18.0	20.0	30.0	40.0	75.0	80.0

2.4 Isotopic enrichment of intracellular amino acids analysis

The biomass was hydrolysed by adding 1.2 mL 6 M HCl and incubating the samples for 16 h at 105°C in tightly closed glass tubes in a dry heat oven. After incubation, 800 µL distilled water were added and the samples centrifuged to remove cell debris. The supernatant was separated in 4 aliquots of 400 µL and were further dried at 105°C until they reached a syrup-like state (4-5 h). These fractions were then utilised for amino acid derivatisation performed as described previously (Bloem et al., 2018; Crépin et al., 2017; Rollero et al., 2017; Rollero et al., 2019). Two different derivatisation agents were used: (a) ethylchloroformate (ECF) derivatisation was carried out by first dissolving the syrup-like hydrolysate in 200 µl of 20 mM HCl and 133 µl of a pyridine- ethanol mixture (1:4), adding then 50 µl ECF. Derivatives

were extracted with 500 µl dichloromethane, and centrifuged at 4 °C at 10 000 g for 5 min. The organic phase was carefully transferred into a 2 mL vial for GC-MS analysis. (b) N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA) derivatisation was performed by adding 200 µl acetonitrile to the hydrolysate, and 200 µl BSTFA was then added to the dissolved hydrolysates to derivatise amino acids. The mixture was incubated 4 h at 135°C and the organic phase carefully transferred to a 2 mL vial for GC-MS analysis.

Derivatised samples were analysed using a Hewlett Packard 6890 gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) equipped with a CTC Combi PAL Autosampler AOC-5000 (Shimadzu, Columbia, SC, USA) and coupled to an HP 5973 mass spectrometer. The gas chromatograph was fitted with a 30 m × 0.25 mm DB-17 ms column with a 0.15 µm film thickness (Agilent Technologies). Different GC-MS programs were used for the analysis of samples derivatised by ECF and BSTFA as described by Crépin et al. (2017) and Bloem et al. (2018). The mass spectrometer quadrupole temperature was set at 150 °C. The source was set at respectively 250 °C and 300 °C for BSTFA and ECF derivatives and the transfer line held at 250 °C. The MS was operated in selected ion monitoring (SIM) mode with positive ion electron impact at 70 eV using the characteristic ions of amino acid fragments reported previously (Crépin et al., 2017; Bloem et al., 2018). For each amino acid fragment, the outcome of the analysis was a cluster of intensities corresponding to its different mass isotopomers. These data were subsequently processed using IsoCor software developed by Millard et al. (2012); they were corrected for natural labelling and assessed for the isotopic enrichment of each amino acid, defined as the labelled fraction of a given compound (expressed as a percentage).

2.5 Isotopic enrichment of volatile compounds analysis

The experiment for isotopic enrichment of volatile compounds analysis was only carried out with fermentations containing ^{13}C-leucine or ^{13}C-valine. The volatile compounds were extracted with dichloromethane from 5 mL samples with deuterated internal standards. Extracted molecules were separated by a HP 6890 gas chromatograph (Agilent Technologies) equipped with a 30 m x 0.25 mm Phenomenex ZB-WAX-fused silica capillary column with a 0.25 µm film thickness (Agilent Technologies) and helium as the carrier gas using the procedure previously described (Rollero et al., 2015). Compounds were detected using an HP 5973 mass spectrometer in SIM mode with positive ion electron impact at 70 eV. The ion clusters reported were used for the quantification and the determination of the labelling patterns of volatile compounds in a previous study (Rollero et al., 2017). These ion clusters were selected on the basis of their high signal-to-noise ratio and low interference from other compounds. The concentration of each volatile molecule was quantified from the sum of the intensities of the corresponding ion cluster (Data Set S4). In parallel, for each ion cluster, intensities were corrected for natural labelling using IsoCor software (Millard et al., 2012) and processed to assess to the isotopic enrichment of volatile compounds, defined as the fraction of labelled molecule with respect to its total production (expressed in percentage). The isotopic enrichment of volatile compounds was determined in duplicate and the concentration of volatile compounds was determined in four replicates.

2.6 Outline of Experiment Design and Data Analysis

The experiment design, previously experienced and validated (Crépin et al., 2017, Rollero et al., 2017), relied on a set of 10 fermentations for each strains, using a synthetic medium with the same chemical composition. Fermentations were carried out labelling specifically one of the nitrogen

sources of interest, namely, ^{15}N-glutamine, ^{15}N-arginine, ^{15}N-NH_4, ^{13}C-valine, and ^{13}C-leucine, each condition being carried out in duplicate. Consumed nitrogen amount was calculated by subtracting the residual nitrogen concentration at each sampling time from the initial nitrogen concentration. Biomass weight, protein concentration, and amino acid composition for each strain were determined from fermentations carried out using the same conditions but without labelled compound, because of the cost of these molecules (Crépin et al., 2017), and allowed to calculate the concentration in proteinogenic amino acids. The labelled fraction of a protinogenic amino acid or a volatile compound was calculated by multiplying its concentration in millimolars by its isotopic enrichment. The difference from the total amount of the compound corresponded to the unlabelled part. Fluxes in the metabolic reactions involved in the synthesis of a target compound (proteinogenic amino acid or volatile molecule) from a labelled nitrogen source were quantified by dividing the labelled fraction of the compound by the total amount of consumed labelled molecule in millimolars. Calculations were done from mean values provided together with raw data and further details on the calculations in Supplementary Materials 1 and 2.

3. Results

To elucidate how *M. pulcherrima* and *T. delbrueckii* manage nitrogen resources during fermentation for growth but also for volatile compounds production, we assessed fluxes distribution in the nitrogen metabolic network using a quantitative approach relying on mass balances and isotopic tracer experiments. For each strain, fermentations were performed under the same conditions (medium, fermentation management), but with a different labelled nitrogen source each time. This design takes advantage of the high reproducibility of fermentations achieved under controlled conditions, and its

relevance was demonstrated with *S. cerevisiae* (Crépin et al., 2017; Rollero et al., 2017).

3.1 Incorporation of nitrogen from glutamine, ammonium, and arginine into proteinogenic amino acids

3.1.1 Consumption of glutamine, ammonium, and arginine during the growth phase

NH_4Cl, arginine and glutamine are the three major nitrogen sources in a synthetic medium, and more generally in grape juice. The ^{15}N labelled form of each compound helped us to explore the way in which the two non-*Saccharomyces* species redistribute nitrogen from these three main sources to fulfil their anabolic requirements.

Arginine, glutamine and ammonium represented more than half of the nitrogen amount consumed by both strains during fermentation, with *T. delbrueckii* consuming nitrogen sources more efficiently than *M. pulcherrima* (Fig. 1A & 2A). When maximum population was reached, NH_4Cl, arginine and glutamine had been completely depleted by *T. delbrueckii*, while *M. pulcherrima* had only consumed respectively 26%, 66% and 79% of the initial NH_4Cl, arginine and glutamine contents. Even if the assimilation of all three sources started at the beginning of fermentation, their relative contribution to the total consumed nitrogen varied throughout the fermentation depending on the species. On one hand, *T. delbrueckii* assimilated glutamine, arginine and ammonium in the same proportion during fermentation, similar to the proportion of these nitrogen sources in the medium. Consequently, ammonium was the largest nitrogen provider for *T. delbrueckii*, accounting for 29% of total consumed nitrogen, followed by glutamine (23%) and arginine (22%). On the other hand, *M. pulcherrima* showed faster consumption of glutamine and arginine than ammonium at the early stages of

growth, although the contribution of ammonium to the intracellular pool gradually increased during growth. At the end of the growth phase, glutamine had provided 34% of total consumed nitrogen by *M. pulcherrima*, followed by arginine (27%), while ammonium was much less consumed than the other two sources, accounting for only 14% of total consumed nitrogen.

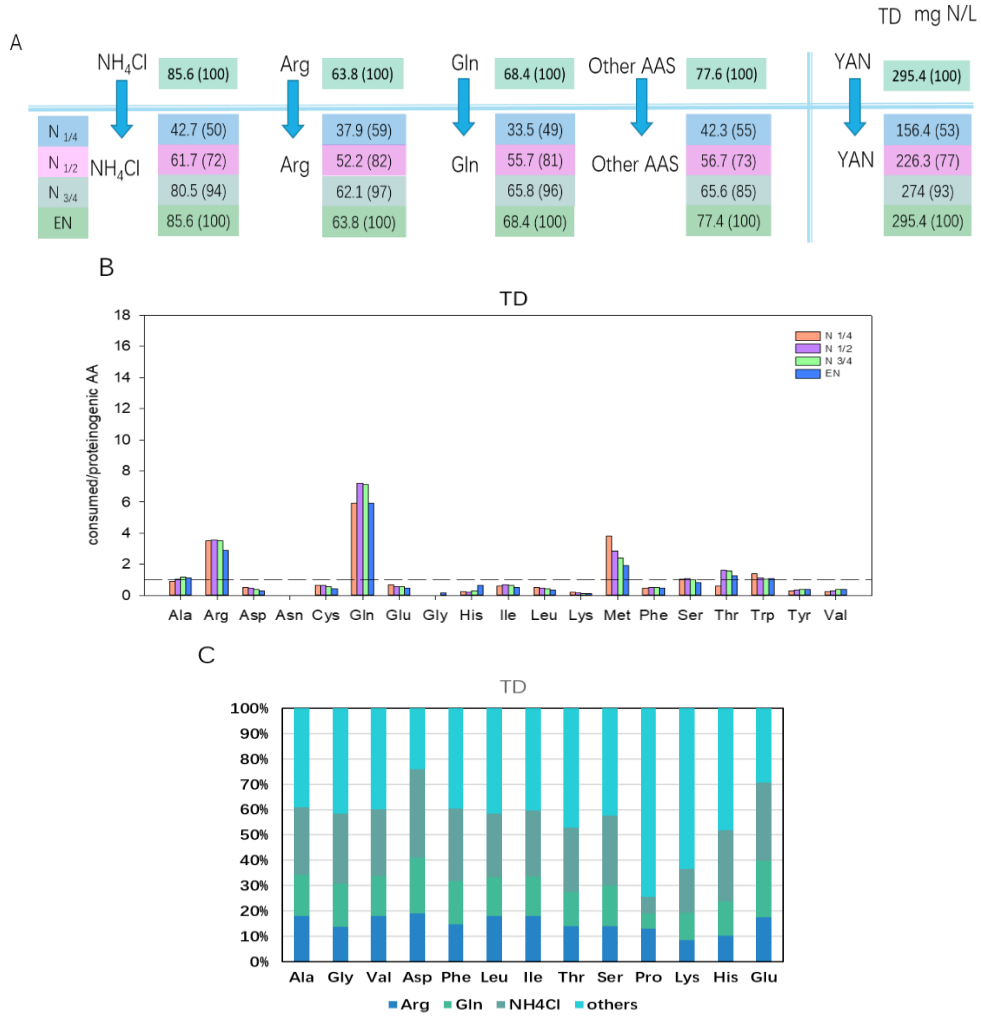


Fig 1. Nitrogen sources consumption and redistribution in *T. delbrueckii*. **A**. Nitrogen consumption (mg N/L) at 4 growth stages: $N_{1/4}$, $N_{1/2}$, $N_{3/4}$, and EN. The values in brackets represent the percentage of consumed nitrogen sources to the initial concentration in the media. **B**. Ratio between the concentration of consumed amino acids and the concentration

of the corresponding proteinogenic amino acids (mM/mM) at different fermentation stages. Ratio>1 indicate that the consumed amino acids are theorically in sufficient quantities in to meet anabolic requirements. **C**. proportion (%) of labelled arginine, glutamine, and NH_4Cl recovered into proteinogenic amino acid at the end of the growth phase (EN).

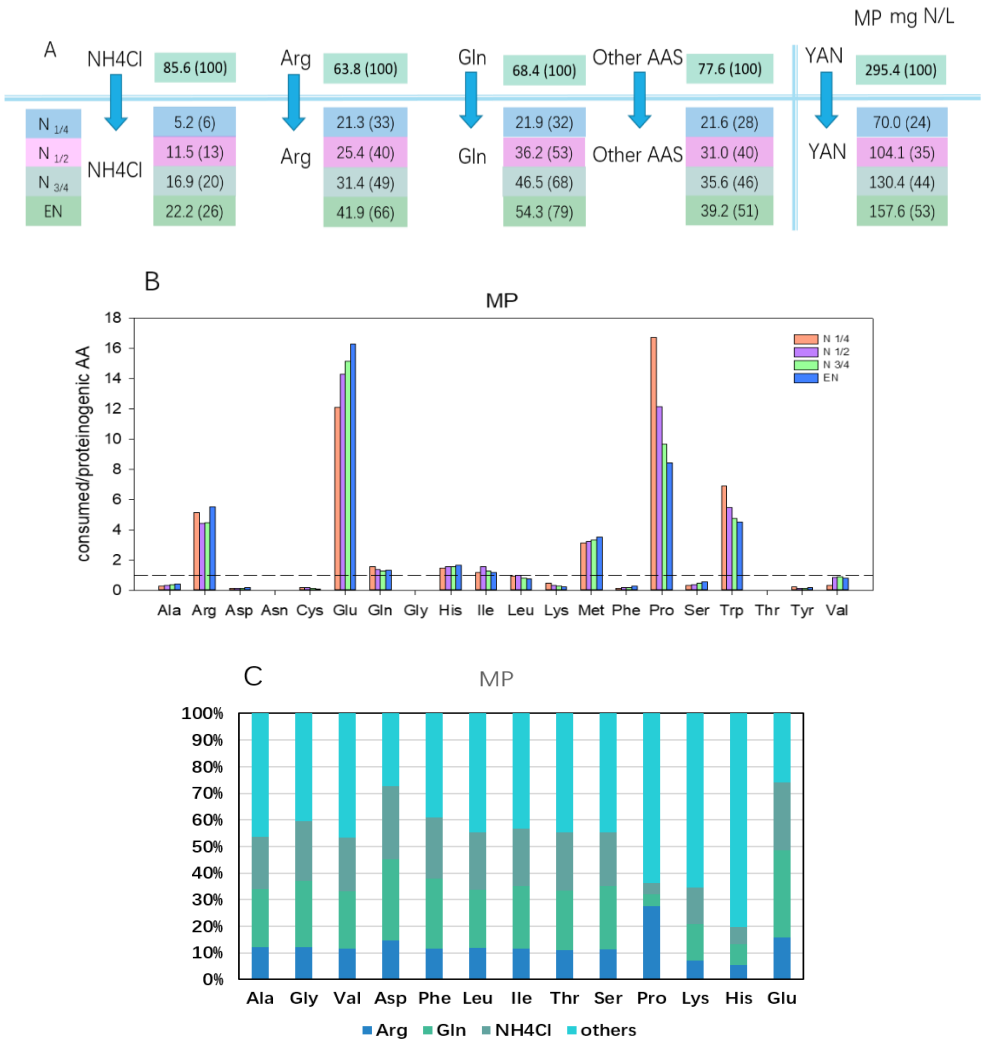


Fig 2. Nitrogen sources consumption and redistribution in *M. pulcherrima*. **A**. Nitrogen consumption (mg N/L) during 4 growth stages: $N_{1/4}$, $N_{1/2}$, $N_{3/4}$, and EN. The values in brackets represent the percentage of consumed nitrogen sources to the initial concentration in the media. **B**. Ratio between the concentration of consumed amino acids and the concentration of the corresponding proteinogenic amino acids (mM/mM) at different stages of fermentation.

Ratio>1 indicate that the consumed amino acids are theorically in sufficient quantities to meet anabolic requirements. **C**. proportion (%) of labelled arginine, glutamine, and NH_4Cl recovered into proteinogenic amino acid at the end of the growth phase (EN).

3.1.2 Relationships between consumption of amino acids and anabolic requirements in proteinogenic amino acids

The first consideration was whether amino acids were always consumed in sufficient amounts to cover the needs of the corresponding proteinogenic amino acids. The ratio between consumed and the relative proteinogenic amino acids showed that, for *T. delbrueckii*, only a few amino acids (alanine, arginine, glutamine, methionine and tryptophan) were consumed in sufficient amounts to cover the anabolic requirements throughout fermentation (ratio>1 in Fig. 1B). The consumption of the other amino acids was too low to cover the needs for protein biosynthesis, emphasising the importance of *de novo* amino acids synthesis and nitrogen redistribution through metabolism. The *M. pulcherrima* strain displayed a similar behaviour, but in this case, additional amino acids (viz. glutamate, leucine, isoleucine, histidine) were consumed in adequate amounts to fulfil anabolic requirements (Fig. 2B). This difference can be explained by the limited growth of *M. pulcherrima*, which consequently resulted in a lesser anabolic requirement for amino acids. The ratio obtained for valine with *M. pulcherrima* was prominent compared to the other amino acids, as this ratio increased from the first stages of the growth phase to be close to 1 at its end. This evolution could be the sign of a decorrelation between the consumption of this amino acid and the anabolic requirement of *M. pulcherrima*.

3.1.3 Redistribution of glutamine, ammonium, and arginine for *de novo* synthesis of other amino acids

Being the most abundant nitrogen sources in the synthetic must, consumed NH_4Cl, arginine and glutamine were catabolised for the synthesis of other amino acids. By combining isotopic enrichment data from the three ^{15}N-labelling experiments, we obtained a comprehensive picture of the main nitrogen distribution into proteinogenic amino acids (except for tryptophan, methionine, cysteine, and tyrosine, which are not detected by the GC-MS method used), and about the contribution of *de novo* synthesis to proteinogenic amino acids against direct incorporation (Fig 1C & 2C). Irrespective of the labelled nitrogen source, ^{15}N isotopic labelling was recovered in all the proteinogenic amino acids measured, even if the amount of amino acids consumed was greater than anabolic requirements. This was true for isoleucine of which more than 56% was synthesised *de novo* by *M. pulcherrima* while this species assimilated a higher amount than its needs for protein synthesis (Fig. 2B & 2C). Interestingly, the ratio between the amount of nitrogen originating from arginine, glutamine and ammonium was similar in all proteinogenic amino acids, with few exceptions only (Fig. 3). First, assessing the contribution of glutamine and arginine compared to ammonium (Arg*/NH4*, Gln*/Arg*), we observed that for *T. delbrueckii*, ammonium was the major contributor to the *de novo* synthesis of other amino acids (ratio<1), followed by glutamine and then arginine. Nevertheless, for *M. pulcherrima*, glutamine was the highest contributor to amino acids *de novo* synthesis, especially during the early growth phase (ratio>1). Ammonium was catabolised in greater amounts than arginine for the synthesis of other amino acids, although, overall, a higher amount of arginine was consumed. Furthermore, no correlation was found comparing the ratio between the

consumption of amino acids and its proteinogenic content on one hand, and their enrichment pattern, on the other hand (Fig. 1B, 2B & 4). From all these observations, taking into account the contribution of amino acid nitrogen to *de novo* synthesis, it appeared that respectively 78 and 80% of *M. pulcherrima* and *T. delbrueckii* proteinogenic amino acids were newly synthesised from a common nitrogen pool.

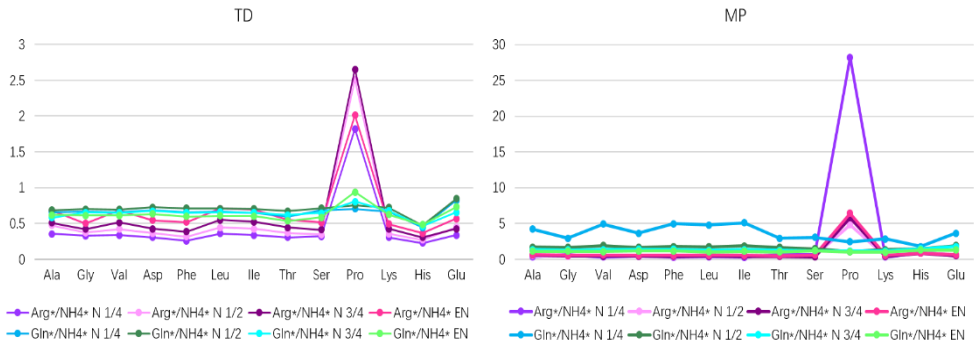


Fig 3. Ratio between arginine and NH_4Cl; glutamine and NH_4Cl in the amount of their redistribution to different amino acids. Ratio>1 indicates a larger contribution than NH_4Cl, ratio<1 represents a lesser contribution.

3.1.4 Different isotopic enrichment patterns of proteinogenic amino acids

The isotopic enrichment for each proteinogenic amino acid changed during all four fermentation stages, with patterns depending on the proteinogenic amino acid, strain and labelled nitrogen source.

Aromatic, aliphatic and hydroxyl amino acids displayed the same general profile of labelling incorporation throughout fermentation. When glutamine or ammonium were used as the labelled nitrogen source, isotopic enrichment in these proteinogenic amino acids in *T. delbrueckii* ranged between 14 % to 20 %, and 22% to 30 %, respectively (Fig. 4). A slight decrease in labelling in the course of fermentation, (less than 5%) was found for all these

compounds. In the presence of labelled arginine, the initial isotopic enrichment in these classes of proteinogenic amino acids was lower, between 6% and 10 %, but increased more substantially, by at least 10%, throughout the process. In the case of *M. pulcherrima*, the initial isotopic enrichment in aromatic, aliphatic and hydroxyl proteinogenic amino acids was higher with ^{15}N-glutamine (from 17% to 25%) compared to ^{15}N-NH_4Cl (from 4% to 10%) or ^{15}N-arginine (between 1% and 7%). Furthermore, for this species, the contribution of nitrogen from glutamine to *de novo* synthesis of proteinogenic compounds decreased during fermentation, while those of ammonium and arginine largely increased, to achieve an average of 22% and 12% respectively at the end of culture.

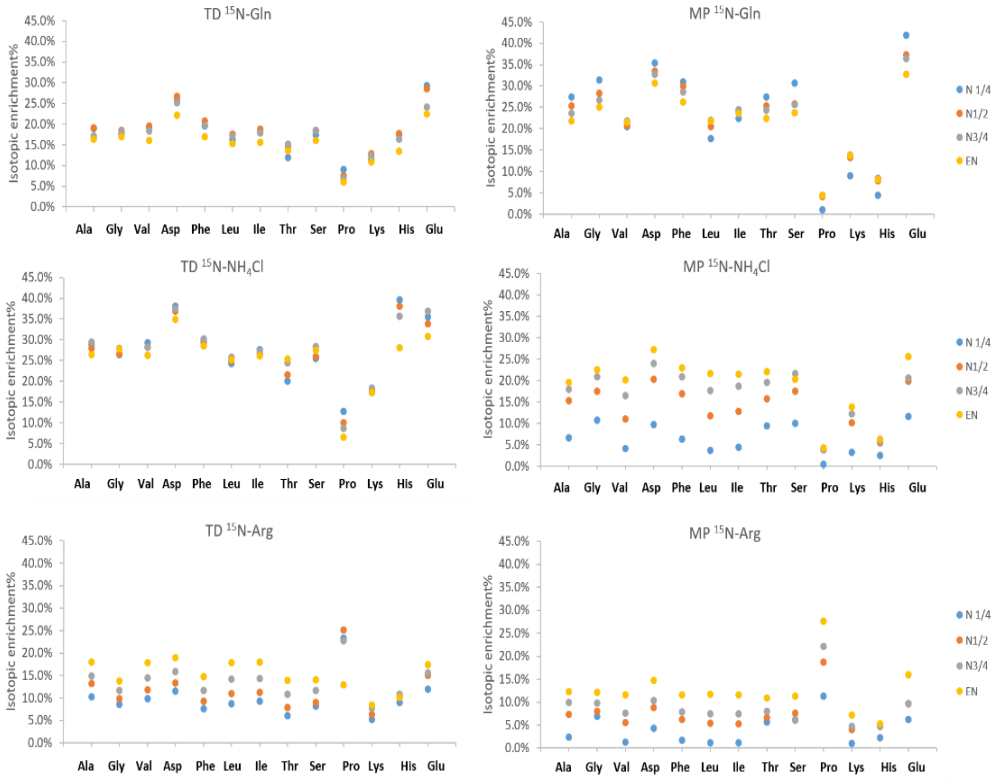
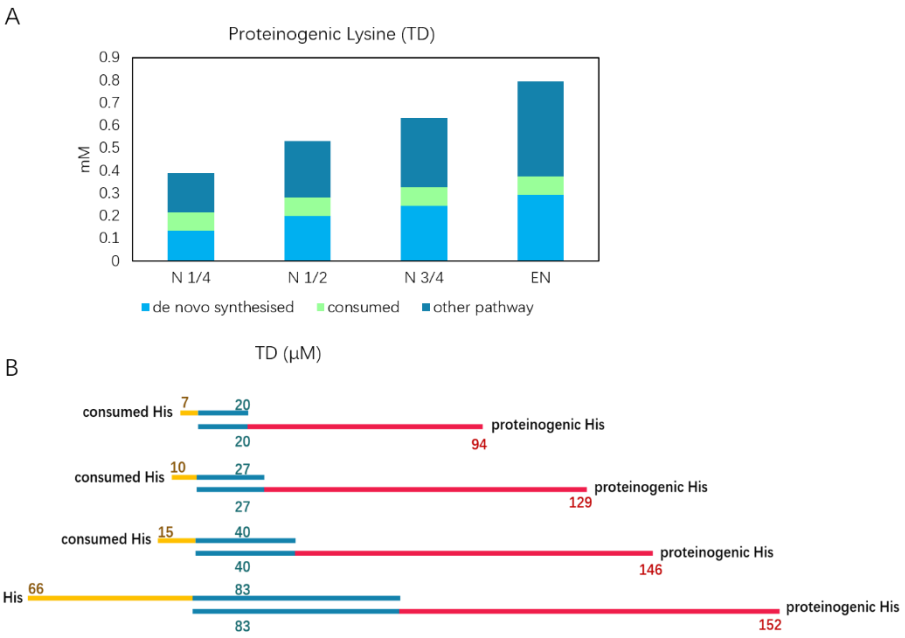


Fig 4. Amino acids isotopic enrichment during four stages of growth when glutamine, NH_4Cl, or arginine is ^{15}N labelled.

In general, the highest isotopic enrichment was measured in glutamate and aspartate, with a proportion of *de novo* synthesis from ammonium, glutamine and arginine accounting for approx. 70% and 75% of the total amount of these proteinogenic amino acids for *M. pulcherrima* and *T. delbrueckii*, respectively (Fig. 1C & 2C). The contribution of glutamine to proteinogenic aspartate (31%) and glutamate (33%) was predominant in *M. pulcherrima* while ammonium was the main nitrogen provider for *de novo* synthesis of aspartate (22%) and glutamate (22%) in *T. delbrueckii*.



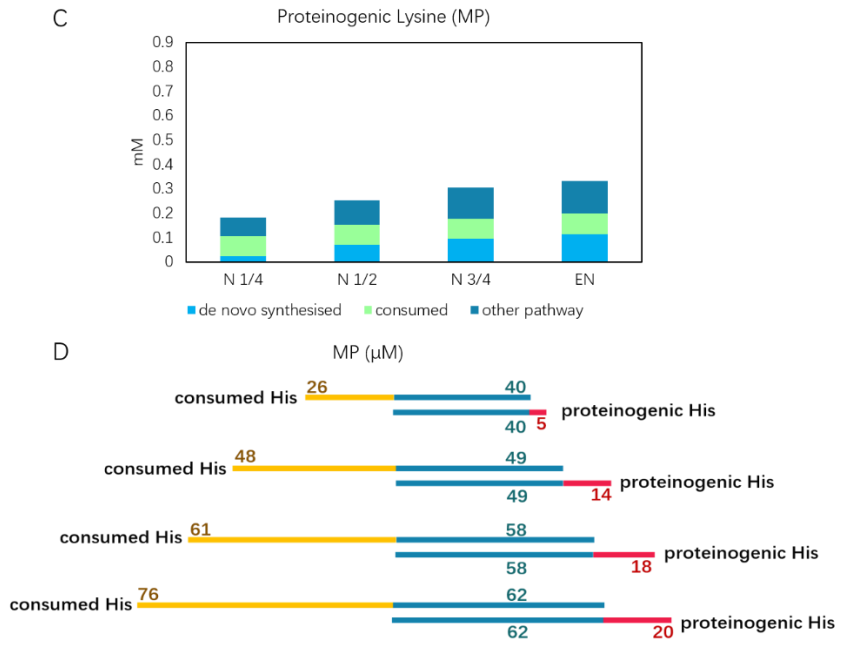


Fig 5. **A**. **C**. Amount of proteinogenic lysine coming from *de novo* synthesis (light blue), directly incorporated from consumed lysine (green) and other pathways in *T. delbrueckii* and *M. pulcherrima* (dark blue). **B**. **D** Fate of histidine in *T. delbrueckii* and *M. pulcherrima*. The amount of direct incorporation from consumed histidine to proteinogenic histidine is in dark blue; rest of consumed histidine is represented in yellow; *de novo* synthesised histidine from other nitrogen sources is in red.

Other exceptions to the general pattern concerned histidine and lysine. The first specificity to be highlighted for both NS strains was their significant anabolic requirements for lysine, this amino acid accounting for a large fraction of proteins (10% of the total weight of proteinogenic amino acids in these species compared with less than 2% in *S. cerevisiae*; Fig. 5A & 5C). As a consequence, even if the nitrogen fraction redistributed towards *de novo* lysine synthesis was relatively high in comparison with the other proteinogenic amino acids, a low incorporation of nitrogen from arginine, glutamine and ammonium was detected in proteinogenic lysine (below 37%

versus over 53% for the other amino acids) (Fig 1C & 2C). Furthermore, the combination of *de novo* synthesis of proteinogenic lysine with the direct incorporation of consumed lysine (2.3 mg/L) was not sufficient to cover the anabolic requirements of either *T. delbrueckii* or *M. pulcherrima*. We also observed differences in the labelling pattern of proteinogenic histidine, revealing differences in the metabolic origin of proteinogenic histidine between *T. delbrueckii* and *M. pulcherrima* (Fig. 5B & 5D). Indeed, at least 52% of proteinogenic histidine was *de novo* synthesised using nitrogen from ammonium, arginine and glutamine in *T. delbrueckii*, as opposed to a much smaller fraction (20%) in *M. pulcherrima*. Differences might correlate with the anabolic requirements for histidine, around 4 times higher in *T. delbrueckii* (234 μM) than in *M. pulcherrima,* (83 μM) along with a delayed histidine consumption by *T. delbrueckii*. Thus, direct incorporation of histidine (83 μM) covered anabolic requirements in *M. pulcherrima* but not in *T. delbrueckii*.

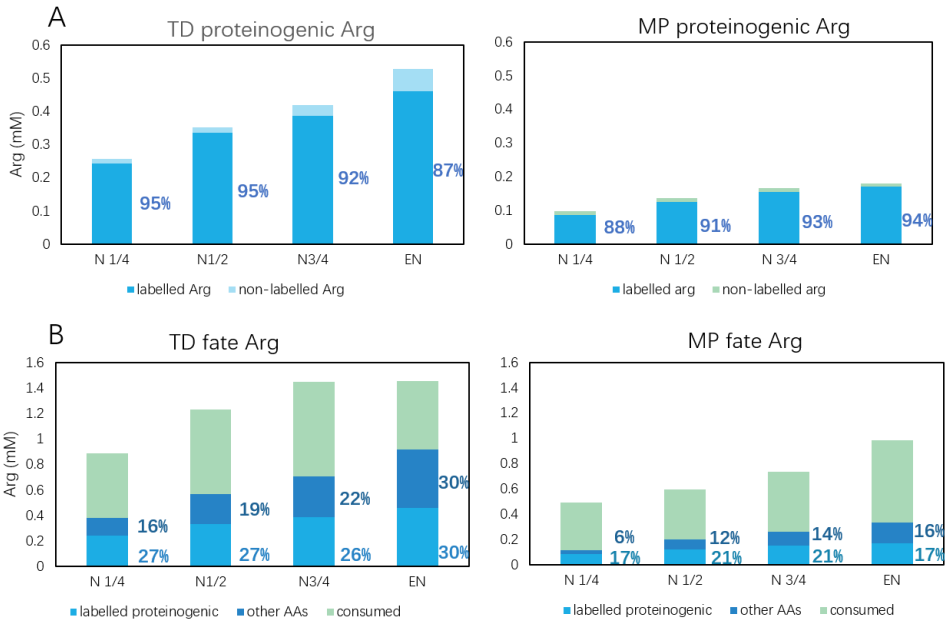


Fig 6. A. Origin of proteinogenic arginine. The labels show the percentage of labelled arginine in total proteinogenic arginine (isotopic enrichment). **B**. Fate of consumed arginine. The labelled arginine consumed was incorporated directly into proteinogenic arginine (light blue bar) or catabolized for the synthesis of other amino acids (dark blue bar). The labels indicate percentages in the total amount of consumed arginine.

3.1.5 Metabolism of arginine and proline during fermentation

The repartition of consumed arginine in the metabolic network was quite different from that of ammonium and glutamine that were efficiently recovered in proteins in the early stages of growth. First, the enrichment percentage of proteinogenic arginine was high (94% for *M. pulcherrima* and 87% for *T. delbrueckii*) when the maximum population was reached on a labelled arginine medium (Fig. 6). This observation demonstrated that the main metabolic origin of proteinogenic arginine is a direct incorporation of consumed arginine, with a low contribution of *de novo* synthesis. However, at the end of the growth phase, a large part of nitrogen from consumed

arginine was not recovered in proteins, but as arginine (direct incorporation: 30% and 17% of consumed arginine for *T. delbrueckii* and *M. pulcherrima* respectively), and other amino acids (redistribution of nitrogen through arginine catabolism and *de novo* synthesis: 30% of consumed arginine for *T. delbrueckii* and 16% for *M. pulcherrima*). Thus, the remaining arginine may have been intracellularly stored as nitrogen stock.

The incorporation pattern of labelled nitrogen in proteinogenic proline was also different from that of the others amino acids (Fig. 1C & 2C). In total, only 25% and 36 % of proteinogenic proline nitrogen originated from the three main sources for *T. delbrueckii* and *M. pulcherrima* respectively. This observation showed that *de novo* proline synthesis remained limited for both strains, suggesting a substantial direct incorporation of consumed proline into proteins. Interestingly, arginine emerged as the major nitrogen provider for proteinogenic proline synthesis (Fig. 3), at the expense of the two other sources, reflecting the formation of proline as an intermediate in arginine catabolism that is not further catabolised to contribute to the intracellular nitrogen pool. Finally, the labelling pattern of proteinogenic proline of *M. pulcherrima* and *T. delbrueckii* reflected the profile of arginine consumption by the two NS strains, with an early arginine uptake by *T. delbrueckii* (providing 25% of nitrogen for proline during the early growth phase, but only 13% at the end of growth phase) and, on the opposite, a delayed arginine assimilation by *M. pulcherrima* resulting in a progressive increase in the proportion of nitrogen from labelled arginine in proteinogenic proline (Fig. 4).

3.2 Origin of the carbon backbone of proteinogenic aliphatic amino acids

Amino acids catabolism not only contributes to the internal nitrogen pool, but

also provides the carbon backbone for cellular biosynthesis as well as precursors for aroma production. The fate of the carbon backbone of the branched chain amino acids valine and leucine was explored using ^{13}C labelled amino acids. These nitrogen sources were totally consumed by both yeast strains during wine fermentation, with an earlier onstart of leucine consumption.

3.2.1 Consumed aliphatic amino acids recovered in proteinogenic amino acids

The amount of labelled carbon in proteinogenic leucine and valine was measured to determine the proportion of these molecules originating from the direct incorporation of their respective consumed amino acids (Fig. 7 & 8). A low isotopic enrichment of proteinogenic valine was observed, indicating a limited direct incorporation of valine into proteins (below 25% and 14% for *M. pulcherrima* and *T. delbrueckii* respectively) and an important contribution of *de novo* synthesis via precursors from CCM. Incorporation profiles differed between the two studied strains: the enrichment percentage stayed constant at approx. 14% during fermentation for *T. delbrueckii*. For *M. pulcherrima* by contrast, the enrichment percentage was higher in the early growth phase, between 31 and 38%, before decreasing to 25% when the population reached its maximum (Fig S1 & S2).

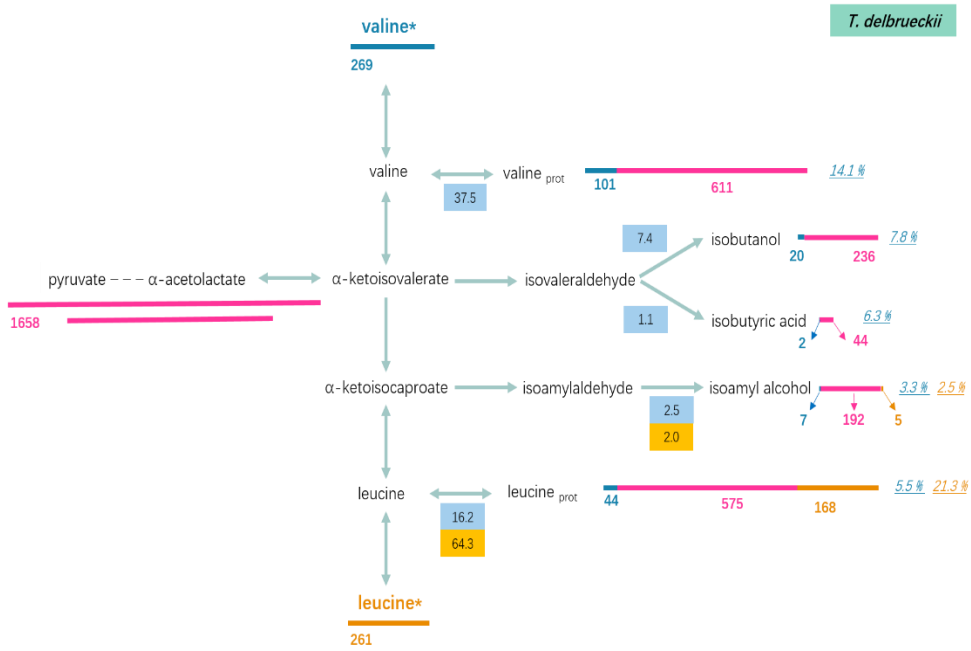


Fig 7. Metabolism of valine and leucine of *T. delbrueckii* at the end of growth phase. Labelled valine (valine*) or leucine (leucine*) were used during fermentations. Length of bars (blue: valine; orange: leucine; pink: central carbon metabolism) is proportional to the compound concentration (µM). Values in blue and orange boxes represent the fraction of consumed valine and leucine (µM) distributed through the pathway, respectively. The italic numbers indicate the percentage of each molecule (proteinogenic amino acid or volatile compound) originated from valine (blue) or leucine (orange). Raw data and details on calculation procedure are provided in Supplementary Material 1.

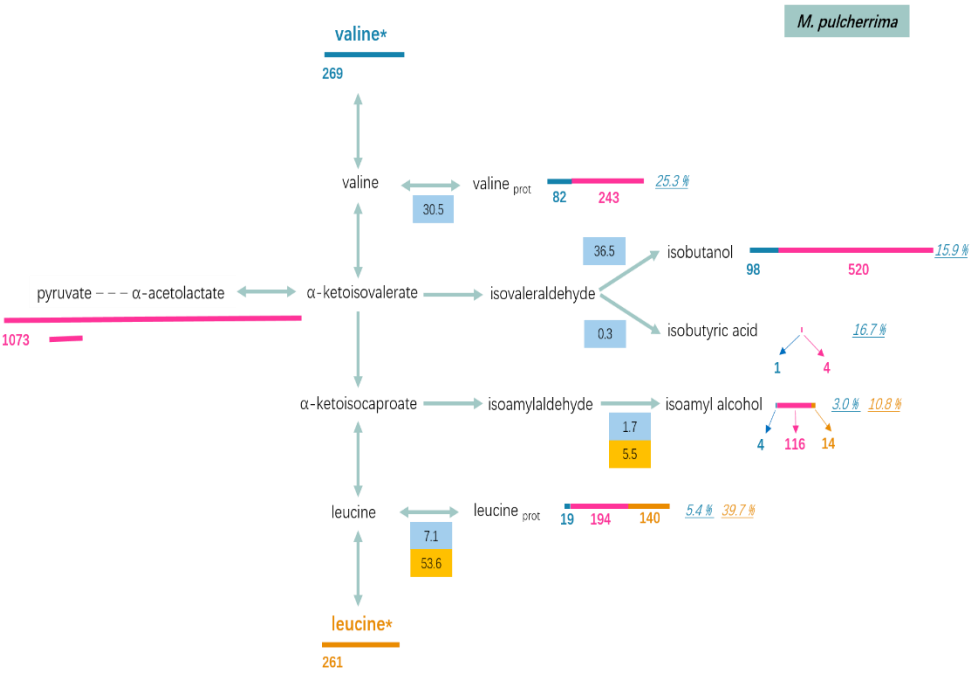


Fig 8. Metabolism of valine and leucine of *M. pulcherrima* at the end of growth phase (µM). Labelled valine (valine*) or leucine (leucine*) were used during fermentations. Length of bars (blue: valine; orange: leucine; pink: central carbon metabolism) is proportional to the compound concentration (µM). Values in blue and orange boxes represent the fraction of consumed valine and leucine (µM) distributed through the pathway, respectively. The italic numbers indicate the percentage of each molecule (proteinogenic amino acid or volatile compound) originated from valine (blue) or leucine (orange). Raw data and details on calculation procedure are provided in Supplementary Material 2.

A large fraction of consumed leucine was incorporated into proteinogenic leucine in both yeasts. Throughout fermentation, the part of consumed leucine directed toward its corresponding proteinogenic amino acid in *T. delbrueckii* and *M. pulcherrima* ranged from 84% to 64%, and from 84% to 54%, respectively (Fig S3 & S4). As a consequence, isotopic enrichment in proteinogenic leucine was substantially higher than for valine, with 40% and 21% enrichment for *M. pulcherrima* and *T. delbrueckii* respectively (Fig 7 & 8). However, CCM contribution was still the main source of carbon for

proteinogenic leucine synthesis, except during *M. pulcherrima* early growth phase in which the direct incorporation of consumed leucine covered up to 78% of leucine anabolic requirements. The absolute amount of consumed leucine recovered in proteins was identical in *M. pulcherrima* and *T. delbrueckii* fermentations. However, the contribution of consumed leucine to the total leucine proteinogenic pool was lower in *T. delbrueckii*, due to higher anabolic requirements, which resulted in a larger *de novo* synthesis of leucine from unlabelled precursors of the CCM.

3.2.2 Formation of volatile compounds through valine and leucine

To draw up a complete picture of the fate of consumed valine and leucine within cells, we measured isotopic enrichment in the volatile compounds deriving from the assimilation of these molecules through the Ehrlich pathway, in addition to that of their proteinogenic counterparts (Fig. 7, 8, S1-S4). Valine catabolism through the Ehrlich pathway accounted for a large part of the fate of this amino acids for the two studied species. For *M. pulcherrima*, more than 38% of consumed valine was directed towards the production of volatile compounds deriving from α-ketoisovalerate (isobutanol, isobutyric acid). As a consequence, the fraction of isobutanol produced from valine catabolism (assessed from the isotopic enrichment) reached up to 40% ($N_{1/2}$), when most labelled valine was consumed. The further decrease in isotopic enrichment of isobutanol to 16% combined with the increase in total isobutanol production revealed that this higher alcohol was synthesised from CCM precursors during the last growth stages. The conversion between α-ketoisovalerate and α-ketoisocaproate was relatively low, which led to a low overall contribution of valine to the production of isoamyl alcohol (isotopic enrichment 3%) and proteinogenic leucine (isotopic enrichment 5.4%) (Fig S2).

Flux distribution through the metabolic network from consumed valine in *T. delbrueckii* was however different. First, in this species, most α-ketoisovalerate produced from the catabolism of consumed valine was directed toward the production of α-ketoisocaproate (18%) at the expense of volatile compounds formation, with isobutanol and isobutyric acid respectively accounting for only 7% and 1% of consumed valine. Furthermore, a large fraction of α-ketoisocaproate from valine assimilation, accounting for 16.2% at the end of the growth phase, was directed towards the production of proteinogenic leucine, while the formation of isoamyl alcohol was limited (3% of consumed valine).

Since leucine was mostly used directly as protein building blocks, only a limited proportion of consumed leucine was catabolised. Interestingly, the proportion of consumed leucine directed towards the production of isoamyl alcohol was higher in *M. pulcherrima* (6% of consumed leucine) than in *T. delbrueckii* (2%). Consequently, isotopic enrichment in isoamyl alcohol during *M. pulcherrima*, of over 11%, was particularly high compared to those measured with *T. delbrueckii* or previously reported with *S. cerevisiae* fermentations (Crépin et al., 2017). A substantial fraction of isoamyl alcohol produced by *M. pulcherrima* originated from consumed leucine, indicating that leucine availability in the medium may directly affect the formation of isoamyl alcohol during the early growth phase.

The analysis of the compounds presented here allowed to recover 66% of leucine consumed by *T. delbrueckii* and only 59% of consumed leucine in *M. pulcherrima* at the end of the growth phase. This suggests that a fraction of leucine may be stored or converted into others compounds.

4. Discussion

Yeast nitrogen metabolism is of significant importance for wine fermentation. Indeed, it influences both fermentation rate and duration and participates in the formation of volatile compounds which in turn determine wine quality. Although there is an increasingly frequent use of non-*Saccharomyces* strains for winemaking, little information is available regarding nitrogen metabolism in these species, because they have long been considered spoilage organisms only and thus not as studied as *S. cerevisiae*. Here, the exploration of the metabolic network of two non-*Saccharomyces* species particularly relevant to winemaking, *T. delbrueckii* and *M. pulcherrima,* by tracing ^{15}N or ^{13}C labelled nitrogen sources partitioning revealed their specificities in the management of nitrogen resource during fermentation.

First, the inability of *M. pulcherrima* to consume 300 mg YAN/L during fermentation (50% consumption only) must be balanced against the capacity of *T. delbrueckii* to deplete all available nitrogen. This has to be considered with respect to the lower anabolic requirement of *M. pulcherrima* (final biomass content: 1.6 g/L) compared to *T. delbrueckii* (4.1 g/L). This lower biomass production by *M. pulcherrima* during fermentation was also observed by Roca-Mesa et al. (2020). Furthermore, while *T. delbrueckii* consumed all three major nitrogen sources (arginine, glutamine, ammonium) with almost the same efficiency (this work, Su et al., 2020; Prior et al., 2019), *M. pulcherrima* first imported glutamine, then arginine, at the expense of ammonium. As a result, only 26% of initial NH_4Cl had been consumed by *M. pulcherrima* at the end of the growth phase. A late and incomplete consumption of NH_4Cl has also been reported in another non-*Saccharomyces* yeast, *Kluyveromyces marxianus* (Rollero et al., 2019) and connected with the genetic background of this species. While in *S. cerevisiae* three ammonium transporters have been identified with high (Mep2p, Mep1p) or low (Mep3p)

affinity (Marini et al., 1994; 1997), only one gene coding for an ammonium transporter, orthologous to *S. cerevisiae MEP3* gene, has been described in *K. marxianus*. Regarding *M. pulcherrima*, automatic annotation approaches have only identified a candidate gene for ammonium transport, orthologous to *MEP2* (Proux-Wéra et al., 2012) and further investigations are required to elucidate why this species displays a poor capacity to uptake this nitrogen source (number of transporters, intrinsic properties, regulation of gene expression).

As previously observed with *S. cerevisiae* (Crépin et al., 2017), the direct incorporation of consumed amino acids into proteins is not in high enough amount to fulfil anabolic requirements in *T. delbrueckii* or *M. pulcherrima*. Therefore, *de novo* synthesis of proteinogenic amino acids takes place in the non-*Saccharomyces* species, involving a redistribution of nitrogen from the consumed nitrogen sources -in particular from the three most abundant ones-, towards the proteinogenic amino acids. Thus, ammonium- and glutamine-derived nitrogen was recovered in all proteinogenic amino acids, at an average level of 18 and 21% (for ammonium and glutamine respectively) of the total concentration of proteinogenic amino acids in *M. pulcherrima* and 25% and 15% (respectively) in *T. delbrueckii*. It is important to point out that *T. delbrueckii* mostly used ammonium-derived nitrogen for the *de novo* synthesis of amino acids preferentially to glutamine, contrary to *S. cerevisiae* that uses both sources equally (22% and 20%, respectively), suggesting a different management of the nitrogen central core between the two species. Conversely, less than 18% of nitrogen in *M. pulcherrima* proteinogenic amino acids originated from consumed ammonium, in line with the low capacity of this species to uptake this compound.

Regarding the third major nitrogen source, we found that most proteinogenic

arginine originated from consumed arginine for both strains, as previously reported for *S. cerevisiae*. However, the isotopic enrichment of proteinogenic arginine in *M. pulcherrima* and *T. delbrueckii*, varying from 87% to 94% depending on fermentation stage, was slightly lower than that previously reported for *S. cerevisiae*, which remained constant at 98% throughout fermentation (Crépin et al., 2017). Furthermore, labelled nitrogen was found in proteinogenic arginine during fermentations carried out in presence of either ^{15}N-glutamine or ^{15}N-ammonium, consistent with a low but effective *de novo* synthesis of arginine (up to 13%) in *M. pulcherrima*. In *S. cerevisiae*, the ArgR/Mcm1 pathway, composed of the *ARG1,3-6,8* genes and responsible for the synthesis of ornithine, citrulline and arginine from glutamine and glutamate, is strongly repressed by cytoplasmic arginine (Ljungdahl and Daignan-Fornier, 2012; Pauwels et al., 2003). Our observations likely indicate a different regulation of this metabolic route, not totally repressed by intracellular arginine concentration in *M. pulcherrima* and *T. delbrueckii,* as previously reported for *K. marxianus* (Rollero et al. 2019). Surprisingly, a part of consumed arginine was not recovered in proteins, accounting for 23% and 72% of arginine consumed by *T. delbrueckii* and *M. pulcherrima,* respectively. The most likely explanation for this discrepancy is a vacuolar storage of part of consumed arginine, combined with its possible further use to support growth after depletion of the other nitrogen sources (Crépin et al., 2014; Gutiérrez et al., 2016). Correspondingly, because of its limited growth, the anabolic requirements of *M. pulcherrima* are lower than those of *S. cerevisiae* or *T. delbrueckii.* Consequently, arginine catabolism (quite costly in ATP, cofactors and NADPH, see Crépin et al., 2014; Cooper, 1982), to provide nitrogen for *de novo* synthesis, appears unnecessary as long as other nitrogen sources are intracellularly available. As regards *T. delbrueckii,* an increase in

the proportion of labelled nitrogen from consumed arginine was observed in proteinogenic amino acids at the end of the growth phase, when most nitrogen compounds were depleted. This is in line with a remobilisation of previously stored arginine to fulfil nitrogen anabolic requirements and sustain further growth.

Overall, compared with the other amino acids, the part of *de novo* synthesised histidine and lysine was low, as shown by the weak isotopic enrichment of these compounds measured throughout *T. delbrueckii* and *M. pulcherrima* fermentations in presence of labelled ammonium, glutamine or arginine. This difference could be explained by a significant direct incorporation into proteins of their counterparts taken from the medium. Indeed, the inability of the two NS species to use histidine to support growth has been previously demonstrated (Su et al., 2019). This work also reported that *T. delbrueckii* displayed reduced fermentative activity when lysine was provided as sole nitrogen source, while *M. pulcherrima* was simply unable to grow under these conditions. These observations suggested that *M. pulcherrima* and *T. delbrueckii* are unable to catabolise lysine or histidine, as already shown for *S. cerevisiae* (Brunke et al., 2014; Ye et al., 1991). Thus the direct incorporation into proteins of consumed lysine and histidine could be more beneficial to yeast than a *de novo* synthesis of these molecules, which is energy and co-factors consuming (Zabriskie and Jackson, 2000; Alifano et al., 1996). This is particularly relevant for proteinogenic histidine in *M. pulcherrima, with* over 75% direct incorporation of consumed histidine. However, this applies to a lesser extent in the case of the metabolic origin of histidine in *T. delbrueckii*, for which isotopic enrichment was higher, suggesting more efficient or alternative pathways for histidine synthesis. Finally, regarding the metabolic origin of proteinogenic lysine, overall

amounts of consumed lysine combined with *de novo* synthesis using glutamine-, arginine- and ammonium-derived nitrogen, which is less efficient compared to the other amino acids, were not sufficient to meet anabolic requirements. This suggests specific alternative pathways for lysine synthesis in these yeasts.

Isotope tracer experiments using ^{13}C-labelled valine and leucine allowed to elucidate their fate, at least in part, in *M. pulcherrima* and *T. delbrueckii*. A general pattern similar to that of *S. cerevisiae* (Crépin et al. 2017) was shown, with a substantial contribution of catabolism accounting for at least 36% of the amino acids consumed. However, the partitioning and fate of consumed leucine and valine in the metabolic network differed between the two strains and with *S. cerevisiae*, reflecting a different management of these nitrogen resources.

First, high yields of isobutanol and isoamyl alcohol were found during *M. pulcherrima* fermentations, combined with a higher isotopic enrichment in these volatile molecules compared with those measured with *S. cerevisiae* and *T. delbrueckii*. These observations showed that, in this strain, consumed amino acids were mostly catabolised. This behaviour is consistent with the limited growth of *M. pulcherrima* during wine fermentation, and consequently, with its low anabolic requirements in terms of amino acids. The first consequence was the decrease in the formation of ketoacids from CCM as precursors of *de novo* synthesis of amino acids, as shown by the higher isotopic enrichment of volatile molecules produced by this strain (10%) compared to that measured during *S. cerevisiae* or *T. delbrueckii* wine fermentations (1%). Then, the formation of volatile molecules through the Ehrlich pathway is likely an efficient way to eliminate ketoacids, toxic compounds for yeasts (Swiegers et al., 2005). The substantial contribution of

leucine and valine catabolism to the production of volatile compounds by *M. pulcherrima* is of technological interest. Therefore, it seems possible to modulate these compounds through nitrogen nutrition management in *M. pulcherrima* when it is used with *S. cerevisiae* (in mixed or sequential inoculation). Finally, the low isotopic enrichment measured in proteinogenic leucine and isoamyl alcohol during *M. pulcherrima* fermentation in presence of labelled valine revealed a limited capacity of this strain to convert α-ketoisovalerate to α-ketoisocaproate. It may be explained by a deficit in this strain in acetyl-coA, which is used as co-substrate in the first step of the conversion of α-ketoisovalerate into α-ketoisocaproate by *LEU4* and *LEU9* (Bell and Henschke, 2005). We had already assumed that the intracellular availability of acetyl-CoA was limited in this strain, because of its low ability to produce acetate, acetate esters, medium-chain fatty acids and their ethyl esters derivatives (Seguinot et al., 2020).

The behaviour of *T. delbrueckii* was quite different and in agreement with its efficient and important growth capacities. Accordingly, this species displayed low production of volatile compounds, and the labelling provided by valine or leucine was mainly recovered in their proteinogenic counterparts, at the expense of higher alcohols. This fluxes partitioning revealed that *T. delbrueckii* showed an optimal management of nitrogen resources to fulfil anabolic requirements, mainly directing consumed nitrogen sources toward protein formation.

Mass and isotopic balances revealed that only 72% and 64% of the labelling originating respectively from consumed leucine and valine during *T. delbrueckii* fermentation were recovered (in proteinogenic amino acids, higher alcohols, acetate esters, and branched amino acids); similarly, small fractions (50% and 65% respectively) were recovered from *M. pulcherrima*

fermentations. To account for these losses, stripping and evaporation of a fraction of the analysed compounds have first to be considered (Mouret et al., 2014). Indeed, up to 0.7% isoamyl alcohol and 21% isoamyl acetate can be lost to evaporation at 24°C, i.e. the fermenting temperature used in our experiments. In addition to these analytical limitations, other metabolic routes may be considered for valine and leucine assimilation, leading to the production of metabolites not measured in this work. Thus, a previous study showed that *M. pulcherrima* was able to produce high concentrations of propyl acetate and ethyl propionate when fermenting with leucine as the sole nitrogen source (Seguinot et al., 2020). Other metabolic pathways described to be involved in valine and leucine degradation in other organisms, may also be operative in non-*Saccharomyces* species: such an example is terpenoid backbone biosynthesis through the mevalonate pathway, connected to leucine degradation in archaea, eukaryote and bacteria (Buhaescu and Izzedine, 2007; VanNice et al., 2014). Valine and leucine degradation can also provide precursors for the biosynthesis of macrolides (molecules such as erythromycin with antibiotic activity) in bacteria and fungi (Hertweck, 2009; Xue et al., 1998). Furthermore, one of the characteristic features of *M. pulcherrima* is its ability to produce pulcherrimin, a red iron-containing pigment with antifungal and antibacterial properties (Lachance, 2016). The formation of its precursor, pulcherriminic acid, involving the cyclisation of two molecules of leucine, may be one of the outcomes of consumed leucine in *M. pulcherrima* (MacDonald, 1965; Krause et al., 2019). Finally, an intracellular storage of leucine and valine, without further degradation, as reported for arginine, cannot be completely ruled out.

5. Conclusions

Isotopic tracer experiments using ^{15}N and ^{13}C-labelled nitrogen sources

allowed us to comprehensively explore nitrogen metabolism in two non-*Saccharomyces* species, *T. delbrueckii* and *M. pulcherrima.* As previously reported for *S. cerevisiae*, consumed nitrogen sources were mainly catabolised for the *de novo* synthesis of amino acids, although arginine, and to a lesser extent histidine and lysine, were mainly incorporated directly into proteins. Differences between strains were observed, as *T. delbrueckii* used ammonium preferentially to glutamine, while ammonium incorporation was low in *M. pulcherrima.* This knowledge is of technological interest, in order to define appropriate nitrogen nutrition strategies during sequential or co-inoculation fermentation of these non-*Saccharomyces species* in combination with *S. cerevisiae.*

Furthermore*,* important differences between strains in the partitioning of nitrogen compounds in the metabolic network were revealed, reflecting distinct nitrogen resource managements by each species adapted to their anabolic requirements. An efficient use of nitrogen from consumed nitrogen sources towards *de novo* synthesis of proteinogenic amino acids in lieu of volatile compounds formation was found in *T. delbrueckii*, in line with this species' high growth capacity. Conversely, in the low biomass-producing species *M. pulcherrima*, amino acids were directed toward catabolism, producing high amounts of ketoacids subsequently transformed into higher alcohols. Overall, these observations underline fluxes distribution significance within the metabolic network to understand the phenotypic behaviour of these yeast species during fermentation. This information is essential for the design of strategies allowing to modulate wine flavour profiles through the use of these species in co- or sequential fermentation with *S. cerevisiae*; however, further investigations are needed to take into account the metabolic consequences of interactions between the non-*Saccharomyces*

species and *S. cerevisiae* strains.

Supplementary material can be downloaded from https://doi.org/10.3390/microorganisms8060904

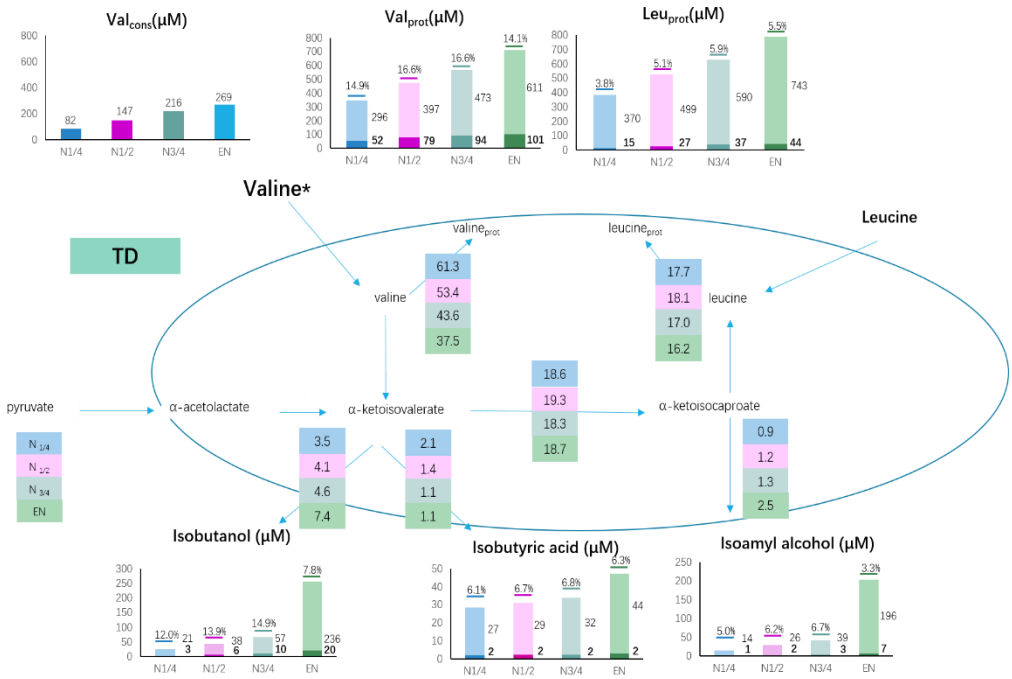


Fig S1. Flux partitioning around ^{13}C labelled valine in *T. delbrueckii*. Val_{cons}, Val_{prot}, and Leu_{prot} represent consumed valine, proteinogenic valine, and proteinogenic leucine respectively. In the bar plots, bars with darker colour represent the isotopic labelled proportion, and the percentage of isotopic enrichment is shown on the top of the bars. values in the coloured boxed are percentages of consumed valine distributed to different compounds during 4 growth stages.

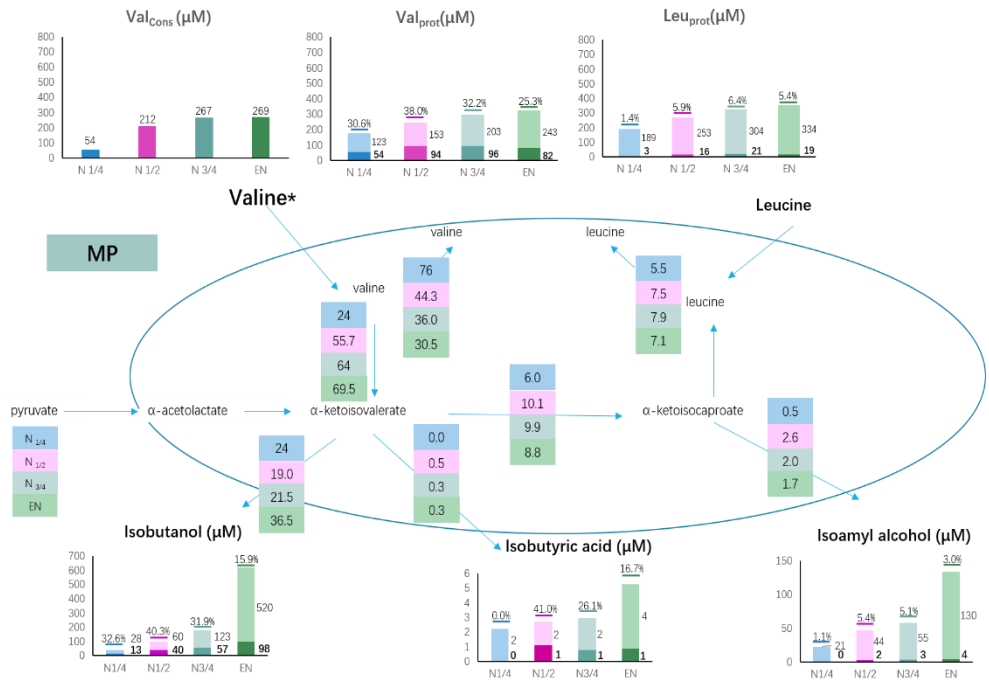


Fig S2. Flux partitioning around ^{13}C labelled valine in *M. pulcherrima*.

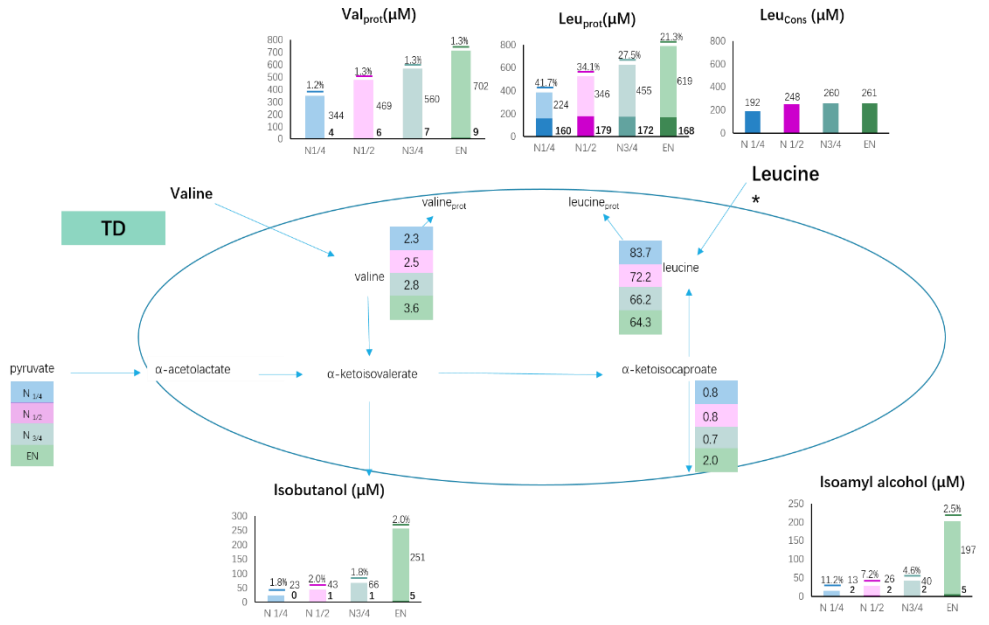


Fig S3. Flux partitioning around ^{13}C labelled leucine in *T. delbrueckii*.

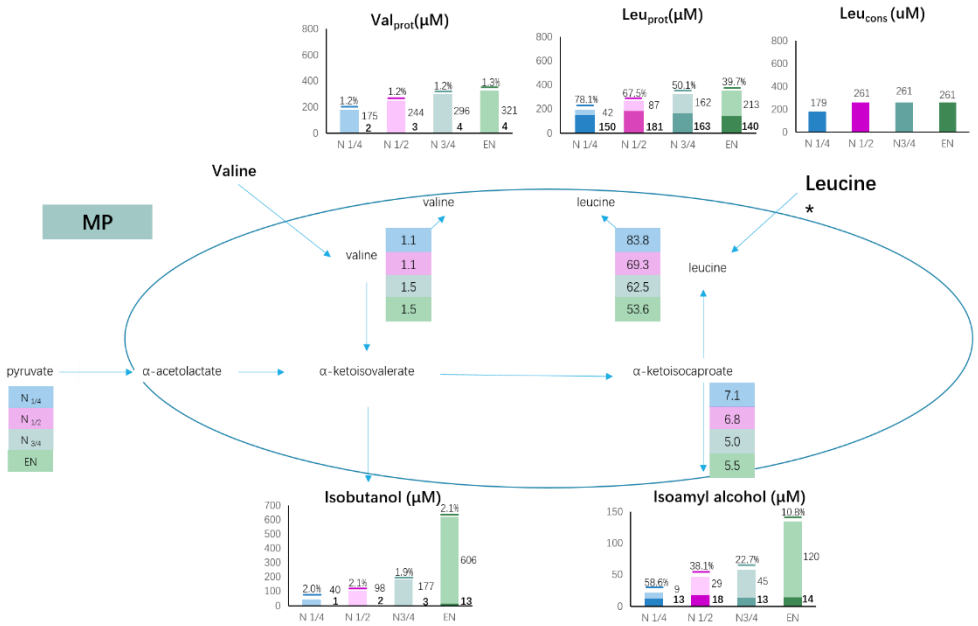


Fig S4. Flux partitioning around ^{13}C labelled leucine in *M. pulcherrima*.

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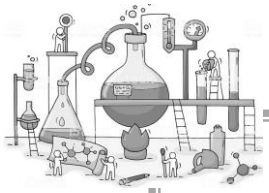

General Discussion

Winemaking is an important biotechnological practice which has been carried out for thousands of years to fulfil the needs of human society. Although the development of modern technologies has largely improved the winemaking process for the production of wines with stable quality, nowadays winemaking is still facing a lot of challenges. Wine as one of the fast-moving consumer goods (FMCG), the market is largely driven by the consumers' preferences. Nowadays, wine with fruity and floral aroma and well balanced ethanol content is favourable by the consumers. However, due to the climate change, the grapes harvested at their phenolic ripeness often have very high sugar content and relatively low nitrogen content. As one of the most important nutrients for yeast, nitrogen has a significant influence on the wine fermentation process in many different aspects. The availability of nitrogen sources not only regulates the yeast biomass yield, the fermentation rate, but also influences the production of volatile compounds and other fermentative metabolites. Previous researches have shed light on nitrogen metabolism by *S. cerevisiae*. However, there are still many questions remained to be answered. Recently, the utilisation of alternative yeast species for winemaking is becoming increasingly popular. These non-conventional yeast species benefits the fermentation by improving the tolerance to certain harsh environmental conditions such as low temperature, as well as by enhancing the sensory profile of wine. Unlike well-studied *S. cerevisiae*, little information is known about the non-conventional yeast species, especially their nitrogen metabolism patterns, which provides important information for conducting successful wine fermentations. This thesis is aiming to explore the nitrogen metabolism of different wine related yeast species including *S. cerevisiae*, *Saccharomyces* non-*cerevisiae* and non-*Saccharomyces* (NS). Nitrogen metabolism can be divided into several specific aspects. The thesis

work mainly focused on the nitrogen requirements during yeast growth and fermentation, nitrogen source preference, nitrogen source assimilation pattern, and nitrogen partitioning in the metabolic network.

Influence of nitrogen concentration on yeast growth and fermentation activity

Assimilable nitrogen is essential for yeast, as it is needed for the synthesis of proteins and enzymes involved in cell growth and metabolism. Nitrogen requirements by yeast to support growth and fermentation is a complex trait and highly species and strain dependent. The influence of nitrogen availability on yeast growth and fermentation can be determined by several interconnected parameters. To determine the influence of nitrogen concentration on yeast growth, lag phase (λ), maximum growth rate (μ_{max}), maximum OD (OD_{max}), and area under the growth curve (AUC) were used as comparison parameters. In general, μ_{max} and OD_{max} increase with YAN concentration until the nitrogen concentration is not the limiting factor. Excessive amount of nitrogen may have an inhibiting effect on cell growth which resulted in a prolonged lag phase time. AUC is a comprehensive parameter that summarises the information of lag phase, μ_{max} and OD_{max}. For *S. cerevisiae* strains, the highest AUC value was observed with 140 mg/L YAN, which further indicated the inhibition effect of excessive nitrogen on yeast growth. Spor et al (2008) studied the influence of genetic and environmental components on the variability of life-history traits and their trade-offs by using media with different glucose concentrations. Their results revealed that when resources are abundant, yeast strains grow slowly, but reache a large population size and have bigger cells. On the other hand, when resources are limited in the medium, the strains have a higher growth rate and display smaller carrying capacity and cell size. In their study, the intrinsic

growth rate and cell size are strongly affected by the glucose concentration. In our case, nitrogen was used as a unique variable factor. When a set of strains are evaluated, the ranking of most strains in terms of growth rate did not change substantially from one nitrogen concentration to another. However, when it comes to the fermentation behaviour, the influence of nitrogen concentration is more strain specific. Some strains are less influenced by low nitrogen concentration and are able to carry out fermentations with a proper rate. Contrarily, other strains have high fermentation capacity with abundant YAN, but the fermentation activity is repressed when nitrogen is limited. From our results, a decorrelation between fermentation rate and growth rate was observed. Since more obvious influences of nitrogen concentration on fermentation capacity were observed, and strains' fermentation behaviours are more relevant to industrial production, they are used as our selection criteria for high and low nitrogen demanding strains.

Yeast species and their ecological niches also influence their nitrogen requirement. In the study of the nitrogen requirements of *Saccharomyces* non-*cerevisiae* strains, we observed that *S. eubayanus* strains showed higher μ_{max} than other *Saccharomyces* species regardless of nitrogen concentration. Moreover, the fermentation rates of *S. eubayanus* strains seemed to be less affected by the increase of nitrogen concentration. On the other hand, the fermentation rates of the industrial *S. uvarum* and *S. cerevisiae* strains were greatly influenced by nitrogen concentrations and the highest fermentation rate was observed with the highest nitrogen concentration. This may be explained by the differences in life-history strategies between environmental and industrial yeast strains (Spor et al., 2009). Yeast strains isolated from the natural environment normally reproduce quickly, reach a large carrying capacity and a small cell size, whereas the industrial strains reproduce slowly,

reach a small carrying capacity but have a big cell size in the fermentation. They are metaphorically described as the strategy of "ants" and "grasshoppers". The *S. eubayanus* strains used in our study were all isolated from the natural environment. They showed "ants" like life-history strategies as described. The *S. uvarum* strains used in our study were isolated from both fermentative and natural environments from the Patagonia region. According to Almeida et al. (2014) and Rodr ǵuez et al. (2017), all of them belong to the South America-A/Holartic phylogeny group. However, they showed different phenotypical behaviours. Therefore, the origin of isolation may play a more important role in yeast physiological activities than the phylogenetic relationships, and this also enhances the important influence of human domestication on shaping yeast behaviours.

Variations in nitrogen requirements were also observed with NS strains. In our study, three different species *T. delbrueckii*, *M. pulcherrima* and *M. fructicola* were studied. During fermentation of synthetic must of 140 mg/L YAN, *T. delbrueckii* strain quickly depleted all the assimilable nitrogen sources, whereas *M. pulcherrima* and *M. fructicola* strains were not able to consume all the assimilable nitrogen sources, even after 48 hours of fermentation. When fermentations were carried out with synthetic must of 300 mg/L YAN, neither *T. delbrueckii* nor *Metschnikowia* strains can deplete the nitrogen sources after 48 hours, however, *S. cerevisiae* consumed all the nitrogen sources within 24 hours. *Metschnikowia* strains clearly have lower nitrogen requirement comparing with *T. delbrueckii* and *S. cerevisiae*. Additionally, it was also observed that under the same fermentation condition, *M. pulcherrima* produces much less biomass than *T. delbrueckii*. Therefore, the low nitrogen requirement of *Metschnikowia* yeast strains may relate to its low growth capacity resulting in a low anabolic requirement.

Nitrogen source preferences of NS strains

From spoilage yeasts to valuable contributors to wine quality, the role of NS has been changed as more knowledge of them is unveiling. NS yeasts are normally used together with *S. cerevisiae* in a sequential or mixed-fermentation manner. Since NS yeasts have a short lag phase, they predominate the early stage of fermentation until *S. cerevisiae* exerts its dominance. One of the out coming problems is the competition for nutrients between different species. In nitrogen limited grape must, NS may deplete the available nitrogen sources which causes difficulties for the implantation of *S. cerevisiae* and therefore lead to uncompleted fermentations. To have better utilisation of these NS strains, their nitrogen source preferences and assimilation order during fermentations were studied. By conducting growth and fermentation experiments in sole nitrogen sources, the differences of nitrogen metabolism between NS and *S. cerevisiae* were revealed. First of all, when growing in single nitrogen sources, NS has a much shorter lag phase time than *S. cerevisiae* in most cases. The short lag phase time may be the life-history strategy of the NS species to compete for nutrients with *S. cerevisiae*. Moreover, NS strains are able to use some of the very poor nitrogen sources for *S. cerevisiae*, such as lysine and proline, to support growth and fermentation. For *S. cerevisiae*, proline can be degraded into glutamate via the proline utilisation pathway in the mitochondria with the presence of oxygen. The proline catabolism is under the regulation of NCR. Under the same growth and fermentation conditions, *S. cerevisiae* was not able to grow by using proline as the sole nitrogen source, whereas *T. delbrueckii* and *Metschnikowia* strains were able to catabolise proline to support growth. Therefore, genetic differences between *S. cerevisiae* and NS strains responsible for the utilisation of proline may exist and it is worth

exploring as future work. Overall, *Metschnikowia* strains have lower growth and fermentation capacity comparing to *S. cerevisiae* and *T. delbrueckii*. However, one of the notable characters of *Metschnikowia* strains is that their preferences for different nitrogen sources are not as clear as the other two species. They are able to use very poor nitrogen sources to grow. On the other hand, the good nitrogen sources do not support a much higher growth capacity. It has been described by Seguinot et al. (2020) that sequential inoculation of *M. pulcherrima* and *S. cerevisiae* positively influence the production of aroma compounds, especially the varietal thiols. Modulation of nitrogen source composition according to the special nitrogen source preference pattern may favour the growth of *Metschnikowia* population, therefore enhances the aroma production.

Nitrogen source assimilation order of different yeast species

During fermentation, nitrogen sources were sequentially assimilated by yeast. The assimilation order is species or strain specific. In *S. cerevisiae*, the consumption of nitrogen sources is mainly regulated by SPS and NCR mechanisms. Early consumed amino acids are transported by permeases under SPS control, whereas most of the late consumed nitrogen sources are transported by permeases under NCR control. For non-*cerevisiae* and NS yeast strains, the mechanisms controlling the nitrogen uptake are still not well known. Our study phenotypically analysed the nitrogen source assimilation order during fermentation by these non-conventional yeast species. Further studies of the relative amino acid transporters and their regulation patterns are still required.

Cryotolerant strains, *S. eubayanus*, and *S. uvarum*, are phylogenetically closely related to *S. cerevisiae*. In general, the nitrogen source assimilation orders of

cryotolerant yeast species are very similar to that of *S. cerevisiae* during fermentation with different nitrogen concentrations and temperatures. Only several exceptions were observed. Cryotolerant strains consume phenylalanine faster than *S. cerevisiae*. This may relate to the higher production of 2-phenylethanol and 2-phenylethyl acetate by these strains. They give pleasant rose-like odour to wine and phenylalanine is the precursor for their production via the Ehrlich pathway (Minebois et al., 2020; Stribny et al., 2015).

More pronounced differences in nitrogen source assimilation order were observed between NS strains and *S. cerevisiae*. The *T. delbrueckii* strain used in our study reproduced fast and were able to deplete the majority of the nitrogen sources in the medium of 300 mg/L YAN within the first 48 hours of fermentation. Due to the low growth capacity of *Metschnikowia* strains, their nitrogen requirement is much lower than *S. cerevisiae* and *T. delbrueckii*. After 48 hours of fermentation, only several amino acids with low concentration in synthetic must of 300 mg/L YAN were completely consumed. *T. delbrueckii* preferentially consumed aspartate, while the consumption of glutamate was slower comparing to *S. cerevisiae*. *Metschnikowia* strains consumed glutamine, tryptophan, valine and isoleucine more rapidly, whereas aspartate, phenylalanine were consumed slower. Understanding the nitrogen source assimilation order is of great importance, since the NS strains are used in sequential inoculation with *S. cerevisiae*. The depletion of nitrogen sources may limit the growth of *S. cerevisiae*. It has been described that sequential fermentation with *M. pulcherrima* and *S. cerevisiae* prolong the fermentation duration up to 250 hours (Seguinot et al., 2020). Moreover, the consumption of nitrogen sources by NS strains may also influence the production of volatile compounds. *M.*

pulcherrima has a low capacity to produce acetate which may resulted in the limitation of cytosolic acetyl-CoA (Seguinot et al., 2020). Consequently, the production of medium-chain fatty acids and their ethyl ester derivatives from acetyl-CoA is low. Therefore, the depletion of some amino acids by *M. pulcherrima* may reduce the formation of these aroma compounds. On another aspect, the production of higher alcohols and acetate esters by *S. cerevisiae* is favoured at low to moderated YAN concentration (Carrau et al., 2015; Rollero et al., 2014). Early inoculation of NS strains may provide a suitable nitrogen environment for the production of higher alcohols and acetate esters.

Gobert et al. (2017) reported that the fermentation temperature influences the nitrogen assimilation of some nitrogen sources such as ammonium by NS strains. In our study, only one fermentation temperature was considered, it could be interesting to explore the effect of temperature on nitrogen assimilation to favour the fermentation of different wine types. Previously, Beltran et al. (2007) reported that low-temperature of fermentation relaxed the nitrogen catabolite repression (NCR) and enhanced the uptake of some amino acids (i.e. arginine and glutamine).

Notably, lysine is prematurely consumed at the very beginning of the fermentation in four different yeast species. Lysine is considered a poor nitrogen source and *S. cerevisiae* is not able to utilise this amino acid as the sole nitrogen source for growth and fermentation. The reason why lysine is early consumed was first believed to be the low concentration of lysine in the synthetic must. However, when different nitrogen sources were supplied in an equal amount in the synthetic must, lysine was still one of the first amino acids to be consumed. One of the possibilities for the rapid uptake of lysine may related to its antioxidant role for cell growth (Olin-Sandoval et al., 2019).

Studies have revealed that yeast cells are able to uptake extracellular lysine to reach concentrations up to one hundred times higher than those required for growth. This phenomenon is referred as "lysine harvesting". The consumed lysine does not accelerate yeast growth, but triggers a reprogramming of redox metabolism. A sufficient amount of intracellular lysine saved the NADPH, which is required for the *de novo* synthesise of lysine, and channelled into glutathione metabolism. The increase of glutathione concentrations reduces levels of reactive oxygen species and increased oxidant tolerance. As described by Olin-Sandoval et al. (2019), lysine harvesting may be one of the most powerful preventative metabolic antioxidant strategies available to microbial cells. Furthermore, lysine consumption sets up an example indicating that the uptake of nitrogen sources may not only be used to support cell growth, but also be used to preventatively reconfigure the cells metabolism to increase tolerance to stress.

Nitrogen source redistribution during fermentation

Once entered the cells, different nitrogen sources can either be used directly as building blocks for proteins synthesis or be catabolised for the *de novo* synthesis of other compounds. To study the nitrogen metabolism of NS strains, the partitioning of the three most abundant nitrogen sources (ammonium, glutamine, and arginine) and two major amino acids related to the Ehrlich pathway, leucine, and valine, was traced by isotopic labelling technique. For high nitrogen demanding species like *T. delbrueckii*, most of the consumed amino acids are not adequate for the anabolic requirements. Therefore, the more abundant nitrogen sources, ammonium, glutamine and arginine, are catabolised to form an intracellular nitrogen pool to serve the needs of *de novo* synthesis of other amino acids. Most of the consumed ammonium and glutamine were recovered in other proteinogenic amino acids, however, the

consumed arginine was not completely recovered. Previous researches revealed that in *S. cerevisiae*, the large amount of the consumed arginine can be stored in the vacuole. This arginine pool is metabolically active and can be effectively utilised during nitrogen starvation (Crépin et al., 2014; Kitamoto et al., 1988). The vacuolar membrane has seven independent amino acid transport systems which are specific for arginine, arginine-lysine, histidine, phenylalanine-tryptophan, tyrosine, glutamine-asparagine, and isoleucine-leucine. In yeast cells growing under nutrient-rich condition approximately 50% of total amino acids are accumulated in the vacuoles (Kawano-Kawada et al., 2018). Under the starvation condition, the transport of amino acids out of the vacuoles plays a critical role in the maintenance of cytosolic amino acid levels. In our study, the partial recovery of arginine is more pronounced with *M. pulcherrima*. Crépin et al. (2014) compared the nitrogen assimilation profiles of high and low biomass producing *S. cerevisiae* strains. They observed that the amount of arginine stored in the vacuole is higher for low biomass producers than for high biomass producers, which indicated a less efficient re-mobilisation of amino acids by low biomass producers. *M. pulcherrima* is a typical low biomass producer, therefore, the explanation for *S. cerevisiae* may also be applicable for *M. pulcherrima*.

In *S. cerevisiae*, branched-chain higher alcohols, isoamyl alcohol, and isobutanol, are synthesised in the yeast cell through the Ehrlich pathway, which involves the degradation of the branched-chain amino acids, leucine, isoleucine and valine (Swiegers et al., 2005). A similar mechanism was observed in *M. pulcherrima* and *T. delbrueckii*. Depending on the intrinsic characters of each species, the flux distribution through the Ehrlich pathway varies. *T. delbrueckii* has a higher growth capacity and reaches high biomass yield. Therefore, a higher amount of amino acids is needed to fulfil the

anabolic requirements. The major part of the consumed leucine and valine were used directly as proteinogenic amino acids or catabolised for the *de novo* synthesis of other amino acids. In *M. pulcherrima*, the anabolic requirement is low due to low biomass production. A much higher percentage of consumed valine and leucine were catabolised for the synthesis of higher alcohols. However, for both species, the catabolism of consumed amino acids only played a minor role in higher alcohol production. The majority of the volatile compounds were produced from catabolism of glucose.

Differences in nitrogen metabolism of wine related yeast species and the possible strategy for industrial utilisation

Our study explored the nitrogen metabolism pattern of several common yeast species used in winemaking covering *S. cerevisiae*, non-*cerevisiae*, and NS. Among them, *S. cerevisiae* is the most important yeast species for wine fermentation and its nitrogen metabolism has been intensively studied during the past decades. A clear illustration of the transporters responsible for nitrogen source uptake, the regulation mechanism of the activation of different transporters, the nitrogen source metabolism has been drawn for *S. cerevisiae*. Cryotolerant yeast species are mainly used in the industry for their capability of conducting fermentations at low temperature. They are phylogenetically close to *S. cerevisiae*. Therefore, the mechanisms for nitrogen metabolism in *S. cerevisiae* may apply to non-*cerevisiae* strains. The nitrogen source preferences and assimilation order of cryotolerant strains were similar to that of *S. cerevisiae* with only several exceptions. When fermentation is conducted with low temperature and low nitrogen concentration, some selected cryotolerant strains may have more advantages to increase their population over *S. cerevisiae* strains. However, the dominance of the non-*cerevisiae* strains in the medium does not guarantee a

successful complete fermentation due to their lower tolerance to ethanol. One of the ideal solutions is to construct hybrid strains of *S. cerevisiae* and cryotolerant species. The hybrid strains are not only low temperature resistance, low nitrogen demanding, but also have good fermentation capacities which can complete the fermentation with high speed and reach the complete dryness. The utilisation of the hybrid strains may help wine makers to solve a lot of practical problems (Querol et al., 2018).

NS strains are gaining increasing popularity in wine making for their role in increasing aroma complexity, enzyme production and ethanol reduction. However, the inoculation in sequential fermentation changes the nitrogen concentration and composition for the implantation of *S. cerevisiae*. Despite the nitrogen source assimilation, the nitrogen sources left in the grape must when *S. cerevisiae* is inoculated maybe not sufficient or suitable for its growth. Therefore, the onset of *S. cerevisiae* strain may be influenced and the fermentation time may be prolonged. The addition of extra nitrogen into the grape must is a common strategy used to prevent sluggish or stuck fermentation caused by nitrogen deficiency. For the sequential fermentation, nitrogen addition may help the implantation of *S. cerevisiae* and shorten the fermentation duration. Some other issues may also influence sequential or mixed-culture fermentation. Some of the *T. delbrueckii* strains are known as killer strains since they produce toxins that may kill *S. cerevisiae* and other NS strains (Ram ŕez et al., 2015). Therefore, if the used *S. cerevisiae* strain is sensitive to the toxins produced by *T. delbrueckii*, the fermentation will be largely influenced. However, some studies use killer *T. delbrueckii* strains on purpose during fermentation to kill *S. cerevisiae* and achieve single culture fermentation only by *T. delbrueckii* (Vel ázquez et al., 2015). It has also been reported that *M. pulcherrima* can have an antagonistic effect on *S. cerevisiae*

which leads to delays in fermentation (Jolly et al., 2014). This is caused by the production of pulcherrimin pigment which depletes iron in the medium, making it not available to the other yeasts. However, Oro et al. (2014) demonstrated no antimicrobial activity of *M. pulcherrima* against *S. cerevisiae*. Instead, it displayed a broad antimicrobial action on spoilage yeasts. Furthermore, wines fermented by NS strains may have different chemical compositions comparing to that of *S. cerevisiae*. Therefore, the following malolactic fermentation will also be influenced (Balmaseda et al., 2018). Finally, as described by Jolly et al. (2013), NS yeasts are like a double edged sword, special care needs to be taken to obtain the desired results.

Genetic resolution for nitrogen requirement

Through the exploration of nitrogen metabolism by *S. cerevisiae*, many approaches have been applied to explore the genetic bases of the difference in nitrogen requirements. QTL mapping combined with hemizygote analysis has revealed many genes related to nitrogen requirements (reviewed by Kessi-Pérez et al., 2020). In our study, high and low nitrogen demanding strains were selected based on their phenotypic characters. The genomic editing tool, CRISPR/Cas9, was applied to verify whether the phenotypical differences of nitrogen requirement is caused by the polymorphisms of *GCN1* gene. However, nitrogen requirement has a broad phenotypic plasticity, and it is a polygenic trait influenced by multiple genes. Therefore, unfortunately, no significant influence was observed from our allele swapping experiment. Most of the methods to explore the nitrogen requirement differences are focused on the polymorphisms within the coding regions. To have a better understanding of the regulation mechanisms, further analysis can also explore the polymorphisms within regulatory regions that may contribute to gene expression variation. Salinas et al. (2016) conducted expression QTL (eQTL)

analysis to test allele-specific expression differences between yeast isolates from distinct geographic and ecological origins to decipher the genetics underlying phenotypic diversity in natural populations. A similar strategy can also be applied for nitrogen requirement difference analysis. Additionally, omics-based analysis is also a powerful tool for deciphering the genetic evidences of nitrogen requirements.

Future perspectives

In grape must, some nitrogen sources are not preferred by yeast, however, they have some potential benefits for yeast cells which worth to be explored. Proline is the most abundant amino acid in grape must. Although proline is the least-preferred nitrogen source for many laboratory yeast strains and although its utilisation results in the slowest growth rates, yeast cells have evolved a regulatory circuit that enables them to use the proline in the environment when preferred nitrogen sources are no longer available (Minebois et al., 2020). The enzymes of proline degradation pathway is encoded by the nuclear genes *PUT1* and *PUT2* and regulated by Put3p. Through the pathway, proline can be degraded into glutamate. In grape juice with low YAN concentration, it will be of great significance if the inoculated yeast strain is able to use proline more efficiently to support the fermentation. One of our future perspectives could be to select *S. cerevisiae* strains with a higher capacity to use proline as nitrogen source. The other option is to improve proline utilisation capacity by directed evolution of *S. cerevisiae* strains. Moreover, it has been revealed in our study that some NS strains can use proline to support growth and fermentation with a higher rate than that of *S. cerevisiae*. It may be worthwhile to explore the proline utilisation mechanism of these NS strains to provide useful information for sequential or mixed-culture fermentations. Except for proline, lysine is another amino

acid which draws our attention. Although it is a poor nitrogen source, *S. cerevisiae* assimilate lysine prematurely before the uptake of other nitrogen sources even when the concentration of lysine was the same as others. Olin-Sandoval et al (2019) revealed the antioxidant role of lysine for cell growth. In our future work, it would be interesting to explore whether the concentration of intracellular lysine will influence the yeast's stress tolerance.

Our study of NS strains has phenotypically determined their nitrogen source preference and nitrogen source assimilation order. Although in general, NS strains have similar nitrogen source utilisation pattern with *S. cerevisiae*, some variations were observed. This indicates that some differences in nitrogen metabolism regulation systems in the molecular level may exist for NS species. However, the nitrogen metabolism regulation system is poorly studied. Many questions remain to be deciphered. Topics focusing on transporters for nitrogen source uptake, the type of regulation mechanisms (such as NCR, SPS in *S. cerevisiae*), and the genetic basis for nitrogen requirement differences are interesting objectives to be further explored.

NS species are used in cooperation with *S. cerevisiae*. The interaction between different species can largely determine the quality of wine. In sequential inoculation fermentation, the early inoculated NS strain changes the fermentation environment for the late inoculated *S. cerevisiae*. Both strains have altered gene expression profiles which lead to different metabolic activities. To investigate the gene expression profiles of both strains in sequential inoculation, RNA-seq analysis can be carried out to compare the gene expression profiles of different strains in the mixed-culture with those obtained in pure cultures. This information can help us to understand how two strains in the fermentation influencing each other on the molecular level.

All in all, nitrogen utilisation by wine related yeast strains is a complex topic rooting down in many interconnected subtopics. There are still many open ends to be explored. A better understanding of this topic will help the winemakers to carry out fermentations in a better controlled way with a smarter utilisation of yeasts and nitrogen sources. This knowledge can also facilitate the yeast providers to make improved versatile yeast strains that can adapt to different nitrogen situations. The outcome of these studies will prevent economic losses of winemakers and guarantee problem free fermentation process and achieve wine with splendid quality.

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Conclusions

1. Nitrogen requirement of industrial *S. cerevisiae* is strain specific and the competitiveness of each strain during wine fermentation can be determined by nitrogen availability in grape-must.
2. High nitrogen-demanding strains showed a lower biomass yield and need more available assimilable nitrogen to keep a constant fermentation rate in the stationary phase.
3. The genome information of our selected low and high nitrogen demanding *S. cerevisiae* strains revealed large differences in heterozygosity terms and stressed the importance of the cell recycling system under nitrogen restriction conditions. However, further genetic analyses are still required to gain a comprehensive understanding of the molecular basis determining this complex yeast feature.
4. Both nitrogen concentration in the medium and fermentation temperature influence the competition capacity of the cryotolerant yeast strains *S. eubayanus* and *S. uvarum* in mixed culture with *S. cerevisiae*.
5. *S. eubayanus* showed better fermentation performance than *S. cerevisiae* at low nitrogen and low temperature condition. The employment of mixed cultures of *Saccharomyces* species can become a strategy for winemakers to overcome the problem of nitrogen-limited musts.
6. An alternative strategy to the mixed cultures is the construction of interspecific hybrid strains of *S. cerevisiae* and selected low nitrogen demanding *S. eubayanus* or *S. uvarum*. These hybrid strains showed higher fermentation capacity in low temperature or low nitrogen fermentation conditions comparing with parental strains.

7. Besides the higher fermentation capacity, the constructed hybrid strains also contributed to wine quality by producing lower amount of acetic acids, and higher amount of volatile compounds than parental strains.
8. Hybrid strains consumed nitrogen sources faster than parental strains, which indicated a higher competition capacity for nutrients. Under the nitrogen-limiting condition, this strategy could ensure a better biomass yield.
9. *Metschnikowia sp.* had lower growth and fermentation rate than *T. delbrueckii* and *S. cerevisiae*. However, these strains can use a wider range of amino acids, especially some poor nitrogen sources for *S. cerevisiae*.
10. *T. delbrueckii* displayed similar growth and fermentation performance to *S. cerevisiae*. Moreover, *T. delbrueckii* strain also had similar preference to nitrogen sources with exception of lysine which can be utilised by *T. delbrueckii* but cannot be used by *S. cerevisiae*.
11. *T. delbrueckii* and *M. pulcherrima* showed similar nitrogen source assimilation order as *S. cerevisiae*. The initial proportion of nitrogen sources in the medium had important influence on nitrogen source assimilation order.
12. In *T. delbrueckii* and *M. pulcherrima*, the consumed major nitrogen sources ammonium, arginine and glutamine are mainly catabolised for the *de novo* synthesis of other amino acids to fulfil the anabolic requirements.
13. The consumed branched-chain amino acids leucine and valine were mainly used by *T. delbrueckii* for *de novo* synthesis of proteinogenic amino acids instead of volatile compounds formation. For the low

biomass-producing species *M. pulcherrima*, higher amount of volatile compounds was produced from the catabolism of these amino acids.

Resumen en español

Introducción

Desde las primeras evidencias, la elaboración de vino tiene una historia de más que 5000 años. El vino está estrechamente relacionado con las actividades humanas e inspiró muchos momentos importantes en la historia de la humanidad. Hoy en día, el consumo de vino sigue siendo una parte importante en nuestra dieta diaria. La producción de vino es más de 270 millones de hectolitros cada año y España es uno de los mayores productores a nivel mundial (Organisation Internationale de la Vigne et du Vin, 2017).

Tradicionalmente, la fermentación del vino se llevaba a cabo de manera espontánea sin conocer las bases científicas que rigen el principio de fermentación. No fue hasta la invención del microscopio por parte de Antoni van Leeuvenhoek y, posteriormente, los trabajos de Louis Pasteur, que se estableció el vínculo entre la levadura y la fermentación de vino. Más tarde, en 1883, el micólogo y fisiólogo danés Emil Christian Hansen aisló el primer cultivo puro de levadura de cerveza, comenzando una nueva época en la investigación de la levadura. En 1890, el enólogo alemán Hermann Müller-Thurgau logró el aislamiento de un cultivo puro de levadura de vino e introdujo el concepto de inoculación en fermentaciones de vino, con cultivos de levaduras especialmente seleccionadas, que se ha convertido en una de las prácticas enológicas comunes en la vinificación moderna.

La fermentación del vino es un proceso ecológico y bioquímico complejo llevado a cabo de forma secuencial por diferentes cepas de levaduras y bacterias. Dentro del grupo de microrganismos relacionados, *S. cerevisiae* es la principal especie responsable de la fermentación alcohólica. *S. cerevisiae* tiene una capacidad de fermentación sobresaliente, que le permite transformar azúcares a etanol de una manera muy eficiente. Además, su alta tolerancia al

etanol permite que las células sobrevivan hasta el final de la fermentación. También es importante mencionar que *S. cerevisiae* puede producir muchos compuestos volátiles utilizando los precursores del mosto de uva y estos compuestos son responsables del aroma del vino.

Hoy en día, las fermentaciones realizadas a temperaturas más bajas son cada vez más populares, especialmente para la fermentación de vinos blancos y rosados. La fermentación a baja temperatura aumenta no solo la retención sino también la producción de algunos compuestos volátiles que mejoran el perfil sensorial del vino. Sin embargo, la baja temperatura no es adecuada para el crecimiento de *S. cerevisiae*. El ambiente frío aumenta la fase de latencia, reduce la tasa de crecimiento e incluso conduce a fermentaciones lentas y paradas. Hay ocho especies en el género *Saccharomyces*, algunas de ellas son conocidas por su excelente tolerancia a la baja temperatura. De hecho, algunas de estas especies, como la criotolerante *S. uvarum*, han sido encontradas en vinos producidos en regiones con climas fríos. También se han aislado híbridos interespecíficos de esta especie y *S. cerevisiae*, e híbridos de tres especies diferentes en los que también se incluye *S. kudriavzevii*, otra levadura muy bien adaptada a bajas temperaturas. La otra especie criotolerante es *S. eubayanus*, que es un miembro relativamente nuevo en el género *Saccharomyces*. No fue sino hasta 2011 en que el primer *S. eubayanus* fue aislado e identificado por Libkind et al. (2011). Estas especies criotolerantes no solo pueden realizar la fermentación a baja temperatura, sino que también mejoran la calidad del vino al aumentar la complejidad del aroma, reducir la concentración de etanol, aumentar la concentración de glicerol, etc. Sin embargo, muchas de estas cepas tienen baja tolerancia al etanol, por lo tanto, no pueden terminar la fermentación. Una posible estratega es la construcción de híbridos interespecíficos entre especies de levadura

criotolerantes con *S. cerevisiae* para obtener cepas de levadura con las características deseadas de ambas cepas parentales.

Tradicionalmente, la fermentación del vino se realizaba de forma espontánea, lo que permitía que las levaduras existentes en las uvas o en el ambiente crecieran y fermentaran juntas. Durante la etapa temprana de la fermentación espontánea, las cepas de levadura no-*Saccharomyces* predominan en el mosto de uva. Con el aumento de la concentración de etanol, *S. cerevisiae* gradualmente supera a las cepas que no son de *Saccharomyces* y se convierte en la especie de levadura dominante hasta el final de la fermentación. Durante mucho tiempo, las especies de levadura que no son *Saccharomyces* en el mosto de uva se consideraron como organismos alterantes, ya que pueden causar fermentaciones problemáticas e influir negativamente en la calidad del vino. Sin embargo, estudios recientes demostraron que algunas especies de no-*Saccharomyces* pueden beneficiar la calidad del vino al mejorar la complejidad del aroma, la sensación en la boca y mostrar caracteres distintivos para cada terruño. Las levaduras no-*Saccharomyces* no son adecuadas para llevar a cabo la fermentación del vino como como única cepa (cultivo puro). A menudo se usan en cooperación con *S. cerevisiae*. La utilización de no-*Saccharomyces* en la fermentación del vino puede reducir el contenido de etanol, aumentar la producción de glicerol y reducir la formación de ácidos volátiles negativos como el acético o acetato de etilo. Además, algunas especies secretan enzimas que ayudan a la liberación de compuestos aromáticos interesantes, como terpenos y tioles, de sus precursores inodoros.

El proceso de fermentación se influye por diferentes parámetros, como pueden ser, la composición del mosto, el microorganismo que se inocule, la

temperatura de fermentación, etc. Durante la fermentación, el nitrógeno es uno de los nutrientes más importantes para la levadura. La carencia de nitrógeno es uno de los principales problemas encontrados en la elaboración del vino, especialmente relacionados con fermentaciones lentas e incompletas. La concentración de nitrógeno regula la formación de biomasa de levadura y, a su vez, la velocidad y la duración de fermentación. El contenido de nitrógeno del mosto también puede afectar a la producción de muchos compuestos volátiles que contribuyen al sabor del vino, ya que algunos aminoácidos son precursores metabólicos directos para la síntesis de alcoholes superiores, ácidos grasos de cadena corta a media y sus derivados de éster etílico o ésteres de acetato. También se ha demostrado que las actividades de las rutas metabólicas involucradas en la producción de glicerol y ácidos orgánicos dependen de la fuente y concentración de nitrógeno.

Hay muchos compuestos que contienen nitrógeno en el mosto de uva, los que pueden ser asimilados por la levadura se le conoce como "nitrógeno fácilmente asimilable". La levadura puede usar alrededor de 30 compuestos distintos que contienen nitrógeno, incluidos aminoácidos, amonio, urea, bases nitrogenadas y derivados de purina. Las fuentes de nitrógeno asimilables se importan a las células de levadura a través de diferentes transportadores específicos o no específicos que están regulados por varios mecanismos diferentes. Uno de ellos es Ssy1p-Ptr3p-Ssy5p (SPS), que es un complejo ubicado en la membrana plasmática con la capacidad de detectar los aminoácidos extracelulares y activar la transcripción de genes que codifican las permeasas de membrana. Otro sistema regulador en *S. cerevisiae* es el conocido como represión catabólica por nitrógeno (NCR, en sus siglas en inglés). El sistema NCR le permite a la célula de levadura utilizar selectivamente fuentes de nitrógeno preferidas y previene o reduce la

transcripción innecesaria de genes que codifican enzimas y permeasas para la utilización de fuentes de nitrógeno más pobres. La ruta TOR ("Target of Rapamycin") es la responsable de la detección intracelular de aminoácidos y de la regulación, a su vez, del sistema NCR. Los dos factores transcripicionales más importantes en la regulación NCR son Ure2 y Gln3. En condiciones de escasez de nitrógeno, la ruta de control general de aminoácidos (GAAC) se activa en *S. cerevisiae*. Estos mecanismos están interconectados y la activación depende de la disponibilidad del nitrógeno en el medio de crecimiento.

Al tratarse de un organismo modelo, el metabolismo de nitrógeno ha sido muy bien estudiado durante las últimas décadas en *S. cerevisiae*. Se han respondido muchas preguntas relevantes. Sin embargo, todavía quedan algunos temas para explorar. Por otro lado, el mundo enológico está tomando en consideración a otras especies de no-*cerevisiae* y no-*Saccharomyces*, las cuales no están muy bien estudiadas, especialmente en lo relativo al metabolismo del nitrógeno. Para estas especies, es de gran importancia comprender su preferencia por las fuentes de nitrógeno, el requerimiento de nitrógeno y los mecanismos moleculares subyacentes que rigen este metabolismo del nitrógeno en una de estas especies en concreto. Este conocimiento permitirá tener un mejor control de la fermentación. Por lo tanto, el objetivo de mi tesis es fortalecer y profundizar el conocimiento que hemos obtenido sobre *S. cerevisiae* y explorar el campo desconocido de las levaduras de vino no-*cerevisiae* y no-*Saccharomyces*. A partir del resultado de la tesis se puede obtener una visión general completa del metabolismo del nitrógeno por especies de levaduras relacionadas con el vino.

Resultados y discusión

Capítulo 1

En la enología moderna, una práctica común es la inoculación de levadura seleccionad en el mosto de uva para garantizar la calidad del vino. Los enólogos eligen las cepas de levadura para que coincida con el potencial del mosto de uva y el tipo de vinos. La concentración de nitrógeno es una de las características más importantes del mosto que se debe determinar antes de la fermentación. Los requerimientos de nitrógeno de *S. cerevisiae* durante la fermentación del vino dependen mucho de la cepa. Para explorar los requisitos de nitrógeno de una colección de 28 levaduras vínicas industriales, se aplicaron diferentes métodos, centrados en la capacidad del crecimiento y de la fermentación con distintas concentraciones de nitrógeno.

Primero, las capacidades de crecimiento y fermentación de todas las 28 cepas se determinaron en mostos sintéticos con 3 diferentes concentraciones (60, 140, and 300 mg/L YAN). Las cepas tienen tasas de crecimiento más altas conforme aumenta la concentración de nitrógeno. Entre el conjunto de cepas, las que mostraron un buen rendimiento con bajas concentraciones de nitrógeno, además de un rendimiento promedio con altas concentraciones de nitrógeno, se consideraron cepas con baja demanda de nitrógeno. Por otro lado, las cepas que mostraron escaso crecimiento y capacidad de fermentación cuando la concentración de nitrógeno era baja, pero que mostraron una capacidad sobresaliente con un alto contenido de nitrógeno, fueron consideradas cepas con alta demanda de nitrógeno.

Después de la determinación del crecimiento y la capacidad de fermentación de una gran cantidad de cepas, se llevaron a cabo fermentaciones en un volumen mayor (80 mL) con cuatro cepas seleccionadas de bajo requerimiento de nitrógeno y tres cepas de alto requerimiento de nitrógeno,

para confirmar aún más sus necesidades de nitrógeno. Los resultados nos ayudaron a reducir los candidatos a 5 cepas, de las cuales tres de ellas son cepas que requieren poco nitrógeno y dos son cepas que requieren mucho nitrógeno.

A continuación, se realizaron fermentaciones en competencia entre las cepas seleccionadas. Para ello, se inocularon las parejas de cepas con alta y baja demanda de nitrógeno en mosto sintético con baja o alta concentración de nitrógeno. Nuestra expectativa era que cuando las fermentaciones se llevaran a cabo en mosto de baja concentración de nitrógeno, la cepa de baja demanda de nitrógeno sería más competitiva con respecto a las cepas de alta demanda de nitrógeno, y viceversa, en el mosto de alta concentración de nitrógeno, las cepas de alta demanda de nitrógeno competirían con la cepa de baja demanda de nitrógeno durante la fermentación. Este método nos permite excluir las influencias de otros factores y solo enfocarnos en la influencia del nitrógeno. Estas fermentaciones en competencia nos han permitido confirmar que la cepa P17 es una cepa con alta necesidad de nitrógeno y la cepa P20 es una cepa con baja necesidad de nitrógeno.

La concentración de nitrógeno también influye en la producción de biomasa. Las cepas de baja demanda de nitrógeno produjeron una mayor cantidad de biomasa en los mostos de uva sintéticos limitados en nitrógeno, mientras que las cepas de alta demanda de nitrógeno lograron un mayor rendimiento de biomasa cuando la concentración de YAN fue superior a 100 mg/L. Además, en un experimento alternativo, las necesidades de nitrógeno de ambas cepas durante la fase estacionaria fueron igualmente testada mediante la determinación de la cantidad de nitrógeno que era necesaria añadir para mantener la velocidad máxima de fermentación constante. Como se esperaba,

la cepa P20, de baja demanda de nitrógeno, solo necesitaba la mitad de la cantidad de nitrógeno que la cepa P17, de alta demanda de nitrógeno, para mantener una fermentación a velocidad constante.

Para determinar las bases genéticas que condicionan los diferentes requerimientos de nitrógeno en las distintas cepas vínicas, se llevó a cabo la secuenciación completa del genoma de las cepas seleccionadas con alto y bajo demanda de nitrógeno. Se observaron muchas diferencias entre el genoma de las dos cepas, incluidas las diferencias en heterocigosidad, un buen número de sustituciones de aminoácidos entre proteínas ortólogas, especialmente en los genes implicados en el sistema de reciclaje de nitrógeno. De los genes que muestran divergencia de aminoácidos, *GCN1*, un regulador positivo de la quinasa Gcn2p, y que forma un complejo con Gcn20p, fue seleccionado como nuestro gen de interés. El intercambio de alelos entre la cepa P20 y P17 se realizó aplicando el método de edición genómica mediado por CRISPR/Cas9, para determinar si este gen es responsable de las diferencias en los requerimientos de nitrógeno de estas dos cepas. Sin embargo, el resultado demostró que el intercambio de alelos no tenía influencias significativas en el comportamiento de fermentación en medios de baja concentración de nitrógeno.

Este estudio combinó diferentes métodos para explorar fenotípicamente el requerimiento de nitrógeno de un conjunto de cepas comerciales de *S. cerevisiae*. Las cepas de baja y alta necesidad de nitrógeno se seleccionaron en base a la alteración en el comportamiento de crecimiento y fermentación a diferentes concentraciones de nitrógeno. Sus requerimientos de nitrógeno se verificaron mediante la fermentación competitiva, la producción de biomasa y la fermentación a velocidad constante a diferentes concentraciones de nitrógeno. La información del genoma de estas dos cepas reveló grandes

diferencias en términos de heterocigosidad y en las sustituciones de aminoácidos entre proteínas ortólogas, lo que subraya la importancia del sistema de reciclaje celular en condiciones de restricción de nitrógeno. La técnica de ingeniería genética CRISPR/Cas9 se implementó para investigar las bases genéticas de las diferencias en los requisitos de nitrógeno. Sin embargo, el intercambio de alelos del gen *GCN1* de la cepa de baja necesidad de nitrógeno a la cepa de alta necesidad de nitrógeno no mostró ningún impacto significativo sobre el comportamiento de fermentación en la fermentación limitada en nitrógeno. Nuestro estudio proporciona una guía para el análisis integral de los requerimientos de nitrógeno por levadura durante la fermentación. En el futuro, se requieren análisis genéticos adicionales para obtener una comprensión integral de esta característica compleja de la levadura.

Capítulo 2

La selección de levaduras con bajo requerimiento de nitrógeno es una necesidad actual en la vinificación. En este trabajo, analizamos los requerimientos de nitrógeno de las cepas pertenecientes a las especies criotolerantes *S. uvarum*, *S. eubayanus* y *S. kudriavzevii*, para evaluar su potencial para llevar a cabo la fermentación de mostos de uva con bajo contenido de nitrógeno.

En primer lugar, la tasa de crecimiento de cepas de diferentes especies se determinó con 13 concentraciones diferentes de nitrógeno (20, 40, 60, 80, 100, 120, 140, 160, 180, 200, 220 and 300 mg/L YAN). En general, las cepas de *S. eubayanus* tienen tasas de crecimiento más altas, con todas las concentraciones de nitrógeno, que las otras especies, mientras que las cepas de *S. kudriavezevii* tuvieron la tasa de crecimiento más baja. Para evaluar si

el comportamiento evidenciado en el crecimiento de la levadura también se observa en el comportamiento fermentativo de las levaduras, seleccionamos cepas con requisitos de nitrógeno altos, bajos e intermedios para ser evaluadas en fermentaciones con concentraciones de nitrógeno baja (60 mg/L YAN), alta (300 mg/L YAN) e intermedia (140 mg/L YAN). Las cepas de *S. eubayanus* se vieron menos afectadas por el aumento de la concentración de nitrógeno, mostrando valores similares en velocidad máxima de fermentación ($V_{máx}$) obtenidos tanto en 140 mg/L como en 300 mg/L. La $V_{máx}$ de las cepas patagónicas de *S. uvarum* (cepas NPCC) mejoró enormemente conforme aumentaba la concentración de nitrógeno, obteniéndose un valor especialmente alto a 300 mg/L. La mayor influencia de la concentración de nitrógeno en la $V_{máx}$ de fermentación se observó en las cepas vínicas *S. uvarum* y *S. kudriavzevii*, que mostraron valores de $V_{máx}$ alrededor del doble en concentraciones altas de nitrógeno en comparación con la observada a 60 mg/L YAN.

Para su potencial de aplicación industrial, se seleccionó una cepa de *S. eubayanus* (NPCC 1285) y una cepa de *S. uvarum* (NPCC 1317), que muestran el mejor rendimiento a bajas concentraciones de nitrógeno (especialmente evidente en los ensayos de crecimiento) para realizar la fermentación en un cultivo mixto con nuestra cepa de referencia de *S. cerevisiae* T73 , con el fin de evaluar su capacidad de competencia en mostos de uva sintéticos con diferentes concentraciones de nitrógeno (60 y 300 mg/L YAN) y a diferentes temperaturas (12 ºC, 20 ºC y 28 ºC). Nuestro resultado mostró que tanto la temperatura de fermentación como las concentraciones de nitrógeno tienen influencias significativas en la cinética de fermentación y en la competitividad de las cepas. Las cepas criotolerantes dominaron las fermentaciones realizadas a 12 ºC, mientras que *S. cerevisiae* prevaleció a

28 ºC, independientemente de la concentración de nitrógeno. A temperatura intermedia, 20 ºC, los cultivos mono y mixtos de *S. eubayanus* mostraron el mejor comportamiento fermentativo, especialmente con concentraciones bajas e intermedias de nitrógeno.

En resumen, las especies de *Saccharomyces* criotolerantes, en particular *S. eubayanus*, podrían ser herramientas interesantes para evitar los bloqueos de fermentación causados por el bajo contenido de nitrógeno en los mostos de uva, especialmente si estas fermentaciones se llevan a cabo a temperaturas por debajo del óptimo para *S. cerevisiae*.

Capítulo 3

La fermentación a temperaturas más bajas se está volviendo cada vez más habitual porque los compuestos volátiles pueden conservarse mejor y, por lo tanto, proporciona al vino notas más frutales y frescas. El problema de esta estrategia es que el tiempo de fermentación se alarga, haciendo que repercuta en la capacidad productiva de las bodegas, y llegando incluso a paradas de fermentación, debido a deficiencias en la adaptación de *S. cerevisiae* a estas temperaturas. Otro problema al que se enfrentan los enólogos es la deficiencia de nitrógeno en el mosto, que provoca fermentaciones muy lentas e incluso paradas antes del consumo total de azúcares.

En nuestro estudio anterior, comparamos los requerimientos de nitrógeno de las cepas patagónicas de *S. eubayanus* y *S. uvarum* con *S. cerevisiae* y nuestros resultados revelaron que ambas especies, especialmente las cepas de *S. eubayanus*, tienen relativamente menores requerimientos de nitrógeno que la cepa de control de *S. cerevisiae*. Aunque revelan algunos caracteres beneficiosos, cuando las cepas criotolerantes se usaron como cultivo puro, a veces no pueden completar las fermentaciones con éxito debido a su baja

tolerancia al etanol. Por lo tanto, el objetivo de nuestro estudio fue mejorar la actividad de fermentación de la levadura a baja temperatura y una concentración limitada de nitrógeno mediante la construcción de híbridos entre *S. cerevisiae* y las cepas criotolerantes con bajo requerimiento de nitrógeno de *S. eubayanus* y *S. uvarum*.

En este estudio, aplicamos el método de hibridación directa de célula a célula, que es simplemente mezclar cultivos celulares de dos padres haploides estables seleccionados de signo sexual distinto (a y α). A pesar de que esto requiere una transformación genética, lo que implica que el híbrido resultante se clasifica como un "Organismo Modificado Genéticamente" (OMG), utilizamos este método de hibridación directo y rápido. El objetivo en esta etapa era obtener una prueba de concepto de que la hibridación de *S. cerevisiae* con *S. eubayanus* y *S. uvarum* era un método factible para obtener cepas con buen rendimiento de fermentación a baja temperatura y condiciones limitadas de nitrógeno.

La capacidad de fermentación de las cepas híbridas se probó en cuatro condiciones, combinando dos temperaturas, 12 °C y 28 °C, y dos concentraciones de nitrógeno, 60 mg/L y 300 mg/L. Las cepas utilizadas en estas fermentaciones fueron cepas parentales *S. cerevisiae* (*Sc*), *S. eubayanus* (*Se*) y *S. uvarum* (*Su*) y dos cepas híbridas, *Sc*x*Se* y *Sc*x*Su*. Para fines de comparación, también se llevaron a cabo las fermentaciones de cultivo mixto entre *S. cerevisiae* y las cepas parentales criotolerantes (*Sc*+*Se* y *Sc*+*Su*).

Los resultados de fermentación indicaron que 12 °C y 60 mg/L de YAN formaron una condición extremadamente estresante y ninguna de las cepas o combinaciones de cepas probadas pudieron completar las fermentaciones dentro de las 500 h del ensayo. A 28 °C, *S. cerevisiae* tuvo un mejor

rendimiento de fermentación que las cepas criotolerantes, que no pudieron fermentar completamente todos los azúcares con 60 mg / L de YAN. Sin embargo, las cepas híbridas mostraron un excelente rendimiento de fermentación, e incluso excedieron positivamente al comportamiento de sus cepas parentales, al completar las fermentaciones en el menor tiempo en todas las condiciones.

Al final de las fermentaciones, se analizaron los principales metabolitos. No se observaron diferencias significativas para los rendimientos de etanol en la mayoría de las condiciones de fermentación. Sin embargo, se observaron diferencias significativas para los rendimientos de glicerol para las diversas condiciones analizadas. En términos generales, *Sc* era un productor bajo en glicerol, mientras que las cepas criotolerantes dieron los mayores rendimientos de glicerol para todas las condiciones. Curiosamente, en condiciones de bajo contenido de nitrógeno a 28 ºC, las cepas híbridas lograron una producción de glicerol significativamente mayor que su cepa parental *Sc*, pero no se encontraron diferencias significativas en las fermentaciones realizadas con 300 mg/L YAN. Además, las cepas híbridas produjeron cantidades significativamente más pequeñas de ácido acético que *Sc*, especialmente cuando el nitrógeno estaba limitado en el mosto sintético (SM). Diferentes cepas también mostraron diversas capacidades en la producción de compuestos volátiles. Las cepas híbridas produjeron grandes cantidades de ésteres de acetato y alcoholes superiores, lo que aumenta la intensidad y la complejidad del aroma en el vino.

Por lo tanto, la hibridación interespecífica entre cepas criotolerantes con bajo contenido de nitrógeno y *S. cerevisiae* es una solución potencial para fermentaciones a baja temperatura o bajo contenido de nitrógeno.

Capítulo 4

Las levaduras no-*Saccharomyces* relacionadas con el vino son cada vez más utilizadas en la práctica enológica por su capacidad de conferir al vino un aroma más complejo y satisfactorio, pero su metabolismo sigue siendo desconocido. Este estudio exploró el perfil de utilización de nitrógeno de tres especies habituales no-*Saccharomyces*: *Torulaspora delbrueckii*, *Metschnikowia pulcherrima* y *Metschnikowia fructicola*. Se investigaron las preferencias de fuente de nitrógeno para apoyar el crecimiento y la fermentación, así como el orden de captación de diferentes fuentes de nitrógeno durante la fermentación del vino.

En primer lugar, las preferencias de fuente de nitrógeno de diferentes especies de levadura se determinaron llevando a cabo experimentos de crecimiento y fermentación en mosto sintético que contenía una sola fuente de nitrógeno. Dependiendo de su capacidad para mantener el crecimiento y la fermentación, los diferentes compuestos de nitrógeno se clasificaron como fuentes de nitrógeno preferidas, intermedias y no preferidas. En general, *S. cerevisiae* tuvo un $\mu_{máx}$ más alto que las cepas no-*Saccharomyces* cuando se cultivaron en buenas fuentes de nitrógeno, mientras que las cepas que no son de *Saccharomyces* mostraron mejores rendimientos de crecimiento que *S. cerevisiae* en las fuentes de nitrógeno más pobres (prolina, cisteína, lisina e histidina). El resultado del crecimiento mostró que amonio, glutamato, glutamina, aspartato, asparagina, GABA, arginina, alanina, leucina y valina se clasificaron como buenas fuentes de nitrógeno para ambos *S. cerevisiae*, y las especies no *Saccharomyces*: *Torulaspora delbrueckii*, *Metschnikowia pulcherrima* y *Metschnikowia fructicola*. Por otra parte, la cisteína, la citosina, la citrulina, la lisina, la glicina, la tirosina y la histidina mantuvieron poco el crecimiento de todas las cepas. Sorprendentemente, la prolina, que siempre

se consideró una fuente de nitrógeno no asimilable para las cepas de *S. cerevisiae* en condiciones anaeróbicas, se agrupó con las fuentes intermedias de nitrógeno porque las cepas no-*Saccharomyces*, especialmente las cepas de *Metschnikowia*, usaron prolina eficientemente para el crecimiento. Mientras que las cepas de *T. delbrueckii* y *S. cerevisiae* compartían las mismas preferencias de fuente de nitrógeno, *Metschnikowia sp.* mostró una menor capacidad para usar eficientemente las fuentes de nitrógeno preferidas para las otras especies, pero pudieron asimilar una gama más amplia de aminoácidos. Curiosamente, aunque las cepas no-*Saccharomyces*, especialmente las cepas *Metschnikowia*, mostraron menos capacidad de crecimiento que *S. cerevisiae*, las cepas no-*Saccharomyces* mostraron aproximadamente una fase de latencia de 10 horas más corta que *S. cerevisiae* para más de la mitad de las fuentes de nitrógeno individuales. Como era esperable, se observó una correlación generalmente buena entre la tasa de crecimiento máxima y la tasa de fermentación máxima para cada fuente de nitrógeno, a excepción de algunas fuentes pobres.

La utilización secuencial de diferentes fuentes de nitrógeno por distintas especies de levadura durante la fermentación proporciona información valiosa para el cultivo mixto o la fermentación secuencial. Durante la fermentación, *S. cerevisiae* y *T. delbrueckii* agotaron rápidamente las fuentes de nitrógeno en el mosto sintético con 140 o 300 mg/L de YAN. Sin embargo, las cepas de *Metschnikowia* no pudieron consumir todo el nitrógeno dentro de las 48 horas posteriores a la fermentación. Las cepas que no son de *Saccharomyces* consumieron diferentes fuentes de nitrógeno en un orden similar al de *S. cerevisiae*, pero con menos velocidad de consumo. Además, cuando todas las fuentes de nitrógeno se suministraron en la misma cantidad, su orden de asimilación se vio afectado de manera similar tanto para las cepas

de *S. cerevisiae* como para las no-*Saccharomyces*. Bajo esta condición, la tasa de consumo de las cepas no *Saccharomyces* y *S. cerevisiae* fue comparable.

En general, este estudio amplía nuestra comprensión sobre las preferencias y las tasas de consumo de las fuentes individuales de nitrógeno por las levaduras no-*Saccharomyces* investigadas en un entorno de fermentación vínica. Este conocimiento proporciona información útil para una explotación más eficiente de las cepas no-*Saccharomyces* que mejora el manejo de la fermentación del vino.

Capítulo 5

Después de la determinación de la preferencia de fuente de nitrógeno y el orden de asimilación de la fuente de nitrógeno por cepas no-*Saccharomyces*, también es de gran importancia comprender el metabolismo intracelular de las fuentes de nitrógeno por estas cepas no-*Saccharomyces*. En las células de levadura, el metabolismo del nitrógeno está regulado por varios mecanismos diferentes. Una vez dentro de las células, los aminoácidos pueden incorporarse directamente en aminoácidos proteinógenos o degradarse para la síntesis *de novo* de otros aminoácidos o para la producción de compuestos volátiles a través de la vía de Ehrlich. En este estudio, un análisis cuantitativo utilizando los tres compuestos nitrogenados más importantes en el mosto, amonio, arginina y glutamina, marcados con ^{15}N, y dos aminoácidos degradados a través de la vía Ehrlich, leucina y valina, marcadas con ^{13}C, fueron utilizados para revelar el patrón de metabolismo del nitrógeno de dos especies no-*Saccharomyces*, *T. delbrueckii* y *M. pulcherrima*.

En ambas cepas, el consumo de muchos aminoácidos en el mosto no es suficiente para cumplir con el requisito anabólico, por lo tanto, es necesaria la síntesis *de novo* de estos aminoácidos. Como las fuentes de nitrógeno más

abundantes en el mosto sintético, el amonio, la arginina y la glutamina se catabolizaron para la síntesis de otros aminoácidos. Independientemente de la fuente de nitrógeno marcada, se recuperó el marcado isotópico ^{15}N en todos los aminoácidos proteinógenos medidos, incluso si la cantidad de aminoácidos consumida fue mayor que los requisitos anabólicos. El 78 y el 80% de los aminoácidos proteinógenos de *M. pulcherrima* y *T. delbrueckii* se sintetizaron a partir de un conjunto común de nitrógeno. En *T. delbrueckii*, el amonio fue el principal contribuyente a la síntesis *de novo* de otros aminoácidos, seguido de glutamina y luego arginina. Sin embargo, para *M. pulcherrima*, la glutamina fue la mayor contribuyente a la síntesis de aminoácidos *de novo*, especialmente durante la fase de crecimiento exponencial. El enriquecimiento isotópico para cada aminoácido proteinogénico cambió durante las diferentes etapas de la fermentación, con patrones que dependen del aminoácido proteinogénico, la cepa y la fuente de nitrógeno marcada. En general, el mayor enriquecimiento isotópico se midió en glutamato y aspartato, con una proporción de síntesis *de novo* a partir de amonio, glutamina y arginina que representa aproximadamente el 70% y 75% de la cantidad total de estos aminoácidos proteinogénicos para *M. pulcherrima* y *T. delbrueckii*, respectivamente. La distribución de la arginina consumida en la red metabólica fue bastante diferente de la del amonio y la glutamina, que se recuperaron eficientemente en las proteínas en las primeras etapas de crecimiento. El enriquecimiento isotópico de la arginina proteinógena fue alto para ambas cepas, lo que indicó que el principal origen metabólico de la arginina proteinogénica es la incorporación directa de la arginina consumida, con un bajo aporte de síntesis *de novo*. Sin embargo, al final de la fase de crecimiento, una gran parte de la arginina consumida no se recuperó en proteínas, sino como arginina. Esto puede deberse a un

almacenamiento de la arginina intracelularmente como reserva de nitrógeno, tal como se ha demostrado anteriormente.

El destino del esqueleto de carbono de los aminoácidos de cadena ramificada valina y leucina se exploró usando aminoácidos marcados con ^{13}C. Se midió la cantidad de carbono marcado en leucina proteinogénica y valina para determinar la proporción de estas moléculas que se originan de la incorporación directa de sus respectivos aminoácidos consumidos. Para ambas cepas, se observó un bajo enriquecimiento isotópico de valina proteinógena, lo que indica una incorporación directa limitada de valina en proteínas y una importante contribución de la síntesis *de novo* a través de precursores del metabolismo central del carbono (CCM). Por otro lado, una gran fracción de leucina consumida se incorporó a la leucina proteinogénica en ambas levaduras. Sin embargo, la contribución de CCM seguía siendo la principal fuente de carbono para la síntesis de leucina proteinogénica.

La valina y la leucina también proporcionan la columna vertebral de carbono como precursores para la producción de aroma. El catabolismo de valina a través de la vía de Ehrlich representó una gran parte del destino de estos aminoácidos para las dos especies estudiadas. Para *M. pulcherrima*, más del 38% de la valina consumida se dirigió hacia la producción de compuestos volátiles derivados del α-cetoisovalerato (isobutanol, ácido isobutírico). Sin embargo, la distribución del flujo a través de la red metabólica desde la valina consumida en *T. delbrueckii* fue diferente. La mayor parte del α-cetoisovalerato producido a partir del catabolismo de la valina consumida se dirigió hacia la producción de α-cetoisocaproato. Además, una gran fracción de α-cetoisocaproato de la asimilación de valina, se dirigió hacia la producción de leucina proteinógena, mientras que la formación de alcohol isoamílico fue limitada. Dado que la leucina se usó principalmente

directamente como bloques de construcción de proteínas, solo se catabolizó una proporción limitada de leucina consumida. La proporción de leucina consumida dirigida a la producción de alcohol isoamílico fue mayor en *M. pulcherrima* que en *T. delbrueckii*.

En general, en *T. delbrueckii*, las fuentes de nitrógeno consumidas se dirigieron principalmente hacia la síntesis *de novo* de aminoácidos proteinogénicos, a expensas de la producción de compuestos volátiles. Este patrón de redistribución estaba en línea con el fenotipo de alta producción de biomasa de esta especie. Por el contrario, en *M. pulcherrima* que mostró capacidades de crecimiento más débiles, una mayor proporción de aminoácidos consumidos fueron catabolizados para la producción de alcoholes superiores a través de la vía de Ehrlich. Esta descripción general de la redistribución de nitrógeno en *T. delbrueckii* y *M. pulcherrima* proporciona información valiosa para un mejor manejo de la fermentación coinoculada o secuencial, que combina estas especies con *S. cerevisiae*.

Conclusiones

1. Las necesidades de nitrógeno en *S. cerevisiae* son específicas de cada cepa y la competitividad de cada una de ellas durante la fermentación del vino puede estar condicionada por la disponibilidad de nitrógeno en el mosto de uva.

2. Las cepas con alta demanda de nitrógeno mostraron un menor rendimiento de biomasa y necesitaron más nitrógeno para mantener una tasa de fermentación constante en la fase estacionaria.

3. La información del genoma de nuestras cepas seleccionadas con baja y alta demanda de nitrógeno reveló grandes diferencias en términos

de heterocigocidad, y subrayó la importancia del sistema de reciclaje celular en condiciones de restricción de nitrógeno. Sin embargo, todavía se necesitan más análisis genéticos para comprender plenamente la base molecular que determina esta compleja característica de la levadura.

4. Tanto la concentración de nitrógeno en el medio como la temperatura de fermentación influyen en la capacidad competitiva de las cepas de levadura criotolerantes *S. eubayanus* y *S. uvarum* en cultivo mixto con *S. cerevisiae*.

5. La cepa de *S. eubayanus* de baja demanda de nitrógeno seleccionada tiene un mejor rendimiento de fermentación con condiciones de bajo nitrógeno y baja temperatura en comparación con las cepas de *S. uvarum* y *S. cerevisiae*.

6. Las cepas híbridas interespecíficas de *S. cerevisiae* y *S. eubayanus* o *S. uvarum* con bajo contenido de nitrógeno seleccionado tienen una mayor capacidad de fermentación en condiciones de baja temperatura o fermentación de bajo nitrógeno en comparación con las cepas parentales.

7. Además de una mayor capacidad de fermentación, en condiciones de baja temperatura o baja concentración de nitrógeno, las cepas híbridas contribuyen a la calidad del vino al producir una menor cantidad de ácido acético y una mayor cantidad de compuestos volátiles que las cepas parentales.

8. Las cepas híbridas consumen fuentes de nitrógeno más rápidamente que las cepas parentales, lo que indica una mayor capacidad de

captación de los nutrientes. Bajo la condición de limitación de nitrógeno, esta estrategia podría garantizar un crecimiento y un tamaño poblacional óptimos.

9. *Metschnikowia sp*. tienen una tasa de crecimiento y capacidad fermentativa más baja que *T. delbrueckii* y *S. cerevisiae.* Sin embargo, pueden usar una gama más amplia de aminoácidos, especialmente algunas fuentes pobres de nitrógeno para *S. cerevisiae*.

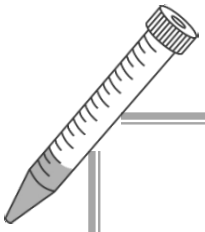
10. *T. delbrueckii* tiene una tasa de crecimiento alta y una capacidad fermentativa que es similar al rendimiento de *S. cerevisiae*. Además, también tiene una preferencia similar por las fuentes de nitrógeno, con la excepción de la lisina que puede ser catabolizada por *T. delbrueckii,* pero no puede ser utilizada por *S. cerevisiae*.

11. *T. delbrueckii* y *M. pulcherrima* tienen un orden de asimilación de las distintas fuentes de nitrógeno similar al de *S. cerevisiae*. La proporción inicial de fuentes de nitrógeno en el medio tiene una influencia importante en el orden de asimilación de la mismas.

12. En *T. delbrueckii* y *M. pulcherrima*, las principales fuentes de nitrógeno consumidas, amonio, arginina y glutamina, se catabolizan principalmente para la síntesis *de novo* de otros aminoácidos y cumplir así con los requisitos anabólicos.

13. Los aminoácidos de cadena ramificada consumidos (leucina y valina) fueron utilizados principalmente por *T. delbrueckii* para la síntesis *de novo* de aminoácidos proteinógenos en lugar de la formación de compuestos volátiles. Para la especie con menor capacidad de producción de biomasa, *M. pulcherrima*, se produce una mayor

cantidad de compuestos volátiles a partir de la catabolización de aminoácidos.



Annex I
Material & Methods

1. Yeast strains

The yeasts used in the present thesis including *S. cerevisiae*, non-*cerevisiae* and non-*Saccharomyces* strains are shown in Tables 1-3.

Table 1. Commercial wine strains belonging to *S. cerevisiae* species used in this work.

Strain	Source
Lalvin®ICVD254 (P1)	Lallemand Inc. (France)
Uvaferm®WAM (P2)	Lallemand Inc. (France)
Lalvin®ICVD80 (P3)	Lallemand Inc. (France)
Lalvin® Rhone2056 (P4)	Lallemand Inc. (France)
Lalvin®ICVGRE (P5)	Lallemand Inc. (France)
Lalvin®EC1118 (P6)	Lallemand Inc. (France)
Lalvin®ICVD47 (P7)	Lallemand Inc. (France)
Uvaferm®CEG (P8)	Lallemand Inc. (France)
Lalvin®Rhone2323 (P9)	Lallemand Inc. (France)
Uvaferm®BC (P10)	Lallemand Inc. (France)
Uvaferm®VRB (P11)	Lallemand Inc. (France)
Uvaferm®43 (P12)	Lallemand Inc. (France)
CrossEvolution® (P13)	Lallemand Inc. (France)
Lalvin®71B (P14)	Lallemand Inc. (France)
Lalvin®BM45 (P15)	Lallemand Inc. (France)
Enoferm®M1 (P16)	Lallemand Inc. (France)
Enoferm®M2 (P17)	Lallemand Inc. (France)

Uvaferm®BDX (P18)	Lallemand Inc. (France)
Uvaferm®CM (P19)	Lallemand Inc. (France)
Lalvin®ICVD21 (P20)	Lallemand Inc. (France)
Lalvin®Rhone2226 (P21)	Lallemand Inc. (France)
Lalvin®CY3079 (P22)	Lallemand Inc. (France)
P23	Lallemand Inc. (France)
P24	Lallemand Inc. (France)
P25	Lallemand Inc. (France)
P26	Lallemand Inc. (France)
P27	Lallemand Inc. (France)
T73 (P28)	Lallemand Inc. (France)

The enological features of the strains can be obtained from the company's website (http://www.lallemandwine.com)

Table 2. Non-*cerevisiae* strains used in this work.

Species	Strain	Origin	Source
S. eubayanus	NPCC1282	Wild	Rodr ǵuez et al., 2014
	NPCC1283	Wild	Rodr ǵuez et al., 2014
	NPCC1284	Wild	Rodr ǵuez et al., 2014
	NPCC1285	Wild	Rodr ǵuez et al., 2014
	NPCC1286	Wild	Rodr ǵuez et al., 2014
	NPCC1287	Wild	Rodr ǵuez et al., 2014

	NPCC1291	Wild	Rodr ǵuez et al., 2014
	NPCC1292	Wild	Rodr ǵuez et al., 2014
	NPCC1294	Wild	Rodr ǵuez et al., 2014
	NPCC1296	Wild	Rodr ǵuez et al., 2014
	NPCC1297	Wild	Rodr ǵuez et al., 2014
	NPCC1301	Wild	Rodr ǵuez et al., 2014
	NPCC1302	Wild	Rodr ǵuez et al., 2014
S.uvarum	NPCC1288	Wild	Rodr ǵuez et al., 2014
	NPCC1289	Wild	Rodr ǵuez et al., 2014
	NPCC1290	Wild	Rodr ǵuez et al., 2014
	NPCC1293	Wild	Rodr ǵuez et al., 2014
	NPCC1298	Wild	Rodr ǵuez et al., 2014
	NPCC1309	Chicha	Rodr ǵuez et al., 2017
	NPCC1314	Chicha	Rodr ǵuez et al., 2017
	NPCC1317	Chicha	Rodr ǵuez et al., 2017
	NPCC1321	Chicha	Rodr ǵuez et al., 2017
	NPCC1322	Chicha	Rodr ǵuez et al., 2017
	NPCC1323	Chicha	Rodr ǵuez et al., 2017
	NPCC1417	Cider	Rodr ǵuez et al., 2017

	NPCC1418	Cider	Rodr ǵuez et al., 2017
	NPCC1419	Cider	Rodr ǵuez et al., 2017
	CBS7001	Wild	Centraal Bureau voor Shimmelcultures
	Velluto BMV58	Wine	Lallemand Inc. (France)
	CECT12600	Wine	The Spanish Type Culture Collection
S. kudriavzevii	CR85	Wild	Lopes et al., 2010
	CR90	Wild	Lopes et al., 2010

Table 3. Non-*Saccharomyces* strains used in this work.

Species	**Strain**	**Source**
T. delbrueckii	Biodiva	Lallemand Inc. (France)
M. pulcherrima	Flavia	Lallemand Inc. (France)
M. fructicola	Gaia	Lallemand Inc. (France)
M. fructicola	LYCC7706	Lallemand Inc. (France)

2. Culture media

2.1 YPD (Yeast peptone dextrose) medium

Glucose	20 g/L
Bacteriological peptone	20 g/L
Yeast extract	10 g/L
Agar (for solid media preparation)	20 g/L

2.2 Sporulation medium: Potassium acetate

Potassium acetate	10 g/L
Agar	20 g/L

2.3 Synthetic grape must (SM)

Synthetic grape must adapted from Riou et al., (1997)

To mimic the wine fermentation conditions synthetic must medium was used that reproduces a standard natural must composition. This medium is very useful to make laboratory micro-fermentations in a reproducible manner.

Media composition for 1 L

<u>Sugars</u>

Glucose	100 g
Fructose	100 g

Organic acids

Malic acid	5 g
Citric acid	0.5 g
Tartaric acid	3 g

Mineral salts

KH_2PO_4	0.75 g
$MgSO_4\ 7H_2O$	0.25 g
$CaCl_2\ H_2O$	0.155 g
NaCl	0.2 g
NH_4Cl	0.46 g (for 300 mg/L YAN)

Add the previous prepared stocking solution of:

Amino acids	13.09 mL (for 300 mg/L YAN)
Oligoelements	1 mL
Vitamins	10 mL

YAN concentration of SM can be adjusted by the amount of NH_4Cl and amino acids supplied.

pH = 3.3 (adjustwith pellets of NaOH)

Add distilled water (up to 1 L).

Filter the whole volume using a 0.22 µm filter.

Amino acids stocking solution (1 L)

Tyrosine (Tyr)	1.5 g
Tryptophan (Trp)	13.4 g
Isoleucine (Ile)	2.5 g
Aspartic acid (Asp)	3.4 g
Glutamic acid (Glu)	9.2 g
Arginine (Arg)	28.3 g
Leucine (Leu)	3.7 g
Threonine (Thr)	5.8 g
Glycine (Gly)	1.4 g
Glutamine (Gln)	38.4 g
Alanine (Ala)	11.2 g
Valine (Val)	3.4 g
Methionine (Met)	2.4 g

Phenylalanine (Phe)	2.9 g
Serine (Ser)	6 g
Histidine (His)	2.6 g
Lysine (Lys)	1.3 g
Cysteine (Cys)	1.5 g
Proline (Pro)	46.1 g

Keep at -20 ℃

Vitamins stocking solution (1 L)

Myo-inositol	2 g
Calcium pantothenate	15 g
Thiamine hydrochloride	0.025 g
Nicotinic acid	0.2 g
Pyridoxine	0.025 g
* Biotin	3 mL

*(stocking biotin solution 100 mg L^{-1})

Keep at -20 ℃

Oligoelements stocking solution (1 L)

$MnSO_4$. H_20	4 g
$ZnSO_4$. $7H_2O$	4 g
$CuSO_4$. $5H_2O$	1 g
KI	1 g
$CoCl_2$. $6H_2O$	0.4 g
H_3BO_3	1 g
$(NH_4)_6Mo_7O_{24}$	1 g

Keep at -20 ℃

3. Culture techniques

3.1 Growth activity analysis

The growth activities were determined by inoculating yeast cells into 96-well plate containing the medium to be used. Growth was monitored by recording OD at 600 nm in a SPECTROstar Omega instrument (BMG Labtech, Offenburg, Germany). Measurements were made every half hour until the growth culture reaches the stationary phase. The measurement was taken after a pre-shaking of the plate for 20 sec.

The wells of the microplate were filled with 0.25 mL of media and inoculated with an initial OD of approximately 0.1 (inoculum level of 10^6 cells mL^{-1}). All experiments were carried out at least in triplicate. Growth parameters

were calculated from each treatment by directly fitting OD measurements versus time to the reparametised Gompertz equation proposed by Zwietering *et al*., (1990):

$$y=D*\exp\{-\exp[((\mu_{max}*e)/D)*(\lambda-t))+1]\}$$

where $y=\ln(OD_t/OD_0)$, OD_0 is the initial OD and OD_t is the OD at time t; $D=\ln(OD_t/OD_0)$ is the asymptotic maximum, μ_{max} is the maximum specific growth rate (h^{-1}), and λ the lag phase period (h).

3.2 Area under the curve determination

The overall yeast growth was estimated as the area under the OD vs. time curve. This parameter was calculated by the R statistical software, v. 3.0., with the growth curve package (Sprouffske, 2018).

3.3 Micro-scale fermentations

To determine the fermentation characters of a large set of yeast strains, micro-scale fermentations were carried out as an efficient high throughput method. Fermentations were carried out in 15 mL tubes with 10 mL of synthetic must. The screw caps of tubes were loosened to allow CO_2 to release. Around 2 x 10^6 cells/mL were inoculated. Fermentations were followed by CO_2 production, which can be represented by the weight loss of fermentation tubes. The tubes filled with 10 mL SM without inoculation were used as the controls for evaporation weight loss (EWL). The weight of fermentation tubes was determined around every 12 h. Fermentations were considered completed when tube weight stopped lowering. Fermentation curves can be obtained by plotting weight loss against time. Data were fitted to the Gompertz model as described in growth parameter analysis. The maximum fermentation rates (V_{max}) were obtained by applying a similar equation to the calculation with

μ_{max}, but by changing only the OD value to the weight loss value. The fermentation experiment was carried out in triplicate.

3.4 Laboratory-scale fermentations

Fermentations were done in laboratory-scale fermenters using 100 mL bottles filled with 80 mL of SM. 2 x 10^6 cells mL^{-1} were inoculated into SM. Fermentations were monitored by measuring density of the SM twice every day. Fermentation was considered to be completed when density was below 998 g/L.

3.5 Density measurements

Density measurement is a good fermentation monitoring approach, because the decrease density during the fermentation is directly proportional to sugar consumption. The grape must density usually is between 1070 and 1120 g L^{-1} and the wine density is between 990 and 998 g/L. The decrease of density is due to the transformation of sugars into ethanol.

To measure the density, cells were removed from the must by centrifugation. Afterwards samples were measured with Densito 30PX densitometer (Mettler Toledo, Switzerland).

The fermentation kinetics were estimated by calculating the time needed to ferment 5% (T5), 50% (T50) and 100% (T100) of the sugars in the synthetic must. The fermentation kinetics were calculated by directly fitting density measurements versus time to the four-parameter logistic equation proposed by Speers et al., (2003). The estimation was done using Sigmaplot software (Systa Software Inc. USA).

$$Pt = P_D / \{1 + e^{[-B * (t - M)]}\} + P\infty$$

where P_t is the Plato values (density values) at time t, $P\infty$ is the Plato values at equilibrium, P_D represents the changes in the Plato during the fermentation (P_0 - P_∞), B is the fermentation rate and M is the time where the exponential fermentation rate is maximal. When the data were fitted to four-parameter logistic equation, we also obtained an estimation of time for every density value. These values were used to calculate the T5, T50 and T100.

3.6 Competition fermentation

In order to determine the competitiveness of two yeast strains in the same fermentation condition, one-to-one competition fermentation can be carried out. Fermentations were performed in 80 mL SM as described previously in laboratory scale fermentation. In competition fermentation, 2×10^6 cells/mL were inoculated with 50% of each strain. At least one of the two strains should carry a selectable marker such as antibiotic marker or temperature sensitivity which allow the identification of the population during the fermentation. The percentage of each strain in the mixed culture can be determined by counting colony-forming units (CFU) on selective and non-selective plates.

3.7 Chemostat cultures (continuous cultures)

A chemostat is a growth vessel into which fresh medium is delivered at a constant rate and cells and spent medium overflow at the same rate. Thus, the culture is forced to divided to keep up with the dilution, and the system exists in a steady state where inputs match outputs (Dunham, 2010).

Continuous cultures were carried out in 0.5 L reactor (MiniBio, Applikon Biotechnology) with a working volume of 0.35 L. pH was measured online and kept constant at 3.3 by the automatic addition of 2 M NaOH and 1 M HCl. The stirrer was set at 150 rpm. The population inoculated in the chemostat

was approximately OD = 0.2. The initial inoculum came from an overnight culture in YPD at 30 ℃. Previously to start the continuous culture, cells were allowed to grow, at the same temperature than the continuous culture, through a normal growth curve to achieve enough biomass (batch phase). The dilution rate (D) was set at 0.2 h^{-1}, which corresponds to the growth rate of the cells during the steady-state.

3.8 Constant rate fermentation by nitrogen addition

Nitrogen requirements during fermentations were also determined by constant rate fermentation as described by Manginot et al. (1997, 1998). Batch fermentations were carried out at 28 ℃ in a 0.5 L bioreactor (MiniBio, Applikon, the Netherlands) with a working volume of 0.35 L. 2×10^6 cells/mL were inoculated in SM at the initial nitrogen concentration of 140 mg/L of YAN. The CO_2 production rate was monitored by a gas monitor (Multi-Gas Monitors INNOVA 1316, LumaSense Technologies) throughout fermentation. When the CO_2 production rate reached the peak value, the automatic addition of nitrogen compounds (40% NH_4Cl and 60% amino acids) was activated to maintain the CO_2 production rate at the maximum value until the residual sugar become limiting. The amount of nitrogen added to fermentation throughout this process was considered to be the nitrogen required to maintain a constant fermentation rate.

3.9 Biomass dry weight determination

Biomass dry weight (g DW L^{-1}) was determined by centrifuging a known volume of culture broth (approximately 30 units of OD_{600}) in pre-weighted tubes that were then washed with 2 volumes of distilled water and dried to constant weight at 70 ℃ for 48 h.

4. Analytical methods

4.1 HPLC analysis for sugars and fermentation metabolites

Glucose, fructose, glycerol and ethanol were analysed in supernatant samples. Analytical HPLC was carried out in a Surveyor Plus Chromatograph (Thermo Fisher Scientific, Waltham, MA) equipped with a refraction index detector, autosampler and a UV-Visible detector. Prior to injection, samples were centrifuged at 13300 rpm for 2 min, and samples were diluted and filtered through 0.22 µm pore size nylon filters (Micron Analitica, Spain). A total volume of 25 µL was injected into a HyperREZ XP Carbohydrate H+8 µm column (Thermo Fisher Scientific) assembled to its correspondent guard. The mobile phase used was 1.5 mM H_2SO_4 with a flux of 0.6 mL min^{-1} and a column temperature of 50 ºC. The concentration of each compound was calculated using external standards. Each sample was analysed in duplicate.

4.2 Nitrogen content analysis for nitrogen compounds

The analysis of concentration of amino acids and ammonium was carried out in an ultimate 3000® UPLC (Thermo Scientific, MA, USA) equipped with a UV-visible detector (Thermo Scientific, MA, USA). The HPLC analysis method is based on Gómez-Alonso et al. (2007), but with some modifications. The 400 µL samples were derivatised with 12 µL diethylethoxymethylenemalonate (DEEMM), together with 300 µL of methanol. The reactions were performed in screw-cap test tubes in an ultrasonic bath for 30 minutes, followed by heating at 80 ºC for 2 h to degrade any excess DEEMM. After derivatisation, samples were filtrated through 0.22 µm nylon syringe filters (Phenomenex, CA, USA). Chromatographic separation was performed with an Accucore® C18 LC column (Thermo Scientific, MA, USA). The binary gradient was applied (phase A: 25 mM

acetate buffer, pH 6.0, and phase B: acetonitrile) at a flow rate of 1.2 mL/min and a column temperature of 30 ℃. The gradient is shown in Table 4.

Table 4. Eluent gradient for the HPLC determination of amino acids.

Time (min)	0.0	3.0	5.0	11.0	12.5	14.0	18.0	21.0	23.0	25.0	26.0
Phase A(%)	95.0	94.0	92.0	90.0	88.0	82.0	80.0	70.0	60.0	25.0	20.0
Phase B(%)	5.0	6.0	8.0	10.0	12.0	18.0	20.0	30.0	40.0	75.0	80.0

Nitrogen compounds were identified by the retention time for the reference compounds. Quantification of nitrogen compounds was determined using the calibration graphs of the corresponding standard nitrogen compounds.

4.3 GC-FIT analysis for volatile compounds

Higher alcohols and esters were analysed by the headspace solid-phase microextraction (HS-SPME) technique using a 100 μm poly-dimethylsiloxane (PDMS) fibre (Supelco, Sigma-Aldrich, Madrid, Spain). The extraction method was the same as that described by Stribny et al. (2016). 2-heptanone (0.005%) was added as an internal standard. A TRACE GC Ultra® gas chromatograph (Thermo Scientific, MA, USA) with a flame ionization detector (FID) was used, equipped with an HP-INNOWax 30 m × 0.25 mm capillary column coated with a 0.25 μm layer of cross-linked polyethylene glycol (Agilent Technologies, CA, USA). The oven temperature programme was: 5 min at 35 ℃, 2 ℃/min to 150 ℃, 20 ℃/min to 250 ℃ and 2 min at 250 ℃. The detector temperature remained constant at 300 ℃. Chromatographs were analysed by the Chrom Quest programme. Volatile compounds were identified by the retention time for the reference compounds.

Quantification of volatile compounds was determined using the calibration graphs of the corresponding standard volatile compounds

5. Molecular techniques

5.1 DNA extraction

The extraction of yeast DNA was carried out from 3 mL overnight culture in YPD at 30 ℃, using the method described by Querol *et al*., (1992). 3 mL of the cell culture was centrifuged at 10000 rpm for 2 min and the YPD was removed. The cell pellet was washed with 1 mL of distilled water and centrifuged at 10000 rpm for 2 min to remove the water. 0.5 mL of Buffer 1 (sorbitol 0.9 M, EDTA 0.1 M pH 7.5) and 30 µL of Zymolyase (Seikagaku Corporation, Japan) were added. The samples were vortexed and the tubes were incubated at 37 ℃ for 20 min. Samples were centrifuged at 10000 rpm for 2 min to remove the supernatant. Protoplasts were re-suspended in 0.5 mL of Buffer 2 (Tris 50 mM pH 7.4, EDTA 20 mM). Afterwards 13 µL of SDS 10% was added and the tubes were incubated at 65 ℃ for 5 min. After the incubation, 0.2 mL of potassium acetate was added, the samples were mixed and incubated in ice for 5 min. Samples were centrifuged 15 min at 12000 rpm at 4 ℃ to ensure the elimination of SDS. Supernatant was added to 0.7 mL of isopropanol (v/v) and incubated at room temperature for 5 min. Samples were centrifuged 10 min at 12000 rpm at 4 ℃. Supernatant was removed and 0.5 mL of ethanol 70% was added. Samples were centrifuged 5 min at 12000 rpm at 4 ℃. Supernatant was removed and samples were dried with a vacuum pump. Finally, DNA was re-suspended in 40 µL of TE (Tris 10 mM pH 7.4, EDTA 1 mM pH 8.0).

5.2 Primers for PCR

Oligonucleotides used in this study are shown in Table 5. Primer pairs were designed using the Web Primer tool and genome sequences from the *Saccharomyces* Genome Database (SGD) (http://www.yeastgenome.org/), with the exception of K2 and K3 described by Güldener *et al.*, (1996). The sequences were sent to Invitrogen to be synthesised. Primers were used to amplify DNA in PCR.

Table 5. Primers used in this work.

Primer	Sequence 5' to 3'	Purpose
galKanMX F	AAAATTGTTAATATACCTCTATACT TTAACGTCAAGGAGAAAAAACTAT Acagctgaagcttcgtacgc	To amplify KanMX4 cassette as donor DNA to replace *GAL1*
galKanMX R	ATTATCATACAATCATTTATTAAGT AGTTGAAGCATGTATGAACTATAA Agcataggccactagtggatc	To amplify KanMX4 cassette as donor DNA to replace *GAL1*
Galcp-F	CGTTCCTGAAACGCAGATG	To check the deletion of *GAL1*
Galcp-R	CTCCTCGCGCTTGTCTACTAA	To check the deletion of *GAL1*
K3	CCTCGACATCATCTGCCC	to check the integration of *Kan*MX4 cassette
K2	GGGACAATTCAACGCGTCTG	to check the integration of *Kan*MX4 cassette
gcnKanMX donor F	GGTAGTTAGATCCGTCTCGCTTCA GTCTATGATAATCTTAcagctgaagcttcg tacgc	To amplify KanMX4 cassette as donor DNA to replace part of *GCN1*
gcnKanMX donor R	TCTTGGACACGCATTCTCCTTTCAA TTGCATCAATCACGCgcataggccactagt ggatc	To amplify KanMX4 cassette as donor DNA replace part of *GCN1*

GCN1 gRNA F	gactttATCATACATTATCAGACTCA	gRNA for CRISPR
GCN1 gRNA R	aaacTGAGTCTGATAATGTATGATaa	gRNA for CRISPR
HO-F	AGACATCGCAAACGTCACGGCTAA CTCTTACGTTATGTGCGCAGATGG CTCGTACGCTGCAGGTCGACG	To amplify the antibiotic resistance cassette to delete *HO* in pair with the reverse primer
HO-R hyg	ACTCTTATGAGGCCCGCGGACAGC ATGAAACTGTAAGATTCCGCCACA TTATATCAGATCCACTAGTGGC	To amplify the *hphMX4* cassette to delete *HO*
HO-R nat	ACTCTTATGAGGCCCGCGGACAGC ATGAAACTGTAAGATTCCGCCACA TTACTAGTGGATCTGATATC	To amplify the *natMX4* cassette to delete *HO*
Se-HOcp-F	TTGCTTCAGTCGAAGTTCG	To check the disruption of the *HO* gene of *S. eubayanus*
Se-HOcp-R	GTACCAATACGGTCACTCCA	To check the disruption of the *HO* gene of *S. eubayanus*
HOcp-F	GAGGTTTGCAGAAGCTTGTTGA	To check the disruption of the *HO* gene of *S. cerevisiae* and *S. uvarum*
HOcp-R	TTGGCGTATTTCTACTCCAGCAT	To check the disruption of the *HO* gene of *S. cerevisiae* and *S. uvarum*
MAT-F	AGTCACATCAAGATCGGTTATGG	Mating type verification
MATα-R	GCACGGAATATGGGACTACTTCG	Mating type verification
MATa-R	ACTCCACTTCAAGTAAGAGTTTG	Mating type verification

5.3 Polymerase chain reaction (PCR)

Standard DNA amplification was performed via PCR in a GenAmp PCR System 2700 (Applied Biosystems, USA) to synthesize the deletion cassettes, to check the deletion and for typing by delta elements amplification. A typical 50 µL reaction was performed, contained 1-5 µL, 0.1-100 ng DNA, 2 µL 200 µM dNTPs, 5 µL 10 µM primers, 5 µL 10 x PCR buffer, 3-5 µL 50 mM $MgCl_2$, 1 µL of Taq polymerase, 0.03 U μL^{-1} and water to complete the 50 µL. PCR products were stored at -20 ℃ until required.

5.4 Deletion cassette amplification

The deletion cassette was amplified from different plasmids (Table 6). Primers used have 50-nucleotide extensions corresponding to regions upstream of the target gene start codon (forward primer) and downstream of the stop codon (reverse primer). The Taq polymerase was used for PCR amplification. Reactions were run with an initial denaturation period of 5 min at 94 ℃, then 30 cycles consisting of denaturation at 94 ℃ for 30 sec, annealing at the correct temperature for each plasmid for 30 sec and elongation at 72 ℃ for 2 min. The amplification terminated with an extended incubation at 72 ℃ for 5 min and cooling to 4 ℃.

Table 6. Plasmids used in this work.

Plasmid	Characteristics/marker
pUG6	*kanMX4*
pAG25	*natMX4*
pAG32	*hygMX4*

5.5 Yeast transformation

Yeast transformation was performed using a lithium acetate protocol describe by Daniel Gietz and Woods, (2002). Yeast cells were inoculated in 50 mL YPD, overnight at 30 ℃ and 200 rpm. This culture was used to inoculate an OD of 0.2 in 5 mL YPD and was incubated at 30 ℃ and 200 rpm until the cells were completed at least 3 divisions (OD of approximately 0.8). Then 5 mL culture was transferred to a new tube and centrifuged at 5000 rpm for 5 min. The media was poured off and cells were resuspended in 2.5 mL of sterile water and centrifuged again. The water was removed and cells were resuspended in 100 µL of 0.1 M LiAc. Cells were pellet a top speed for 15 sec and LiAc was removed with a micropipette. Cells were resuspended in 40 µL of 0.1 M LiAc, centrifuged and LiAc was removed again with a micropipette. Then transformation mix was added in this order: 240 µL of PEG (50% w/v), 36 µL of 1 M LiAc, 50 µL of ss-DNA 2 mg mL^{-1} (previously boiled for 5 min and quickly chilled in ice) and 34 µL of deletion cassette. Each tube was vigorously vortexed until the cell pellet had been completely mixed. Tubes were incubated at 30 ℃ for 30 min, then 42 ℃ for 30-60 min. Tubes were centrifuged at 7000 rpm for 15 sec and the transformation mix was removed with a micropipette. Into each tube 1 mL of water was added and pellet was resuspended by pipetting it up and down gently. Then tubes were centrifuged at 7000 rpm for 15 sec and water was removed. Pellet was resuspended in 500 µL YPD and incubated at 30 ℃ for 3 h with a gentle shaking at 125 rpm. 200 µL (twice for each transformation) were plated onto selection plates and incubated at 30 ℃ until colonies appeared (2-3 days).

5.6 Deletion verification

The genomic DNA of possible deletion mutants was analyzed by PCR using primers upstream and downstream of the deleted region, and a combination with primers of the *Kan*MX gene (K2 and K3, Table 5). The Taq polymerase was used for PCR amplification. Reactions were run with an initial denaturation period of 5 min at 94 ℃, then 30 cycles consisting of denaturation at 94 ℃ for 30 sec, annealing at 50-55 ℃ (depending on the different primers) for 30 sec and elongation at 72 ℃ for 2-5.5 min (depending on the different PCR product length). The amplification terminated with an extended incubation at 72 ℃ for 5 min and cooling to 4 ℃.

5.7 Ascus Dissection and mating type determination

Potassium acetate medium was used to induce the sporulation of strains. The plates were incubated at 30 ℃ during 5 days. After this time, we tested that the yeast cells have sporulated by examining them under optical microscope. Yeast strains differed in their efficiency and the required time in sporulation. When we can see the tetrad of spores, we continued with the microdissection. Asci were digested with 0.5 mg/mL zymolyase at 37 ℃ for 30 minutes. The dissection of spores was done with micromanipulator equipment (Singer instruments, United Kingdom) on YPD plates. After, the spores were grown in YPD plates with antibiotic resistance. To test the mating type of each spore, we carried out a PCR with MAT, MATa and MATα primer (Huxley et al., 1990). The specific primers produce a characteristic PCR product that differentiates if the strain is a/α haploid or diploid. The size of the band is 544 bp (haploid a) or 404 bp (haploid α). If it is a diploid we observed both bands. PCR was done under the following conditions: 94 ℃ for 5 min, 30 cycles at 94 ℃ for 1 min, 58 ℃ for 2 min and 72 ℃ for 2 min, and 72 ℃ for 7 min.

5.8 Genome sequencing and analysis of two *S. cerevisiae* strains

Two of the selected strains in our study were sequenced by Illumina HiSeq with 150 bp paired-ends reads. Trimmomatic v.0.33 (Bolger, Lohse, and Usadel 2014) was used to remove adapters, while Sickle (version 1.33, available at https://github.com/najoshi/sickle) was run to read trimming by quality. *De novo* genome assemblies were obtained using SPAdes v.3.1.0 (Bankevich et al. 2012). Annotation was done by transferring the annotation from the *S. cerevisiae* S288c reference strain (R64-2-1) to our assemblies using RATT (Otto et al. 2011). Then a *de novo* gene prediction with Augustus (Stanke and Waack 2003) was performed. Finally, both annotations were merged and manually corrected.

Phylogeny reconstruction was performed using the genome sequencing data from 90 representative strains of different *S. cerevisiae* lineages (Peter et al., 2018). In all, 1732 orthologous genes were translated into proteins, aligned with MAFFT v.7.2 (Katoh and Standley, 2013) and backtranslated into codons. The concatenated codon alignment was carried out for maximum-likelihood phylogeny reconstruction using RAxML v.8.2 (Stamatakis, 2014) with 100 rapid bootstraps and the GTRGAMMA model.

To perform a chromosome copy number variation analysis, reads were mapped against the reference assembly of *S. cerevisiae* S288C (version R64-2-1) using bowtie2 v2.3.0 (Langmead and Salzberg, 2012). The position read depth was computed using an in-house Python script of a sliding-window method in which the mean mapping reads was calculated and represented in 10 kb windows along each chromosome.

A variant calling analysis was performed using FreeBayes v1.2. (Garrison and Marth 2012) with default parameters. Low quality SNPs were removed with vcftools (Danecek et al. 2011).

To analyse the differences between genomes of these two strains at the coding sequence level, pair-wise amino acid substitutions were calculated using aligned pairs of orthologous proteins between two strains. In this way, a total number of 6035 orthologs were found and analysed using an in-house Python script.

5.9 CRISPR/Cas9 sgRNA design and assembly

CRISPR/Cas9-mediated genomic editing was performed to explore the genetic basis for the differences in nitrogen requirement. CRISPR/Cas9 plasmid pWS174 (Addgene plasmid # 90961; http://n2t.net/addgene:90961; RRID: Addgene 90961) was used in our study. This plasmid contains *NatR* as a yeast marker, *KanR* as an *E. coli* marker and GFP, which is replaced with the designed guide RNA (gRNA). The 20 bp gRNA target was designed by following the instructions described in Benchling (https://www.benchling.com). A *Bsm*BI restriction site (GACT overhang at the 5' and GTTT at the 3', and an additional TT included between the 5' overhang and the gRNA sequence) was added to the target sequence. The oligonucleotides were assembled into pWS174 as described by Shaw et al., (2019). They were firstly phosphorylated by T4 PNK, by using 1 µL oligo, 10 µL ligase buffer 1 µL T4 PNK and 7 µL H_2O. The mixture is incubated for 1 h at 37 ℃. Then 10 µL of each phosphorylated oligo was mixed together and the volume was brought to 200 µL with water. The annealing was carried out by taking 50 µL of the mixture and running the following program on the thermocycler: 96 ℃ for 6 min, 0.1 ℃ per second ramp down to 23 ℃, and hold

at 23 ℃. Then the phosphorylated oligos were assembled to the vector in a 10 μL *Bsm*BI golden gate assembly. The reaction mixture containing 2 μL of annealed oligos, 0.5 μL of vector, 1 μL 10x ligase buffer, 1 μL *Bsm*BI, and 1 μL T4 DNA ligase. The reaction was carried out by the thermocycler protocol: 42 ℃ for 2 min, 16 ℃ for 5 min, repeat these two steps for 10 times, then 60 ℃ for 10 min and finally 80 ℃ for 10 min. The reaction mix was transformed into *E. coli* and plated on LB medium with kanamycin. In the successfully constructed plasmids, GFP should be replaced with gRNA. Therefore, the *E. coli* colonies without florescent were selected, and plasmids were extracted and verified by PCR and sequencing. The gRNA sequences and primers are shown in Table 4.

5.10 CRISPR/Cas9 mediate allele swapping in diploid *S. cerevisiae* strain

Industrial *S. cerevisiae* strains were normally diploids and genetic modifications of two copies of certain gene are often laborious. CRISPR/Cas9 mediated technology can facilitate the process. To accomplish a precise genome modification, the homology directed repair (HRD) method was applied for double strand break (DSB) repair. Donor DNA was transformed into yeast cells together with the constructed CRISPR plasmids pWS174. Yeast transformation was carried out by the lithium acetate method (Gietz and Woods, 2002). For each transformation, around 100-200 ng of CRISPR plasmid and more than 2 μg of donor DNA were used as illustrated by Shaw et al. (2019).

To facilitate transformants selection, two successive CRISPR events were carried out with the gene *GCN1* of P17 to achieve allele swapping as described by Biot-Pelletier et al. (2016). In the first step, the CRISPR-Cas9

system introduced DSB in *GCN1* and the *KanMX4* cassette obtained from the pUG6 plasmid (Güldener et al, 1996) with 40 bp homology arms of the target was used as donor DNA to repair DSB. 628 bp of the target gene were replaced with *KanMX4*. Transformants were selected by double resistance of G418 and nourseothricin. Primers up- and down-stream of the *KanMX4* was designed and the insertion was verified by sequencing. In the second step, the new gRNA that targeted the inserted *KanMX4* was designed. CRISPR plasmid introduced the DSB in the *KanMX4* gene, and part of *GCN1* gene including the mutation sites (762 bp) from P20 was used as the donor DNA for DSB repairing. Transformants were selected by replicate plate from YPD-clonNAT to YPD-G418. The transformants without resistance to G418 were further confirmed by sequencing.

AnnexII Publications

Chapter 2

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Fermentative behaviour and competition capacity of cryotolerant *Saccharomyces* species in different nitrogen conditions

Ying Su[a], Andrea Cecilia Origone[b], María Eugenia Rodríguez[b,c], Amparo Querol[a], José Manuel Guillamón[a,*], Christian Ariel Lopes[b,d,**]

[a] Departamento de Biotecnología, Instituto de Agroquímica y Tecnología de los Alimentos, CSIC, Carrer del Catedràtic Agustín Escardino Benlloch, 7, 46980 Paterna, Valencia, Spain
[b] Instituto de Investigación y Desarrollo en Ingeniería de Procesos, Biotecnología y Energías Alternativas, PROBIEN, Consejo Nacional de Investigaciones Científicas y Técnicas de la República Argentina, Universidad Nacional del Comahue, Buenos Aires 1400, 8300, Neuquén, Argentina
[c] Facultad de Ciencias Médicas, Universidad Nacional del Comahue, 8324 Cipolletti, Río Negro, Argentina
[d] Facultad de Ciencias Agrarias, Universidad Nacional del Comahue, 8303 Cinco Saltos, Río Negro, Argentina

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ABSTRACT

The selection of yeasts with low nitrogen requirement is a current need in winemaking. In this work, we analysed nitrogen requirements of strains belonging to the cryotolerant species *S. uvarum*, *S. eubayanus* and *S. kudriavzevii*, in order to evaluate their potential for conducting the fermentation of low nitrogen content grape musts. Our result demonstrated that *S. eubayanus* is the species less influenced by the increasing nitrogen concentrations in both growth and fermentation conditions. Strains showing the best behaviours, *S. eubayanus* NPCC 1285 and *S. uvarum* NPCC 1317, were selected to be tested in mixed cultures with *S. cerevisiae* T73 at different temperatures (12 °C, 20 °C and 28 °C) in synthetic grape must with different nitrogen concentrations (60, 140 and 300 mg/L YAN). The cryotolerant strains dominated the fermentations carried out at 12 °C while *S. cerevisiae* prevailed at 28 °C independently from the nitrogen concentration. At intermediate temperature, 20 °C, *S. eubayanus* mono and mixed cultures showed the best fermentative behaviour especially with low and intermediate nitrogen concentration. In summary, cryotolerant *Saccharomyces* species, particularly *S. eubayanus*, could be interesting tools to avoid fermentations stucks caused by low nitrogen content in grape musts.

1. Introduction

Nitrogen is a key nutrient during wine fermentation, affecting both fermentation kinetics and the formation of wine aroma. It is the major limiting nutrient for growth under oenological conditions. Although alternative nitrogen sources, such as oligopeptides, amides, biogenic amines and nucleic acids, can be found and might constitute a substantial nitrogen resource in grape juice (Ough, 1991; Henschke and Jiranek, 1993; Perry et al., 1994; Marsit et al., 2015), yeast assimilable nitrogen (YAN) is mainly composed of ammonium and amino acids (Henschke and Jiranek, 1993). Different factors including grape variety, geographical origin, climate conditions and some technological processes affect the YAN content in musts and thus the fermentation kinetics (Butzke, 1998; Dubois et al., 1996; Nicolini et al., 2004). Previous works have determined that, in general, a minimum of 140 mg/L of YAN is required for yeast to complete alcoholic fermentation (Bell and Henschke, 2005; Bely et al., 1990; Butzke, 1998). Nevertheless, it strongly depends on the yeast species developing during the fermentation process.

Saccharomyces cerevisiae is the main yeast species used in the wine industry. Its favoured characteristics such as high fermentation efficiency, high ethanol tolerance and consistency of wine quality help *S. cerevisiae* to maintain its dominant position (Mas et al., 2016). However, the context of the worldwide Oenology has altered with the climate change, which affects the grape composition and ends up with grape musts with low nitrogen and high sugar concentrations (van Leeuwen and Darriet, 2016). This situation gives the wine industry a big challenge to meet consumers' preference for wines with lower alcohol and fruitier aromas. One of the oenological practices applied by winemakers is to use lower fermentation temperatures, as far as

* Corresponding author.
** Corresponding author at: Instituto de Investigación y Desarrollo en Ingeniería de Procesos, Biotecnología y Energías Alternativas, PROBIEN, Consejo Nacional de Investigaciones Científicas y Técnicas de la República Argentina, Universidad Nacional del Comahue, Buenos Aires 1400, 8300, Neuquén, Argentina.
E-mail addresses: guillamon@iata.csic.es (J.M. Guillamón), clopes@conicet.gov.ar (C.A. Lopes).

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Interspecific hybridisation among diverse *Saccharomyces* species: A combined biotechnological solution for low-temperature and nitrogen-limited wine fermentations

Ying Su[a], Amparo Gamero[a,b], María Eugenia Rodríguez[c,d], Christian Ariel Lopes[c,d], Amparo Querol[a], José Manuel Guillamón[a,*]

[a] Departamento de Biotecnología, Instituto de Agroquímica y Tecnología de los Alimentos, CSIC, Carrer del Catedràtic Agustín Escardino Benlloch, 7, 46980 Paterna, Valencia, Spain
[b] Área de Tecnología de Alimentos, Facultad de Farmacia, Universitat de València, Avda. Vicent Andrés Estellés s/n, 46100 Burjassot, Valencia, Spain
[c] Instituto de Investigación y Desarrollo en Ingeniería de Procesos, Biotecnología y Energías Alternativas (PROBIEN, Consejo Nacional de Investigaciones Científicas y Técnicas de la República Argentina – Universidad Nacional del Comahue), Buenos Aires 1400, 8300 Neuquén, Argentina
[d] Facultad de Ciencias Agrarias, Universidad Nacional del Comahue, 8303 Cinco Saltos, Río Negro, Argentina

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ABSTRACT

Lack of the prezygotic barrier in the *Saccharomyces* genus facilitates the construction of artificial interspecific hybrids among different *Saccharomyces* species. Hybrids that maintain the interesting features of parental strains have been applied in industry for many beneficial purposes. Two of the most important problems faced by wine makers is nitrogen deficiency in grape must and low-temperature fermentation. In our study, hybrids were constructed by using selected low nitrogen-demanding cryotolerant *S. eubayanus*, *S. uvarum* strains and *S. cerevisiae*. The fermentation capacity of the hybrid strains was tested under four conditions by combining two temperatures, 12 °C and 28 °C, and two nitrogen concentrations, 60 mg/L and 300 mg/L. The hybrid strains obtained combined characters of both parental strains and conferred better fermentation rates under low-temperature or low-nitrogen conditions. The hybrid strains also produced larger amounts of acetate esters and higher alcohols, which increase aroma intensity and complexity in wine. Nitrogen sources were more rapidly consumed by the hybrid strains, which allows greater competition ability under nitrogen-deficiency conditions. Therefore, the interspecific hybridisation between low nitrogen-demanding cryotolerant strains and *S. cerevisiae* is a potential solution for low-temperature or low-nitrogen fermentations.

1. Introduction

Nitrogen is one of the substantial nutrients for yeasts that regulates biomass formation and fermentation activity, and provides important precursors for wine aroma-related compounds, such as higher alcohols, esters and volatile fatty acids (Bely et al., 1990; Bisson, 1991; Swiegers et al., 2005). Lack of nitrogen is one of the main causes of sluggish or stuck wine fermentation and H_2S production (Bell and Henschke, 2005; Giudici and Kunkee, 1994). While nitrogen plays a significant role in fermentation processes and sensory wine characters, yeast assimilable nitrogen (YAN) deficiency in grape must has been well-identified in many wine regions in Europe and elsewhere in the world (Butzke, 1998; Hagen et al., 2008; Henschke and Jiranek, 1993; Nicolini et al., 2004). Many studies have revealed that at least 140 mg/L YAN is needed to achieve complete wine dryness (Bely et al., 1990, Mendes-Ferreira et al., 2004). Technically speaking, a higher YAN concentration is needed to match a higher sugar concentration in grape must. Nitrogen addition to grape must can be one solution, but it is often difficult to handle. Excessive nitrogen additions may lead to the presence of non-assimilated residual nitrogen at the end of fermentation, which leads to microbial instability and ethyl carbamate accumulation in wine (Ough and Amerine, 1988). Therefore, it is of much interest to develop yeast strains with lower nitrogen requirements to complete fermentations.

The *Saccharomyces* genus has been used as an ideal paradigm for hybridisations. In spite of the fact that eight identified species (*S. cerevisiae*, *S. paradoxus*, *S. mikatae*, *S. kudriavzevii*, *S. arboricola*, *S. uvarum*, *S. eubayanus* and *S. jurei*) are highly divergent in nucleotide sequences, they barely show any prezygotic barriers, which enables them to mate and form viable diploids (Morales and Dujon, 2012). Natural hybrids have been isolated from different fermentation processes. The most

* Corresponding author.
E-mail address: guillamon@iata.csic.es (J.M. Guillamón).

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Nitrogen sources preferences of non-*Saccharomyces* yeasts to sustain growth and fermentation under winemaking conditions

Ying Su[a,1], Pauline Seguinot[b,1], Isabelle Sanchez[c], Anne Ortiz-Julien[d], José Maria Heras[e], Amparo Querol[a], Carole Camarasa[b], José Manuel Guillamón[a,*]

[a] Departamento de Biotecnología de Alimentos, Instituto de Agroquímica y Tecnología de Alimentos, Consejo Superior de Investigaciones Científicas (CSIC), Valencia, Spain
[b] UMR SPO: INRA, Universite Montpellier, Montpellier SupAgro, 34060, Montpellier, France; Lallemand SAS, 31700, Blagnac, France
[c] UMR MISTEA: INRA, Montpellier SupAgro, 34060, Montpellier, France
[d] Lallemand SAS, 31700, Blagnac, France
[e] Lallemand Bio, 28521, Madrid, Spain

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ABSTRACT

Wine-related non-*Saccharomyces* yeasts are becoming more widely used in oenological practice for their ability to confer wine a more complex satisfying aroma, but their metabolism remains unknown. Our study explored the nitrogen utilisation profile of three popular non-*Saccharomyces* species, *Torulaspora delbrueckii*, *Metschnikowia pulcherrima and Metschnikowia fructicola*. The nitrogen source preferences to support growth and fermentation as well as the uptake order of different nitrogen sources during wine fermentation were investigated. While *T. delbrueckii* and *S. cerevisiae* strains shared the same nitrogen source preferences, *Metschnikowia* sp. Displayed a lower capacity to efficiently use the preferred nitrogen compounds, but were able to assimilate a wider range of amino acids. During alcoholic fermentation, the non-*Saccharomyces* strains consumed different nitrogen sources in a similar order as *S. cerevisiae*, but not as quickly. Furthermore, when all the nitrogen sources were supplied in the same amount, their assimilation order was similarly affected for both *S. cerevisiae* and non-*Saccharomyces* strains. Under this condition, the rate of nitrogen source consumption of non-*Saccharomyces* strains and *S. cerevisiae* was comparable. Overall, this study expands our understanding about the preferences and consumption rates of individual nitrogen sources by the investigated non-*Saccharomyces* yeasts in a wine environment. This knowledge provides useful information for a more efficient exploitation of non-*Saccharomyces* strains that improves the management of the wine fermentation.

1. Introduction

In the past 50 years, the inoculation of selected *Saccharomyces cerevisiae* strains has become increasingly prevalent in winemaking. It is an excellent way to control alcoholic fermentation by ensuring the complete exhaustion of sugars and avoiding the formation of undesirable off-flavours. During wine fermentation, the predominant non-*Saccharomyces* yeasts in grape juice are rapidly outcompeted by *S. cerevisiae* because they poorly adapt to increasing ethanol concentrations and to low levels of dissolved oxygen (Bauer and Pretorius, 2000; Boulton et al., 1995; Bisson, 1999). Some of these non-conventional yeasts have attracted more attention as they positively modify the wine chemical composition and, consequently, improve wine flavour and bouquet. (Padilla et al., 2016). However, as a result of their low ethanol tolerance, these species are unable to achieve complete sugar consumption. Consequently, in order to take advantage of their phenotypic specificities while avoiding stuck or sluggish fermentations, they are usually combined in industry with *S. cerevisiae* in sequential or co-inoculation. In this context, both the growth and the metabolic activity of non-*Saccharomyces* yeasts may impact *S. cerevisiae* performance in mixed culture fermentation mainly due to their nutrient consumption (Medina et al., 2012; Taillandier et al., 2014).

Nitrogen availability in grape must is a key parameter for the wine fermentation progress as it acts at many levels. Firstly, nitrogen is an important nutrient for sustaining yeast growth and, for the vast majority of fermentations, it is the limiting factor for biomass production (Bell and Henschke, 2005; Martínez-Moreno et al., 2012; Mendes-Ferreira et al., 2004; Varela et al., 2004). Nitrogen also plays a key role

* Corresponding author.
E-mail address: guillamon@iata.csic.es (J.M. Guillamón).
[1] These authors contributed equally to this work.

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Isotopic Tracers Unveil Distinct Fates for Nitrogen Sources during Wine Fermentation with Two Non-*Saccharomyces* Strains

Ying Su [1], Pauline Seguinot [2,3], Audrey Bloem [2], Anne Ortiz-Julien [3], José María Heras [4], José Manuel Guillamón [1] and Carole Camarasa [2,*]

1 Departamento de Biotecnología de Alimentos, Instituto de Agroquímica y Tecnología de Alimentos (IATA), Consejo Superior de Investigaciones Científicas (CSIC), 46980 Paterna-Valencia, Spain; ying.su@iata.csic.es (Y.S.); guillamon@iata.csic.es (J.M.G.)
2 UMR SPO, Université Montpellier, INRAE, Montpellier SupAgro, 34060 Montpellier, France; seguinot.pauline@gmail.com (P.S.); audrey.bloem@umontpellier.fr (A.B.)
3 Lallemand SAS, 31700 Blagnac, France; ajulien@lallemand.com
4 Lallemand Bio, 28521 Madrid, Spain; jmheras@lallemand.com
* Correspondence: carole.camarasa@inrae.fr

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Abstract: Non-*Saccharomyces* yeast strains have become increasingly prevalent in the food industry, particularly in winemaking, because of their properties of interest both in biological control and in complexifying flavour profiles in end-products. However, unleashing the full potential of these species would require solid knowledge of their physiology and metabolism, which is, however, very limited to date. In this study, a quantitative analysis using ^{15}N-labelled NH_4Cl, arginine, and glutamine, and ^{13}C-labelled leucine and valine revealed the specificities of the nitrogen metabolism pattern of two non-*Saccharomyces* species, *Torulaspora delbrueckii* and *Metschnikowia pulcherrima*. In *T. delbrueckii*, consumed nitrogen sources were mainly directed towards the *de novo* synthesis of proteinogenic amino acids, at the expense of volatile compounds production. This redistribution pattern was in line with the high biomass-producer phenotype of this species. Conversely, in *M. pulcherrima*, which displayed weaker growth capacities, a larger proportion of consumed amino acids was catabolised for the production of higher alcohols through the Ehrlich pathway. Overall, this comprehensive overview of nitrogen redistribution in *T. delbrueckii* and *M. pulcherrima* provides valuable information for a better management of co- or sequential fermentation combining these species with *Saccharomyces cerevisiae*.

Keywords: ^{15}N- and ^{13}C-isotope labelling; quantitative analysis of metabolism; nitrogen resource management; *Torulaspora delbrueckii*; *Metschnikowia pulcherrima*

1. Introduction

Traditionally, wine fermentation is a process in which sugars are converted into ethanol through interactions between microorganisms, mainly yeast species, also imparting pleasant notes to wines [1]. However, some spoilage microorganisms may cause problematical wine fermentations. In 1883, Emil Christian Hansen successfully isolated the first pure yeast culture [2], which started a new era for yeast research and utilisation. In modern oenology, inoculation of pure cultures of *Saccharomyces cerevisiae* is widely used as an efficient way to prevent the growth of spoilage non-*Saccharomyces* species, thus ensuring the completion of fermentation and a stable wine quality [3–5]. Nevertheless, in the last decade, more attention has been paid to non-*Saccharomyces* yeasts for their technological properties of interest in winemaking. Two of the most studied and commercialised of these species are *Torulaspora delbrueckii* and *Metschnikowia pulcherrima* [6], used to reduce the production

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