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Influence of nitrogen status in wine alcoholic fermentation

Antoine Gobert[a,*], Raphaëlle Tourdot-Maréchal[a], Céline Sparrow[b], Christophe Morge[b], Hervé Alexandre[a]

[a] *UMR Procédés Alimentaires et Microbiologiques, Université de Bourgogne Franche-Comté/ AgroSup Dijon - Equipe VAlMiS (Vin, Aliment, Microbiologie, Stress), Institut Universitaire de la Vigne et du Vin Jules Guyot, Université de Bourgogne, Dijon, France*

[b] *SAS Sofralab, 79, Av. A.A. Thévenet, BP 1031, Magenta, France*

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ABSTRACT

Nitrogen is an essential nutrient for yeast during alcoholic fermentation. Nitrogen is involved in the biosynthesis of protein, amino acids, nucleotides, and other metabolites, including volatile compounds. However, recent studies have called several mechanisms that regulate its role in biosynthesis into question. An initial focus on *S. cerevisiae* has highlighted that the concept of "preferred" *versus* "non-preferred" nitrogen sources is extremely variable and strain-dependent. Then, the direct involvement of amino acids consumed in the formation of proteins and volatile compounds has recently been reevaluated. Indeed, studies have highlighted the key role of lipids in nitrogen regulation in *S. cerevisiae* and their involvement in the mechanism of cell death. New winemaking strategies using non-*Saccharomyces* yeast strains in co- or sequential fermentation improve nitrogen management. Indeed, recent studies show that non-*Saccharomyces* yeasts have significant and specific needs for nitrogen. Moreover, sluggish fermentation can occur when they are associated with *S. cerevisiae*, necessitating nitrogen addition. In this context, we will present the consequences of nitrogen addition, discussing the sources, time of addition, transcriptome changes, and effect on volatile compound composition.

1. Introduction

The main sources of yeast assimilable nitrogen (YAN) in grape must are ammonium and amino acids. Their concentrations vary depending on geographical location (Rapp and Versini, 1995), climate (Ribéreau-Gayon et al., 2006), cultivar or rootstock (Schreiner et al., 2017; Stines et al., 2000), and viticulture techniques (Schreiner et al., 2017; Spayd et al., 1994). During alcoholic fermentation, yeast take up and metabolize YAN and other nutrients to support growth and produce biomass, as well as volatile compounds (Vilanova et al., 2007). YAN deficiency can sometimes lead to sluggish or stuck fermentation (Alexandre and Charpentier, 1998; Bisson, 1999). Under enological conditions, a concentration of approximately 140 mg N/L of YAN is necessary to complete fermentation within a reasonable period of time (Beltran et al., 2005; Bely et al., 1990; Bisson, 1999; Henschke and Jiranek, 1993; Jiranek et al., 1995; Kemsawasd et al., 2015), depending on sugar concentration and winemaking practices.

During alcoholic fermentation, the consumption of YAN by yeast is regulated by several molecular mechanisms, which have been well described in *Saccharomyces cerevisiae.* The most recent reviews on the subject date back to 2005 and 2012 (Bell and Henschke, 2005; Ljungdahl and Daignan-Fornier, 2012) and only one review on nitrogen regulation was been recently published (Zhang et al., 2018). The authors examined several studies on the role of nitrogen metabolism under enological conditions. Most studies have classified YAN as "preferential or non-preferential sources", depending on the alcoholic fermentation conditions, strains used, and classification method (Beltran et al., 2006; Crépin et al., 2012; Gobert et al., 2017; Jiranek et al., 1995; Kemsawasd et al., 2015; Rollero et al., 2018). The comparison of the studies previously cited highlighted significant differences. Such differences could be due to the complexity of nitrogen regulation, which depends on substrate availability, strain phenotype and matrix.

Recent interest in non-*Saccharomyces* (NS) yeasts in spontaneous fermentation (Combina et al., 2005; Cordero-Bueso et al., 2013; Jolly et al., 2014; Liu et al., 2016) and co- or sequential fermentation (Anfang et al., 2009; Ciani et al., 2010; Clemente-Jimenez et al., 2005; Englezos et al., 2018; Medina et al., 2013; Padilla et al., 2017; Soden et al., 2000) add a second level of complexity to nitrogen management under enological conditions. Despite the large body of literature concerning the use of NS yeasts to increase the aromatic complexity of wine (Azzolini et al., 2014; Escribano et al., 2018; Lambrechts and Pretorius, 2000; Liu

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[*] Corresponding author.
E-mail address: antoinegobert1@gmail.com (A. Gobert).

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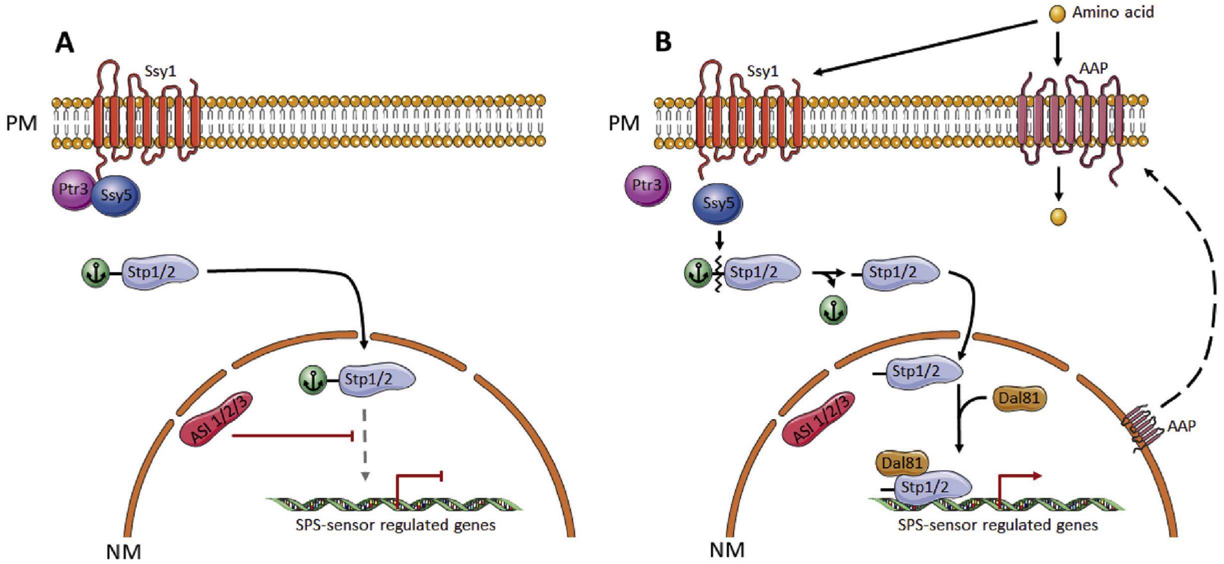

Fig. 1. (A) The non-induced resting state in the absence of inducing amino acids. In the absence of inducing amino acids, low levels of AAPs are present in the plasma membrane (PM). NM, nuclear membrane. (B) The induced state in the presence of extracellular amino acids. The derepressed AAP gene expression leads to increased levels of AAP in the PM and enhanced rates of amino acid uptake (adapted from Ljungdahl et al. (2012)).

et al., 2016; Sadoudi et al., 2017, 2012; Swiegers and Pretorius, 2005), improve ethanol reduction (Canonico et al., 2016; Ciani et al., 2016; Contreras et al., 2015, 2014; Englezos et al., 2016; Gobbi et al., 2014; Röcker et al., 2016; Rolle et al., 2017), or act as bio-protection/control agents in winemaking (Cordero-Bueso et al., 2017; Fernandes Lemos Junior et al., 2016; Qin et al., 2015; Simonin et al., 2018; Wang et al., 2018), little data on nitrogen needs, sources, and preferences are available and have never been reviewed (Andorrà et al., 2010; Gobert et al., 2017; Kemsawasd et al., 2015). In addition, only one study (Englezos et al., 2018) has highlighted specific features of one NS yeast, *Starmerrella bacillaris*, concerning the management of nitrogen.

Aromatic complexity is an essential aspect of wine quality and largely influences consumer acceptance (King et al., 2011; Lattey et al., 2010). Volatile compounds, an essential element of overall wine flavor, are formed during alcoholic fermentation. The relationship between nutrient availability and the production of desirable volatile compounds is one of the main goals in enology and industry. Some YAN sources have been reported to be precursors of volatile compounds in *S. cerevisiae* (Carrau et al., 2008; Fairbairn et al., 2017; Hazelwood et al., 2008; Ribéreau-Gayon et al., 2006) and non-*Saccharomyces* (González et al., 2018) principally *via* the Ehrlich pathway (González et al., 2018; Hazelwood et al., 2008). However, contrary to the generally accepted view, recent studies have shown that the catabolism of consumed branched amino acids (leucine, isoleucine, threonine and valine) plays an indirect role in the formation of some volatile compounds in *S.cerevisiae* (Crépin et al., 2017; Rollero et al., 2017). Precisely, Crépin et al. (2017) demonstrated the low contribution of the carbon skeletons of consumed amino acids to the production of volatile compounds derived from α-keto acids. Lipid and nitrogen metabolism are interconnected and affect the production of some volatile compounds (Rollero et al., 2017, 2016). The aforementioned studies have shown that there may be a direct relationship between nitrogen sources and the production of volatile compounds and that it may involve more complex mechanisms than those reported to date.

Although a large diversity of YAN content and concentration in must can be found during enological processes, ammonium phosphate or ammonium sulfate are commonly added to YAN deficient must. YAN supplementation directly affects biomass production and the performance of alcoholic fermentation (Martínez-Moreno et al., 2012; Varela et al., 2004). However, although it is clear that YAN impact volatile compounds production, the mechanisms implicated seem to be indirect and need to be more investigated. It has been demonstrated that the use of an amino-acid mix for must supplementation increases the rate of alcoholic fermentation more than ammonium phosphate or sulfate addition, with sometimes less production of undesirable volatile compounds (Fairbairn et al., 2017; Kevvai et al., 2016; Martínez-Moreno et al., 2012).

2. YAN metabolism in yeasts

2.1. *Saccharomyces cerevisiae*

2.1.1. *Transporters and general regulation in Saccharomyces cerevisiae*

Although diverse yeast species are used in the first step of winemaking (Barata et al., 2012; Capozzi et al., 2015; Gilbert et al., 2014), many studies have focused on the nitrogen metabolism of *S. cerevisiae* (Crépin et al., 2017, 2012; Grenson et al., 1974; Hazelwood et al., 2008; Jiranek et al., 1995; Mitchell, 1985; Stanbrough and Magasanik, 1995) and many reviews have focused on its metabolism (Bell and Henschke, 2005; Henschke and Jiranek, 1993; Horák, 1997; Ljungdahl and Daignan-Fornier, 2012; Ramos et al., 2016). Here, we will only provide a brief general description of *S. cerevisiae* nitrogen metabolism, with a focus on recent studies. The readers can refer to the above-cited articles for an in-depth description of *Saccharomyces cerevisiae* nitrogen metabolism.

In *S. cerevisiae*, YAN is transported into the cell by various specific or non-specific permeases. Ammonium, which represents a significant proportion of nitrogen sources, is transported by three permeases: Mep1p, Mep2p, and Mep3p. The Mep2 protein displays the highest affinity for ammonium, followed closely by Mep1p and finally Mep3p, of which the affinity is much lower (Marini et al., 1997). These transporters consist of uniport systems.

Amino acids are assimilated by various, more or less, selective transporters. Amino-acid permeases (AAPs) are active symport systems (Kotyk, 1994; Ramos et al., 2016). Among them, the general amino-acid permease (Gap1) allows the transport of all amino acids. Other AAPs are more selective and transport only one or a group of amino acids (Ramos et al., 2016). These permeases are regulated by several mechanisms. The first is located on the plasma membrane and forms a complex called Ssy1p-Ptr3p-Ssy5p (SPS). This complex induces an endoproteolytic processing event in response to the extracellular amino-acid status, which activates the transcription of AAP genes (Fig. 1)

(Andréasson and Ljungdahl, 2004; Ljungdahl, 2009).

Nitrogen catabolite repression (NCR) is another regulatory system used by *S. cerevisiae*. Currently, *GAP1, CAN1, PUT4, DIP5, UGA4* (amino acids permeases) and *MEP1, MEP2, MEP3* (ammonium permease) are known to be under the control of this regulatory system (Ljungdahl and Daignan-Fornier, 2012). This system leads the yeast to selectively utilize preferred sources of nitrogen when they are available. Conversely, general de-repression of the genes regulated by the NCR system leads the cell to nonspecifically use other sources of nitrogen in the absence of a preferential nitrogen source. The classification of nitrogen source preferences is not absolute and their repressive effects can vary substantially between yeast strains (Magasanik and Kaiser, 2002). The expression of genes encoding NCR-sensitive AAPs is regulated by complex pathways involving multiple transcription factors with activating or inhibitory effects. The target of these transcription factors is the UAS_{NTR} activation sequence, located upstream of the promoter of genes encoding NCR-sensitive AAPs. This element consists mainly of two distinct dodecanucleotide sites with a pentanucleotide consensus sequence 5′-GATATA-3 '. A pair of Dal80/Uga43 proteins and three Gat1, Ure2, Gln3 proteins have been identified and reported to participate in the regulation of NCR-sensitive gene expression across the UAS_{NTR} element (Cunningham et al., 1996). However, results from a more recent study suggests that Ure2p and GATA transcription factors (Gln3, Gat1, Dal80 and Gzf3 transcription factors) are involved. Thus, in the study of Cunningham et al. (1996), Gln3p and Gat1p were factors that allowed the activation of NCR-sensitive gene transcription. The factors Dal80/Uga43 are proteins that block the binding site of the Gln3 and Gat1 factors. Ure2 sequesters Gln3 and Gat1 proteins in the cytoplasm. In the study of Georis et al., Gln3 and Gat1 were shown to be activators of NCR-sensitive gene expression. However, there are differences at the level of the repression systems. Georis et al. showed that Dal80 and Gzf3, but not Uga43, were blocking proteins of the UAS_{NTR} binding site. Ure2 was shown to have the same role in both studies (Fig. 2).

In *S. cerevisiae*, the NCR system is controlled by the target of the rapamycin pathway (TOR), which is comprised of two complexes, TOR complex 1 (TORC1) and TOR complex 2 (TORC2). Only TORC1 is involved in the control of the NCR system. This aspect will not be discussed further here, as nitrogen metabolism, including the regulation of sensing, transport, and catabolism was recently extensively reviewed by Zhang et al. (2018). However, a study showed that *TORC1*-mediated control of NCR is only partial (Fayyad-Kazan et al., 2016). The authors investigated the regulation of the NCR system by glutamate and glutamine, major nitrogen sources for biosynthesis. The results showed a negative role of the anabolic glutamate dehydrogenase (Gdh1) on Gat1 and Gln3 (transcriptional activators of NCR genes) activity under repressive nitrogen conditions. Thus, preferred nitrogen sources may trigger NCR-sensitive gene repression or *TORC1* activation, probably through transient glutamine accumulation.

More broadly, global gene expression is strongly influenced by the concentration of nitrogen during fermentation. In synthetic must under conditions of low nitrogen, genes mainly associated with protein synthesis and RNA and nucleic acid metabolism are downregulated at the beginning of fermentation (24–48 h) (Mendes-Ferreira et al., 2007). In contrast, genes involved in energy generation, carbohydrate metabolism, oxidoreductase activity, respiratory chain phosphorylation, transporter activity, respiration, response to oxidative stress, oxygen, and reactive oxygen species metabolism are upregulated. This expression profile is sustainable, as the genes expressed at the end of fermentation are fundamentally the same as those expressed early in the yeast cell response (Mendes-Ferreira et al., 2007). Under the same conditions, Barbosa et al. (2015) showed that gene expression varies highly among yeast strains, depending on nitrogen availability (low or high concentration), fermentation stage, and the interaction of the two factors. In particular, the high fermenter yeast strain (*S. cerevisiae* UCD522) used in this study showed high expression of 333 genes involved in transport and phosphate metabolism throughout fermentation under conditions of low nitrogen. In contrast, 246 genes were more highly expressed, including flocculation genes, under conditions of high nitrogen (Barbosa et al., 2015). In addition, transcriptional regulation can be affected by the nitrogen source. In 2006, Boer et al., evaluated the transcriptional response of *S. cerevisiae* in glucose-limited chemostat culture in synthetic must. Phenylalanine, leucine, methionine, and proline were used as “non-preferred” nitrogen sources and asparagine and ammonium as “preferred” nitrogen sources. A group of 23 genes was upregulated during growth on “non-preferred” sources. Among them, six are involved in the metabolism of the “non-preferred” nitrogen sources allantoin and urea (*DAL1*, *DAL2*, *DAL5*, *DUR1*, *DUR2*, *DUR3*) and five encode transporters for nitrogen-containing compounds (*GAP1, PTR2, MEP2, MEP3* and *OPT2*). This group also included the GATA factor. From these 23 genes, 14 were established as NCR targets (Boer et al., 2006).

2.1.2. Classification of “preferred” and “non-preferred” nitrogen sources in Saccharomyces cerevisiae

The phenotypic diversity of *S. cerevisiae* concerning YAN preferences correlates with the presence of genetic variants (Cubillos et al., 2017; Gutiérrez et al., 2013a; Salinas et al., 2012). Currently, three methods are used to classify YAN as “preferred” or “non-preferred” nitrogen sources. The first, used since the 80's and 90's, considers the consumption rate, specific growth rate, and kinetics of fermentation (Grenson, 1992, 1983; Henschke and Jiranek, 1993; Jiranek et al.,

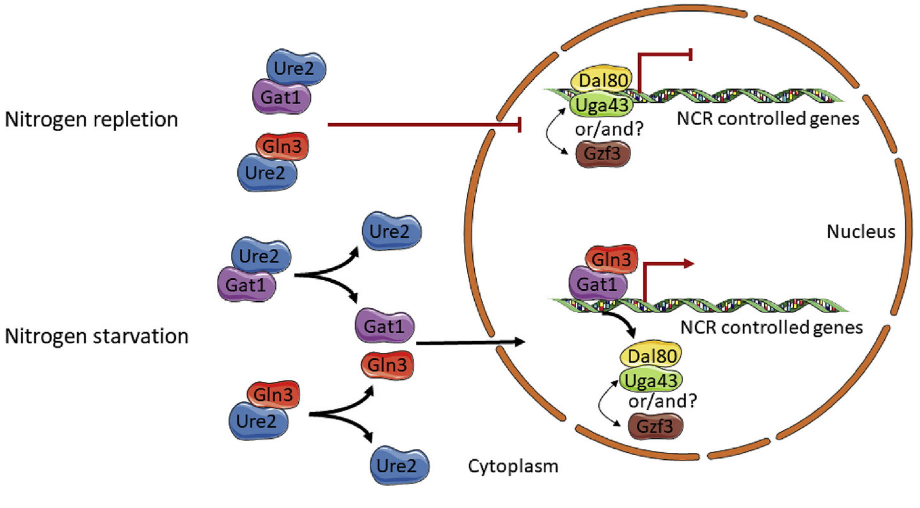

Fig. 2. Translocation into the nucleus of Gat1p/Gln3p complex depending on nitrogen status. In nitrogen repletion condition, Gat1p and Gln3p are sequestered by Ure2p in the cytoplasm. Dal80p and/or Gzf3p block the UAS_{NTR} binding site. In nitrogen starvation condition, Ur2p releasesUre2p release Gat1p and Gln3p and the complex migrates into the nucleus. Dal80p/Uga43p and/or Gzf3p release the UAS_{NTR} binding site and Gat1p/Gln3p complex activates the transcription of NCR controlled genes.

Table 1
Nitrogen compounds considered to be "preferred" nitrogen sources in *S. cerevisiae*.

Amino acids	References
Alanine	8
Ammonium	1, 2, 3, 6, 7, 8, 11, 13
Arginine	2, 3, 8
Asparagine	1, 3, 4, 5, 6, 7, 8, 9, 11, 12, 13
Aspartate	3, 8
Aspartic acid	4, 12
GABA	8
Glutamine	1, 3, 5, 6, 8, 11, 12, 13
Glutamate	8, 11, 13
Glutamic acid	4
Histidine	2
Isoleucine	3, 12
Leucine	2, 3, 12
Lysine	3, 9, 12
Methionine	3
Serine	2, 3, 9, 10, 12
Threonine	2, 3, 9, 10, 12

1: (Grenson, 1992), 2: (Henschke and Jiranek, 1993), 3: (Jiranek et al., 1995), 4: (Albers et al., 1996), 5: (Magasanik and Kaiser, 2002), 6: (Görgens et al., 2005), 7: (Boer et al., 2006), 8: (Ljungdahl and Daignan-Fornier, 2012), 9: (Crépin et al., 2012), 10: (Pinu et al., 2014), 11: (Tesnière et al., 2015), 12: (Gobert et al., 2017), 13: (Brice et al., 2018).

1995). The second is based on the response of the NCR system. YAN that induce the activation of transcription of permeases is considered as "preferred" sources. Crépin et al. (2012) used this method to show that classification was possible according to the order of assimilation of YAN by *S. cerevisiae*. The last method is based on the response of the SPS system (Boer et al., 2006; Ljungdahl, 2009). YAN sources that induce the activation of transcription of permeases under the control of the SPS mechanism are considered to be "preferred", as for the method based on NCR regulation.

Studies to classify nitrogen sources preferences have yielded varying results throughout the years. Table 1 reviews all studies that have attempted to rank YAN preferences. Three YAN sources (ammonium, asparagine, and glutamine) have been significantly more often classified as preferred nitrogen sources, regardless of the classification method. In contrast, alanine, GABA, glutamic acid, histidine, and methionine have been the least often classified. Overall, 17 YAN sources can theoretically be considered to be "preferred". This comparison highlights the difficulty in comparing and determining a pool of preferential YAN. Indeed, in winemaking, the metabolic activity of *S. cerevisiae* can be strongly influenced by the matrix. Authors have used different synthetic grape musts or real musts, coming from different grape varieties. YAN assimilation may be influenced by other nutrients, such as lipids or vitamins, not present at the same concentration under all conditions. Without standardization of the matrix, it is difficult to draw definitive conclusions on the YAN that are preferentially assimilated. In addition, multifactorial abiotic factors (discussed later in this review) need to be considered when categorizing YAN. The effect of the strain is also a variable that must be considered. Each strain of *S. cerevisiae* shows differences in terms of their YAN consumption capacities (Brice et al., 2018; Crépin et al., 2012; Cubillos et al., 2017).

Several studies have focused on arginine under fermentation conditions. Although arginine is not considered to be a "preferred" nitrogen source in most studies, it plays a major role in nitrogen metabolism. During the first stage of fermentation, part of the amino-acid content, particularly arginine, localizes to the vacuole. After the complete exhaustion of nitrogen sources at the end of the growth phase, arginine stored in the vacuole is remobilized to maintain the biomass concentration, particularly for low biomass-producing *S. cerevisiae* strains (Crépin et al., 2014). Cheng et al. (2016) showed a relationship between the response of *S. cerevisiae* to arginine and ethanol stress. They showed that the addition of 250 mg/L of arginine under stressful conditions increased the cell density by 2.3-fold over that of the control (Cheng et al., 2016). The same observation was made by Noti et al. (2018). They demonstrated that arginine exerted a positive effect (relative to other amino acids) on the growth and fermentation rate of *S. cerevisiae*. They suggested that arginine exerts this beneficial effect on growth not only because of its nutritional role as a nitrogen source, but also because of its effect as an osmoprotectant (Noti et al., 2018).

2.1.3. Nitrogen and volatile compounds metabolism in Saccharomyces cerevisiae

The metabolic network involved in the production of volatile compounds has been well mapped by Styger et al. (2011). They studied genetic factors that affect the conversion of amino acids to aroma compounds, focusing on regulation of the Ehrlich pathway (Hazelwood et al., 2008). They showed that the expression of a cluster of ten genes could be significantly correlated with the production of specific volatile compounds. This cluster could be divided into three categories encoding five dehydrogenases, three decarboxylases, and two reductases. Among the dehydrogenases, *GPD2*, *ADH3,* and *OYE2* play an important role in cellular redox maintenance reactions, whereas *AAD6* and *HOM2* appear to be directly involved in Ehrlich reactions. In particular, *HOM2* catalyzes the second step in the common pathway for methionine and threonine biosynthesis. Its expression is regulated by Gcn4 and the general control of amino-acid synthesis. Other genes, including *PRO2,* which catalyzes the second step of proline biosynthesis, can perform their functions directly on the pathway (Styger et al., 2011). The function of the genes was verified by deletion of those that appeared to most strongly affect the Ehrlich pathway. Deletion of *HOM2* led to the greatest reduction in the concentration of higher alcohols and volatile acids, suggesting that *HOM2* plays a central and direct role in the formation of both the higher alcohols and volatile acids. *PRO2* deletion led to an increase in the levels of fatty acids in strains grown on medium supplemented with branched-chain amino acids. The authors suggested that *PRO2* plays a different role in the Ehrlich pathway than the other selected genes.

Other molecules, such as volatile thiols, also play an important organoleptic role, especially in white wine. The regulation of production of two volatile thiols, 4-methyl-4-sulfanylpentan-2-one (4MSP) and 3-sulfanyl- hexan-1-ol (3SH), was investigated by Thibon et al. (2008) in synthetic medium. The authors demonstrated that *IRC7*, a gene encoding a putative cystathionine β-lyase, was one of the main genes encoding the protein that catalyzes 4MSP and 3SH release under enological conditions. They also demonstrated that transcriptional regulation of *IRC7* is associated with the NCR system (Thibon et al., 2008). The NCR system is also involved in the regulation of the production of another thiol, 3-mercapto-hexanol (3 MH), by modulating the activity of Gap1 (Subileau et al., 2008).

Although many studies have shown a significant correlation between YAN and volatile compounds production (Carrau et al., 2008; Fairbairn et al., 2017; Hernandez-Orte et al., 2006; Hernández-Orte et al., 2006, 2004), recent studies suggest that this link may be much weaker than previously thought (Crépin et al., 2017; Rollero et al., 2017). In a first part, Crépin et al. (2017) showed that the main role of the catabolism of most of the consumed amino acids is to supply nitrogen for the *de novo* synthesis of proteinogenic amino acids in accordance with anabolic requirements. This *de novo* synthesis (provided approximately 60–80% of the demand for nearly all amino acids) achieved with the carbon backbones of precursors (mainly α-keto acids) in addition to ammonium donors. In a second part, ^{13}C tracer used by authors provided evidence that the carbon intermediates originated primarily from the catabolism of sugars through the central carbon metabolism, while the catabolism of and interconversions between amino acids accounted for a limited portion of the formation of α-keto acids. Furthermore, a low contribution of the carbon skeletons of consumed amino acids to the production of volatile compounds derived from α-keto acids was demonstrated (Crepineet al., 2017). Rollero et al. (2017) complemented this study by investigating how variations in nitrogen and lipid resources can influence the contributions of both nitrogen and carbon metabolism to the production of fermentative aromas using filiation experiments with ^{13}C-labelled leucine and valine nitrogen sources. They showed that only a small fraction of higher alcohols was synthetized using the carbon skeletons of amino acids. Moreover, the quantity of YAN available before fermentation had a strong impact on both metabolic protein synthesis and higher alcohol flux. Under conditions of low nitrogen (70 mg/L), the direct incorporation of exogenous leucine into biomass was limited to 30%. This observation is in accordance with the results of Crépin et al. (2017), which show that consumed amino acids provide an intracellular nitrogen pool for *de novo* synthesis. However, under conditions of moderate (250 mg/L) to high nitrogen (425 mg/L), the direct incorporation of consumed leucine into biomass reached 70%. This result appears to contradict that of Crépin et al. (2017). However, in this study, a low available YAN condition was tested (180 mg/L of YAN for 240 g/L of glucose). Variations in the concentration of YAN appeared to influence the percentage of direct incorporation of amino acids into biomass formation. Simultaneously, the total production of higher alcohols changed differently, depending on nitrogen availability. Rollero et al. (2017) showed that an increase of nitrogen from 70 to 250 mg/L increases the synthesis of α-ketoacids via central carbon metabolism, which results in a marked increase in the flux towards the formation of higher alcohols. However, at high concentration of YAN (425 mg/L), intracellular α-ketoacids are, to a large extent, directed towards the synthesis of amino acids at the expense of higher alcohol formation, resulting in a decrease in the formation of aromas relative to the previous conditions. In opposite, it is indeed well known that the production of higher alcohols is highly affected by the nitrogen concentration and composition of grape must (Beltran et al., 2005; Carrau et al., 2008; Vilanova et al., 2007). Nitrogen starvation increases the production of higher alcohols, probably due to the higher levels of α-keto acids produced on those conditions, which cannot be transaminated and are in turn converted into higher alcohols.

Nevertheless, the strain under study, use of synthetic medium, concentration of nitrogen sources, temperature, and anaerobic and stirring conditions are all elements to consider in validating the redistribution of YAN into the cell for the *de novo* synthesis of proteinogenic amino acids and the consequences on the production of higher alcohols.

2.2. Non-Saccharomyces yeasts

Nitrogen metabolism of NS yeasts is a recent topic of interest in enology. Little data is available and published studies have never been reviewed. There is also recent biotechnological interest in NS yeasts in winemaking, including their needs, in particular that of nitrogen. The use of NS yeasts in co- or sequential fermentation generally leads to YAN consumption and competition for nitrogen sources with *S. cerevisiae*. These types of fermentation can ultimately lead to sluggish or stuck fermentation (Medina et al., 2012). Consequently, a better comprehensive understanding of the metabolic nitrogen requirements of NS yeasts and their regulation will aid in the control of fermentation. The first studies focused on preferential nitrogen sources of NS yeasts (Andorrà et al., 2010; Gobert et al., 2017; Kemsawasd et al., 2015). As for *S. cerevisiae*, the term "preferential" is questionable for NS yeasts. The difficulty in identifying "preferential" sources lies in that it depends on the strain, fermentation method and classification method (Andorrà et al., 2010; Gobert et al., 2017; Kemsawasd et al., 2015). Available studies provide a first overview of "preferential" nitrogen sources, mainly according to the capacity of NS yeasts to assimilate nitrogen and its rate of consumption. No molecular classification based on the regulation of nitrogen assimilation, such as the NCR or SPS systems (known in *S. cerevisiae*) is currently available. Currently, only one study indicated the presence of the NCR mechanism in *Hanseniaspora vineae* (Lleixà et al., 2019). Table 2 shows the first proposed classification of the various "preferential" nitrogen sources according to NS yeast strain, although such a comparison is not evident. Six NS yeasts have been studied: *H. uvarum*, *L. thermotolerans*, *M. pulcherrima*, *S. bacillaris*, *T. delbrueckii*, and *P. membranifaciens*. Most publications have reported glutamine as the preferred nitrogen source for *H. uvarum*, *L. thermotolerans*, *M. pulcherrima*, and *T. delbrueckii* and leucine for *H. uvarum*, *L. thermotolerans*, and *M. pulcherrima*. However, Gobert et al. (2017) showed a significant effect of fermentation temperature on the assimilation and consumption rates of nitrogen sources. For example, leucine was classified as a "preferred" source at 28 °C for *M. pulcherrima*, whereas it was classified as an "intermediate" source at 20 °C. This effect has also been observed for *S. cerevisiae* (Beltran et al., 2006). No studies are available to explain this phenomenon, but the recent study of Englezos et al. (2018) on NS yeast *S. bacillaris* provides a first answer. Surprisingly, the strains studied showed poor assimilation of amino acids during alcoholic fermentation relative to that of ammonium, which was entirely consumed. In addition, the production of several amino acids, such as alanine, glutamic acid, glycine, and valine was observed, whereas precedent studies have never reported such production. These results showing the ability of *S. bacillaris* to preferentially consume ammonium suggest less efficient SPS-dependent regulation (based on the *S. cerevisiae* model) of amino-acid permeases or an inhibitory mechanism mediated by ammonium. A significant proportion of YAN can be assimilated by NS yeast strains before the predominance of *S. cerevisiae* in the fermentation process. As a consequence, the growth and fermentation kinetics of *S. cerevisiae* may be

Table 2
Nitrogen compounds considered to be "preferred" nitrogen sources in NS yeasts depending on the conditions of fermentation.

Amino acids	NS yeasts	Condition of fermentation	Ref.
Alanine	Hu, Mp, Sb, Td	Grape juice 20 °C Grape juice 28 °C Synthetic medium 25 °C	2, 3
Ammonium	Mp, Td	Synthetic medium 25 °C	2
Arginine	Lt, Hu, Td	Synthetic medium 25 °C	2
Asparagine	Lt, Pm, Td	Grape juice 28 °C Synthetic medium 25 °C	2, 3
Aspartic acid	Hu, Lt	Synthetic medium 25 °C	2
Cysteine	Hu	Must 20 °C stirring	1
Glutamic acid	Hu, Lt, Mp	Synthetic medium 25 °C Must 20 °C stirring	1, 2
Glutamine	Hu, Lt, Mp, Td	Grape juice 20 °C Grape juice 28 °C Synthetic medium 25 °C Must 20 °C stirring	1, 2, 3
Glycine	Sb, Pm	Grape juice 20 °C Must 20 °C stirring	1, 3
Histidine	Mp, Pm	Grape juice 20 °C	3
Isoleucine	Lt, Mp, Td	Grape juice 28 °C Synthetic medium 25 °C	2, 3
Leucine	Hu, Lt, Mp	Grape juice 28 °C Synthetic medium 25 °C Must 20 °C stirring	1, 2, 3
Lysine	Mp, Sb, Pm	Grape juice 20 °C Grape juice 28 °C	3
Methionine	Hu, Mp	Grape juice 28 °C Must 20 °C stirring	1, 3
Phenylalanine	Hu, Lt	Synthetic medium 25 °C	2
Serine	Lt, Pm	Grape juice 28 °C	3, 2
Threonine	Mp, Pm	Grape juice 20 °C	3,
Tryptophan	Sb	Must 20 °C stirring	1
Tyrosine	Lt	Synthetic medium 25 °C	2
Valine	Hu	Synthetic medium 25 °C	2

Abbreviations of NS yeasts: Hu: *H. uvarum*, Lt: *L. thermotolerance*, Mp: *M. pulcherrima*, Sb: *S. bacillaris*, Pm: *P. membranifaciens* and Td: *T. delbrueckii*. References 1: (Andorrà et al., 2010), 2: (Kemsawasd et al., 2015), 3 (Gobert et al., 2017):

negatively affected (Gobert et al., 2017; Kemsawasd et al., 2015; Medina et al., 2012; Rollero et al., 2018; Wang et al., 2015). In particular, Gobert et al. (2017) showed that NS yeasts can consume between 66 and 215 mg/L of YAN, depending on the species. Of course, the rate of consumption can vary, depending on the winemaking methods (spontaneous fermentation, co-inoculation, sequential fermentation, temperature, aeration) and matrix (YAN must composition). Thus, nitrogen deficiencies need to be prevented by nitrogen supplementation to cover the needs of *S. cerevisiae* in the must for optimal alcoholic fermentation.

3. Factors that influence YAN assimilation

3.1. Sugar - nitrogen balance

Sluggish or stuck fermentations can be prevented by nitrogen supplementation when using deficient must. Several studies have been performed to determine the optimal nitrogen concentration in must to guarantee complete fermentation (Alexandre and Charpentier, 1998; Bely et al., 1990; Bisson, 1999; Henschke and Jiranek, 1993; Jiranek et al., 1995; Mendes-Ferreira et al., 2004). It is generally accepted that 120 at 140 mg N/L is sufficient (Bely et al., 1990) to complete the fermentation of 200 g/L of sugar. However, the needs may be higher, depending on winemaking practices (Mendes-Ferreira et al., 2004). The ratio of nitrogen to carbon sources during fermentation needs to be balanced to ensure good metabolic activity of the yeast. Several studies have associated nitrogen deficiency with a high turnover rate of sugar transporters in nitrogen-deficient must, resulting in a loss of sugar uptake capacity by the cells (Bisson, 1999; Salmon, 1989). Varela et al. (2004) showed that the rate of carbon uptake in “normal” fermentation was 3.6-fold higher during the exponential phase and 10-fold higher during the late stationary phase than for “sluggish” fermentation. The authors found that nitrogen deficiency affects the glucose transporter turnover rate and the expression of at least one: *HXT1* (low-affinity glucose transporter). Surprisingly, under conditions in which sugar uptake is low, they showed that adding biomass from sluggish cultures (from another tank) not only reduced the time to finish a problematic fermentation but was also less likely to affect the quality of the resulting wine. The higher the concentration of biomass, the quicker the fermentation was completed, even if the cells were grown in nitrogen-deficient medium (Maisonnave et al., 2013; Varela et al., 2004). Biomass formation coupled with macro- and micronutrients, including YAN, appear to be essential parameters involved in the mechanism of sluggish fermentation.

3.2. *S. cerevisiae* strain effect

In 2012, Crépin et al. showed that the kinetics of YAN consumption were strongly strain-dependent. Fourteen *S. cerevisiae* strains were tested in synthetic medium. The maximum rate of nitrogen consumption was between 16.8 and 27.8 mg N/L/h, which represented 40% of the variability in terms of the kinetics of nitrogen consumption. More recently, Lemos Junior et al. estimated the YAN needs of seven *S. cerevisiae* vineyard strains in synthetic must using three different YAN concentrations (70 mg N/L, 150 mg N/L, and 300 mg N/L). The authors showed no significant differences in fermentation kinetics at 150 and 300 mg N/L for all but one strain. However, there was a strong decrease in the rate of fermentation at 70 mg N/L. Thus, the fermentation kinetics appears to strongly correlate with the *S. cerevisiae* strain in conventional winemaking. In addition, although YAN supplementation reduced the duration of fermentation in this study, a concentration of 300 mg N/L appears to be the lower limit required for the maximum rate of fermentation. Brice et al. (2018) evaluated the diverse nitrogen needs of *S. cerevisiae* by determining the specific quantity of nitrogen consumed in an environment containing excess nitrogen, eliminating the nitrogen limitation/starvation factor that is generally observed during the fermentation processes. The authors demonstrated that the differences in the capacity of the strains to consume YAN were a result of differences in their ability to uptake specific nitrogen sources. The differences between the strains tested in this study to import nitrogen sources could be explained by mutations in the coding sequences that modulate the activities of the permeases or differences in the expression pattern of genes encoding these transporters.

3.3. Importance of lipids

Lipids, another nutrient present in must, affect nitrogen metabolism and this topic is reviewed here for the first time. The major fatty acids found in must were variable depending on studies (Gallander and Peng, 1979; Yunoki et al., 2005). However, palmitic, stearic, arachidic, behenic, linoleic and linolenic acids are predominant. Phytosterols are also present in grape berries, predominantly β-systerol. Although lipids (sterols and fatty acids) have been shown to play a key role in maintaining membrane integrity and viability during alcoholic fermentation, no link has yet been reported between lipid and nitrogen assimilation. In 2013, Tesnière et al. evaluated the consequences of nutritional lipid/nitrogen imbalances on wine yeast survival during alcoholic fermentation. The authors showed that yeast cell death during lipid-limited fermentation (using ergosterol as lipid source) was strongly influenced by the nitrogen content of the medium, with high nitrogen availability leading to high rates of cell death. Several amino acids were implicated as toxic precursors: arginine, glutamate, and glutamine. These observations correlated with the presence of the TOR-associated protein Tco89. High viability during lipid-limited fermentation was restored when the gene encoding this protein was deleted. This study highlights a relationship between the *TORC1* nitrogen signaling pathway and lipid limitation during alcoholic fermentation. Similarly, a study by Rollero et al. (2016) highlighted the role of lipid management in nitrogen metabolism. The addition of phytosterols to low-nitrogen medium influenced the consumption of various nitrogen sources. In particular, the nitrogen sources were most rapidly depleted at the lowest lipid concentration, especially valine, phenylalanine, and leucine. Consistent with these results, in nitrogen-rich medium, phytosterol addition (8 mg/L) increased the consumption rate of nitrogen sources, in particular tyrosine, tryptophan, glutamine, and ammonium. However, this was not true for all tested strains. In the same study, one strain consumed nitrogen more rapidly when the must contained a low concentration of phytosterols (2 mg/L). This effect may be explained by changes in the plasma membrane due to the incorporation of phytosterols. Indeed, the phospholipid composition of the membrane can be influenced and could affect transporter activity, particularly that of the arginine permease Can1p and Gap1p (Lauwers and André, 2006; Malinska et al., 2003).

3.4. Temperature

Temperature influences the expression of several genes involved in nitrogen metabolism, particularly that of several transporters. For example, *GAP1* is 5.2-fold down-regulated at 12.5 °C relative to 25 °C (Deed et al., 2015). However, the transcription of several other genes, such as *ARO9* and *ARO10* (involved in catabolism of phenylalanine and methionine and catabolism of multiple amino acids) is induced (Deed et al., 2015; Romagnoli et al., 2012). In 2006, Beltran et al. showed that *S. cerevisiae* grown at a low temperature (13 °C) consumed less nitrogen than at 25 °C. Indeed, the consumption of ammonium was 79 mg/L at 25 °C and 59 mg/L at 13 °C and that of amino acids 67 mg/L at 25 °C and 59 mg/L at 13 °C. The expression of *GAP1* and *MEP2* was not affected by the difference in temperature during fermentation, except at the end of the fermentation at 13 °C, at which point the gene expression levels were higher (Beltran et al., 2006).

Table 3
Comparison between the impact of the timing of addition and nitrogen source on fermentation.

Time of addition	N Source	Biomass	Growth rate	Fermentation time	Fermentation rate	Ref.
Must	AC	↗	↗	↘	↗	7, 14
	AC	nd	nd	↘	↗	9
	AC	↘	↘	→	↘	15
	AC	→	→	↘	↗	17
	AS	↗	nd	↘	↗	1
	AS	↗	→	↘	↗	3
	AS	↗	↗	↘	↗	5
	DAP	→	↗	→	→	2
	DAP	nd	nd	↘	↗	8, 12
	DAP	→	nd	↘	↗	10
	DAP	nd	nd	→	→	13, 16
	DAP	↗	nd	↘	↗	18
	AA	→	↗	→	→	2
	AA	nd	nd	↘	→	8
	AA	nd	nd	→	→	11
	AA	↘	↘	→	↘	15
	AA	↗	nd	↘	↗	17
	AC + AA	↗	↗	↘	↗	14
	AC + AA	↗	nd	↘	nd	4
	DAP + AA	nd	nd	↘	↗	6
Stationary growth phase	AC	→	→	↘	↗	7
	AC	↗	→	↘	↗	17
	AS	→	nd	↘	↗	1
	AS	↗	↗	↘	↗	3, 5
	DAP	nd	nd	→	→	13
	DAP	↗	nd	↘	↗	18
	AA	→	→	↘	↗	7
	AA	↗	nd	↘	↗	18
	AC + AA	→	→	↘	↗	7
	AC + AA	→	nd	↗	nd	4

1: Bely et al. (2003), 2: Hernández-Orte et al. (2004), 3: Mendes-Ferreira et al. (2004), 4: Beltran et al. (2005), 5: Hernandez-Orte et al. (2006), 6: Arias-Gil et al. (2007), 7: Jiménez-Marti et al. (2007), 8: Miller et al. (2007), 9: Vilanova et al. (2007), 10: Carrau et al. (2008), 11: Garde-Cerdan et al. (2008), 12: Ugliano et al. (2008), 13: Adams et al. (2010) 14: Torrea et al. (2011), 15: Martínez-Moreno et al. (2012), 16: Vilanova et al. (2012), 17: Martínez-Moreno et al. (2014), 18: Seguinot et al. (2018). nd: no data. Abbreviations: N, nitrogen; AC, ammonium chloride; AS, ammonium sulfate; DAP, diammonium phosphate; AA, amino acids. Upward arrows (↗) indicate an increase of the corresponding parameter relative to control, neutral arrows (→) no significant effect, and downward arrows (↘) a decrease. Several studies that concern the impact of nitrogen addition during fermentation are intentionally not mentioned in the table (Albers et al., 1996; Barbosa et al., 2012, 2009; Manginot et al., 1998). In these studies, the impact of increasing the nitrogen concentration was evaluated, but no data for controls without nitrogen addition were available. The experimental conditions in reference to the studies (matrix, initial sugar concentration, initial nitrogen concentration and nature) are described in Table 6.

3.5. Oxygen

Under anaerobiosis, yeast growth normally requires oxygen in order to favour the synthesis of sterols and unsaturated fatty acids. However, in such conditions, superfluous oxygen consumption by yeast cells is observed. The superfluous oxygen consumed by the yeast cells appeared not to be related to classical respiration, but mainly to the operation of several mitochondrial alternative respiration pathways, which were linked to the cell cytochrome contents (Salmon et al., 1998). It was calculated that approximately 124 μmol oxygen/g biomass were necessary for the synthesis of unsaturated fatty acids (Verduyn et al., 1990). In 2000, Blateyron and Sablayrolles showed that the combined additions of oxygen and DAP during the fermentation was more efficient to avoid a sluggish fermentation that a DAP addition alone. Authors justified that nitrogen had an immediate effect on the kinetics of fermentation by reactivating protein synthesis, particularly sugar transporters, while adding oxygen mainly influenced the kinetics at the end of fermentation, by synthesizing unsaturated fatty acids and sterols and thus increasing the yeast's resistance to ethanol. However, the precedent section showed that lipids and nitrogen metabolisms were interconnected (Rollero et al., 2016; Tesnière et al., 2013). Thus, oxygen by participating in lipid synthesis, influence indirectly nitrogen assimilation by the yeast. This observation was confirmed in a recent study where in low lipids condition in a must, the oxygen addition inducing the biosynthesis of ergosterol and unsaturated fatty acid by yeasts promoting nitrogen assimilation (Ochando et al., 2016). Therefore oxygen plays an important role in the regulation of assimilation of nitrogen.

4. Management of nitrogen addition

4.1. Ammonium salts and biotin

During winemaking, ammonium sulfate, ammonium phosphate, or diammonium phosphate are most commonly added. Ammonium salts are generally supplemented with biotin. This vitamin, among others, is required by the urea carboxylase involved in the catabolism of arginine (Cooper, 1982), one of the most abundant amino acids in must (Bell and Henschke, 2005; Boulton et al., 1996; Bouzas-Cid et al., 2017; Rapp and Versini, 1995).

Biotin also plays a role as a cofactor for pyruvate carboxylase, which catalyzes the transformation of pyruvate to oxaloacetate. Oxaloacetate is a precursor of both α-ketoglutarate and aspartic acid, the key intermediates of nitrogen assimilation, and the synthesis of other nitrogenous compounds (Cooper, 1982; Ljungdahl and Daignan-Fornier, 2012). Although biotin has been reported to be involved in nitrogen metabolism and is largely used in industry, only one study has investigated the influence of nitrogen and biotin interactions on the performance of *S. cerevisiae* in alcoholic fermentation (Bohlscheid et al., 2007). Two strains of *S. cerevisiae* were tested in synthetic must with different biotin (0, 1, and 10 μg/L) and YAN (60 and 250 mg/L) concentrations. Both strains exhibited poor growth and very low fermentation rates without biotin. An increase in nitrogen concentration resulted in higher fermentation rates, whereas adjusting biotin from 1 to

10 μg/L had no effect. Based on this single study, coupled biotin/nitrogen sources appear to be essential for fermentation with nitrogen addition. However, only a low concentration of biotin appears to be necessary (1 μg/L).

4.2. Consequences of nitrogen addition on fermentation parameters

It is generally agreed that at least 120–140 mg N/L YAN is required for satisfactory fermentation kinetics and final product quality (Bely et al., 1990; Henschke and Jiranek, 1993). However, several studies have shown that the needs can be higher (Coleman et al., 2007; Martínez-Moreno et al., 2012; Mendes-Ferreira et al., 2004) and can reach 267 mg N/L YAN to ferment 200 g/L glucose. Taillandier et al. (2007) showed that there is an optimal requirement that varies from 0.62 to 0.91 mg N/g of sugar, depending on the strain of *S. cerevisiae*. However, the authors found no correlation between sugar assimilation rates and nitrogen requirement (Taillandier et al., 2007). Thus, in the context of nitrogen addition, the yeast strain, matrix, time of addition and nitrogen sources appear to be critical elements for the optimization of enological parameters. We have collected the main conclusions of the studies concerning nitrogen addition in Table 3. This overview highlights the contradictory nature of the results.

Most studies included ammonium salts as the principal supplementary YAN source. In must or synthetic must, ammonium chloride addition generally decreases the fermentation time and increases the fermentation rate (Jiménez-Martí et al., 2007; Martínez-Moreno et al., 2014; Torrea et al., 2011; Vilanova et al., 2007). Nevertheless, this is not always true (Martínez-Moreno et al., 2012). No trends are observable concerning biomass production and growth rate (Table 3). The addition of ammonium sulfate to must appeared to decrease the fermentation time and increase the fermentation rate and biomass in all cases (Bely et al., 2003; Hernández-Orte et al., 2006; Mendes-Ferreira et al., 2004). Only the growth rate parameter was divergent (Hernandez-Orte et al., 2006; Mendes-Ferreira et al., 2004). More data are available concerning DAP addition to must. In four studies, DAP addition decreased the fermentation time and increased the fermentation rate (Carrau et al., 2008; Miller et al., 2007; Seguinot et al., 2018; Ugliano et al., 2008). In contrast, three studies showed no effect on the same parameters (Adams and van Vuuren, 2010; Hernández-Orte et al., 2004; Vilanova et al., 2012). Data concerning biomass production and growth rates are surprisingly limited. Only one study showed an increase in biomass after DAP addition to must (Seguinot et al., 2018), whereas two others showed no significant effect (Carrau et al., 2008; Hernández-Orte et al., 2004). Hernàndez-Orte et al. (2004) evaluated the impact of DAP addition to must on yeast growth rate. They observed an increase, but it is impossible to make a generalization based on a single study. All of this data shows that ammonium salts have not the same effect. This might be explained by the diversity of the matrix and *S. cerevisiae* strains used. Testing the effects of all ammonium salts on different *S. cerevisiae* strains in a single conventional synthetic medium could be a good approach to evaluate nitrogen addition impact.

Amino-acid addition to must as a source of nitrogen supplementation has become increasingly common. The effect varies widely, as for ammonium salts. In general, no impact on fermentation time has been reported (Garde-Cerdán and Ancín-Azpilicueta, 2008; Hernández-Orte et al., 2004; Martínez-Moreno et al., 2012) except for Miller et al. (2007). Neutral (Garde-Cerdán and Ancín-Azpilicueta, 2008; Hernández-Orte et al., 2004; Miller et al., 2007), decrease (Martínez-Moreno et al., 2012) or increase (Seguinot et al., 2018) effects on fermentation rate can be observed. The results concerning the effect on biomass production were also contradictory (Hernández-Orte et al., 2004; Martínez-Moreno et al., 2012; Seguinot et al., 2018) as for growth rate (Hernández-Orte et al., 2004; Martínez-Moreno et al., 2012). Coupled with ammonium chloride or DAP, amino acid addition appears to decrease fermentation time while increasing fermentation rate (Arias-Gil et al., 2007; Beltran et al., 2005; Torrea et al., 2011) and probably biomass production (Beltran et al., 2005) and growth rate (Torrea et al., 2011). As ammonium salts, the variability of the results might be linked to the diversity of the matrix and *S. cerevisiae* strains used (Fairbairn et al., 2017).

The addition of nitrogen during the stationary growth phase does not have the same consequences on enological parameters as its addition to must; it decreases the fermentation time and increases the fermentation rate, regardless of the study and nitrogen source (Bely et al., 2003; Hernández-Orte et al., 2006; Jiménez-Martí et al., 2007; Martínez-Moreno et al., 2014; Seguinot et al., 2018), with two exceptions for which there were no significant (Adams and van Vuuren, 2010) or opposite effect (Beltran et al., 2005). Generally, nitrogen addition during the stationary growth phase has no significant effect on growth rate (Hernandez-Orte et al., 2006; Martínez-Moreno et al., 2014), excepted in the study of Hernandez-Orte et al. (2006) in which it increased. Half of the published studies showed no significant effect on biomass production (Beltran et al., 2005; Bely et al., 2003; Hernandez-Orte et al., 2006), whereas the other half showed an increase, regardless of the nitrogen source (Hernandez-Orte et al., 2006; Martínez-Moreno et al., 2014; Seguinot et al., 2018).

Overall, it appears to be very complicated to compare the effect of YAN addition on essential fermentation parameters: maximum cell biomass, growth rate, fermentation time and fermentation rate. Indeed, the time of nitrogen addition, sources, the use or not of NS yeasts, the strain of *S. cerevisiae* used, the initial sugar concentration, temperature and the YAN already present in the must appear to influence the fermentation profile. This has led to several contradictions, depending on the experiments (Table 3). For example, Albers et al. (1996) showed that the addition of an amino-acid mixture to must resulted in greater maximum cell biomass than the addition of ammonium sulfate. However, in 2005, Beltran et al. showed that ammonium was the preferred nitrogen source for biomass production. The timing of addition can also affect biomass production. In 2006, Hernandez-Orte et al. showed that ammonium sulfate added at the beginning of fermentation significantly increased the maximum biomass. However, the results were compared to those obtained with nitrogen deficient synthetic medium. Thus, it is difficult to conclude whether the addition of an amino acid mixture to the must has a better potential to increase biomass formation (Albers et al., 1996) than ammonium sulfate added during the first part of fermentation (Hernandez-Orte et al., 2006). In addition, Hernandez-Orte et al. (2006) reported that adding ammonium sulfate to synthetic must always resulted in lower volatile acidity. Similar results were reported by Bely et al. (2003). However, ammonium sulfate added in excess increased the production of volatile acidity. Although this phenomenon is strain-dependent, it is difficult to draw conclusions on the effect of ammonium sulfate addition on volatile acidity without considering the amount added. The link between sugar and nitrogen metabolism has been established. However, comparing recent to previous studies using different sugar concentrations to compare the effect of nitrogen addition may not be informative. For example, Albert et al. (1996) used 20 g/L of sugar, whereas Taillandier et al. (2007) used 240 g/L and Bely et al. (2003) 360 g/L. Comparing the effect of nitrogen addition between these studies without considering the initial sugar concentration may be misleading. A multiparametric approach appears to be essential to resolve these issues. A compilation of similarly represented data would provide a first comprehensive view of the impact of nitrogen addition on fermentation. This would show if there are identifiable trends or if the effect of strain is predominant.

4.3. Consequences of nitrogen addition on volatile compounds produced by *S. cerevisiae*

Table 4 provides the first overview of studies concerning the impact of nitrogen addition on volatile compounds and glycerol produced by *S. cerevisiae*.

The addition of ammonium chloride (Jiménez-Martí et al., 2007;

Table 4
Impact of the addition of YAN sources on volatile compounds and glycerol produced by *S. cerevisiae*.

Time of addition	N Source	Higher alcohols	Esters	Fatty acids	Volatile acidity	Glycerol	Ref.
Must	AC	↘	↘	nd	nd	nd	6
	AC	↘	↗	→	→	→	8
	AC	↘	↗	↗	↗	→	12
	AC	nd	nd	nd	nd	→	13
	AC	nd	↘	nd	↗	↗	15
	AS	nd	nd	nd	↘	→	1
	AS	↘	→	↗	↘	nd	4
	DAP	↘	→	↗	nd	nd	2
	DAP	nd	↗	nd	nd	nd	7
	DAP	↘	↗	↗	nd	nd	9
	DAP	→	↗	↗	↘	↗	11
	DAP	↘	↗	→	→	→	14
	DAP	↗	↗	nd	nd	→	16
	AA	↘	→	→	nd	nd	2
	AA	nd	↘	nd	nd	nd	7
	AA	↗	↗	→	↘	nd	10
	AA	nd	nd	nd	nd	→	13
	AA	↘	↗	nd	nd	→	16
	AC + AA	↘	↗	↗	↗	→	12
	AC + AA	↘	↗	→	nd	→	3
	DAP + AA	nd	nd	↗	nd	nd	5
Stationary growth phase	AC	↘	↘	nd	nd	nd	6
	AC	nd	↘	nd	↗	↗	15
	AS	nd	nd	nd	↗	→	1
	AS	↘	→	↘	↘	nd	4
	DAP	↘	↗	nd	nd	→	16
	AA	↗	↘	nd	nd	nd	6
	AA	→	↗	nd	nd	→	16
	AC + AA	↘	↘	nd	nd	nd	6
	AC + AA	↗	↘	→	nd	↘	3

1: Bely et al. (2003), 2: Hernández-Orte et al. (2004), 3: Beltran et al. (2005), 4: Hernandez-Orte et al. (2006), 5: Arias-Gil et al. (2007), 6: Jiménez-Marti et al. (2007), 7: Miller et al. (2007), 8: Vilanova et al. (2007) 9: Carrau et al. (2008), 10: Garde-Cerdan et al. (2008), 11: Ugliano et al. (2008), 12: Torrea et al. (2011), 13: Martínez-Moreno et al. (2012), 14: Vilanova et al. (2012), 15: Martínez-Moreno et al. (2014), 16: Seguinot et al. (2018), nd: no data. Abbreviations: N, nitrogen; AC, ammonium chloride; AS, ammonium sulfate; DAP, diammonium phosphate; AA, amino acids. Upward arrows (↗) indicate an increase in the concentration of the corresponding metabolite relative to control, neutral arrows (→) no significant effect, and downward arrows (↘) a decrease. Several studies that concern the impact of nitrogen addition during fermentation are intentionally not mentioned in the table (Albers et al., 1996; Barbosa et al., 2012, 2009; Manginot et al., 1998). In these studies, the impact of increasing the nitrogen concentration was evaluated, but no data for controls without nitrogen addition were available. The experimental conditions in reference to the studies (matrix, initial sugar concentration, initial nitrogen concentration and nature) are described in Table 6.

Martínez-Moreno et al., 2014, 2012; Torrea et al., 2011; Vilanova et al., 2007) or ammonium sulfate (Bely et al., 2003; Hernandez-Orte et al., 2006) before fermentation appears to decrease higher alcohol production. The impact on the production of other metabolites is less clear (Hernandez-Orte et al., 2006; Jiménez-Martí et al., 2007; Martínez-Moreno et al., 2014; Torrea et al., 2011; Vilanova et al., 2007). The preceding paragraph underlines that there is more data concerning DAP than for other ammonium salts. No significant trends have been reported concerning higher alcohol production (Carrau et al., 2008; Hernández-Orte et al., 2004; Seguinot et al., 2018; Ugliano et al., 2008; Vilanova et al., 2012). For ester production, only Hernández-Orte et al. (2004) reported no effect, whereas other studies (Carrau et al., 2008; Miller et al., 2007; Seguinot et al., 2018; Ugliano et al., 2008; Vilanova et al., 2012) reported a general increase. The same trend was found for fatty acids, except in the study of Vilanova et al. (2012). Concerning the effect of nitrogen addition on volatile acidity, a decrease (Vilanova et al., 2012) or a neutral effect were reported (Ugliano et al., 2008). For glycerol, two studies showed no significant effect (Seguinot et al., 2018; Vilanova et al., 2012), whereas Ugliano et al. (2008) reported an increase. DAP addition also affects hydrogen sulfide production. The addition of DAP to Shiraz must fermented with *S. cerevisiae* led to greater hydrogen sulfide formation than non-supplemented fermentation. Moreover, DAP addition induced prolonged formation of hydrogen sulfide during the latter stage of fermentation, which was associated with higher hydrogen sulfide content in the final wines (Ugliano et al., 2009). Other volatile sulfur compounds were also present in significantly higher concentrations in the wine, including sulfides, disulfides, mercaptans and mercaptoesters (Ugliano et al., 2009). Once again, the strain effect seems to be involved. The study of the effect of ammonium salts on the production of volatile compounds on a large panel of *S. cerevisiae* strains could be relevant to identify possible trends.

The addition of amino acids before fermentation also leads to significantly different outcomes. Most studies have shown a decrease in higher alcohol production (Hernández-Orte et al., 2004; Seguinot et al., 2018), except one in which an increase was reported (Garde-Cerdán and Ancín-Azpilicueta, 2008). Similar contradictions were reported in ester production (Garde-Cerdán and Ancín-Azpilicueta, 2008; Miller et al., 2007; Seguinot et al., 2018). All available studies reported no significant difference in fatty-acids (Garde-Cerdán and Ancín-Azpilicueta, 2008; Hernández-Orte et al., 2004) or glycerol production (Martínez-Moreno et al., 2012; Seguinot et al., 2018). Only one study reported a decrease in volatile acidity (Garde-Cerdán and Ancín-Azpilicueta, 2008). Coupled with ammonium salts, amino-acid addition appears to increase fatty acids production (Arias-Gil et al., 2007; Torrea et al., 2011) excepted for Beltran et al. (2005). For other metabolites, only Beltran et al. (2005) and Torrea et al. (2011) reported an increase in ester production and volatile acidity, whereas there was no significant effect on glycerol production and a decreased production of higher alcohols. In addition, the addition of DAP coupled with amino acids appears to drastically reduce the production of hydrogen sulfide (Barbosa et al., 2012). This study, in which the authors evaluated three strains of *S. cerevisiae*, showed that methionine, in particular, suppressed hydrogen-sulfide production under all conditions.

Less data are available concerning nitrogen addition during the stationary growth phase. A decrease in higher alcohol production has been reported, regardless of YAN source, except for amino-acid addition alone (Jiménez-Martí et al., 2007; Seguinot et al., 2018). Ammonium chloride addition during the stationary growth phase appears to generally decrease ester production (Jiménez-Martí et al., 2007; Martínez-Moreno et al., 2014) and increase volatile acidity and glycerol production (Martínez-Moreno et al., 2014). The two studies concerning ammonium sulfate addition during the stationary growth phase reported no significant effect on ester production (Bely et al., 2003; Hernandez-Orte et al., 2006), whereas fatty acid production was decreased. The results concerning volatile acidity are contradictory (Bely et al., 2003; Hernandez-Orte et al., 2006). Only DAP appears to increase ester production, in contrast to other ammonium salts. However, there is no significant effect on glycerol concentration (Seguinot et al., 2018). The results obtained after amino-acid addition during the stationary growth phase are also contradictory about ester concentration (Jiménez-Martí et al., 2007; Seguinot et al., 2018). Finally, only Jiménez-Martí et al. (2007) evaluated the impact of the addition of ammonium chloride coupled with amino acids during the stationary growth phase.

The addition of amino acids at the beginning of the stationary phase significantly increases the production of isoamyl acetate relative to the control or DAP addition (Seguinot et al., 2018). In the same study, the authors assessed the influence of these parameters on the production of the higher alcohol propanol. Propanol is considered to be a marker of nitrogen availability in must (Mouret et al., 2014). After the complete consumption of YAN, the production of propanol restarted immediately upon the addition of nitrogen during the stationary phase and stopped again when no more YAN remained in the medium. Propanol

Table 5
Influence of YAN status on the transcriptional activity of gene families in *S. cerevisiae* during alcoholic fermentation.

N Source & condition	Gene family upregulated	Gene family downregulated	Ref.
AS Supplemented versus non-supplemented	RNA processing Ribosome biogenesis Amino-acid biosynthesis	Protein catabolic processes Stress response Oxidation-reduction processes	4
DAP High versus low level	Cell wall functions Glucose metabolism Protein folding Energy pathways Alcohol metabolism Glycolysis	Ribosomal proteins Small nucleolar ribonucleoprotein complex Nucleolus and RNA processing	1
DAP High versus low level	Flocculation Ribosomal proteins RNA processing Autophagy	nd nd nd nd	2
AA Supplemented versus non-supplemented	Biosynthesis of aroma compounds Yeast growth Environmental stress response	nd nd nd	3

1: Mendes-Ferreira et al. (2007), 3: Barbosa et al. (2015), 3: Liu et al. (2017), 4: Tesnière et al. (2015), nd: no data. Abbreviations: AS, ammonium sulfate; DAP, diammonium phosphate; AA, amino acids. The experimental conditions in reference to the studies (matrix, initial sugar concentration, initial nitrogen concentration and nature) are described in Table 6.

production is strongly dependent on the nature of the added nitrogen source (inorganic or organic). Mouret et al. (2014) showed that DAP addition induced greater production of propanol than amino-acid addition, in particular after addition during the stationary phase. In this study, final propanol production increased 4.3-fold after DAP addition during the stationary phase *versus* a two-fold increase after the addition of amino acids. It would be interesting to study the metabolic fluxes impacted by nitrogen addition in detail, especially the production of propanol from threonine, in order to explain the link between production differences observed and nitrogen sources.

The data concerning the impact of YAN addition on volatile compound and glycerol production by *S. cerevisiae* highlight several contradictions, as for enological parameters. The highly variable results cast doubt on the direct link between YAN sources and volatile compounds production. This was confirmed by the recent study of Fairbairn et al. (2017) in which the addition of a single amino acid resulted in the predictable production of volatile compounds. The authors proved a linear correlation between amino-acid concentration and the concentration of aromatic compounds directly derived from this amino acid. However, linear correlations were lost and volatile compounds production became unpredictable with the use of more complex nitrogen sources (mix) (Fairbairn et al., 2017). The diversity of methods used, including different matrices, fermentation condition, and strains leads to the lack of consensus, requiring reconsideration of how to approach the issue.

Such considerations are supported by the study of Crépin et al. (2017) who demonstrated that. YAN is mainly catabolized and involved in the *de novo* synthesis of proteinogenic compounds but not in the formation of volatile compounds. Nonetheless, YAN addition has consistently influenced volatile compounds production. Thus, these data suggest that there may be another indirect mechanism involving YAN sources and sugar metabolism for the production of volatile compounds.

4.4. *Sensory profile impact*

The role of nitrogen during wine fermentation has been extensively studied, particularly the effect of nitrogen on the production of volatile compounds. Surprisingly, few studies evaluated its impact of nitrogen addition on wine sensory profiles wines. In Shiraz wines, where DAP was added to a final YAN concentration of 250 or 400 mg/L in must, the scores for the descriptors “confectionary”, “red fruit”, and “dark fruit” are higher for the high-nitrogen fermentations. These descriptors are strongly positively associated with acetate esters and medium chain fatty acids ethyl esters (Ugliano et al., 2010). Along with DAP became the clear influence on perceived fruitiness, the other major effect of nitrogen addition was a suppression of the perceived intensity of descriptors such as “cheese”, “earth”, and “yeast” (Ugliano et al., 2010). In the same way, wines from Airen musts supplemented with DAP become more “citric” and less sulphurous, independently of the yeast strain used (Hernández-Orte et al., 2004). In another study, a mix of amino acids addition (phenylalanine, aspartic acid, threonine and alanine) in Merlot must affect sulphured, vegetal, fusel, floral, lactic and reduction notes. The least altered notes were spices and sweet fruits (Hernández-Orte et al., 2006, 2004). In their study, Torrea et al. (2011) have shown that the addition of either organic or inorganic nitrogen influence differently the formation of yeast volatile compounds. Consequences of ammonium nitrogen addition are an increase in ethyl acetate and acetic acid while a mix of amino acids and ammonium nitrogen led to a higher production of acetate and medium chain fatty acids ester. From a sensorial point of view, addition of nitrogen sources to reach 320 mg N/L in must led to wines rated higher in intensity for most of the fruity and floral attributes. However, the type of nitrogen added has no significant effect on the sensorial profile. Yet, sensory properties of wines from musts supplemented with amino acids seem to depend on the yeast strain. No study describes sensory impact about nitrogen addition coupled with lipids and/or vitamins.

4.5. *Transcriptome changes in response to nitrogen addition*

The expression of several genes that reflects nitrogen limitation under enological conditions has been shown to predict nitrogen deficiency, including that of *CAR1*, an arginase that catabolizes arginine to ornithine and urea (Carrasco et al., 2003); *ACA1,* which is important for carbon source utilization (Jiménez-Martí et al., 2007); *FSP2,* an alpha-glucosidase; *RGS2*, a negative regulator of glucose-induced cAMP signaling; *AQY1*, a spore-specific water channel; *AGX1,* an alanine glyoxylate aminotransferase (Backhus et al., 2001); *DAL4,* allantoin permease; *GAP1*, general amino acid permease (Gutiérrez et al., 2013b); and *ICY1*, which affects the consumption of ammonium during fermentation (Martínez et al., 2014). However, the studies focused mainly on the impact of low nitrogen medium on the transcriptional activity of *S. cerevisiae*. Little data is currently available concerning the impact of nitrogen addition. Table 5 provides the first comparison between gene families up- and downregulated after YAN addition before fermentation. Under low nitrogen conditions, the addition of DAP to synthetic grape juice medium during fermentation downregulated the expression of genes associated with ribosomal proteins, the small

Table 6
Experimental conditions used in Tables 3–5.

References	Table 3	Table 4	Table 5	Matrix	Initial sugar concentration (g/L)	Initial nitrogen concentration (mg/L)	Nature of nitrogen source
Bely et al. (2003)	1	1		Must	320 to 370	92	Naturally present in must
Hernández-Orte et al. (2004)	2	2		Must	nd	175	Naturally present in must
Mendes-Ferreira et al. (2004)	3			GJM	200	66	AS
Beltran et al. (2005)	4	3		SM	200	60 or 300	AC + AA
Hernandez-Orte et al. (2006)	5	4		SM	210	270	AS + AA
Arias-Gil et al. (2007)	6	5		Must	nd	285	Naturally present in must + DAP
Jiménez-Marti et al. (2007)	7	6		SM	200	60 to 300	AS + AA
Mendes-Ferreira et al. (2007)			1	GJM	200	66 to 267	DAP
Miller et al. (2007)	8	7		Must	213	2095	Naturally present in must
Vilanova et al. (2007)	9	8		CDGJ	225	117	AC + AA
Carrau et al. (2008)	10	9		CDGJ	120	75 to 400	DAP + AA
Garde-Cerdan et al. (2008)	11	10		Musts	nd	285 to 1173	Naturally present in must
Ugliano et al. (2008)	12	11		Musts	215	87 to 312	Naturally present in must
Adams et al. (2010)	13			Must	nd	361	Naturally present in must
Torrea et al. (2011)	14	12		Must	200	160	Naturally present in must
Martínez-Moreno et al. (2012)	15	13		SM	200 to 280	0 to 220	AC or AA or AC + AA
Vilanova et al. (2012)	16	14		Must	nd	250	Naturally present in must
Martínez-Moreno et al. (2014)	17	15		SM	240	100 to 200	AC + AA
Barbosa et al. (2015)			3	GJM	200	67 to 670	DAP
Liu et al. (2017)			4	SM	200	300	AC+AA
Tesnière et al. (2015)			5	YNB	20	0	Ammonium (source unspecified)
Seguinot et al. (2018)	18	16		SM	200	100	AC + AA

Abbreviations:GJM, grape must modified; SM, synthetic must; CDGJ, chemically defined grape juice; YNB, yeast nitrogen base.

nucleolar ribonucleoprotein complex, the nucleolus, and RNA processing (Mendes-Ferreira et al., 2007). In contrast, genes involved in the functions of the cell wall, glucose metabolism, protein folding, energy pathways, alcohol metabolism, and glycolysis were upregulated (Mendes-Ferreira et al., 2007). Similarly, Barbosa et al. (2015), evaluated the consequences of the transcriptomic response of various *S. cerevisiae* strains to DAP addition to synthetic must before fermentation. Surprisingly, genes involved in ribosomal protein and RNA processing were upregulated, which is opposite of the results of Mendes-Ferreira et al. (2007). In addition, genes involved in flocculation were the most highly over-represented category in the annotation of the most highly expressed genes during high-nitrogen fermentation, in particular, *FLO10,* which participates in the fermentation stress response (FSR). Thus, Barbosa et al. (2015) suggested that such overexpression is an adaptation of the yeast to stress conditions and allows the yeast to conserve a good capacity of fermentation. In contrast to that of Mendes-Ferreira et al. (2007), the study of Barbosa et al. (2015) demonstrated that the addition of DAP before fermentation leads to the upregulation of the expression of genes involved in ribosomal protein and RNA processing.

The addition of ammonium sulfate during fermentation, when yeast are in nitrogen starvation, leads to global transcriptome changes. Tesnière et al. (2015) showed that 30 min after ammonium sulfate replenishment, a set of 1410 genes enriched for those related to RNA processing, ribosome biogenesis, translation, and amino-acid biosynthesis was upregulated. At the same time, 1564 genes related to protein catabolic processes (ubiquitin-dependent and vacuolar protein degradation), stress responses, and oxidation-reduction processes were downregulated. Under similar conditions, DAP addition downregulated genes including those involved in RNA processing and ribosomal protein synthesis (Mendes-Ferreira et al., 2007). This contradiction was not noted by Tesnière et al. (2015). Conway et al. (2012) also evaluated the impact of the addition of ammonium sulfate coupled with amino acids. In this study, they obtained contradictory results compared to Mendes-Ferreira et al. (2007) (Table 5). Thus, it appears that the transcriptional activity related to RNA processing is affected depending on the ammonium salts. However, it is difficult to compare the two conditions as the strains and medium and fermentation parameters were different.

As already discussed, amino acids are another potential supplementary nitrogen source. High nitrogen concentrations, including that from exogenous amino-acids and ammonium, can unbalance the general composition of the must, particularly under conditions of high nitrogen content and low micronutrients. In 2017, Duc et al. showed that, among the seven micronutrients evaluated in this study (ergosterol, oleic acid, pantothenic acid, nicotinic acid, thiamin, biotin, and inositol), four trigger yeast cell death when the yeast are in growth-restricting amounts in a nitrogen-rich medium. Two (ergosterol, oleic acid) are lipid growth factors, conditional to anaerobic growth, and the other two vitamins (pantothenic acid and nicotinic acid). They showed that the *TORC1/SCH9* signaling pathway was a key complex in this effect. Indeed, the authors observed that decreasing *TORC1/SCH9* signaling restored high cell viability under several micronutrient-limited fermentation conditions. Viability was also restored by *SCH9* deletion under conditions of oleic acid, ergosterol, and pantothenic acid starvation. Global gene expression experiments showed that two clusters distinguish the conditions that preserve viability from those that lead to a high rate of cell death. The first included genes involved in mitochondrial respiration, electron transport processes, the TCA cycle, membrane transport, and detoxification. The second included genes involved in nitrogen catabolism, purine metabolism, and membrane transport, including that of nitrogen substrates. Significant differences in gene expression were specifically observed in these clusters under conditions of high nitrogen content and limited-micronutrients (Duc et al., 2017).

The addition of amino acids alone as a nitrogen source to must also affects global gene expression (Liu et al., 2017). L-valine, L-leucine, and L-isoleucine added in a non-limiting nitrogen medium resulted in the up- or downregulation of 25 genes during fermentation. Some genes were upregulated throughout fermentation, including: *ACT1,* a structural protein involved in cell polarization; *PDA1*, which catalyzes the direct oxidative decarboxylation of pyruvate to acetyl-CoA; *SER1*, required for serine and glycine biosynthesis; *FOX2*, a multifunctional enzyme of the peroxisomal fatty acid beta-oxidation pathway; *MEP2*, ammonium permease; *BAP3*, which is involved in the uptake of cysteine, leucine, isoleucine, and valine; *THR4*, a conserved protein that catalyzes the formation of threonine from O-phosphohomoserin; *AGX1*, which catalyzes the synthesis of glycine from glyoxylate; *STL1*, a glycerol proton symporter of the plasma membrane; and *GAP1*, general amino acid permease), whereas others genes were upregulated only during the exponential growth phase, including *GIP1*, a meiosis-specific regulatory subunit of the Glc7p protein phosphatase; *DIT2*, N-formyltyrosine oxidase; *RCK1*, a protein kinase involved in the oxidative stress response; *SPO1* a meiosis-specific prospore protein; and *MEK1*, meiosis-specific serine/threonine protein kinase. The addition of this mix of amino acids also up-regulated the expression of *SUT1*, which positively regulates sterol uptake genes under anaerobic conditions; *RRI2*, a subunit of the COP9 signalosome complex; and *CIN5*, a basic leucine zipper transcription factor of the yAP-1 related to the environmental stress response. Surprisingly, the expression of few genes related to the biosynthesis of volatile compounds was also affected. Among 412 genes, only the expression of *AAD3, AAD4, AAD14, ADH2*, and *PDC5*, involved in the biosynthesis of aroma substances, was significantly up-regulated (Liu et al., 2017).

5. Concluding remarks

From grape to wine, the role of nitrogen in winemaking is complex. This review focuses on fermentation and highlights new considerations concerning the impact of nitrogen on yeast metabolism and how biotic, abiotic, and exogenous addition can influence enological parameters. Here, the review of recent studies showed that YAN preferentially consumed by NS yeast strains during the first stage of fermentation vary and other factors, such as competition can be involved. There are no studies that explain why some YAN sources are preferentially consumed by NS yeasts. Moreover, the effect on the expression of genes involved in nitrogen regulation of *S. cerevisiae* in the presence of NS yeasts is still unexplored. The vast majority of studies have referred to "preferred" or "non-preferred" YAN sources, but a close examination in this review shows that this categorization varies widely, even if the same parameters of comparison were used. An exploration of genetic variation appears to be necessary to obtain a better understanding of the preferential assimilation of YAN. Much genetic data are available for *S. cerevisiae.* In contrast, current genetic data for NS yeast are too limited to develop significant research on nitrogen regulation. Enological nitrogen addition using amino acids is being increasingly performed, but the impact on *S. cerevisiae* in terms of fermentation parameters and volatile compound production is not clear. Indeed, recent studies show that only 5% of amino acids present in the must are directly involved in the synthesis of volatile compounds. Moreover, transcriptomic analysis has shown that the addition of nitrogen does not lead to the significant overexpression of genes involved in volatile compound production. Thus, the significant modification of the volatile compound profiles in wine caused by the addition of amino acids needs to be further investigated. Deciphering the link between nitrogen metabolism and volatile compound synthesis will provide useful information to monitor the fermentative aroma profile of wine.

Future studies that will lead to a better comprehension of YAN dynamics, from grape to wine, interconnected with other pathways (vitamins and lipids), will help to explain the production of volatile compounds and allow better control of fermentation by avoiding sluggish fermentation, providing new tools to winemakers. Most of the studies examining the link between nitrogen and volatile compound production were carried out in synthetic must. Given the complexity of the composition of must it will be necessary to include real must in future studies aiming to understand the influence of nitrogen on fermentation kinetics and aroma production.

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Appendix A. Supplementary data

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