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Occurrence of yeast cell death associated with micronutrient starvation during wine fermentation varies with nitrogen sources

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ABSTRACT

Aim: Nitrogen availability is an essential parameter for wine alcoholic fermentation. Moreover, recent results have shown that it plays a key role in yeast cell death in interaction with micronutrients limitations such as lipids or vitamins. We found that yeast cell death was triggered by starvation for a set of micronutrients, including oleic acid, ergosterol, pantothenic acid and nicotinic acid whenever the level of nitrogen was high, but not in low nitrogen conditions. We examined here the impact of the nature of the nitrogen source supplementation in the light of these previous results.
Methods and results: 19 amino acids or NH_4^+ were added, in amounts corresponding to 354 mg/L assimilable nitrogen, to an oenological medium that was low in nitrogen and oleic acid. Yeast viability in function of the fermentation progress was assessed and showed differences in cell death during the alcoholic fermentation in function of the amino acid added. The addition of NH_4^+ was also tested at two different times during wine fermentation. The results obtained show that various nitrogen sources (amino acids, ammonium) can trigger cell death but with different intensities.
Conclusion: It appears that some amino acids are preferable to others in alcoholic fermentation because they do not trigger cell death. We also provide evidence that the timing of nitrogen addition has a strong impact on cell death in musts with micronutrient limitations: an early nitrogen addition is more likely to trigger cell death than a late addition.
Significance and impact of the study: Our results provide a novel frame for managing nitrogen supplementation of grape musts and to avoid stuck fermentation.

KEYWORDS

Saccharomyces cerevisiae, oenological fermentation, amino acids, ammonium, micronutrient starvation, cell death

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Nitrogen availability is an essential parameter of wine alcoholic fermentation. Indeed, it is usually considered as the main parameter controlling both the fermentation rate and the duration of fermentation (Bely *et al.*, 1990a). Low levels of assimilable nitrogen in grape must can lead to sluggish or stuck fermentation, a situation which is generally avoided by supplementing musts with nitrogen salts (Sablayrolles, 2009). It is generally believed that high nitrogen content in grape musts enhances the fermentation process. Recent work has also focused on the impact of must supplementation with organic nitrogen sources (i.e. amino acids) during wine fermentation and shown the importance of understanding the distinct effects of the different nitrogen sources (Kemsawasd *et al.*, 2015), which has been rarely investigated in oenological conditions. However, high nitrogen levels are not necessarily sufficient to ensure a high-quality fermentations process, as other nutrients can impact fermentation outcomes and can interact with nitrogen availability (Tesnière *et al.*, 2013). We have recently shown that high nitrogen amount in musts could trigger yeast cell death when some micronutrients were in growth-limiting concentrations (Duc *et al.*, 2017). We found that yeast cell death in alcoholic fermentation was triggered by starvation for a set of micronutrients, including oleic acid, ergosterol, pantothenic acid and nicotinic acid, whenever the level of nitrogen was high but not in low nitrogen conditions. This showed the importance of nitrogen in regulating cell death during wine fermentation. We also demonstrated that yeast cell death under micronutrient limitation is controlled by the availability of residual nitrogen, and that this involved the nitrogen signaling pathways, mainly the TOR pathway that controls the triggering of an appropriate stress response. These findings are consistent with those of Boer *et al.* (2008), which showed that the capacity of survival during the stationary phase was regulated by the first limiting nutrient.

Some of these micronutrient limitations can occur in winemaking situations depending on the practices used. For example, grape must clarification, aimed at the removal of solid particles, can deplete musts in lipids, especially sterol and unsaturated fatty acids that are critical nutrients for the yeast in alcoholic fermentation (Houtman and Plessis, 1986). The impact of the nitrogen content of grape musts has thus to be considered for potential interaction with micronutrient limitations in wine alcoholic fermentation management. As nitrogen supplementation – usually performed with ammonium salt but more complex organic sources are also proposed – is a common practice in oenology, a better knowledge of the effect of supplementation with different nitrogen sources and considering the interactions with micronutrient limitations is required for enhanced nitrogen management. In this work we examined the impact of individual nitrogen sources (amino acids and NH_4^+) and of the timing of their addition; we showed a strong impact of both the nature of the nitrogen source and time of addition on yeast cell death and fermentation outcome.

MATERIALS AND METHODS

1. Strains

We used the commercial wine yeast strain Lalvin EC1118®, a *Saccharomyces cerevisiae* strain isolated in Champagne (France) and manufactured by Lallemand (Montreal, Canada).

2. Synthetic culture media

Unless otherwise specified, a synthetic fermentation medium (SM71) with 71 mg/L yeast assimilable nitrogen (YAN) and 23% glucose + fructose (1/1), adjusted to pH 3.3 and simulating one-third nitrogen and amino acid concentrations of a standard grape juice was routinely used (Bely *et al.*, 1990b). Per liter, this medium contained the following: 115 g glucose, 115 g fructose, 6 g citric acid, 6 g DL-malic acid, 750 mg KH_2PO_4, 500 mg K_2SO_4, 250 mg $MgSO_4.7H_2O$, 155 mg $CaCl_2.2H_2O$, 200 mg NaCl, 4 mg $MnSO_4.H_2O$, 4 mg $ZnSO_4.7H_2O$, 1 mg $CuSO_4.5H_2O$, 1 mg KI, 0.4 mg $CoCl_2.6H_2O$, 1 mg H_3BO_3, 1 mg $(NH_4)_6Mo_7O_{24}.4H_2O$, 20 mg myo-inositol, 2 mg nicotinic acid, 1.5 mg calcium pantothenate, 0.25 mg thiamine-HCl, 0.25 mg pyridoxine and 0.003 mg biotin. As nitrogen sources, it also contained ammoniacal nitrogen and amino acids as follows (per liter): 77 mg NH_4Cl, 102 mg L-proline, 84.5 mg L-glutamine, 30 mg L-tryptophan, 24 mg L-alanine, 20 mg L-glutamic acid, 13 mg L-serine, 12.5 mg L-threonine, 8 mg L-leucine, 7.5 mg L-aspartic acid, 7.5 mg L-valine, 6.5 mg L-phenylalanine, 62.5 mg L-arginine, 5.5 mg L-histidine, 5.5 mg L-isoleucine, 5 mg L-methionine, 3 mg L-glycine, 3 mg L-lysine, 3 mg L-tyrosine and 2 mg L-cysteine. The medium was heat-sterilized (100°C, 10 min). Vitamins were filtered-sterilized (0.22 µm) and were added to the medium after sterilization. Lipid factors (LF) were added to the fermentation medium after sterilization, to a final concentration of 15 mg/L ergosterol and 18 mg/L oleic acid (brought as Tween 80®, which is mainly a mix of oleate esters (Zhang *et al.*, 2012)) that correspond to an oleic acid limitation (Ole-). For the fermentations performed under

ergosterol limitation (Erg-), the ergosterol concentration was fixed at 1.5 mg/L. For the fermentations performed under pantothenic acid limitation (Pan-), the pantothenic acid concentration was fixed at 0.02 mg/L. For the fermentations performed under nicotinic acid limitation (Nic-), the nicotinic acid concentration was fixed at 0.08 mg/L. When added, p-aminobenzoate (PABA) concentration was 1 mg/L.

3. Fermentation conditions and kinetics

The yeast strain Lalvin EC1118® used in this study was pre-cultured for 24 h at 28°C in a yeast nitrogen base medium (YNB) (6.7 g/L) without amino acids (Becton, Dickinson and Company) and glucose (20 g/L) in Erlenmeyer flasks. The fermentation medium was then inoculated from this pre-culture to reach 10^6 cell/mL medium. Fermentations were carried out in 350 mL fermenters (containing 300 mL medium), with fermentation air locks. Fermentation media were routinely de-aerated prior to inoculation by bubbling pure argon for 15 min. Filling conditions were controlled and fermentations were carried out under anaerobic and isothermal conditions (28°C), with permanent stirring (300 rpm). The amount of CO_2 released was calculated from manual weighing of 350 mL fermenters (Sablayrolles *et al.*, 1987). Residual sugar amount was calculated from released CO_2 using linear regression (1 g of sugar consumed = 0.47 g of CO_2 (Bely *et al.*, 1990b)). All the fermentations were performed in biological duplicate and standard deviations were calculated from these.

4. Cell population densities and cell viability determinations

In all the experiments, cell populations were counted using a Beckman-Coulter electronic particle counter. Cell viability was determined by flow cytometry using a C6 cytometer (Accuri, BD Biosciences): propidium iodide (PI) (Calbiochem) was added to the cell suspension (5 µL of a 0.1 mg/mL solution), and the samples were mixed by gentle shaking. PI is a fluorescent nucleic acid stain (excitation 488 nm, emission 575 nm) that cannot penetrate intact cell membranes. PI flow cytometry analysis was performed 15 min after staining. Fluorescence data for PI-stained cells were collected in channel FL3. Viability was determined as the percentage of intact and fragile cells among all cells (Delobel *et al.*, 2012). All the measurements were performed from the biological duplicate and standard deviations were calculated.

5. Nitrogen sources assay

To test the effect of nitrogen sources on yeast viability, we added the equivalent of 354 mg/L YAN provided by a single amino acid or by ammonium to the synthetic must before inoculation or at 30 g of CO_2 produced. Nitrogen sources were added to fermenters prior to argon bubbling when used before the start of fermentation or simultaneously with argon bubbling to ensure anaerobiosis. The corresponding concentrations of each nitrogen source are listed in Table 1. Although proline is not metabolized under anaerobic conditions, we tested this amino acid (considering it could provide one nitrogen atom) because it is present in high quantities in natural grape musts (Henschke and Jiranek, 1993). In the case of arginine, three of the four nitrogen atoms were considered as metabolizable by yeast (Middelhoven, 1964). In order to specify some nitrogen source effects, other concentrations were tested in the same way. All the conditions were performed in biological duplicate.

RESULTS AND DISCUSSION

1. Nitrogen sources have different impacts on yeast viability in oleic acid-limited fermentations

TABLE 1. Nitrogen sources concentration equivalent to 354 mg/L YAN.

Nitrogen sources	Concentration equivalent of 354 mg/L YAN (mg/L)
Alanine	2254
Arginine	1469
Asparagine	1671
Aspartic acid	3367
Cysteine	3065
Glutamine	1848
Glutamic acid	3722
Glycine	1899
Histidine	1308
Isoleucine	3318
Leucine	3318
Lysine	1849
Methionine	3775
Phenylalanine	4179
Proline	2912
Serine	2658
Threonine	3013
Tryptophan	2583
Valine	2963
Ammonium	406

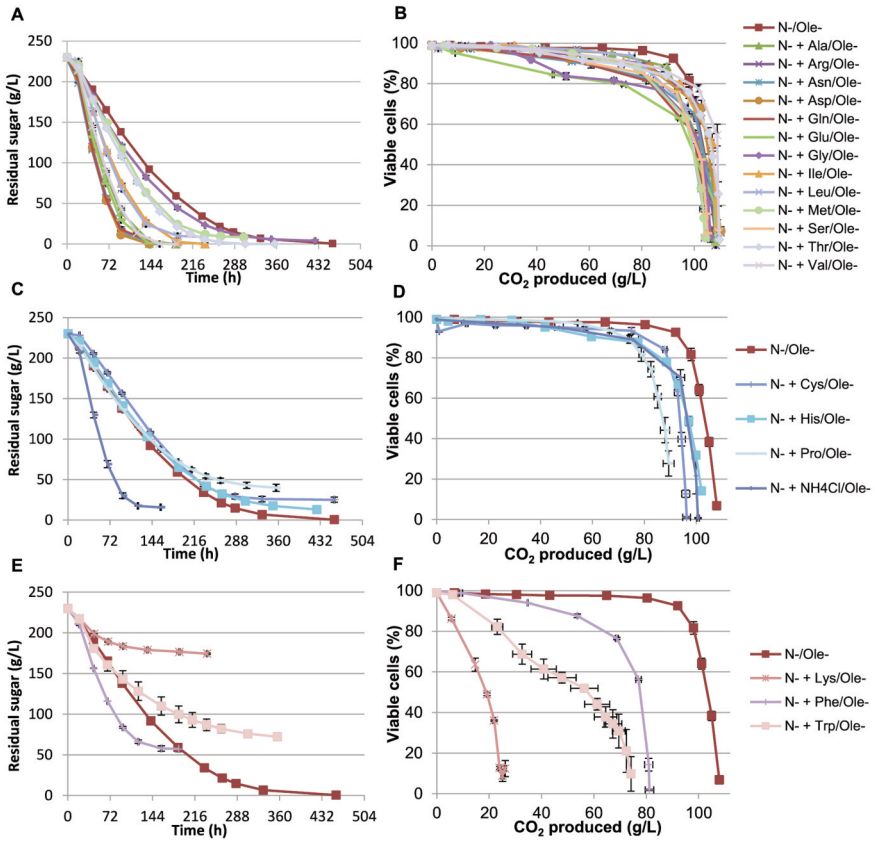

FIGURE 1. Effects of supplementation of 354 mg/L YAN equivalent of various nitrogen sources.
Effects produced in (A, C, E) residual sugars and (B, D, F) yeast viability during wine fermentation under oleic acid starvation (18 mg/L of oleic acid in the fermentation medium). Nitrogen supplementations were separated in three groups according to their impact on yeast viability: the first group led to the lowest loss of viability (A, B); the second group led to a moderate loss of viability (C, D); and the third group led to the highest loss of viability (E, F). Fermentations were performed in duplicate. Error bars correspond to the standard deviation.

In order to address the impact of individual nitrogen source (amino acids and NH_4^+) supplementation, we used a basal fermentation medium containing a low level (71 mg/L) of assimilable nitrogen provided by a combination of amino acids and ammonium. We previously showed that, in this medium, there was no loss of viability until the end of the fermentation (Duc *et al.*, 2017). The oleic acid content of the medium was set to a level supporting an identical growth compared to that allowed by the available nitrogen. In these conditions, growth is thus simultaneously restricted by nitrogen and oleic acid (N- /Ole- medium). Yeast cells maintained high viability throughout the alcoholic fermentation, while the fermentation progression was consistently low in presence of a low nitrogen level (Figure 1). To assess the impact of individual nitrogen sources, each of 19 amino acids or NH_4^+ were added to this "N- /Ole- medium" in amounts corresponding to 354 mg/L assimilable nitrogen (see Table 1) – finally resulting in 425 mg/L YAN amount corresponding to a nitrogen-rich must (Figure 1). In these conditions, the oleic acid is the limiting nutrient and residual nitrogen is present in the medium during the stationary phase (Duc *et al.*, 2017). The effects of each nitrogen source addition are displayed in Figure 1 and Figure S1. In order to consider the impact of ethanol on cell viability, this parameter is displayed in relation with the fermentation progress (amount of CO_2 released) rather than in relation to time (see Figure S2 for viability against the time).

Regarding cell viability, most of the nitrogen supplementations led to a loss of viability higher than the N-/Ole- condition. This is consistent with a loss of viability controlled by the nitrogen content under micronutrient starvation, with cell death enhanced by high nitrogen concentrations, as previously reported (Tesnière *et al.*, 2013; Duc *et al.*, 2017). The kinetics of cell death differed strongly depending on the nitrogen source available. As the fermentation stopped when the viability highly reduced, we used the CO_2 value produced at this point as the criteria to define the three groups of amino acids for their capacity to trigger cell death: no or a small amount of cell death (Figure 1B), moderate cell death

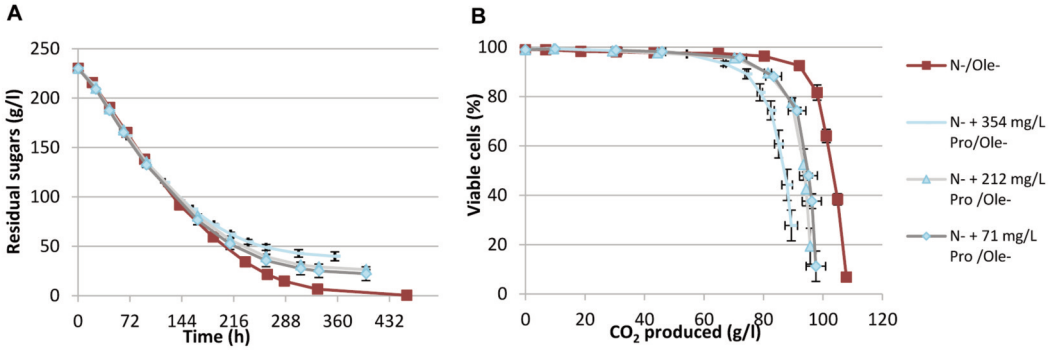

FIGURE 2. Effects of supplementation of 354, 212 and 71 mg/L "YAN equivalent" of proline one.
Effects produced in (A) residual sugars and (B) yeast viability during the wine fermentation under oleic acid starvation (18 mg/L of oleic acid in the fermentation medium). Fermentations were performed in duplicate. Error bars correspond to the standard deviation.

(Figure 1D), and extensive cell death (Figure 1F). Most of the supplementation in amino acids only led to a minimal loss of viability, which allows a production of CO_2 over 104 g/L, including Ala, Arg, Asn, Asp, Gln, Glu, Ile, Leu, Met, Ser, Thr, and Val, which correspond to the first group (Figures 1A & B). In fact, all the fermentation kinetics were enhanced by nitrogen addition and all sugars were more rapidly fermented. Glutamine, glutamic acid and valine were the more efficient nitrogen sources, leading to a strong reduction in fermentation duration (145 h versus 440 h for the non-supplemented must). The second group (Figure 1C and D) comprised cysteine, histidine, proline and ammonium supplementation that led to some loss of viability associated with stuck fermentations, with residual sugars varying from 13 g/L to 40 g/L with a CO_2 production between 89 and 101 g/L. The final yeast population was lower after proline, cysteine and histidine addition, pointing to a negative effect of these nitrogen sources on cell growth. The third amino acids group, allowing CO_2 production below 82 g/L and associated with a high loss of viability, includes phenylalanine, tryptophan and lysine (Figures 1E and F). Lysine supplementation triggered an early loss of viability that could be observed as soon as fermentation started. The maximal population was low (20.10^6 cells/mL), suggesting a toxic effect of this amino acid on yeast cells. It led to a stuck fermentation before 30 g of CO_2 produced with about 174 g/L residual sugar. Tryptophan supplementation strongly impacted yeast viability with a constant loss throughout fermentation that stopped at 74 g of CO_2 produced with approximatively 72 g/L residual sugars. Phenylalanine also led to a stuck fermentation at 81 g of CO_2 produced with 58 g/L of residual sugar, consistent with a high loss of viability. These results show that oleic acid limitation can lead to very different outcomes depending on the nitrogen sources used for supplementation.

It is also worthwhile to note that, in our experiment, supplementations in cysteine and histidine led to lower cell growth than in the non-supplemented condition (32.10^6 cells/mL and 45.10^6 cells/mL, respectively). Thus, they also appear to inhibit cell growth. This is consistent with the findings of Schultz and Pomper (1948), Cooper (1982) and Ye *et al.* (1991) who found a similar behavior for cysteine, histidine and lysine used as sole nitrogen sources.

2. Proline supplementation enhances cell death under oleic acid starvation

Interestingly, we found that proline supplementation at the start of the fermentation process enhanced the loss of viability and led to stuck fermentation at 90 g of CO_2 produced (Figure 1D). In order to specify its effect, we tested different levels of proline supplementation of the medium (Figure 2). Supplementation with lower concentrations of proline (71 mg/L or 212 mg/L "YAN equivalent") led to a loss of viability (Figure 2B) and stuck fermentations, as shown by the presence of residual sugars (Figure 2A). There was also a dose effect as the condition with the highest amount of proline (354 mg/L "YAN equivalent") led to much enhanced cell death and to a stuck fermentation occurring sooner than in the 212 and 71 mg/L "YAN equivalent" conditions. This shows that the supplementation in proline results in yeast cell death in the concentration range examined here. Although proline is not catabolized in such condition of anaerobiosis, and thus does not provide any assimilable nitrogen, it seems that the sole presence of proline in the medium

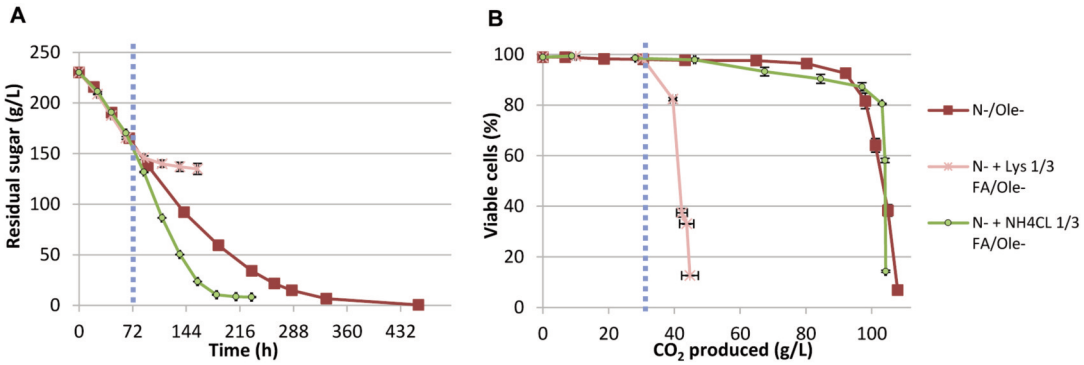

FIGURE 3. Effect of supplementation of 354 mg/L YAN equivalent of lysine at 30 g of CO_2 produced. Effects produced in (A) residual sugars and (B) yeast viability during the wine fermentation under oleic acid starvation (18 mg/L of oleic acid in the fermentation medium). Fermentations were performed in duplicate. Error bars correspond to the standard deviation. Dotted line indicates the nitrogen supplementation at 30 g of CO_2 produced.

decreases yeast viability. A recent work by Crépin *et al.* (2017) showed that some proline uptake likely occurs during alcoholic fermentation. However, Stracka *et al.* (2014) have shown that proline does not activate the TOR pathway. Thus, the pathway by which the cell death is triggered is certainly not the TOR pathway and the mechanisms involved remain unclear. The limited literature on proline does not report any negative proline impact during wine fermentation. On the contrary, some recent works (Takagi, 2008; Sasano *et al.*, 2012a; Sasano *et al.*, 2012b; Takagi *et al.*, 2016) report that intracellular proline accumulation affords some protection from ethanol stress. More importantly, the concentrations of proline used in these experiments (580–2900 mg/L) are within the range encountered in natural musts (60–3100 mg/L) (Long *et al.*, 2012).

3. Lysine supplementation is toxic for yeast under oleic acid starvation

As we previously described, lysine supplementation under oleic acid starvation led to a high loss of viability and inhibited cell growth (Figure 1E and Figure 1F). Indeed, immediately after lysine supplementation yeast cells started dying, with an arrest of fermentation at 30 g of CO_2 produced. This result highlights the importance of lysine in yeast metabolism. In fact, it is known that *Saccharomyces cerevisiae* cannot grow on a simple lysine medium (Walters and Thiselton, 1953; Ye *et al.*, 1991). Similarly, Kemsawasd *et al.* (2015) found that lysine supplementation of a medium limited in nitrogen leads to low cell viability, and Thomas and Ingledew, (1992) reported that in solid medium fermentation lysine supplementation was toxic to yeast. In order to specify the effect of lysine supplementation and mimic the effect of an addition of nitrogen in oenological conditions, we added lysine at 30 g of CO_2 produced when yeast cells were in the stationary phase (Figure S1E; Figure 3). We found that wine fermentation stopped just after this supplementation (Figure 3A), with a high loss in cell viability (Figure 3B), highlighting the extremely toxic effect of lysine. Even though it has been shown that intracellular lysine content lead to a regulation of the TOR pathway and modulated the cell death (Powers *et al.*, 2006), our results, i.e. the limited cell growth and rapid cell death, suggest that the mechanisms involved in yeast cell death are different from an effect of nitrogen signaling and more consistent with a toxic effect of lysine or of a lysine-derived compound.

4. Aromatic amino acids supplementation can lead to stuck fermentation

We found that supplementation of the medium in tryptophan and phenylalanine leads to similar yeast cell death during wine fermentation (Figures 1D and 1F). Tyrosine supplementation was tested and led to the same response as tryptophan and phenylalanine supplementations (results not shown here). As these are part of the aromatic amino acids family, it appears thus that this family plays a key role in yeast viability. These amino acids have a specific biosynthetic pathway, termed "super pathway of aromatic amino acids", in which a negative feedback control is applied by the aromatic amino acids on their own biosynthetic pathway (Braus, 1991) (Figure 4). Thus, this negative feedback could be triggered by an initial supplementation in these amino acids. Moreover, this pathway is connected to the methionine and purine metabolism via the chorismate pathway, an intermediate of aromatic amino acid synthesis that leads to a set of methyl-donor compounds. As neither chorismate nor p-aminobenzoate were present in the synthetic medium we used, we hypothesized that high aromatic amino acid

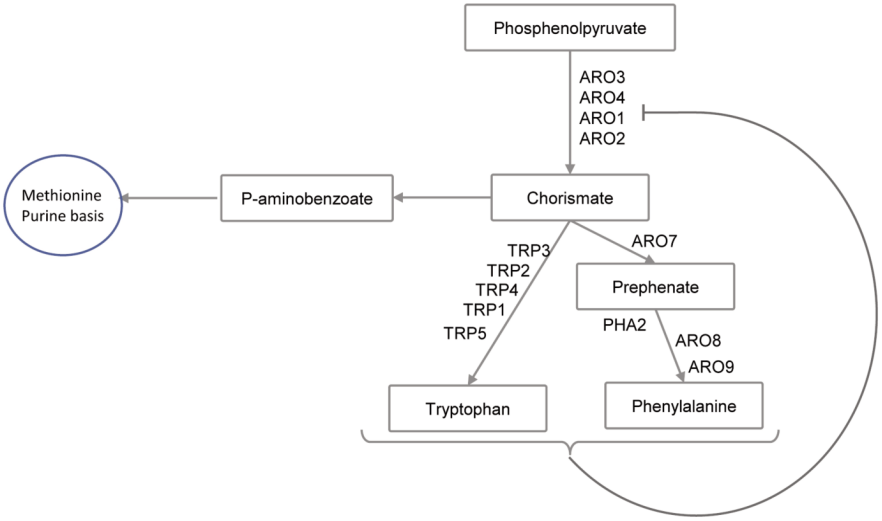

FIGURE 4. Super pathway of the aromatic amino acids and its feedback control. (Source: Braus, 1991)

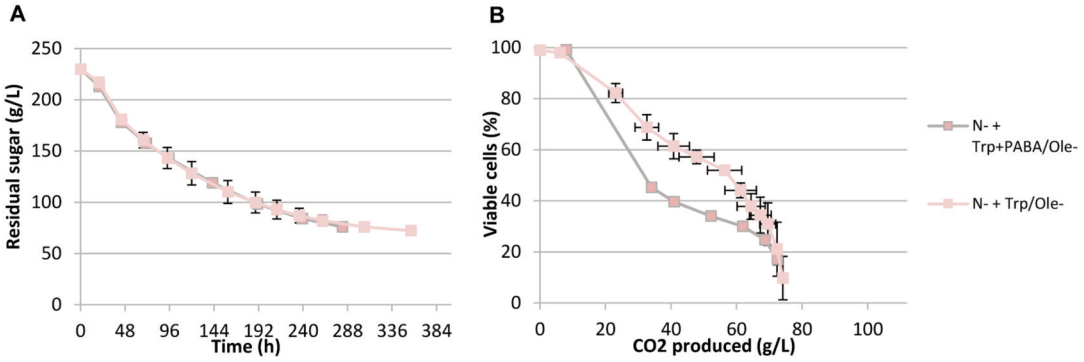

FIGURE 5. Effect of supplementation of 1 mg/L of p-aminobenzoic acid (PABA) and 354 mg/L YAN equivalent of tryptophan.
Effects produced in (A) residual sugars and (B) yeast viability during wine fermentation under oleic acid starvation (18 mg/L of oleic acid in the fermentation medium). Fermentations were performed in duplicate. Error bars correspond to the standard deviation.

concentrations could have repressed the chorismate pathway, therefore leading to a specific p-aminobenzoate requirement. Therefore, we checked the effect of an initial supplementation in p-aminobenzoate and tryptophan on yeast viability. As shown in Figure 5, p-aminobenzoate supplementation did not restore yeast viability, which shows that this enhanced cell death is not the result of a limitation in p-aminobenzoate. In fact, the loss of cell viability from 20 to 70 g of CO_2 produced is even higher in the presence of PABA, which may be due to a dose-dependent effect (Figure5A and Figure S4). To specify the effect of these amino acids, we added tryptophan and phenylalanine at 30 g of CO_2 produced under oleic acid starvation (Figure 6), and found that these amino acids, when added in stationary phase, trigger only little cell death. This suggests that these amino acids have a toxicity associated to yeast growth and intense nitrogen metabolism. In starvation, a lower metabolic activity may explain a weaker cell death. Moreover, upon entry into stationary phase, the triggering of a stress response that protects yeast cells may contribute to reducing yeast cell death.

5. Ammonium supplementation leads to cell death and stuck fermentations with several micronutrient limitations

We found that initial ammonium supple mentation led to a small amount of cell death during alcoholic fermentation under oleic acid

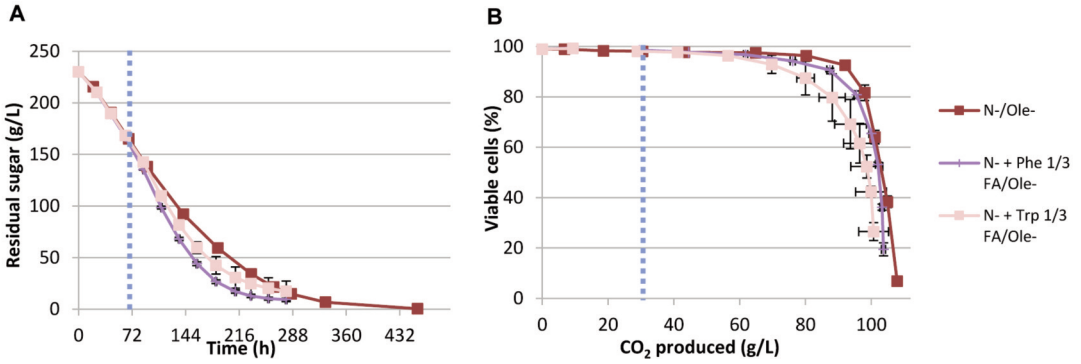

FIGURE 6. Effects of supplementation of 354 mg/ YAN equivalent of tryptophan and phenylalanine 30 g of CO_2.

Effects produced in (A) residual sugars and (B) yeast viability during wine fermentation under oleic acid starvation (18 mg/L of oleic acid in the fermentation medium). Fermentations were performed in duplicate. Error bars correspond to the standard deviation. Dotted line indicates the nitrogen supplementation at 30 g of CO_2 produced.

starvation (Figure 1D). As ammonium is the most commonly used nitrogen source in oenological conditions, we checked its impact under various micronutrient starvation conditions: ergosterol, pantothenic acid and nicotinic acid starvation. These starvation conditions are relevant as they lead to cell death in function of the nitrogen concentration of the medium during alcoholic fermentation (Duc *et al.*, 2017). As shown in Figure 7, high initial levels of NH_4Cl led to high cell death levels under all micronutrient starvation conditions examined. This loss of cell viability is associated with stuck fermentations in each situation with high residual sugars levels under pantothenic and nicotinic acid limitations (150 and 100 g/L, respectively). Under oleic acid and ergosterol limitations, even though fermentations were initially more rapid, cell death also led to stuck fermentations. These results show that high initial NH_4^+ concentrations in the must can be detrimental to fermentation outcome when a micronutrient is limiting. We therefore examined the impact of a similar amount of ammonium supplementation with delayed addition at 30 g of CO_2 produced (Figure 8). In such conditions, cell viability patterns were not strongly altered by nitrogen addition. Viability remained high in all situations and only addition of NH_4^+ to the ergosterol-limited medium triggered a loss of viability (Figure 8D). Moreover, though cell death was observed under ergosterol limitation, it did not lead to a stuck fermentation as shown by the complete consumption of sugars (Figure 8C). Thus, yeast cells were able to better manage ammonium supplementation when it was added at 30 g of CO_2 produced. This result is in line with the theory that yeast acquires some protection through the triggering of a stress response when entering into the stationary phase (Duc *et al.*, 2017). Also, these observations support a role of the TOR pathway in the control of the cell death in the medium condition we studied. Indeed, it has been described that NH_4^+ nitrogen could provoke cell death through the TOR pathway (Santos *et al.*, 2012).

GENERAL DISCUSSION

The supplementation of grape musts with nitrogen sources is a common practice to compensate for deficiencies in assimilable nitrogen. However, the potential interactions between nitrogen and other micronutrients are poorly understood. We examined here the impact of nitrogen supplementation in the light of previous results evidencing that nitrogen sources could trigger yeast cell death when combined with some micronutrient limitations. We show here that a diversity of nitrogen sources, including amino acids and ammonium, can trigger cell death when a synthetic must is limited in oleic acid. In fact, not all nitrogen sources display the same ability to trigger cell death. Our results show that a set of amino acids (group 1) do not trigger cell death but, on the contrary, have a beneficial impact on the fermentation rate. By contrast, other amino acids and ammonium are liable to promote cell death but probably through different molecular mechanisms. Lysine is obviously toxic to yeast through an unknown mechanism, as already observed (Kemsawasd *et al.*, 2015; Thomas and Ingledew, 1992). Other amino acids and ammonium promote cell death with a behavior that is consistent with an effect through nitrogen signaling. It is noticeable, however, that although the TOR sensing system is considered to play a key role in nitrogen sensing, the ability to trigger cell death is not directly linked to the known capacity of an amino acid to activate the Tor kinase Torc1 (González and Hall, 2017).

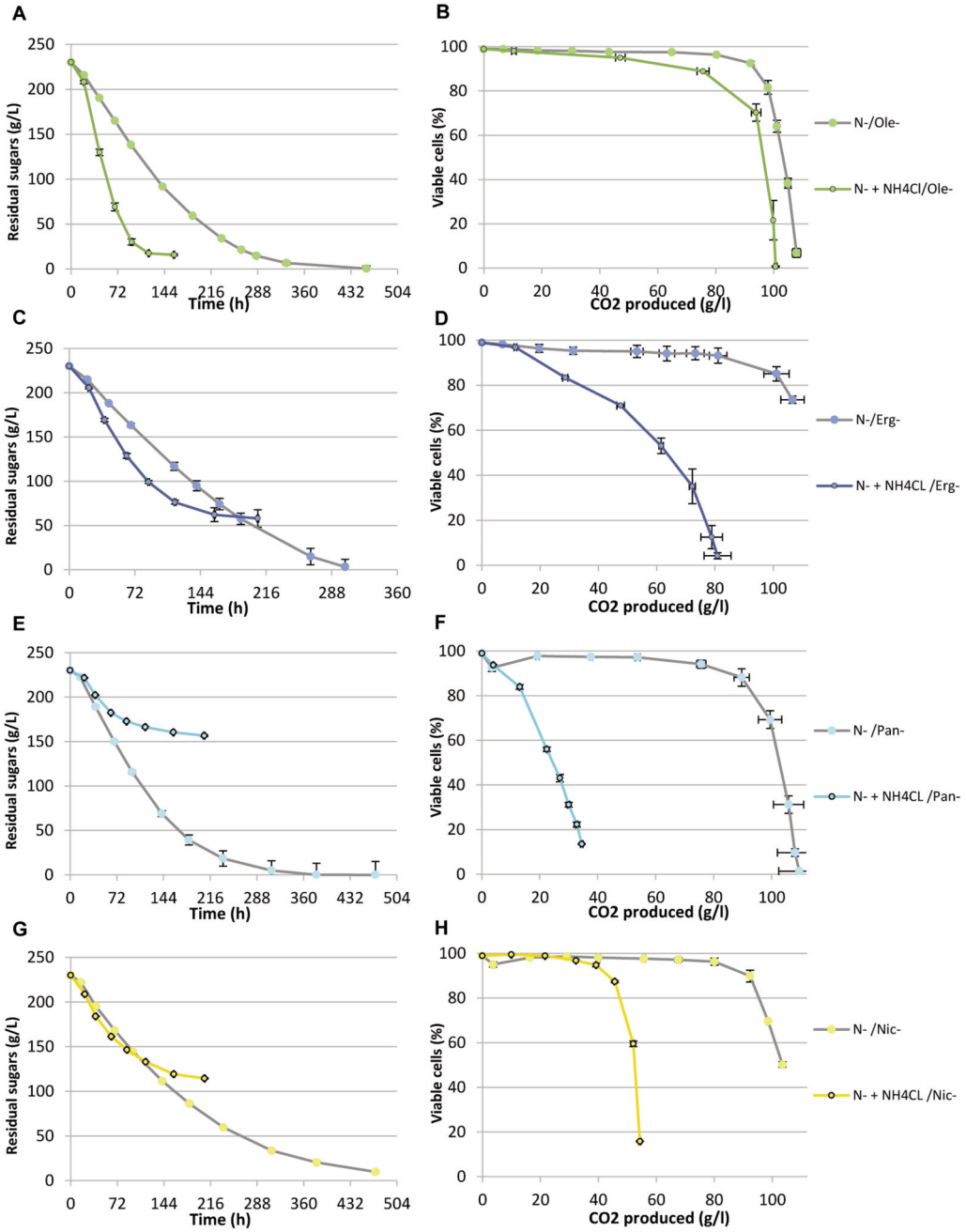

FIGURE 7. Effect of a supplementation of 354 mg/L YAN equivalent of ammonium.
Effects produced in (A, C, E, G) residual sugars and (B, D, F, H) yeast viability during the wine fermentation under (A, B) oleic acid (18 mg/L of oleic acid in the fermentation medium), C, D) ergosterol (1.5 mg/L of ergosterol in the fermentation medium), (E, F) pantothenic acid (0.02 mg/L of pantothenic acid in the fermentation medium) and (G, H) nicotinic acid starvations (0.08 mg/L of nicotinic acid in the fermentation medium). Fermentations were performed in duplicate. Error bars correspond to the standard deviation.

Notably, glutamine and leucine, which are both sensed by TorC1, did not lead to cell death in our conditions. Conversely, low TORC1 activators such as phenylalanine or tryptophan (Stracka *et al*., 2014) were found to lead to the highest loss of cell viability. Moreover, it has been previously described that other nitrogen pathways are involved in cell death regulation, for example the SPS pathway or the general amino acid control pathway (Tsang *et al.*, 2015; Codogno & Meijer 2005). Whether this system is involved in cell death triggered by these amino acids deserves additional experiments with an assessment of the signaling pathway activity.

Surprisingly, we showed that proline, which is not considered as a source of assimilable

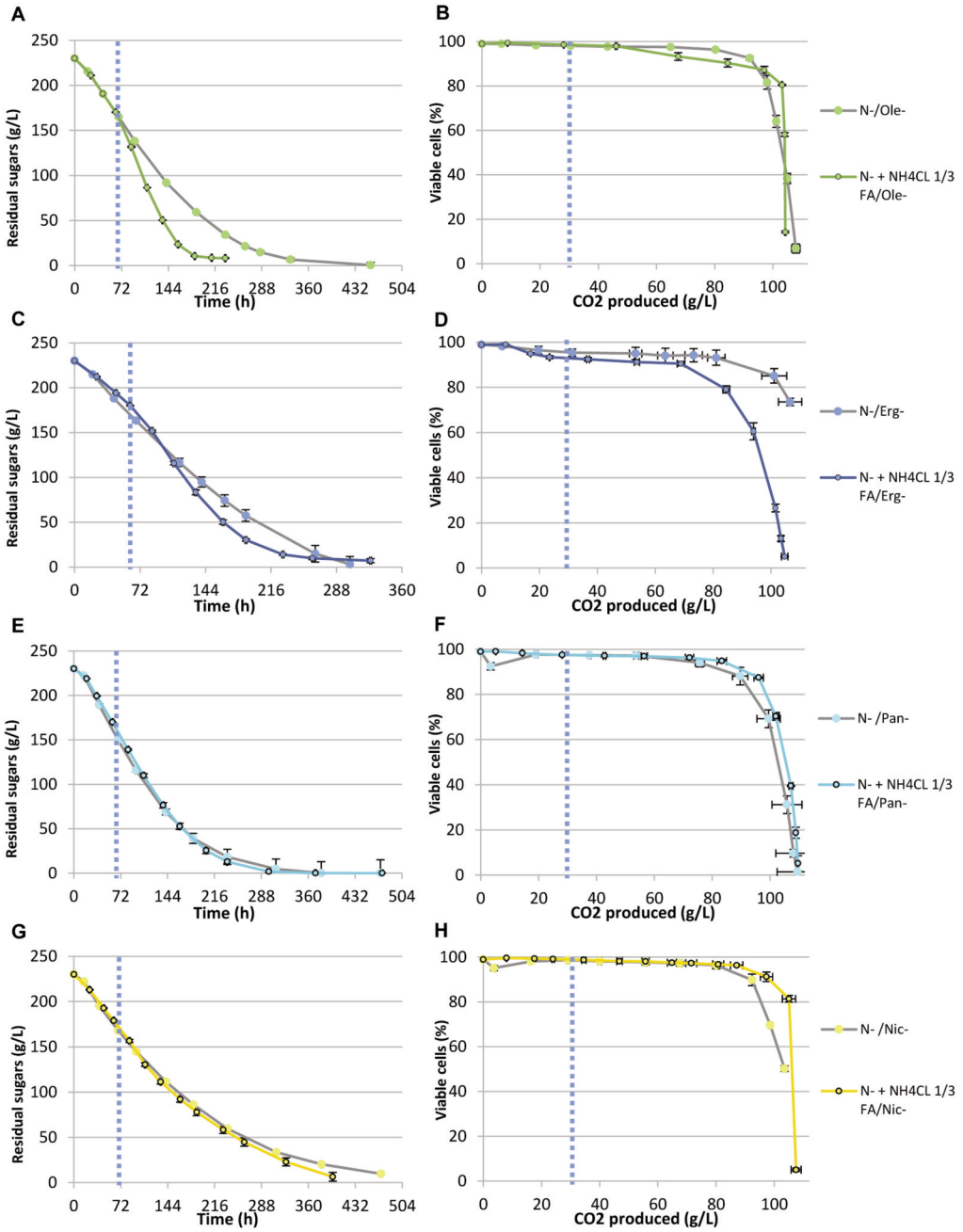

FIGURE 8. Effect of a supplementation of 354 mg/L YAN equivalent of ammonium at 30 g of CO_2.
Effects produced in (A, C, E, G) residual sugars and (B, D, F, H) yeast viability during the wine fermentation under (A, B) oleic acid, (C, D) ergosterol, (E, F) pantothenic acid and (G, H) nicotinic acid starvations. Fermentations were performed in duplicate. Error bars correspond to the standard deviation. Dotted line indicates the nitrogen supplementation at 30 g of CO_2 produced.

nitrogen for yeast during wine fermentation, is liable to promote cell death. Proline remains at a high level in wine after grape must fermentation because it cannot be metabolized in anaerobic conditions. The death-promoting capacity of proline was unexpected as it is available in high amounts in grape musts and is considered as a cell protectant. Proline content increases during ripening and can indeed reach very high levels in mature berries (Stines *et al.*, 1999). Our data suggest that grape musts obtained from fully ripe berries could be more sensitive to micronutrient deficiencies, which can be triggered by processes such as grape must clarification that removes lipids (sterols and unsaturated fatty acids).

Our results show that the timing of nitrogen addition can strongly impact cell death. While early addition of ammonium to the must triggers cell death, a later addition in fermentation is not

associated to such a cell death. These differences are in line with a role of nitrogen in modulating the stress response when cells are entering into stationary phase. Indeed, we previously found that yeast under micronutrient limitation does not set up a stress response at the entry of the stationary phase, while this is the case when both limited by nitrogen and micronutrient (Duc *et al*., 2017). Consistent with that, we assume that when nitrogen is added late in the fermentation process, yeast cells have already developed a stress response, and cells keep their viability while an early addition prevents the triggering of such response. Indeed, the outcome of the fermentation is different and underscores the advantage of a late nitrogen supplementation.

CONCLUSION

Twenty-one sources of nitrogen were screened for their capacity to trigger cell death during wine fermentation under micronutrient starvation that could be encountered in oenological conditions. Our data show that nitrogen supplementation of grape must is not systematically associated with an improvement of the fermentation process. Several nitrogen sources display an ability to trigger cell death, including NH_4^+. We showed that in alcoholic fermentation the nitrogen sources in the first group of amino acids we identified are preferable to others, i.e. the amino acids of the second and third groups, as they will not trigger yeast cell death. Those amino acids did not display such cell death inducing capacities, suggesting that in some circumstances organic nitrogen may be more suitable for nitrogen addition than NH_4^+. In fact, in some specific situations we have shown that adding high amounts of NH_4^+ could have a detrimental impact on the fermentation kinetic. Our results highlight instead the importance of considering the micronutrient status when managing nitrogen supplementation during wine fermentation, so as to avoid stuck fermentations. Also, we show that the timing of nitrogen addition during wine fermentation is an essential parameter to avoid cell death and stuck fermentation. These results invite reflections on the oenological practices and on the formulation of novel oenological products for yeast nutrition, such as amino acids mixes that also contain micronutrients.

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