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Camille Duc, Martine Pradal, Isabelle Sanchez, Jessica Noble, Bruno Blondin, et al.. Specific gene regulations of unusual micronutrient starvations leading to cell death during wine fermentation. *OENO One*, 2020, 54 (2), pp.359-371. 10.20870/oeno-one.2020.54.2.2970 . hal-02871928

HAL Id: hal-02871928

<https://hal.inrae.fr/hal-02871928>

Submitted on 17 Jun 2020

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Specific gene regulations of unusual micronutrient starvations leading to cell death during wine fermentation

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ABSTRACT

Yeast cell death can occur during wine alcoholic fermentation, leading to stuck fermentations, which are a major issue for winemakers. Cell death is generally considered to result from ethanol stress that negatively affects membrane integrity. However, it has been recently found that yeast cell death is also related to nitrogen metabolism. Indeed, nitrogen starvation is one of the most frequently encountered starvations in oenological conditions, and yeast is able to cope with such deficiencies. However, cell death can also result from the inability of yeast to implement an appropriate stress response under some conditions of nutrient limitations which the yeast is unlikely to encounter in the wild. More specifically, a set of micronutrients (oleic acid, ergosterol, pantothenic acid and nicotinic acid) which was linked to cell death when present in low, growth-restricting concentrations was identified. Upon examination of gene expression under conditions of imbalance between nitrogen and these nontraditional micronutrients, it appeared that, in addition to already identified mechanisms of gene regulation in relation to nitrogen metabolism, some genes had specific deficiency regulations. This can also explain some of the observed cell mortality. Our data include specific regulations of certain key genes of lipid metabolism, as well as others related to DNA stability under unusual deficiency conditions. Our work allows us to propose a model for the mechanisms involved in controlling yeast mortality under oenological fermentation conditions.

KEYWORDS

Saccharomyces cerevisiae, Micronutrients starvation, transcriptomic, wine fermentation, lipids, cell death

Supplementary data can be downloaded through: <https://oenone.eu/article/view/2970>

INTRODUCTION

Grape must is a complex medium composed of many nutrients such as nitrogen, sugars, lipids or vitamins (Duc *et al.*, 2017; Gobert *et al.*, 2019; Tesnière, 2019). Nitrogen is one of the most important and most studied nutrients, as it is required for yeast biomass production and is directly related to the ability of yeast to perform sugar fermentation. A low nitrogen concentration in the must has been shown to be responsible for stuck or sluggish fermentations (Bely *et al.*, 1990). These situations are often explained by a loss of yeast viability, usually attributed to insufficient lipid availability in the must. Indeed, a deficiency in lipids - some of the principal components of the yeast membrane - has been shown to lead to cell death due to the inability of yeast to cope with ethanol stress at the end of the fermentation stationary phase (Alexandre *et al.*, 1994; Casalta *et al.*, 2016).

However, the importance of the first limiting nutrient was shown to be a key parameter for yeast survival during the stationary phase (Boer *et al.*, 2008). For oenological fermentation conditions, it has thus been shown that the balance between nitrogen and the other nutrients is crucial for this survival (Duc *et al.*, 2017; Tesnière *et al.*, 2013). The nature of the nitrogen has also been shown to influence the occurrence of cell death under these conditions (Duc *et al.*, 2019).

In a previous study (Duc *et al.*, 2017), we found that ergosterol, and oleic, pantothenic and nicotinic acids are key nutrients in the control of yeast cell death during wine fermentation. We also showed in the same study that during alcoholic fermentation cell death occurs when these nutrients become limiting before nitrogen does. This was explained by a failure of stress survival response at a post-transcriptional level. Our data pointed to the fact that cell death results from the inability of yeast to set up appropriate stress response under such conditions of nutrient limitation which are most unlikely to be encountered by yeast in the wild.

In addition, we found some specificities in gene expression, which, depending on the limiting nutrient factor, also explained a part of cell death in these conditions. In addition, they provide a new perspective on cell death, because it takes into account nutrient factors other than nitrogen. The aim of the present paper is to explore these

complementary results which involve unusual starvation conditions that, to our knowledge, have not been previously investigated.

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).

2. Synthetic culture media

Unless otherwise specified, a synthetic fermentation medium with 425 mg/L assimilable nitrogen (SM425) and 23 % glucose + fructose (1/1), strictly buffered to pH 3.3 and simulating one third nitrogen and amino acid concentrations of a standard grape juice was routinely used (Bely *et al.*, 1990). This medium contained, per liter: 115 g glucose, 115 g fructose, 6 g citric acid, 6 g DL-malic acid, 750 mg KH₂PO₄, 500 mg K₂SO₄, 250 mg MgSO₄·7H₂O, 155 mg CaCl₂·2H₂O, 200 mg NaCl, 4 mg MnSO₄·H₂O, 4 mg ZnSO₄·7H₂O, 1 mg CuSO₄·5H₂O, 1 mg KI, 0.4 mg CoCl₂·6H₂O, 1 mg H₃BO₃, 1 mg (NH₄)₆Mo₇O₂₄, 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. It also contained ammoniacal nitrogen and amino acids as nitrogen sources (per liter): 460 mg NH₄Cl, 612 mg L-proline, 505 mg L-glutamine, 179 mg L-tryptophane, 145 mg L-alanine, 120 mg L-glutamic acid, 78 mg L-serine, 76 mg L-threonine, 48 mg L-leucine, 45 mg L-aspartic acid, 45 mg L-valine, 38 mg L-phenylalanine, 374 mg L-arginine, 33 mg L-histidine, 33 mg L-isoleucine, 31 mg L-methionine, 18 mg L-glycine, 17 mg L-lysine, 18 mg L-tyrosine and 13 mg L-cysteine. The medium was heat-sterilised (100 °C, 10 min). Lipid factors (LF) were added to the fermentation medium after sterilisation to a final concentration of 530 mg/L oleic acid and 15 mg/L ergosterol. To evaluate yeast adaptation to nutrient limitations, eight main fermentation conditions were established with different concentrations in the culture medium of: ergosterol at 1.5 mg/L (Erg-: low ergosterol conditions), oleic acid at 18 mg/L (bought as Tween 80[®], which is mainly a mix of oleate esters (Zhang *et al.*, 2012) (Ole-: low oleic acid conditions), pantothenic acid at 0.02 mg/L (Pan-: low pantothenic acid conditions) and nicotinic acid at 0.08 mg/L (Nic-: low nicotinic

acid conditions) with high: N⁺ (425 mg/L of yeast assimilable nitrogen) or low: N⁻ (71 mg/L of yeast assimilable nitrogen) nitrogen level. Thiamin, biotin and inositol starvations were also tested in condition of excess nitrogen with a concentration of respectively 15 µg/L, 0.06 µg/L and 0.2 mg/L.

3. Fermentation conditions and kinetics

The yeast strain was precultured for 24 h at 28 °C in a nutrient medium containing Yeast Nitrogen Base (YNB) (6.7 g/L) without amino acids and glucose (20 g/L) in Erlenmeyer flasks. The fermentation medium was then inoculated at 10⁶ cell/mL from this preculture. Yeast cultures were carried out in fermenters (1.2 L containing 1 L medium), with fermentation locks (CO₂ bubbling outlets filled with water). Fermentation media were routinely de-aerated prior to inoculation by bubbling pure argon for 5 min. Filling conditions were controlled and fermentations were carried out under anaerobic and isothermal conditions (24 °C), with permanent stirring (300 rpm). The amount of CO₂ released was calculated from automatic weight measurements (taken every 20 min) (Sablayrolles *et al.*, 1987). The CO₂ production rate was calculated by sliding-window second-order polynomial fitting on the last 11 measurements using a custom-developed Labview application.

4. Experimental design for assessing the stress response under micronutrient starvations

For assessing stress response, we performed four experiments: a heat-shock sensitivity assay, a glycogen accumulation assay, a cell cycle assay and a transcriptomic analysis, all performed following a kinetic approach. We thus selected four time-points across fermentation: T1, T2, T3 and T4 (Figure 1). To perform the transcriptomic analysis, 10⁹ cells were harvested at each time-point. The first time-point, T1, corresponds to a growth of 20x10⁶ cells/mL, during exponential growth (i.e., when no nutrient is limiting). The second time-point, T2, was taken at 12 g of CO₂ produced, which corresponds to entry into the stationary phase. Time-points T3 and T4 were taken at 40 g and 75 g respectively of CO₂ produced and corresponded to the stationary phase.

We tested 6 nutrient conditions: two conditions that allowed yeast cells to maintain their viability during wine fermentation and that served as

controls, and 4 conditions of unusual nutrient starvations that led to cell death.

The two chosen control conditions were nitrogen starvation (N⁻) and nitrogen plus ergosterol starvation (N⁻/Erg⁻), because we had shown in the first part of the results that, in our conditions, yeast viability depended on nitrogen concentration availability. The other four conditions all had excess nitrogen, but were starved of oleic acid (N⁺/Ole⁻), ergosterol (N⁺/Erg⁻), pantothenic acid (N⁺/Pan⁻) or nicotinic acid (N⁺/Nic⁻).

5. RNA extraction and microarray assay

Total RNAs were isolated from culture samples taken at the selected time-points in the previously described conditions, using the TRIzol® method according to Chomczynski and Sacchi (1987). Aliquots of 10⁹ cells were harvested and quickly washed using 750 mL of cooled (4 °C) DEPC-treated water. Cells were pelleted, frozen in a -80 °C methanol bath and mechanically lysed through vortexing with glass beads (d=0.3 mm) in 400 mL TRIzol® (GIBCO BRL) at 4 °C for 15 min. The liquid phase was then collected and TRIzol® was added to a 4 mL final volume. The samples were mixed and incubated for 5 min at room temperature, and 800 mL chloroform was then added. The mixture obtained was vortexed, incubated for 3 min and then centrifuged (9,000 g for 15 min). The supernatant was centrifuged again (2,000 g for 2 min) in swing-out buckets. RNAs were pelleted from 2 mL aliquots of the supernatant by the addition of 2 mL cooled isopropanol (-20 °C) and incubated for 10 min. The samples were

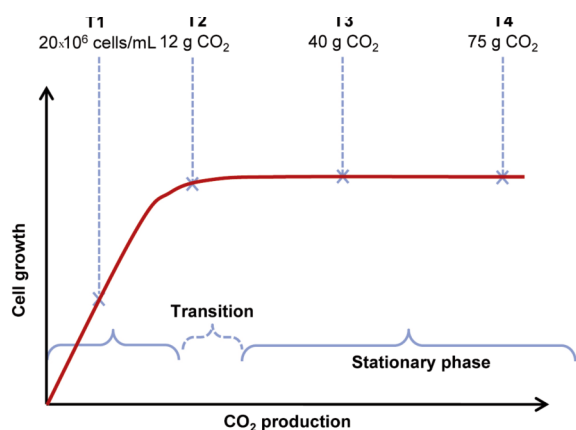


FIGURE 1. Representation of the different time-points at which transcriptomic analysis for stress response assessment was performed.

centrifuged (9,000 g for 10 min) and the resulting nucleic acid pellet was washed twice with 750 mL 75 % ethanol/DEPC-treated water and then dissolved in 150 µL nuclease-free water (Qiagen). Total RNA from 100 µg aliquots of these preparations was purified with an RNeasy® mini kit (Qiagen) following the RNA cleanup protocol, including membrane DNase digestion. RNAs were eluted with 2 x 30 µL of the provided RNase-free water and their quality was verified through capillary electrophoresis using an RNA 6000 Nano LabChip Kit (Agilent Technologies). Samples of 100 ng purified RNA were labelled with Low input Quick Amp Labelling one-colour kit (Agilent Technologies) according to the manufacturer's recommendations (indirect labelling of mRNAs with Cyanin 3 dCTP dye). RNAs were hybridised on 8 x 15 k array Agilent standard Yeast V2 Gene Expression Microarrays (Agilent Technologies) for 17 h in a rotating oven at 65 °C following the manufacturer's recommendations. A Genepix 4000B scanner was used for array digitalisation: laser voltage was set to avoid signal saturation and data were extracted with GenePix® Pro 7 software (Molecular Devices).

6. Statistical analysis of microarray data

The R3.1.3 software was used for statistical analyses (R Core Team, 2014). The raw microarray data were imported and normalised according to the quantile method for normalisation between arrays using the limma package (Smyth and Speed, 2003). On the normalised data set, we analysed gene expression changes over time using the maSigPro package and the single series approach. maSigPro is a regression-based approach for identifying genes with temporal expression changes (Conesa *et al.*, 2006). For the first step of this method, we defined a binomial regression model for each gene expression over the 4 time-points:

$$Y = b_0 + b_1 \text{ time} + b_2 \text{ time}^2 + \varepsilon$$

with:

- Y: normalized expression value
- time: the quantitative variable of the time-points (min)
- b_0 : start value
- b_1 : the slope estimation (induction or repression of the gene) – linear effect
- b_2 : the shape estimation (first change in the temporal profile) – quadratic effect
- ε are independent $N(0, \sigma^2)$ error terms

We adjusted this model by applying the least-squared technique for each gene, and we only selected genes with significant changes over time (i.e., with an adjusted p-value threshold of 0.005 corrected by the Benjamini-Hochberg method). A variable selection procedure was then applied using stepwise regression to find significant coefficients for each gene (step.method="backward", alpha=0.01). The list of differentially expressed genes according to the slope was generated and allowed us to define patterns of changes in expression over time (Rsquared cutoff=0.8). These patterns were then clustered using a hierarchical classification analysis with correlation distance and a complete linkage method using cluster v3.0. They were displayed with JavaTreeView v1.1.5r2 (de Hoon *et al.*, 2004; Saldanha, 2004).

For a functional analysis of the defined clusters, each cluster was analysed using the web-based tool Funspec (<http://funspec.med.utoronto.ca/>; adjusted p.value = 0.05 with a Bonferroni correction method) and genes were classified into functional categories, biological processes and protein cellular localisations using the GO database (Robinson *et al.*, 2002). In order to obtain individual patterns for some stress (or stress-related) gene expressions (*OSH3*, *OSH6*, *ISC1*, *SIR2*), the corresponding data were extracted from the transcriptomic analysis.

The complete microarray data set was deposited in the Gene Expression Omnibus (GEO) public repository (accession number GSE95152). Microarray description is under GEO accession number GPL17690.

RESULTS AND DISCUSSION

As presented in Duc *et al.*, (2017), we designed the fermentation experiments to assess whether micronutrient limitations could trigger yeast cell death during alcoholic fermentation. Since fermentation rates were highly variable among the situations examined, and given the impact of ethanol on cell viability, cell viability is displayed in relation to fermentation progress (amount of CO₂ released), rather than in relation to time. As a result of these assays, we were able to find that an ergosterol, oleic acid, pantothenic acid or nicotinic acid starvation can lead to cell death during wine fermentation (see Supplementary data 1).

As shown in our previous paper (Duc *et al.*, 2017), 1946 genes were found and classified into

28 clusters having a significant dependence on time for the 6 nutrient conditions tested. In that study, we aimed at finding the gene regulation changes linked to nitrogen metabolism inasmuch as we found it related to cell death control in these fermentation conditions.

However, among all these clusters, some transcriptomic changes were found to be specific to the different unusual starvation conditions. These data are presented in this paper. They seem to be related to cell death and could therefore, in addition to the mechanisms related to nitrogen disequilibrium, explain a part of the mortality observed in these conditions.

1. Lipid metabolism is affected by different micronutrient starvations

Pantothenic acid as a precursor of acetyl-CoA is related to lipid metabolism as well as, obviously, oleic acid and ergosterol. By looking for the elements common to the starvation of these 3 micronutrients related to lipid metabolism, it was possible to identify a cluster of 26 genes (Figure 2 and Supplementary data 2). The genes present in this cluster displayed low expression under both of the conditions which allow yeast to survive (i.e., N-, N-/Erg-), whereas they showed a modification of their profiles under the 3 lipid-related starvation conditions (i.e, oleic acid or ergosterol or pantothenic acid). For these 3 conditions in this cluster, we observed overexpression for two genes involved in lipid metabolism: *INO1* encoding inositol-3-phosphate synthetase, taking part in the biosynthesis phosphatidylinositol and derived molecules, and *FAS2* encoding the alpha subunit of fatty acid synthetase. The strong expression of these genes under lipid-linked micronutrient starvations probably corresponds to a response to the resulting imbalance in lipid metabolism and the corresponding need for lipid biosynthesis. In the same cluster, and more specifically for the oleic acid and ergosterol starvations, there was also a strong expression of the *MCA1* gene coding for a metacaspase, which plays a key role in apoptosis phenomena (40 % of known apoptotic deaths depend on *MCA1* (Falcone and Mazzoni, 2016)). The relative superposition of *MCA1* expression with yeast mortality suggests that *MCA1* may play a role in the yeast cell death we observed.

2. Phospholipid and sphingolipid metabolisms are specifically affected by oleic acid starvation

The transcriptomic analysis (Figure 3 and Supplementary data 3) also revealed a cluster of 18 genes specifically overexpressed under oleic acid starvation conditions. It is enriched with genes involved in the phospholipid biosynthesis pathway: two genes encoding acyltransferases of this pathway (*ALE1* and *CST26*) and *ECT1* encoding an ethanolamine-phosphate cytidylyl-transferase, as well as genes involved in sterol trafficking and homeostasis, and *OSH3* and *OSH6* encoding two oxysterol binding proteins. It is also possible to note the overexpression of *ISC1*, which is involved in the catabolism of sphingolipids (Sawai *et al.*, 2000). These three genes (*OSH3*, *OSH6* and *ISC1*) are also overexpressed under pantothenic acid limitation conditions (Figure 4). Oleic acid starvation in particular, but less for pantothenic acid starvation, visibly causes lipid metabolism

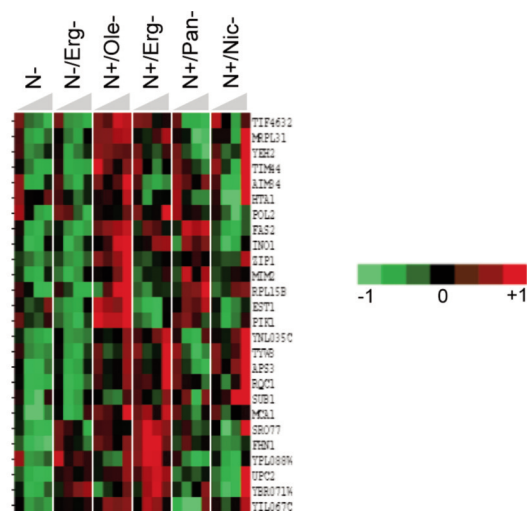


FIGURE 2. Cluster with overexpression of genes involved in lipid metabolism under oleic, ergosterol or pantothenic acid deficiency conditions.

For : N-: low nitrogen, 71 mg/L YAN; N-/Erg- : low nitrogen/low ergosterol, 71 mg/L YAN, 1.5 mg/L ergosterol; N+/Ole-: high nitrogen/low oleic acid, 425 mg/L YAN, 18 mg/L oleic acid; N+/Erg-: high nitrogen/low ergosterol, 425 mg/L YAN, 1.5 mg/L ergosterol; N+/Pan-: high nitrogen/low pantothenic acid, 425 mg/L YAN, 0.02 mg/L pantothenic acid and N+/Nic-: high nitrogen/low nicotinic acid, 425 mg/L YAN, 0.08 mg/L nicotinic acid; transcriptomic assays were performed at four time-points during alcoholic fermentation (T1, 20 10⁶ cells/mL; T2, 12 g CO₂ produced; T3, 40 g CO₂ produced; T4, 75 g CO₂ produced) indicated by the grey triangle. Results show the mean of biological triplicates.

disruptions. In particular, it is noted that the level of oleic acid influences the expression of genes involved in ergosterol homeostasis. Phospholipids are the main structural compounds in membranes. A modulation of the expression of the genes in their biosynthesis leads to a reorganisation of these compounds in the yeast. These changes, therefore, directly affect membrane structure, weakening the yeasts' resistance to the ethanol stress they face and thus increasing their mortality during alcoholic fermentation. In addition, sphingo-lipids play an important role in yeast signaling processes, notably in protein trafficking (Cowart and Obeid, 2007). Other types of sphingolipids (those in C26) are toxic to yeast (Epstein *et al.*, 2012). Thus, overexpression of *ISC1* (a central gene in sphingolipid metabolism) would lead to a modification of the sphingolipid pool and could have a negative effect on yeast metabolism leading to a decrease in their survival capacity.

In addition, *ISC1* has been shown to be involved in yeast mortality control in certain situations, particularly those of stress (Almeida *et al.*, 2008). Indeed, *ISC1* deletion caused increased sensitivity to stress and accelerated aging, but lower sensitivity to acetic acid (Rego *et al.*, 2014; Rego *et al.*, 2012). The roles of this gene are therefore complex, and the impact of the observed overexpression of *ISC1* on the sphingolipid pool should be further evaluated.

There is also a strong expression of the *ATF1* gene encoding an alcohol acetyltransferase which plays a major role in the formation of esters during alcoholic fermentation (Verstrepen *et al.*, 2003). This gene is regulated by anaerobiosis and unsaturated fatty acids (Fujii *et al.*, 1997), and its strong expression in an oleic acid-deficient environment is therefore consistent with the above elements. The fact that the anaerobiosis is identical under our different conditions highlights the key role of oleic acid in controlling these genes.

3. Sterol metabolism is affected by ergosterol starvation and is not related to cell death

Our analysis of the transcriptome also revealed a cluster related to sterol metabolism with no direct link to viability, but which corresponds to important regulations in alcoholic fermentation. This cluster is specific to ergosterol-deficient conditions (Figure 5 & Supplementary data 4), with genes overexpressed independently of the

nitrogen level. Analysis of the enrichment of genes belonging to this group shows that a large number of them are involved in the biosynthesis of ergosterol (*ERG28*, *ERG26*, *ERG25*, *ERG1*, *ERG11*, *NCP1*, *ERG9*, *ERG3*, *ERG27*, *ERG6*, *ERG2*, *ERG24*) and also contains genes encoding mannoproteins potentially involved in sterol importation (*TIR1*, *TIR3*, *DAN1*, *DAN4*, *TIR4*, *TIR2*). These genes are usually known for their anaerobically inducible expression (Abe, 2007; Baumann *et al.*, 2011; Rachidi *et al.*, 2000; Zitomer *et al.*, 1997). However, we observed in our work that, although all conditions were strictly anaerobic, ergosterol starvation led to their overexpression, which shows that in addition to anaerobic conditions, the availability of sterols in the environment regulates their expression. However, this reorganisation of expression cannot be linked to the viability phenotype, since it is observed both in conditions of yeast viability and conditions leading to cell mortality.

In addition to lipid metabolism as sterol, phospholipid and sphingolipid, other specific changes in gene expressions including cell cycle and DNA stability were observed.

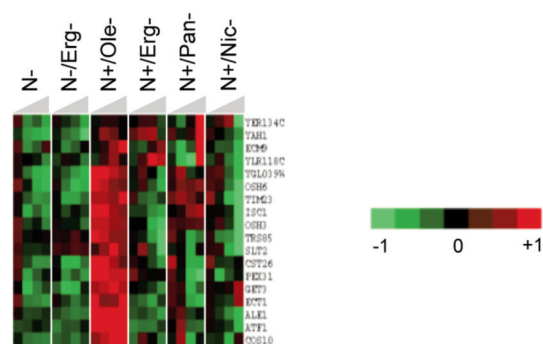


FIGURE 3. Cluster with overexpression of genes involved in lipid metabolism under oleic acid deficiency conditions.

For : N-: low nitrogen, 71 mg/L YAN; N-/Erg-: low nitrogen/low ergosterol, 71 mg/L YAN, 1.5 mg/L ergosterol; N+/Ole-: high nitrogen/ low oleic acid, 425 mg/L YAN, 18 mg/L oleic acid; N+/Erg-: high nitrogen/ low ergosterol, 425 mg/L YAN, 1.5 mg/L ergosterol; N+/Pan-: high nitrogen/ low pantothenic acid, 425 mg/L YAN, 0.02 mg/L pantothenic acid and N+/Nic-: high nitrogen/ low nicotinic acid, 425 mg/L YAN, 0.08 mg/L nicotinic acid; transcriptomic assays were performed at four time-points during alcoholic fermentation (T1, 20 10⁶ cells/mL; T2, 12 g CO₂ produced; T3, 40 g CO₂ produced; T4, 75 g CO₂ produced) indicated by grey triangle. Results show the mean of biological triplicates.

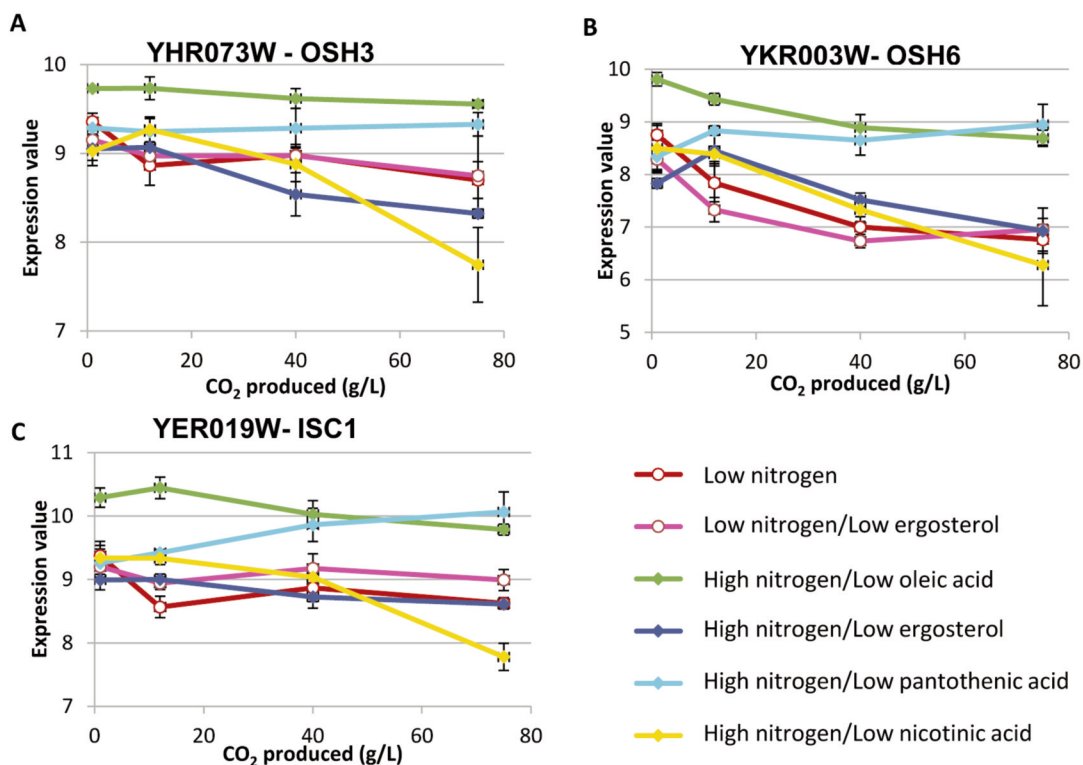


FIGURE 4. Expression kinetics of (A) OSH3, (B) OSH6 and (C) ISC1 during alcoholic fermentation under micronutrient deficiency conditions.

(A) *OSH3*, (B) *OSH6*, (C) *ISC1*, for: (—) N-: Low nitrogen (71 mg/L YAN) (viability control 1); (—) N-/Erg-: Low nitrogen/ Low ergosterol (71 mg/L YAN, 1.5 mg/L ergosterol) (viability control 2); (—) N+/Ole-: High nitrogen/ Low oleic acid (425 mg/L YAN, 18 mg/L oleic acid); (—) N+/Erg-: High nitrogen/ Low ergosterol (425 mg/L YAN, 1.5 mg/L ergosterol); (—) N+/Pan-: High nitrogen /Low pantothenic acid (425 mg/L YAN, 0.02 mg/L pantothenic acid); and (—) N+/Nic-: High nitrogen/ Low nicotinic acid (425 mg/L YAN, 0.08 mg/L nicotinic acid). Data were obtained from the transcriptomic analysis. Curves show the mean of fermentations performed in triplicate, error bars correspond to standard deviation.

4. Alteration in cell cycle function genes is under pantothenic acid starvation

Under pantothenic acid starvation conditions, it was possible to determine two groups of overexpressed genes (with 33 and 44 genes respectively) (Figure 6A & B) (Supplementary data 5 & 6), as well as a 3rd cluster of 77 under-expressed genes (Figure 6C & Supplementary data 7).

The analysis of these clusters showed an enrichment of genes related to the cell cycle. Indeed, there was an overexpression of genes involved in DNA condensation (*TOF2*, *ISW2*, *HST2*, *SWI1*) and in nucleus division regulation (*IME2* and *WTM2*). As for the enrichment of repressed genes, it targets in particular genes known for their function in the mitotic cell cycle (*MPS1*, *SPC105*, *BUB1*, *CEP3*) and cell division (*MPS2*, *NBP1*).

These results seem to reflect a deregulation of the yeast cell cycle during fermentation under conditions of pantothenic acid starvation. The identified genes are also consistent with the absence of cell cycle arrest, which is observed when yeasts enter the stationary phase in this same condition (see Duc *et al.* (2017)). This observation is consistent with the work of Stolz *et al.* (2004) on *Schizosaccharomyces pombe*, which showed that a starvation in pantothenic acid led to septation defects during cell division. Although such a mechanism has not been reported in *S. cerevisiae*, the transcriptional response of yeasts under pantothenic acid starvation conditions clearly shows a deregulation of the genes that control cell division. In addition, pantothenic acid is an intermediate of acetyl-CoA, a coenzyme that has been shown to be essential for cell division (Shi and Tu, 2013). It therefore seems consistent to find differences in the expression of yeast genes

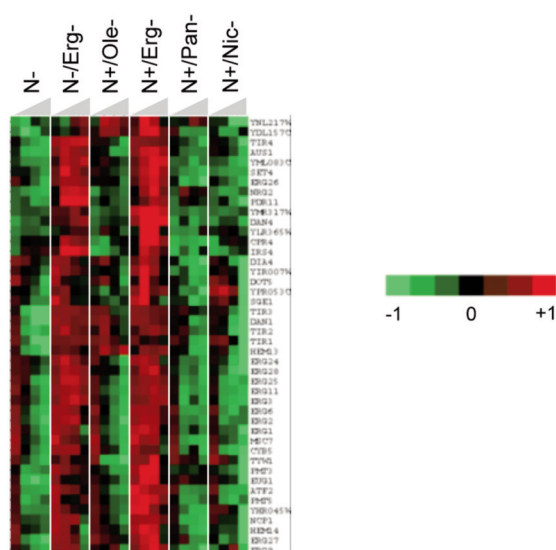


FIGURE 5. Genes overexpressed in ergosterol deficiency conditions

For : N-: low nitrogen, 71 mg/L YAN; N-/Erg- : low nitrogen/low ergosterol, 71 mg/L YAN, 1.5 mg/L ergosterol; N+/Ole-: high nitrogen/low oleic acid, 425 mg/L YAN, 18 mg/L oleic acid; N+/Erg-: high nitrogen/low ergosterol, 425 mg/L YAN, 1.5 mg/L ergosterol; N+/Pan-: high nitrogen/low pantothenic acid, 425 mg/L YAN, 0.02 mg/L pantothenic acid and N+/Nic-: high nitrogen/low nicotinic acid, 425 mg/L YAN, 0.08 mg/L nicotinic acid; transcriptomic assays were performed at four time-points during alcoholic fermentation (T1, 20 10⁶ cells/mL; T2, 12 g CO₂ produced; T3, 40 g CO₂ produced; T4, 75 g CO₂ produced) indicated by the grey triangle. Results show the mean of biological triplicates.

regulating the cell cycle during alcoholic fermentation, as well as to find a problem of cell cycle arrest in the G0/G1 phase under pantothenic acid starvation conditions.

5. Deregulation of genes involved in maintaining DNA stability under nicotinic acid starvation

Finally, a gene cluster of 66 genes specific to nicotinic acid starvation was identified (Figure 7 & Supplementary data 8), in which gene overexpression was observed to be in relation to genome regulation and DNA metabolism. Indeed, there is enrichment in genes responsible for meiosis, sporulation, transcription regulation, histone deacetylation or chromatin modification. This enrichment could be the result of DNA alterations.

Nicotinic acid is involved in the synthesis of NAD⁺, a cofactor in many metabolic reactions. In anaerobic conditions, yeast is unable to

directly synthesise nicotinic acid and can therefore only synthesise NAD⁺ by recycling certain intermediates or by incorporating nicotinic acid present in the fermentation medium (Panozzo *et al.*, 2002). The NAD⁺ cofactor is essential for the activity of sirtuin Sir2, a NAD⁺-dependent deacetylase that has been shown to be involved in the control of aging (Fabrizio *et al.*, 2005; Kaerberlein *et al.*, 1999).

It should also be noted that the expression of the *SIR2* gene is higher under nicotinic acid starvation conditions than in the other tested starvations (Figure 8). This is consistent with a decrease in Sir2 activity compensated by overexpression of its encoding gene. Indeed, it can be assumed that a deficiency in nicotinic acid - and thus in NAD⁺ - will lead to a decrease in Sir2 histone activity and, therefore, to DNA instability and defects in DNA compaction, resulting in mortality phenomena (Anderson *et al.*, 2003). We did not quantify nucleotides in this study; it is therefore not possible to draw conclusions regarding the metabolic changes associated with nicotinic acid starvation. However, transcriptomics data with overexpression of both *RNR4* (which codes for a ribonucleotide-diphosphate reductase) and *NRT1* (which codes for a high-affinity ribosyl nicotinamide transporter), are consistent with a response to a synthesis need and nucleotide import. These results would, therefore, be consistent with mortality related to a lack of Sir2 activity. This Sir2 activity will have to be quantified to assess this hypothesis.

CONCLUSION

We had previously been able to show the importance of nutritional limitations and their relationship with nitrogen metabolism in controlling yeast viability during alcoholic fermentation. In particular, starvation in ergosterol, oleic acid, pantothenic acid and nicotinic acid appears to be critical. The involvement of these starvations in the occurrence of cell death is consistent with Boer's work (Boer *et al.*, 2008) and the role of the first limiting nutrient in the cells' ability to resist stress. Beyond the involvement of the TOR pathway in the control of yeast viability under these conditions (Duc *et al.*, 2017), specificities related to nutritional starvation appear.

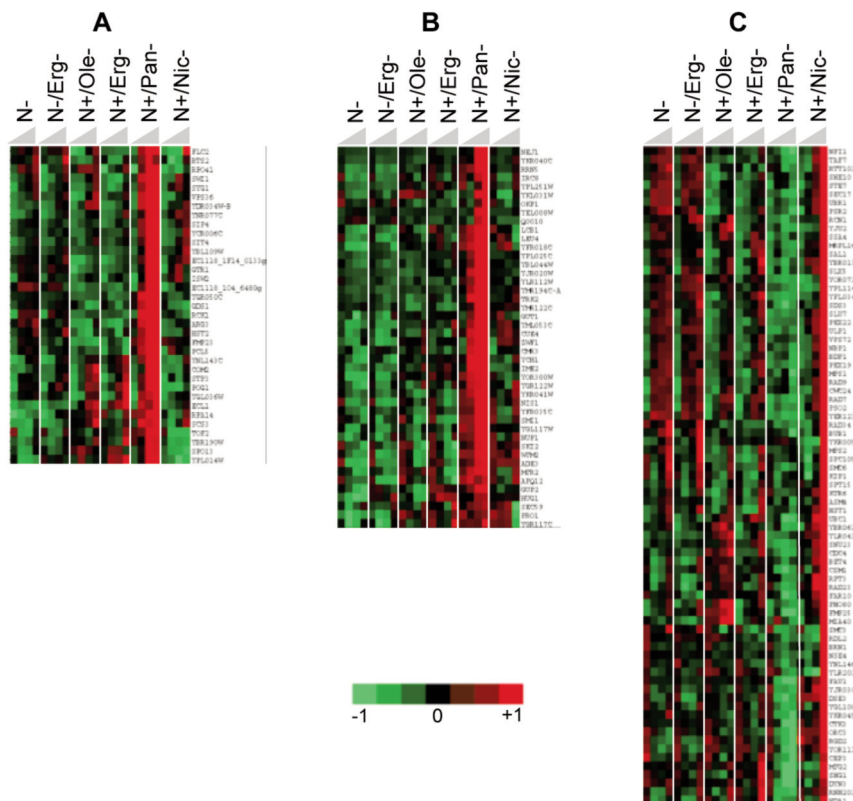


FIGURE 6. Clusters with overexpression (A and B) and repression (C) of genes in pantothenic acid deficiency conditions.

For: N-: low nitrogen, 71 mg/L YAN; N-/Erg-: low nitrogen/low ergosterol, 71 mg/L YAN, 1.5 mg/L ergosterol; N+/Ole-: high nitrogen/low oleic acid, 425 mg/L YAN, 18 mg/L oleic acid; N+/Erg-: high nitrogen/low ergosterol, 425 mg/L YAN, 1.5 mg/L ergosterol; N+/Pan-: high nitrogen/low pantothenic acid, 425 mg/L YAN, 0.02 mg/L pantothenic acid and N+/Nic-: high nitrogen/low nicotinic acid, 425 mg/L YAN, 0.08 mg/L nicotinic acid; transcriptomic assays were performed at four time-points during alcoholic fermentation (T1, 20 10⁶ cells/mL; T2, 12 g CO₂ produced; T3, 40 g CO₂ produced; T4, 75 g CO₂ produced) indicated by the grey triangle. Results show the mean of biological triplicates.

Therefore, other mechanisms more specific to micronutritional deficiencies controlling the viability of yeasts cannot be ruled out. Indeed, transcriptomic analysis has made it possible to determine certain modifications in the expression of genes specific to certain limitations. In particular, the *MCA1* gene is overexpressed under conditions of ergosterol and oleic acid limitation. This gene is a key gene in the regulation of apoptosis, a type of regulated cell death. However, although it was not easy to determine sphingolipid and ceramide content in yeasts, transcriptomic analysis under conditions of restricted oleic acid showed overexpression of *ISC1*, a gene involved in sphingolipid synthesis. This lipid class has been shown to modulate yeast mortality, particularly through the induction of apoptosis by ceramides; i.e., sphingolipid derivatives (Eisenberg and Büttner, 2014). Sphingolipids with 26 carbons have also been shown to be toxic (Epstein *et al.*, 2012). Thus, a modification of the sphingolipid pool in

yeast can have significant effects on yeast metabolism, as well as a direct effect on mortality, which could therefore reduce their ability to survive. Regarding pantothenic acid starvation, there appears to be a major defect in cell cycle arrest, which is controlled at the transcriptional level, as evidenced by the specific enrichment of cell cycle regulation genes in these starvation conditions. Finally, this pantothenic starvation seems to also induce specific mechanisms: its impact on viability appears to be linked to Sir2 activity via the decrease in NAD⁺ availability. Therefore, in addition to the mechanism for regulating nitrogen-dependent viability, which was highlighted in our previous paper (Duc *et al.*, 2017) and which is common to all micronutrient starvations tested, each starvation seems to lead to specific responses.

Our study allows us to propose a model for the regulation of cell viability under the

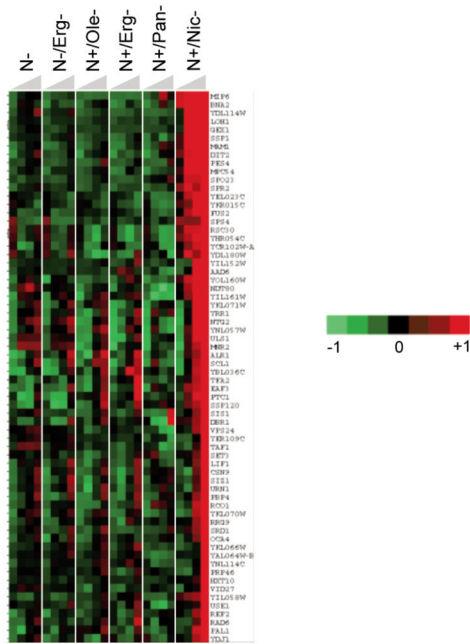


FIGURE 7. Overexpression of genes in nicotinic acid deficiency conditions. For : N-: low nitrogen, 71 mg/L YAN; N-/Erg-: low nitrogen/low ergosterol, 71 mg/L YAN, 1.5 mg/L ergosterol; N+/Ole-: high nitrogen/low oleic acid, 425 mg/L YAN, 18 mg/L oleic acid; N+/Erg-: high nitrogen/low ergosterol, 425 mg/L YAN, 1.5 mg/L ergosterol; N+/Pan-: high nitrogen/low pantothenic acid, 425 mg/L YAN, 0.02 mg/L pantothenic acid and N+/Nic-: high nitrogen/low nicotinic acid, 425 mg/L YAN, 0.08 mg/L nicotinic acid; transcriptomic assays were performed at four time-points during alcoholic fermentation (T1, 20 10⁶ cells/mL; T2, 12 g CO₂ produced; T3, 40 g CO₂ produced; T4, 75 g CO₂ produced) indicated by the grey triangle. Results show the mean of biological triplicates.

micronutrient starvation conditions tested (Figure 9). The contribution of the different mechanisms to mortality still need to be characterised, in particular by specifying the possible additive or interaction effects of these different nutritional factors.

Acknowledgments: We would like to thank Anne Ortiz-Julien from Lallemand SAS for her support of this work. Our thanks also go to Philippe Chatelet for his re-reading of the manuscript.

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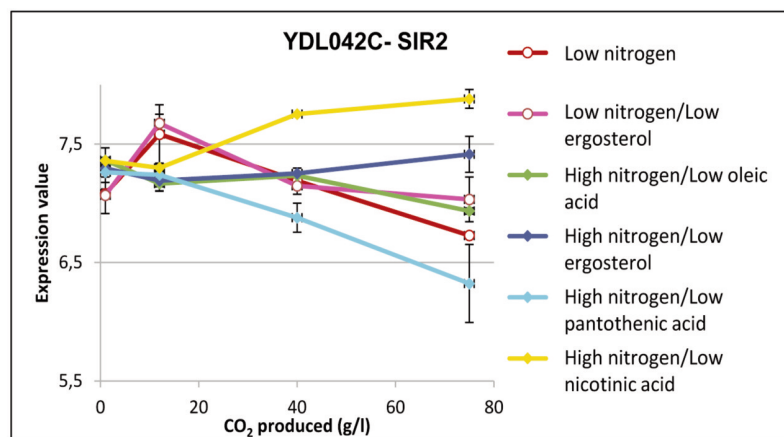
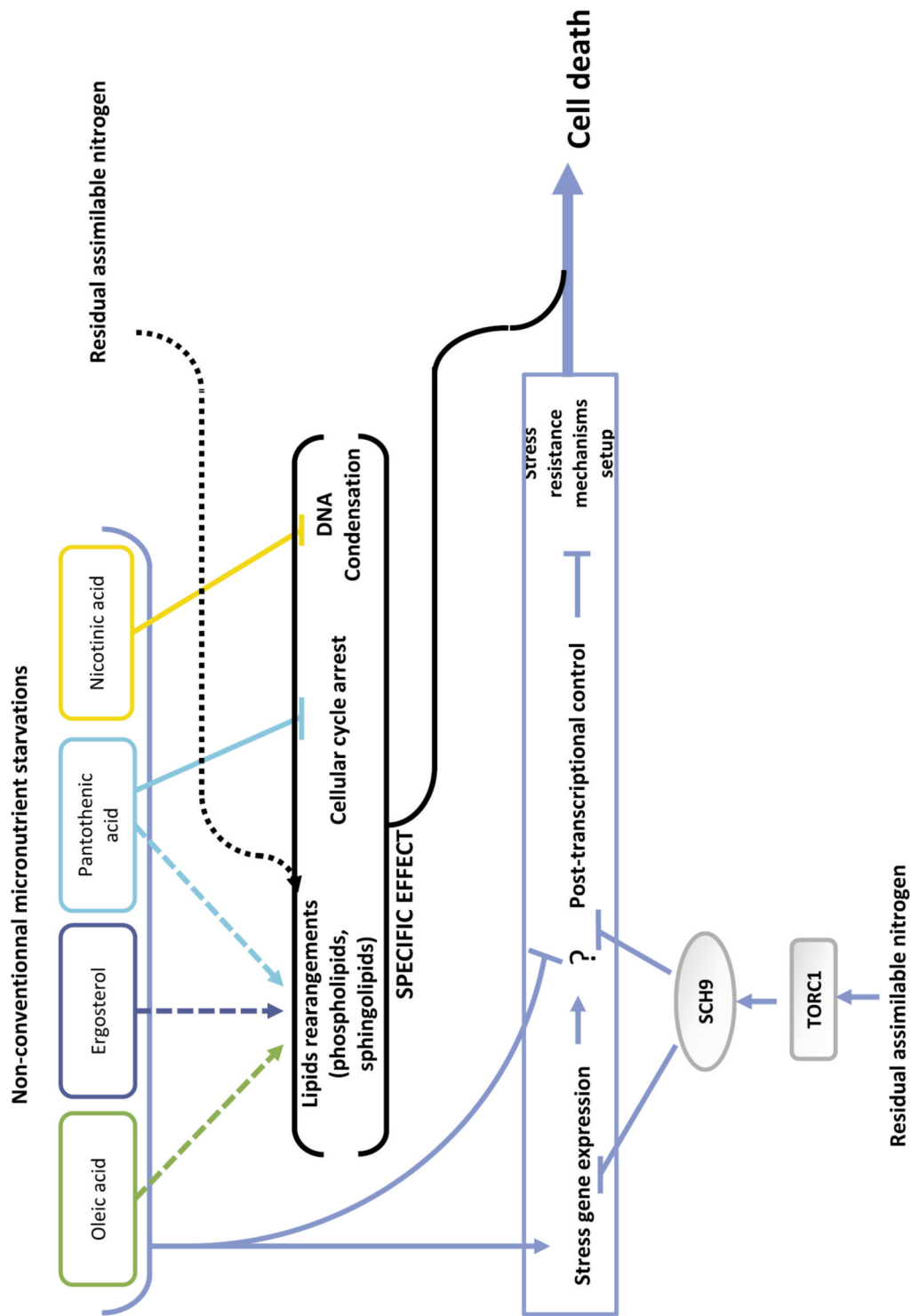


FIGURE 8. Expression of the SIR2 gene during alcoholic fermentation under micronutrient deficiency conditions (A) *OSH3*, (B) *OSH6*, (C) *ISC1*, for: (—) N-: Low nitrogen (71 mg/L YAN) (viability control 1); (—) N-/Erg-: Low nitrogen/ Low ergosterol (71 mg/L YAN, 1.5 mg/L ergosterol) (viability control 2); (—) N+/Ole-: High nitrogen/ Low oleic acid (425 mg/L YAN, 18 mg/L oleic acid); (—) N+/Erg-: High nitrogen/Low ergosterol (425 mg/L YAN, 1.5 mg/L ergosterol); (—) N+/Pan-: High nitrogen /Low pantothenic acid (425 mg/L YAN, 0.02 mg/L pantothenic acid); and (—) N+/Nic-: High nitrogen/Low nicotinic acid (425 mg/L YAN, 0.08 mg/L nicotinic acid). Data were obtained from the transcriptomic analysis. Curves show the mean of fermentations performed in triplicate, error bars correspond to standard deviation.

FIGURE 9. Proposal for additional regulations of yeast viability during alcoholic fermentation in the event of micronutritional starvation.



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