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Impact of initial lipid content and oxygen supply on alcoholic fermentation in Champagne-like musts

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Abstract

Available nitrogen, lipids, or oxygen are nutrients with major impact on the kinetics of winemaking fermentation. Assimilable nitrogen is usually the growth-limiting nutrient which availability determines the fermentation rate and therefore the fermentation duration. In some particular cases, as in Champagne, grape musts have high available nitrogen content and low turbidity, i.e., below 50 Nephelometric Turbidity Unit (NTU). In the case of low turbidity, the availability of lipids, particularly phytosterols, becomes limiting. In this situation, control of oxygenation, which is necessary for lipid synthesis by yeast, is particularly crucial during fermentation.

To mimic and understand these situations, a synthetic medium simulating the average composition of a Champagne must was used. This medium contained phytosterol (mainly β-sitosterol) concentrations ranging from 0 to 8 mg/L corresponding to turbidity between 10 – 90 NTU. Population reached during the stationary phase and the maximum fermentation rate are conditioned by the initial phytosterol concentration determining the amount of nitrogen consumption. An early loss of viability was observed when the lipid concentrations were very low. For example, the viability continuously decreased during the stationary phase to a final value of 50% for an initial phytosterol concentration of 1 mg/L. In some fermentations, 10 mg/L oxygen were added at the end of the growth phase to combine the effects of initial content of phytosterols in the musts and the de novo synthesis of ergosterol and unsaturated fatty acids induced by oxygen addition. Effect of oxygen supply on the fermentation kinetics was particularly significant for media with low phytosterol contents. For example, the maximum fermentation rate was increased by 1.4-fold and the fermentation time was 70 hours shorter with oxygen addition in the medium containing 2 mg/L of phytosterols. As a consequence of the oxygen supply, for the media containing 3, 5 and 8 mg/L of phytosterols, the assimilable nitrogen was completely exhausted and the
fermentation kinetics, as well as the final populations and viabilities (greater than 90%), were identical for the 3 conditions. The impacts of the lipid content and additional oxygen on acetate, glycerol and succinate synthesis were also studied. The phytosterols decreased the acetate and increased the succinate synthesis, and oxygenation resulted in a decrease in succinate formation.

This work highlights the similarities and differences between the effects of lipids and oxygen on fermentation kinetics and yeast metabolism. This research highlights the need for an optimal combined management of lipid content in the must via turbidity and oxygenation, particularly in nitrogen-rich musts.

**Keywords:**

Winemaking fermentation; Lipids; Nitrogen; Oxygen supply; Viability; Acetic acid.
Introduction

Yeast Available Nitrogen (YAN) is an essential nutritional factor during alcoholic fermentation. Assimilable nitrogen is used by yeasts for protein synthesis and cell growth. Under winemaking conditions, nitrogen is usually the limiting nutrient, and it is highly correlated with fermentation kinetics, especially the maximum CO$_2$ production rate and the final yeast population (M. Bely, Sablayrolles, and Barre 1990).

Therefore, observing slow fermentation under oenological conditions for Champagne is paradoxical because Champagne musts, from chardonnay and pinot noir/pinot meunier grape varieties, usually contain large concentrations of nitrogen compounds. For example, between 1982 and 1989, the YAN levels in Champagne musts were between 200 and 420 mg N/L (Ribéreau-Gayon et al. 2012). This last observation suggests that nitrogen is probably not the limiting factor in Champagne musts and that another nutrient is responsible for the long fermentation durations obtained here.

During the white winemaking process, variable amounts of grape solids (which are composed of heterogeneous particle deposits) can be found depending on the pressing process and the clarification level. Grape solids are an important source of nutrients for yeasts (Casalta et al. 2013; Casalta et al. 2016). They have been shown to contain phytosterols ($\beta$-sitosterol, campesterol, and stigmasterol) and fatty acids (palmitic acid, oleic acid, linoleic acid, and $\alpha$-linoleic acid), which are localized in the coating of grape berries and are assimilated by yeasts (Miele, Bouard, and Bertrand 1993). $\beta$-sitosterol represents more than 70% of total phytosterols (Le Fur et al. 2015). The absorption of phytosterols allows yeasts to compensate, at least partially, for a lack of oxygen (Andreasen and Stier 1953) and a deficiency in long-chain fatty acids during fermentation (Lorenz et al. 1986; Rodriguez et al. 1985). The assimilation of these phytosterols leads to the increased viability of yeasts at the end of fermentation (Luparia et al. 2004). The
Champagne winemaking practice consists in clarifying the must with a pectolytic enzyme to reach a turbidity level that is usually lower than 50 NTU at the end of the clarification step. The excessive clarification of the must consists in an important removal of solid particles (coming from grape skins) that are the primary sources of lipids and, more precisely, of phytosterols. This removal results in lower yeast proliferation and a significant decrease in the fermentation rate (Houtman and Du Plessis 1986).

The objective of this work is to better understand the oenological situation of Champagne, that is characterized by high YAN contents and low phytosterol concentration in the musts. Our final aim is to improve the management of fermentation by increasing the fermentation rate and limiting the risks of sluggish fermentation caused by cell mortality by adding phytosterols and/or oxygen during fermentation.

Materials and methods

Strain

The yeast strain used is a *Saccharomyces cerevisiae* strain isolated from Champenois-Champagne vineyard. It is a non-commercial dry active strain used by Moët & Chandon. Fermentation flasks were inoculated with 10 g/hl active dry yeast previously rehydrated for 30 min at 30 °C in a 50 g/l glucose solution (1 g of dry yeast diluted in 10 ml of this solution).

Fermentation media

Fermentations were performed on a synthetic medium (SM) mimicking Champagne must and derived from standard grape juice as described by Bely (Bely et al. 1990). This culture medium contained 180 g/L of sugars (90 g/L glucose and 90 g/L fructose), 7 g/L malic acid, 7 g/L tartric acid, salts (0.75 g/L KH$_2$PO$_4$, 0.50 g/L K$_2$SO$_4$, 0.25 g/L MgSO$_4$, 0.155 g/L CaCl$_2$, ...
and 0.20 g/L NaCl), vitamins (20 mg/L myo-inositol, 1.5 mg/L pantothenic acid, 0.25 mg/L thiamine, 2 mg/L nicotinic acid, 0.25 mg/L pyridoxine, and 0.003 mg/L biotin), and trace elements (4 mg/L MnSO$_4$, 4 mg/L ZnSO$_4$, 1 mg/L CuSO$_4$, 1 mg/L KI, 0.4 mg/L CoCl$_2$, 1 mg/L H$_3$BO$_3$, and 1 mg/L (NH$_4$)$_6$Mo$_7$O$_24$). The pH of the medium was adjusted to 3.1 with 10 M NaOH.

The source of nitrogen was a mixture of ammonium (30%) and amino acids (70%) as commonly found in pinot noir must. The assimilable nitrogen concentration was 360 mg N/L. The composition of the amino acid solution was as follows (in mg/L): tyrosine (8.3), tryptophan (6.5), isoleucine (15.9), aspartate (15.1), glutamate (56.9), arginine (550.3), leucine (20.5), threonine (124.3), glycine (3.5), glutamine (180), alanine (221.3), valine (30.9), methionine (5.9), phenylalanine (20.8), serine (74.7), histidine (12.6), lysine (4.6), cysteine (15.1) and proline (100.5). This solution was established after an assay of natural Champagne grape musts from pinot noir. To obtain 360 mg/L of assimilable nitrogen in the SM, 14.6 mL of an amino acid stock solution and 413 mg of NH$_4$Cl were added to 1 litre of medium.

The SM medium was initially supplemented with different concentrations of phytosterols (CAS: 85.451, Sigma Aldrich, Saint Quentin Fallavier, France). The stock solution was composed of 15 g/L of β-sitosterol in Tween 80 and absolute ethanol (1:1, v/v). To obtain 2 mg/L of initial phytosterols, 1 mL/L of 10 times-diluted stock solution was added to the medium. Total desorption of oxygen was monitored by using a PreSens® oxygen probe.

**Fermentation conditions**

The fermentations were performed in 1.2-L glass fermenters with 1.1 L of medium containing 360 mg/L of assimilable nitrogen and various levels of phytosterols (0 – 8 mg/L) at 20 °C. CO$_2$ released was measured from an accurate and automatic online monitoring of weight loss (Sablayrolles et al., 1987).
Cell population

During fermentation, the total cell population was determined using a Beckman Coulter counter (Model Z2, Beckman-Coulter, Margency, France) that was fitted with a 100 μm-aperture probe.

Cell viability

The cell viability was determined by flow cytometry using a BD Accuri™ C6 cytometer (BD Biosciences, Le Pont de Claix, France). The samples were centrifuged (5 min at 14,000 x g). The supernatants were removed and the pellets were then diluted with Phosphate-Buffered Saline (1X) to reach 5 to 10 x 10^6 cells/mL. For staining, propidium iodide (PI) was added to the cell suspension (5 µL of PI at 100 mg/mL in 500 mL of centrifuged and diluted sample), mixed by gentle shaking and incubated for 10 min at room temperature in the dark, then placed on ice before the fluorescence measurement by flow cytometry. Propidium iodide is a fluorescent nucleic acid stain that cannot penetrate intact cell membranes. Propidium iodide fluorescence (with excitation using a 488-nm laser and emission at 575 nm) was collected via a 670-nm large-pass filter (Fluorescence 3). Viability was determined as the percentage of intact and fragile cells among all the cells (Delobel et al. 2012).

Bubble-free oxygenation system

Oxygen was added by using the bubble-free oxygenation system developed by Moenne et al. 2013. To add 10 mg/L of oxygen over 120 minutes, the conditions were set up as follows: length of the silicone tube: 0.4 m, liquid flow rate: 120 mL/min, and maximum Oxygen Transfer Rate (OTRm): 5 mg/L.h. The oxygen addition was performed at the maximum rate of CO₂ production corresponding to the end of the cellular growth phase (Sablayrolles and Barre 1986).
Measurement of assimilable nitrogen

The ammonium concentration was determined enzymatically (RBiopharm AG™, Darmstadt, Germany). The free amino acid content of the must was determined by cation exchange chromatography, with post-column ninhydrin derivatization (Biochrom 30, Biochrom™, Cambridge, UK) as described by (Crepin et al. 2012).

Determination of metabolite concentrations

The ethanol, glycerol, succinate and acetate concentrations were determined by HPLC (HPLC 1290 Infinity, Agilent™ Technologies, Santa Clara, California, USA) on a Phenomenex Rezex ROA column (Phenomenex™, Le Pecq, France) at 60 °C. The column was eluted with 0.005 N H$_2$SO$_4$ at a flow rate of 0.6 mL/min. The acetic acid concentration was determined with a UV meter at 210 nm; the concentrations of the other compounds were determined with a refractive index detector. The analysis was performed with an Agilent™ EZChrom software package.

Results

In the present work, we have evaluated the effects of the initial phytosterol concentration in the must, with and without oxygen supplementation during fermentation, on the fermentative kinetic profiles, population levels, nitrogen consumption and viability in the synthetic medium mimicking Champagne must conditions. We have used different levels of the initial phytosterol concentrations ranging between 0 – 8 mg/L to simulate different levels of turbidity between 10 – 90 NTU.

Effects of the initial phytosterol concentration on fermentation kinetics

In this section, we focused on the effect of the initial phytosterol concentration in the must, from 0 to 8 mg/L, simulating different levels of turbidity.
Fermentation kinetics and cell population

As shown in figure 1, when the initial phytosterol concentration increased, the maximum fermentation rate of CO$_2$ production ($V_{\text{max}}$) increased from 0.4 to 1.3 gCO$_2$/L.h, and the fermentation duration was reduced from stuck fermentation to 150 h. The increase in the phytosterol content also resulted in higher final population levels. The population level during the stationary phase was much lower during slow fermentation, with 20 million cells/mL corresponding to fermentation without phytosterols. By contrast, the maximum amount of initial phytosterols (8 mg/L) led to a larger population, reaching 120 million cells/mL. Consequently, the $V_{\text{max}}$ value can be correlated to the total cell number value during the stationary phase with a regression coefficient of 0.989 (data not shown). The initial phytosterol concentration also directly impacted the duration of the fermentation and the cell number that was reached at the end of the growth phase. These data confirm the key role of phytosterol content in our fermentation conditions.

Cellular viability

An early loss of viability was observed in musts containing very low lipid concentrations (figure 2). When the phytosterol content is higher than 2 mg/L, the viability value remained higher than 80% during the major part of the fermentation process. For phytosterol concentrations equal to 5 and 8 mg/L, the kinetics of cellular viability were similar to that obtained at 3 mg/L (data not shown).

Nitrogen consumption

Assimilable nitrogen was monitored at 60% fermentation, i.e., during the stationary phase, when nitrogen consumption has ceased (Crepin et al. 2012), and before nitrogen release occurs as a result of mortality. The assimilable nitrogen was not exhausted, with the exception of the fermentation performed with 8 mg/L of phytosterols. These results confirm previous observations of natural musts (Casalta, Cervi, Salmon, & Sablayrolles, 2013).

The number of cells during the stationary phase is correlated with nitrogen consumption.
(R²=0.987), as shown in figure 3. Moreover, the initial nitrogen concentration is identical under each condition. Thus, the initial concentration of phytosterols determined the amount of nitrogen that was assimilated by the yeast.

Table 1 details the residual ammonium and major amino acid concentrations for different initial levels of phytosterols. The sum of the arginine and alanine concentrations represents 46% of the initial assimilable nitrogen, and they represent more than 60% of the final residual assimilable nitrogen without phytosterols. When the amount of residual nitrogen decreases, the proportion of residual arginine and alanine increases and reaches over 90% at 5 mg/L phytosterol. This result confirms that these two amino acids are consumed late, as previously observed by (Crepin et al. 2012). We also noted an increase in residual proline with the increase in the initial phytosterol concentration (table 1). This observation can be explained by the fact that even if proline cannot be assimilated under anaerobic conditions (Brandriss and Magasanik 1979), it is produced from arginine degradation (Martin et al. 2003).

**Effects of oxygen supplementation on fermentation**

By inducing the biosynthesis of ergosterol and unsaturated fatty acid by yeasts, the addition of oxygen during fermentation is one of the levers that is used to improve fermentation (Andreasen and Stier 1953; Rosenfeld et al. 2003; Sablayrolles and Barre 1986). The best timing for an oxygen supply during fermentation is towards the end of the growth phase (Sablayrolles and Barre 1986), i.e., close to the maximum rate of CO₂ production.

**Fermentation kinetics and cell population**

The oxygen supply had a positive impact on each condition (figure 4). This addition increased the maximum rate of CO₂ production and the cell number, resulting in a reduction of the fermentation duration. The efficacy of oxygen addition was much higher for musts that were highly deficient in phytosterols. Without any initial phytosterol, the
addition of 10 mg/L of oxygen made the complete consumption of sugars possible, for a
high increase in the maximum rate of CO$_2$ production (0.3 to 1.1 gCO$_2$/L) and the cell
number (20 to 90 million cells/mL). When the phytosterol content is 3 mg/L, the increase in
these two parameters is lower: from 1.0 to 1.4 g CO$_2$/L for the maximum rate of CO$_2$
production; and from 90 to 140 million cells/mL for the cell number. Finally, above a
threshold phytosterol content (3 mg/L), the fermentation kinetics and cell numbers during
the stationary phase were identical as a consequence of oxygen added at 10 mg/L.

**Nitrogen consumption**

Following the addition of 10 mg/L of oxygen, the nitrogen consumption was
systematically increased (figure 5) by the addition of 10 mg/L of oxygen (figure 5). When
oxygen was added, at a phytosterol concentration of 3 mg/L or above, the YAN was
exhausted. These modalities can be classified into two groups, in which the first is
composed of fermentations with residual nitrogen; and the second is without residual
nitrogen and with the equivalent fermentation kinetics and numbers of cells. This result
indicates that under Champagne oenological conditions (low turbidity and high assimilable
nitrogen levels), the oxygen supply had two effects: lipid synthesis activation and increase
of nitrogen consumption.

**Cellular viability**

The addition of 10 mg/L of oxygen to the culture medium that contained no phytosterol resulted in
cell viability maintenance at its maximum value throughout the fermentation process (figure
2). This observation is also true for all the conditions involving phytosterols (dataset not shown).

**Effects of the phytosterol concentration and oxygen on metabolite production**

The analysis of some primary metabolites shows that the effects of phytosterols and
oxygen have some similarities. Even if the final concentrations of acetic acid, succinic acid
and glycerol are different, we can distinguish two global evolutions: (i) under 3 mg/L, in
which a strong evolution is observed; and (ii) above 3 mg/L, in which the evolution is limited, as shown in figure 6.

First, we noticed that the increase in the initial amount of phytosterols strongly reduced acetate production, as observed in previous studies (Delfini and Costa 1993; Luparia et al. 2004). The production of this organic acid was divided by 1.8 between 0.5 mg/L and 3 mg/L of phytosterols. A good correlation was established between the final acetate concentration and the level of nitrogen assimilated by the yeast ($R^2 = 0.98$). In addition we did not find any effect of added oxygen on the final acetate concentration.

Second, we observed that the increase of initial phytosterols increased the final succinate concentration. Between 1 and 8 mg/L, the final succinate doubled. In addition, we also found that the final succinate concentration was correlated with the amount of nitrogen that was assimilated by the yeast ($R^2 = 0.979$). However, with oxygen, the amount of succinate is stable (0.3 mg/L) and independent of the initial phytosterol concentration.

Finally, from 0 to 3 mg/L phytosterols, a reduction of 1 g/L in the final glycerol concentration was obtained. Above 3 mg/L, the final glycerol concentration was stable at approximately 6 g/L. Moreover; the added oxygen did not have any effect on the final glycerol concentration.

**Discussion**

Must turbidity, and oxygen are essential for white wine alcoholic fermentation, and Champagne conditions provide an excellent example for distinguishing among the different mechanisms that are involved. These conditions are usually characterized by high levels of Yeast Available Nitrogen (YAN) in musts and low amounts of solid particles, which is the result of dramatic clarification, with turbidity levels lower than 50 NTU. Grape solids provide reserves of sterols and fatty acids for yeast during anaerobic fermentation (Le Fur et al. 2015; Casalta et al. 2016; Ruggiero et al. 2013). Therefore, Champagne musts are
characterized by lipid deficiencies, and assimilable nitrogen is usually the limiting nutrient in wine-making fermentation, including in white winemaking.

In a synthetic medium that was used for simulating the average composition of Champagne musts, we observed slow fermentation when the culture medium was deprived of lipid resources and oxygen. Under this condition, the consumption of assimilable nitrogen and the final population were very low (figures 1 and 5 and table 2). The population was limited by the amount of sterols and fatty acids available to synthesize the plasmic membrane; low turbidity induces low lipid availability, and under strict anaerobic conditions, yeast is not able to synthesize ergosterol and unsaturated fatty acids (Andreasen and Stier 1953; Andreasen and Stier 1954). Increasing the initial content of lipids in the must (turbidity) led to higher nitrogen consumption during the alcoholic fermentation process. Total nitrogen depletion was obtained for an initial phytosterol content of 8 mg/L. This value depends on the strain and on the initial nitrogen content (Rollero et al. 2015). As a consequence of the higher nitrogen consumption, the cell population and the maximum CO$_2$ production rate were increased and the fermentation duration was reduced. Adjusting the turbidity appears to be necessary for improving the fermentation kinetic of white wine fermentations (Casalta et al. 2013). Using another approach, providing an oxygen supply during fermentation favours the synthesis of ergosterol by yeast (Andreasen and Stier 1953; Andreasen and Stier 1954) and allows for the correction of lipid deficiency (figure 4 and 5 and table 1). We added 10 mg/L oxygen at the end of the growth phase, which is considered the best timing for an oxygen supply (Sablayrolles and Barre 1986; Blateyron and Sablayrolles 2001; Fornairon-Bonnefond et al. 2003; Rosenfeld et al. 2003). Following the addition of oxygen, a switch from lipid-limited to nitrogen-limited fermentation occurred for a 3 mg/L phytosterol content. Above this lipid concentration, the total depletion of assimilable nitrogen resulted in a “standardization” of the fermentation kinetics with similar values for the fermentation
duration, maximum CO₂ production rate and population. This effect is confirmed visible on figure 7, where principal component analysis (PCA) allows evaluating the significance effect of lipids (turbidity) and oxygen on the fermentation kinetics (duration, nitrogen consumption, total cell, viability) and organoleptic aspects, the synthesis of some key metabolites (acetic acid, glycerol). It is thus necessary to combine the management of turbidity and oxygen to deplete the assimilable nitrogen, especially in the case of nitrogen-rich musts.

Champagne conditions are also an excellent model for studying the impact of nitrogen-lipid balance management on the modulation of cell viability during the fermentation process. Ethanol is the primary cause of mortality because it affects the integrity of the cell membrane, damages the permeability of numerous ionic species, and decreases the fluidity of the plasma membrane, leading to the dissipation of the transmembrane electrochemical potential, and subsequently acidifies the intracellular and vacuolar conditions (Salgueiro, Sá-Correia, and Novais 1988; Teixeira et al. 2009). Increasing the initial phytosterol concentration and/or the addition of oxygen dramatically improved the cell viability (figure 2). This positive effect of phytosterols on viability has also been observed in natural musts (Casalta et al. 2013). The effects of phytosterols and oxygen on viability are comparable. However, the mechanisms are slightly different. Under anaerobic conditions, yeast is able to: (i) incorporate exogenous phytosterols and fatty acids as an external source of lipids that is directly provided by the medium (Daum et al. 1998; Vanegas et al. 2012; Luparia et al. 2004); and (ii) synthesize endogenous unsaturated fatty acids and sterols in the presence of oxygen, but, in that case, the major sterol is ergosterol. Molecular oxygen is required for squalene cyclization to sterols and the subsequent demethylation of lanosterol, and for unsaturated fatty acid biosynthesis (Parks 1978). Ergosterol induces the formation of a liquid-ordered phase (Sankaram and Thompson 1991; Marsh 2009) and plays an important role in modulating the membrane
dynamic structure and mechanical properties (Bacia, Schwille, and Kurzchalia 2005; Hung et al. 2007). Unsaturated fatty acids and sterols play an important role in protecting the yeast plasma membrane against ethanol-induced interdigitation (Vanegas et al. 2012). However, ergosterol is certainly more efficient than phytosterols at maintaining yeast membrane integrity (Luparia et al. 2004).

Many other metabolic pathways are also influenced by the management of the phytosterol content in musts and/or oxygen addition. We focused on the production of three of the primary metabolites in central carbon metabolism: acetic acid, glycerol and succinic acid. Acetic acid plays a crucial role in the organoleptic balance of wine; it is the primary component of volatile acidity. This compound is produced by the oxidation of acetaldehyde via the cytosolic pyruvate dehydrogenase (PDH) bypass (Remize, Andrieu, and Dequin 2000). Under our experimental conditions, when lipids were the limiting nutrient, we observed a negative correlation between the final acetic acid concentration and the initial phytosterol content. Acetyl-CoA carboxylase is generally considered the rate-limiting step in lipogenesis (Donaldson 1979). Thus, it can be hypothesized that supplementing the culture medium with lipids decreases the cellular demand in lipids and thus the intensity of the metabolic flux from acetyl-CoA to lipid synthesis. The decrease in this specific metabolic flux results in the lower production of acetic acid, which is an intermediate of this metabolic pathway (figure 6 and 7) (Moreno-Arribas and Polo 2009). In the case of the oxygen supply, no effect on acetic acid production was noted (figure 6). This phenomenon can be explained by the demand of lipid synthesis and the redox balance (NADPH/NADP) turnover. The synthesis of ergosterol and unsaturated fatty acids requires both oxygen and NADPH, whereas only NADPH is necessary to synthesize diacylglycerol or saturated fatty acids (Parks 1978). Therefore, in the presence of oxygen, the NADPH demand is still high for lipid biosynthesis; thus, the turnover of NADPH to NADP is high. The conversion of acetaldehyde to acetic acid allows for the renewal of the NAPD to NADPH (Remize,
Andrieu, and Dequin 2000). When oxygen is added, most of the acetyl-CoA is used for lipid synthesis, without modification of the acetic acid excretion. Oxygen allows for the complete utilization of acetyl-CoA for lipid synthesis whereas turbidity (phytosterol + fatty acids) provides key components directly, which does not require acetyl-CoA.

Glycerol, which may contribute positively to the quality of wine, is produced by yeasts to maintain the redox stability (NADH/NAD turnover) and as a response to cellular stress (Vriesekoop, Haass, and Pamment 2009; Nevoigt 1997), in particular osmotic stress (Hohmann 1997). Glycerol formation results from the L-Glycerol 3-phosphate dephosphorylation. L-Glycerol 3-phosphate is also the first step of triglyceride biosynthesis (Zheng 2001). In our conditions where lipids are the limiting nutrient, glycerol is negatively correlated to the phytosterol content (figure 6). Whereas, in 'standard' oenological situations, i.e. when nitrogen is the limiting nutrient, glycerol production increases with the lipid content (Luparia et al. 2004). This difference can be explained by the modulation of the activation of triglyceride pathway. In the case of strong lipid deficiency, biosynthesis of triglyceride is strongly activated. Yeast produces L-Glycerol 3-phosphate and the excessive flow is converted into glycerol. When the initial lipid content increases, the amount of fatty acids increases and triglyceride biosynthesis is triggered. Thus, the accumulation of glycerol in the media is lower, figure 7. When oxygen is added to the media, we do not observe a difference in glycerol production (such as acetic acid production) because turbidity (phytosterol + fatty acids) provides key components which facilitated triglyceride production.

Succinic acid is the predominant non-volatile organic acid formed during fermentation, and it is a good metabolic marker of the Krebs cycle. In our study, succinic acid production was increased by phytosterols and therefore dependent on the amount of nitrogen consumed and biomass produced, as already observed in previous studies (Muratsubaki 1987;
Verduyn et al. 1990; Albers et al. 1996). The biosynthesis of most amino acids involves transamination reactions in which glutamate serves as an amino group donor to form α-ketoglutarate, which can be converted into succinic acid via two metabolic pathways: the GABA bypass, or via α-ketoglutarate dehydrogenase and succinyl-CoA synthetase (Camarasa, Grivet, and Dequin 2003). Consequently, when nitrogen consumption is higher, the glutamate demand for transamination increases, resulting in a higher accumulation of α-ketoglutarate and a more important synthesis of succinic acid. Thus, succinic acid evolution is an indirect consequence of the effect of phytosterols on nitrogen consumption under lipid-limiting conditions. The effect of oxygen is slightly different. Under oxygenated conditions, succinate synthesis remained almost stable and independent of the nitrogen consumption quantity. A possible explanation is a lower flux from acetyl-CoA to α-ketoglutarate because of the greater mobilization of acetyl-CoA for lipid production, regardless of the initial phytosterol content.

Conclusion

This work highlights the similarities but also the differences in the effects of lipids and oxygen on fermentation kinetics and yeast metabolism, figure 7. It addresses the optimal combined management of turbidity and oxygenation, in particular in nitrogen-rich musts. The best strategy is a combination of: (i) a moderate amount of solids in the must, corresponding to 2-3 mg/L phytosterols, to permit enough nitrogen consumption and yeast growth without having a negative effect on the wine quality, especially acetic acid formation; and (ii) an addition of approximately 10 mg/L oxygen at the end of the growth phase as suggested by Blateyron and Sablayrolles (2001), to maintain the viability of the yeasts. The application of this strategy may not be easily applicable at an industrial scale. There is a need for the complete control of the quantity of oxygen transfer, which is difficult in large tanks. Another difficulty is to quantify precisely the relationship between
phytosterols and turbidity. Even if it is well-known that turbidity is related to the amount of
phytosterols that are available in the must, this relationship depends on the pressing and
clarification process and also on the grape varieties (Casalta et al., 2016).

In the future, the two major challenges will be: (i) the combined optimization of oxygen,
solid grape particles and nitrogen in the case of nitrogen-poor musts; and (ii) the control of
the wine aroma profile (Rollero et al. 2015).
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**Figures captions:**

**Fig.1:** Impact of the initial phytosterol concentration on the fermentation kinetics (A) and total population (B). The phytosterol range is between 0 – 8 mg/L with 0 mg/L (dark), 1 mg/L (orange), 2 mg/L (violet), 3 mg/L (green), 5 mg/L (red) and 8 mg/L (blue).

**Fig.2:** Evolution of cell viability during the fermentation process for different initial phytosterol concentrations and for the oxygen supply on a fermentation without phytosterols. The phytosterol range is between 0 and 3 mg/L with 0 mg/L (dark), 0.5 mg/L (orange), 0.75 mg/L (violet), 1 mg/L (green), 2 mg/L (red), 3 mg/L (blue), and 0 mg/L + 10 mg/L of O₂ (grey).

**Fig.3:** Population level during the stationary phase according to the quantity of nitrogen consumed at 60% fermentation. The tags correspond to the initial phytosterol concentration and the dotted line represents the linear regression (R²=0.9873).

**Fig.5:** Residual nitrogen, at 60% fermentation progress, of the oxygenated conditions (blue) and without added oxygen (red), for different initial phytosterol concentrations.

**Fig.4:** Effect of adding 10 mg/L of oxygen at the Vmax during two hours under different phytosterol concentrations. The kinetics and population (blue squares) under oxygenated conditions are shown in blue, whereas the kinetics and the population (red squares) for the same condition without added oxygen are presented in red. The different phytosterol
concentrations studied here are 0 mg/L (A), 2 mg/L (B), 3 mg/L (C), 5 mg/L (D) and 8 mg/L (E).

**Fig.6**: Final concentrations of acetic acid (A), succinic acid (B) and glycerol (C) for different initial phytosterol concentrations, with the conditions without oxygen in red and the oxygenated conditions in blue.

**Fig.7**: Principal component analysis (PCA) of kinetics parameters (duration, maximum growth rate (Vmax), Total cells number, viability and residual nitrogen) and primary metabolites (acetic acid, glycerol and succinic acid). On the individual factor maps, oxygenated modalities are represented in red and non-oxygenated modalities in blue.