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

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12

13 Abstract

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

22 To mimic and understand these situations, a synthetic medium simulating the average
23 composition of a Champagne must was used. This medium contained phytosterol (mainly
24 β -sitosterol) concentrations ranging from 0 to 8 mg/L corresponding to turbidity between 10
25 – 90 NTU. Population reached during the stationary phase and the maximum fermentation
26 rate are conditioned by the initial phytosterol concentration determining the amount of
27 nitrogen consumption. An early loss of viability was observed when the lipid concentrations
28 were very low. For example, the viability continuously decreased during the stationary
29 phase to a final value of 50% for an initial phytosterol concentration of 1 mg/L. In some
30 fermentations, 10 mg/L oxygen were added at the end of the growth phase to combine the
31 effects of initial content of phytosterols in the musts and the de novo synthesis of
32 ergosterol and unsaturated fatty acids induced by oxygen addition. Effect of oxygen supply
33 on the fermentation kinetics was particularly significant for media with low phytosterol
34 contents. For example, the maximum fermentation rate was increased by 1.4-fold and the
35 fermentation time was 70 hours shorter with oxygen addition in the medium containing 2
36 mg/L of phytosterols. As a consequence of the oxygen supply, for the media containing 3,
37 5 and 8 mg/L of phytosterols, the assimilable nitrogen was completely exhausted and the

38 fermentation kinetics, as well as the final populations and viabilities (greater than 90%),
39 were identical for the 3 conditions. The impacts of the lipid content and additional oxygen
40 on acetate, glycerol and succinate synthesis were also studied. The phytosterols
41 decreased the acetate and increased the succinate synthesis, and oxygenation resulted in
42 a decrease in succinate formation.

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

47 **Keywords:**

48 | Winemaking fermentation; Lipids; Nitrogen; Oxygen supply; Viability; Acetic acid.

49

50 **Introduction**

51 Yeast Available Nitrogen (YAN) is an essential nutritional factor during alcoholic
52 fermentation. Assimilable nitrogen is used by yeasts for protein synthesis and cell growth.
53 Under winemaking conditions, nitrogen is usually the limiting nutrient, and it is highly
54 correlated with fermentation kinetics, especially the maximum CO₂ production rate and the
55 final yeast population (M. Bely, Sablayrolles, and Barre 1990).
56 Therefore, observing slow fermentation under oenological conditions for Champagne is
57 paradoxical because Champagne musts, from chardonnay and pinot noir/pinot meunier
58 grape varieties, usually contain large concentrations of nitrogen compounds. For example,
59 between 1982 and 1989, the YAN levels in Champagne musts were between 200 and 420
60 mg N/L (Ribéreau-Gayon et al. 2012). This last observation suggests that nitrogen is
61 probably not the limiting factor in Champagne musts and that another nutrient is
62 responsible for the long fermentation durations obtained here.
63 During the white winemaking process, variable amounts of grape solids (which are
64 composed of heterogeneous particle deposits) can be found depending on the pressing
65 process and the clarification level. Grape solids are an important source of nutrients for
66 yeasts (Casalta et al. 2013; Casalta et al. 2016). They have been shown to contain
67 phytosterols (β -sitosterol, campesterol, and stigmasterol) and fatty acids (palmitic acid,
68 oleic acid, linoleic acid, and α -linoleic acid), which are localized in the coating of grape
69 berries and are assimilated by yeasts (Miele, Bouard, and Bertrand 1993). **β -sitosterol**
70 **represents more than 70% of total phytosterols (Le Fur et al. 2015)**. The absorption of
71 phytosterols allows yeasts to compensate, at least partially, for a lack of oxygen
72 (Andreasen and Stier 1953) and a deficiency in long-chain fatty acids during fermentation
73 (Lorenz et al. 1986; Rodriguez et al. 1985). The assimilation of these phytosterols leads to
74 the increased viability of yeasts at the end of fermentation (Luparia et al. 2004). The

75 Champagne winemaking practice consists in clarifying the must with a pectolytic enzyme
76 to reach a turbidity level that is usually lower than 50 NTU at the end of the clarification
77 step. The excessive clarification of the must consists in an important removal of solid
78 particles (coming from grape skins) that are the primary sources of lipids and, more
79 precisely, of phytosterols. This removal results in lower yeast proliferation and a significant
80 decrease in the fermentation rate (Houtman and Du Plessis 1986).

81

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

87 **Materials and methods**

88 **Strain**

89 | The yeast strain used is a *Saccharomyces cerevisiae* strain isolated from Champenois-
90 Champagne vineyard. It is a non-commercial dry active strain used by Moët & Chandon.
91 Fermentation flasks were inoculated with 10 g/hl active dry yeast previously rehydrated for
92 30 min at 30 °C in a 50 g/l glucose solution (1 g of dry yeast diluted in 10 ml of this
93 solution).

94 **Fermentation media**

95 Fermentations were performed on a synthetic medium (SM) mimicking Champagne must
96 and derived from standard grape juice as described by Bely (Bely et al. 1990). This culture
97 medium contained 180 g/L of sugars (90 g/L glucose and 90 g/L fructose), 7 g/L malic acid,
98 7 g/L tartaric acid, salts (0.75 g/L KH₂PO₄, 0.50 g/L K₂SO₄, 0.25 g/L MgSO₄, 0.155 g/L CaCl₂,

99 and 0.20 g/L NaCl), vitamins (20 mg/L myo-inositol, 1.5 mg/L pantothenic acid, 0.25 mg/L
100 thiamine, 2 mg/L nicotinic acid, 0.25 mg/L pyridoxine, and 0.003 mg/L biotin), and trace
101 elements (4 mg/L MnSO₄, 4 mg/L ZnSO₄, 1 mg/L CuSO₄, 1 mg/L KI, 0.4 mg/L CoCl₂, 1
102 mg/L H₃BO₃, and 1 mg/L (NH₄)₆Mo₇O₂₄). The pH of the medium was adjusted to 3.1 with 10
103 M NaOH.

104 The source of nitrogen was a mixture of ammonium (30%) and amino acids (70%) as
105 commonly found in pinot noir must. The assimilable nitrogen concentration was 360 mg
106 N/L. The composition of the amino acid solution was as follows (in mg/L): tyrosine (8.3),
107 tryptophan (6.5), isoleucine (15.9), aspartate (15.1), glutamate (56.9), arginine (550.3),
108 leucine (20.5), threonine (124.3), glycine (3.5), glutamine (180), alanine (221.3), valine
109 (30.9), methionine (5.9), phenylalanine (20.8), serine (74.7), histidine (12.6), lysine (4.6),
110 cysteine (15.1) and proline (100.5). This solution was established after an assay of natural
111 Champagne grape musts from pinot noir. To obtain 360 mg/L of assimilable nitrogen in the
112 SM, 14.6 mL of a amino acid stock solution and 413 mg of NH₄Cl were added to 1 litre of
113 medium.

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

119 **Fermentation conditions**

120 The fermentations were performed in 1.2-L glass fermenters with 1.1 L of medium
121 containing 360 mg/L of assimilable nitrogen and various levels of phytosterols (0 – 8 mg/L)
122 at 20 °C. CO₂ released was measured from an accurate and automatic online monitoring
123 of weight loss (Sablayrolles et al., 1987).

124 **Cell population**

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

128 **Cell viability**

129 The cell viability was determined by flow cytometry using a BD Accuri™ C6 cytometer (BD
130 Biosciences, Le Pont de Claix, France). The samples were centrifuged (5 min at 14,000 x
131 g). The supernatants were removed and the pellets were then diluted with Phosphate-
132 Buffered Saline (1X) to reach 5 to 10 x 10⁶ cells/mL. For staining, propidium iodide (PI)
133 was added to the cell suspension (5 µL of PI at 100 mg/mL in 500 mL of centrifuged and
134 diluted sample), mixed by gentle shaking and incubated for 10 min at room temperature in
135 the dark, then placed on ice before the fluorescence measurement by flow cytometry.
136 Propidium iodide is a fluorescent nucleic acid stain that cannot penetrate intact cell
137 membranes. Propidium iodide fluorescence (with excitation using a 488-nm laser and
138 emission at 575 nm) was collected via a 670-nm large-pass filter (Fluorescence 3).
139 Viability was determined as the percentage of intact and fragile cells among all the cells
140 (Delobel et al. 2012).

141 **Bubble-free oxygenation system**

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

148 **Measurement of assimilable nitrogen**

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

153 **Determination of metabolite concentrations**

154 The ethanol, glycerol, succinate and acetate concentrations were determined by HPLC
155 (HPLC 1290 Infinity, Agilent™ Technologies, Santa Clara, California, USA) on a
156 Phenomenex Rezex ROA column (Phenomenex™, Le Pecq, France) at 60 °C. The
157 column was eluted with 0.005 N H₂SO₄ at a flow rate of 0.6 mL/min. The acetic acid
158 concentration was determined with a UV meter at 210 nm; the concentrations of the other
159 compounds were determined with a refractive index detector. The analysis was performed
160 with an Agilent™ EZChrom software package.

161

162 **Results**

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

169 **Effects of the initial phytosterol concentration on fermentation kinetics**

170 In this section, we focused on the effect of the initial phytosterol concentration in the must,
171 from 0 to 8 mg/L, simulating different levels of turbidity.

172 **Fermentation kinetics and cell population**

173 As shown in figure 1, when the initial phytosterol concentration increased, the maximum
174 fermentation rate of CO₂ production (V_{\max}) increased from 0.4 to 1.3 gCO₂/L.h, and the
175 fermentation duration was reduced from stuck fermentation to 150 h. The increase in the
176 phytosterol content also resulted in higher final population levels. The population level
177 during the stationary phase was much lower during slow fermentation, with 20 million
178 cells/mL corresponding to fermentation without phytosterols. By contrast, the maximum
179 amount of initial phytosterols (8 mg/L) led to a larger population, reaching 120 million
180 cells/mL. Consequently, the V_{\max} value can be correlated to the total cell number value
181 during the stationary phase with a regression coefficient of 0.989 (data not shown). The
182 initial phytosterol concentration also directly impacted the duration of the fermentation and
183 the cell number that was reached at the end of the growth phase. These data confirm the
184 key role of phytosterol content in our fermentation conditions.

185 **Cellular viability**

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

191 **Nitrogen consumption**

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

198 ($R^2=0.987$), as shown in figure 3. Moreover, the initial nitrogen concentration is identical
199 under each condition. Thus, the initial concentration of phytosterols determined the
200 amount of nitrogen that was assimilated by the yeast.

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

212 **Effects of oxygen supplementation on fermentation**

213 By inducing the biosynthesis of ergosterol and unsaturated fatty acid by yeasts, the
214 addition of oxygen during fermentation is one of the levers that is used to improve
215 fermentation (Andreasen and Stier 1953; Rosenfeld et al. 2003; Sablayrolles and Barre
216 1986). The best timing for an oxygen supply during fermentation is towards the end of the
217 growth phase (Sablayrolles and Barre 1986), i.e., close to the maximum rate of CO_2
218 production.

219 **Fermentation kinetics and cell population**

220 The oxygen supply had a positive impact on each condition (figure 4). This addition
221 increased the maximum rate of CO_2 production and the cell number, resulting in a
222 reduction of the fermentation duration. The efficacy of oxygen addition was much higher
223 for musts that were highly deficient in phytosterols. Without any initial phytosterol, the

224 addition of 10 mg/L of oxygen made the complete consumption of sugars possible, for a
225 high increase in the maximum rate of CO₂ production (0.3 to 1.1 gCO₂/L) and the cell
226 number (20 to 90 million cells/mL). When the phytosterol content is 3 mg/L, the increase in
227 these two parameters is lower: from 1.0 to 1.4 g CO₂/L for the maximum rate of CO₂
228 production; and from 90 to 140 million cells/mL for the cell number. Finally, above a
229 threshold phytosterol content (3 mg/L), the fermentation kinetics and cell numbers during
230 the stationary phase were identical as a consequence of oxygen added at 10 mg/L.

231 **Nitrogen consumption**

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

241 **Cellular viability**

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

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

246 The analysis of some primary metabolites shows that the effects of phytosterols and
247 oxygen have some similarities. Even if the final concentrations of acetic acid, succinic acid
248 and glycerol are different, we can distinguish two global evolutions: (i) under 3 mg/L, in

249 which a strong evolution is observed; and (ii) above 3 mg/L, in which the evolution is
250 limited, as shown in figure 6.

251 First, we noticed that the increase in the initial amount of phytosterols strongly reduced
252 acetate production, as observed in previous studies (Delfini and Costa 1993; Luparia et al.
253 2004). The production of this organic acid was divided by 1.8 between 0.5 mg/L and 3
254 mg/L of phytosterols. A good correlation was established between the final acetate
255 concentration and the level of nitrogen assimilated by the yeast ($R^2= 0.98$). In addition we
256 did not find any effect of added oxygen on the final acetate concentration.

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

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

266 Discussion

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

274 characterized by lipid deficiencies, and assimilable nitrogen is usually the limiting nutrient
275 in wine-making fermentation, including in white winemaking.

276 In a synthetic medium that was used for simulating the average composition of
277 Champagne musts, we observed slow fermentation when the culture medium was
278 deprived of lipid resources and oxygen. Under this condition, the consumption of
279 assimilable nitrogen and the final population were very low (figures 1 and 5 and table 2).

280 The population was limited by the amount of sterols and fatty acids available to synthesize
281 the plasmic membrane; low turbidity induces low lipid availability, and under strict
282 anaerobic conditions, yeast is not able to synthesize ergosterol and unsaturated fatty acids
283 (Andreasen and Stier 1953; Andreasen and Stier 1954). Increasing the initial content of
284 lipids in the must (turbidity) led to higher nitrogen consumption during the alcoholic
285 fermentation process. Total nitrogen depletion was obtained for an initial phytosterol
286 content of 8 mg/L. This value depends on the strain and on the initial nitrogen content
287 (Rollero et al. 2015). As a consequence of the higher nitrogen consumption, the cell
288 population and the maximum CO₂ production rate were increased and the fermentation
289 duration was reduced. Adjusting the turbidity appears to be necessary for improving the
290 fermentation kinetic of white wine fermentations (Casalta et al. 2013). Using another
291 approach, providing an oxygen supply during fermentation favours the synthesis of
292 ergosterol by yeast (Andreasen and Stier 1953; Andreasen and Stier 1954) and allows for
293 the correction of lipid deficiency (figure 4 and 5 and table 1). We added 10 mg/L oxygen at
294 the end of the growth phase, which is considered the best timing for an oxygen supply
295 (Sablayrolles and Barre 1986; Blateyron and Sablayrolles 2001; Fornairon-Bonnefond et
296 al. 2003; Rosenfeld et al. 2003). Following the addition of oxygen, a switch from lipid-
297 limited to nitrogen-limited fermentation occurred for a 3 mg/L phytosterol content. Above
298 this lipid concentration, the total depletion of assimilable nitrogen resulted in a
299 “standardization” of the fermentation kinetics with similar values for the fermentation

300 duration, maximum CO₂ production rate and population. This effect is confirmed visible on
301 figure 7, where principal component analysis (PCA) allows evaluating the
302 significance effect of lipids (turbidity) and oxygen on the fermentation kinetics (duration,
303 nitrogen consumption, total cell, viability) and erganeleptic aspee the synthesis if some key
304 metabolites -(acetic acid, glycerol). It is thus necessary to combine the management of
305 turbidity and oxygen to deplete the assimilable nitrogen, especially in the case of nitrogen-
306 rich musts.

307 Champagne conditions are also an excellent model for studying the impact of nitrogen-lipid
308 balance management on the modulation of cell viability during the fermentation process.
309 Ethanol is the primary cause of mortality because it affects the integrity of the cell
310 membrane, damages the permeability of numerous ionic species, and decreases the
311 fluidity of the plasma membrane, leading to the dissipation of the transmembrane
312 electrochemical potential, and subsequently acidifies the intracellular and vacuolar
313 conditions (Salgueiro, Sá-Correia, and Novais 1988; Teixeira et al. 2009). Increasing the
314 initial phytosterol concentration and/or the addition of oxygen dramatically improved the
315 cell viability (figure 2). This positive effect of phytosterols on viability has also been
316 observed in natural musts (Casalta et al. 2013). The effects of phytosterols and oxygen on
317 viability are comparable. However, the mechanisms are slightly different. Under anaerobic
318 conditions, yeast is able to: (i) incorporate exogenous phytosterols and fatty acids as an
319 external source of lipids that is directly provided by the medium (Daum et al. 1998;
320 Vanegas et al. 2012; Luparia et al. 2004); and (ii) synthesize endogenous unsaturated fatty
321 acids and sterols in the presence of oxygen, but, in that case, the major sterol is
322 ergosterol. Molecular oxygen is required for squalene cyclization to sterols and the
323 subsequent demethylation of lanosterol, and for unsaturated fatty acid biosynthesis (Parks
324 1978). Ergosterol induces the formation of a liquid-ordered phase (Sankaram and
325 Thompson 1991; Marsh 2009) and plays an important role in modulating the membrane

326 dynamic structure and mechanical properties (Bacia, Schwille, and Kurzchalia 2005; Hung
327 et al. 2007). Unsaturated fatty acids and sterols play an important role in protecting the
328 yeast plasma membrane against ethanol-induced interdigitation (Vanegas et al. 2012).
329 However, ergosterol is certainly more efficient than phytosterols at maintaining yeast
330 membrane integrity (Luparia et al. 2004).

331 Many other metabolic pathways are also influenced by the management of the phytosterol
332 content in musts and/or oxygen addition. We focused on the production of three of the
333 primary metabolites in central carbon metabolism: acetic acid, glycerol and succinic acid.
334 Acetic acid plays a crucial role in the organoleptic balance of wine; it is the primary
335 component of volatile acidity. This compound is produced by the oxidation of acetaldehyde
336 via the cytosolic pyruvate dehydrogenase (PDH) bypass (Remize, Andrieu, and Dequin
337 2000). Under our experimental conditions, when lipids were the limiting nutrient, we
338 observed a negative correlation between the final acetic acid concentration and the initial
339 phytosterol content. Acetyl-CoA carboxylase is generally considered the rate-limiting step
340 in lipogenesis (Donaldson 1979). Thus, it can be hypothesized that supplementing the
341 culture medium with lipids decreases the cellular demand in lipids and thus the intensity of
342 the metabolic flux from acetyl-CoA to lipid synthesis. The decrease in this specific
343 metabolic flux results in the lower production of acetic acid, which is an intermediate of this
344 metabolic pathway (figure 6 and 7) (Moreno-Arribas and Polo 2009). In the case of the
345 oxygen supply, no effect on acetic acid production was noted (figure 6). This phenomenon
346 can be explained by the demand of lipid synthesis and the redox balance (NADPH/NADP)
347 turnover. The synthesis of ergosterol and unsaturated fatty acids requires both oxygen and
348 NADPH, whereas only NADPH is necessary to synthesize diacylglycerol or saturated fatty
349 acids (Parks 1978). Therefore, in the presence of oxygen, the NADPH demand is still high
350 for lipid biosynthesis; thus, the turnover of NADPH to NADP is high. The conversion of
351 acetaldehyde to acetic acid allows for the renewal of the NADP to NADPH (Remize,

352 Andrieu, and Dequin 2000). When oxygen is added, most of the acetyl-CoA is used for
353 lipid synthesis, without modification of the acetic acid excretion. Oxygen allows for the
354 complete utilization of acetyl-CoA for lipid synthesis whereas turbidity (phytosterol + fatty
355 acids) provides key components directly, which does not require acetyl-CoA.

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

373

374 Succinic acid is the predominant non-volatile organic acid formed during fermentation, and
375 it is a good metabolic marker of the Krebs cycle. In our study, succinic acid production was
376 increased by phytosterols and therefore dependent on the amount of nitrogen consumed
377 and biomass produced, as already observed in previous studies (Muratsubaki 1987;

378 Verduyn et al. 1990; Albers et al. 1996). The biosynthesis of most amino acids involves
379 transamination reactions in which glutamate serves as an amino group donor to form α -
380 ketoglutarate, which can be converted into succinic acid via two metabolic pathways: the
381 GABA bypass, or via α -ketoglutarate dehydrogenase and succinyl-CoA synthetase
382 (Camarasa, Grivet, and Dequin 2003). Consequently, when nitrogen consumption is
383 higher, the glutamate demand for transamination increases, resulting in a higher
384 accumulation of α -ketoglutarate and a more important synthesis of succinic acid. Thus,
385 succinic acid evolution is an indirect consequence of the effect of phytosterols on nitrogen
386 consumption under lipid-limiting conditions. The effect of oxygen is slightly different. Under
387 oxygenated conditions, succinate synthesis remained almost stable and independent of
388 the nitrogen consumption quantity. A possible explanation is a lower flux from acetyl-CoA
389 to α -ketoglutarate because of the greater mobilization of acetyl-CoA for lipid production,
390 regardless of the initial phytosterol content.

391 **Conclusion**

392 This work highlights the similarities but also the differences in the effects of lipids and
393 oxygen on fermentation kinetics and yeast metabolism, **figure 7**. It addresses the optimal
394 combined management of turbidity and oxygenation, in particular in nitrogen-rich musts.
395 The best strategy is a combination of: (i) a moderate amount of solids in the must,
396 corresponding to 2-3 mg/L phytosterols, to permit enough nitrogen consumption and yeast
397 growth without having a negative effect on the wine quality, especially acetic acid
398 formation; and (ii) an addition of approximately 10 mg/L oxygen at the end of the growth
399 phase as suggested by Blateyron and Sablayrolles (2001), to maintain the viability of the
400 yeasts. The application of this strategy may not be easily applicable at an industrial scale.
401 There is a need for the complete control of the quantity of oxygen transfer, which is difficult
402 in large tanks. Another difficulty is to quantify precisely the relationship between

403 phytosterols and turbidity. Even if it is well-known that turbidity is related to the amount of
404 phytosterols that are available in the must, this relationship depends on the pressing and
405 clarification process and also on the grape varieties (Casalta et al., 2016).

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

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611 **Figures captions:**

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

615

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

621

622 **Fig.3:** Population level during the stationary phase according to the quantity of nitrogen
623 consumed at 60% fermentation. The tags correspond to the initial phytosterol
624 concentration and the dotted line represents the linear regression ($R^2=0.9873$).

625

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

628

629 **Fig.4:** Effect of adding 10 mg/L of oxygen at the V_{max} during two hours under different
630 phytosterol concentrations. The kinetics and population (blue squares) under oxygenated
631 conditions are shown in blue, whereas the kinetics and the population (red squares) for the
632 same condition without added oxygen are presented in red. The different phytosterol

633 concentrations studied here are 0 mg/L (A), 2 mg/L (B), 3 mg/L (C), 5 mg/L (D) and 8 mg/L
634 (E).

635

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

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

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