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1 **Influence of cell-cell contact between *L. thermotolerans* and *S. cerevisiae* on yeast**
2 **interactions and the exo-metabolome.**

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16

17 **Summary:**

18 Sequential fermentation of grape must inoculated with *L. thermotolerans* and then *S.*
19 *cerevisiae* 24 h later (typical wine-making practice) was conducted with or without cell-cell
20 contact between the two yeast species. We monitored cell viability of the two species
21 throughout fermentation by flow cytometry. The cell viability of *S. cerevisiae* decreased
22 under both conditions, but the decrease was greater if there was cell-cell contact. An
23 investigation of the nature of the interactions showed competition between the two species for
24 nitrogen compounds, oxygen, and must sterols. Volatile-compound analysis showed
25 differences between sequential and pure fermentation and that cell-cell contact modifies yeast
26 metabolism, as the volatile-compound profile was significantly different from that of
27 sequential fermentation without cell-cell contact. We further confirmed that cell-cell contact
28 modifies yeast metabolism by analyzing the exo-metabolome of all fermentations by FT-ICR-
29 MS analysis. These analyses show specific metabolite production and quantitative metabolite
30 changes associated with each fermentation condition. This study shows that cell-cell contact
31 not only affects cell viability, as already reported, but markedly affects yeast metabolism.

32 Keywords: interactions, *S. cerevisiae*, *L. thermotolerans*, cell-cell contact, flow cytometry,
33 metabolomics.

34 I. Introduction

35 Alcoholic fermentation in grape must is mainly performed by the well-known yeast
36 *Saccharomyces cerevisiae*. However, *S. cerevisiae* is not the only yeast present on grape
37 berries and in grape must. Other interesting yeasts, called non-*Saccharomyces*, are
38 increasingly being studied because of their ability to improve the complexity of the wine
39 aroma by increasing the concentration of certain aromatic molecules, such as terpenoids or
40 higher alcohols (Ciani, 1997; Esteve-Zarzoso et al., 1998; Rojas et al., 2001; Jolly et al., 2006;
41 Fleet, 2008; Benito, 2018; Zhang et al., 2018), or other molecule of interest, such as glycerol
42 (Romano et al., 1992; Barbosa et al., 2015). The production of these molecules is mostly due
43 to enzymatic activities present in non-*Saccharomyces* yeasts, which are lower or absent from
44 *S. cerevisiae* strains (Esteve-Zarzoso et al., 1998; Strauss et al., 2001; Jolly et al., 2014).
45 *Lachancea thermotolerans* (*L. thermotolerans*) is naturally present in grape must (Torija et
46 al., 2001; Kapsopoulou et al., 2005) and has been reported to enhance the overall acidity of
47 wine due to the high production of L-lactic acid (Mora et al., 1990; Gobbi et al., 2013). This
48 characteristic may be desirable for wine with a low acidity (Balikci et al., 2016). Moreover, *L.*
49 *thermotolerans* is able to increase the concentration of interesting aromatic molecules in co-
50 fermentation with *S. cerevisiae* than when *S. cerevisiae* is used alone. These molecules
51 include ethyl esters and terpenes (Benito et al., 2015; Balikci et al., 2016; Benito et al., 2016),
52 as well as glycerol (Kapsopoulou et al., 2006). The biotechnological interest of co-
53 fermentation with non-*Saccharomyces* and *S. cerevisiae* in wine making is now clear (García
54 et al., 2016), but co-fermentation is not well controlled. Indeed, the presence of non-
55 *Saccharomyces* yeasts with *S. cerevisiae* during alcoholic fermentation leads to interactions
56 between these different species (Ciani et al., 2016) which are highly dependent on the species
57 and strains used (Wang et al., 2016). Among such interactions, competition for nutrients, such
58 as nitrogen and oxygen, can be among the earliest that occur during co-fermentation,
59 especially in sequential fermentations (inoculation with *S. cerevisiae* a minimum of 24 h after
60 inoculation with the non-*Saccharomyces* yeast). Indeed, the non-*Saccharomyces* consume
61 nutrients before *S. cerevisiae* inoculation, leading sometimes to sluggish fermentation
62 (Sablayrolles et al., 1996; Alexandre and Charpentier, 1998; Taillandier et al., 2007). Among
63 these nutrients, phytosterols may be good candidates to study these interactions. Indeed, under
64 conditions of aerobiosis, yeast are able to synthesize unsaturated fatty acids (UFA) and sterols

65 for membrane integrity and energy production, with a succession of reactions involving
66 oxygen-dependent enzymes (Tehlivets et al., 2007). However, in the absence of oxygen, these
67 enzymes are not active and yeast use the fatty acids and sterols present in the must (Salmon,
68 2006). It is important to know the proportion of the phytosterols consumed by non-
69 *Saccharomyces* before inoculation with *S. cerevisiae* when performing sequential
70 fermentation.

71 Competition for nutrients is not the only interaction that occurs during alcoholic fermentation,
72 because direct physical contact between non-*Saccharomyces* and *S. cerevisiae* cells could lead
73 to cell-cell interactions. Indeed, several studies have shown early growth arrest of *Lachancea*
74 *thermotolerans* in co-fermentations with *S. cerevisiae*. They concluded that this is due to a
75 cell-cell contact mechanism (Nissen and Arneborg, 2003; Nissen et al., 2003), whereas
76 Albergaria *et al.* (2009) and Branco *et al.* (2014) showed that antimicrobial peptide secretion
77 is responsible for the early death of this non-*Saccharomyces* yeast. Several other molecules
78 are involved in yeast-yeast interactions and affect the growth of yeast, such as tyrosol,
79 tryptophol, and phenylethanol, which are *quorum sensing* molecules, especially under
80 nitrogen-limiting conditions (Zupan et al., 2013; González et al., 2018; Valera et al., 2019).
81 Additional interaction mechanisms have been reported and reviewed recently (Liu et al.,
82 2015). Although cell-cell contact may explain some interactions between yeast, the effect of
83 cell-cell contact on yeast metabolism relative to when the different species are physically
84 separated has never been investigated.

85 Here, we exhaustively studied the interactions between *L. thermotolerans* and *S. cerevisiae* by
86 comparing pure fermentations of each species with sequential fermentation, with and without
87 their physical separation. The competition for yeast assimilable nitrogen (YAN), oxygen, and,
88 for the first time, phytosterols was also studied. We analyzed the consequences of such
89 interactions on volatile compound profiles and report, their impact on the exo-metabolome.

90

91 **II. Materials and methods**

92 1. Yeast strains

93 A modified *S. cerevisiae* strain supplied by INRA/SupAgro Montpellier was used in this
94 study: *S. cerevisiae* 59A-GFP MATa ho *AMNI::TEF2Pr-GFP-ADH1-NATMX4*, a haploid
95 derivative of the commercial wine strain EC1118, modified to strongly express eGFP(S65T)
96 (Marsit et al., 2015).

97 *L. thermotolerans* BBMCZ7-FA20 (previously isolated and identified by Sadoudi et al.
98 (2012)) was used as the non-*Saccharomyces* yeast strain.

99

100 2. Growth conditions

101 All yeast strains were grown at 28°C in modified YPD medium (20 g.L⁻¹ glucose, 10 g.L⁻¹
102 peptone, and 5 g.L⁻¹ yeast extract with 18 g.L⁻¹ of agar for Petri dish cultivation),
103 supplemented with 0.1 g.L⁻¹ chloramphenicol. For fermentation inoculation, yeasts were pre-
104 cultured in 250-mL sterile Erlenmeyer flasks, closed with dense cotton plugs, containing 150
105 mL modified YPD medium and incubated with agitation (100 rpm) at 28°C for 24 or 48 h.

106

107 3. Fermentation conditions

108 Fermentations were carried out in triplicate in white must containing 212.1 ± 4.81 g.L⁻¹
109 glucose/fructose, pH 3.41 ± 0.02 , as well as 251.2 ± 20.5 mg.L⁻¹ total assimilable nitrogen.
110 The must was centrifuged at 7,000 x g for 7 min at 4°C before use. Sugar concentration and
111 ethanol production were monitored by Fourier transformed infra-red spectroscopy (FTIR,
112 OenoFOSS™, FOSS, Hilleroed, Denmark). The detection cell was filled with 200 µL
113 centrifuged (12,000 g for 5 min at 4°C) supernatant from cultures and the analysis run using
114 FOSS User Interface software.

115 3.1. Pure fermentations

116 Pure fermentations were carried out in 1-L test tubes containing 800 mL white must and
117 closed with specific silicon caps. Each test tube was inoculated with 10⁶ cells.mL⁻¹ from a
118 YPD-medium pre-culture of *S. cerevisiae* or *L. thermotolerans* and incubated at 20°C without
119 agitation.

120 3.2. Sequential fermentations in flasks

121 Sequential fermentations were carried out in 2-L pasteurized (2 h at 70°C) test tubes, closed
122 with specific silicon caps. Two different fermentation conditions were tested: without (i) and
123 with (ii) cell-cell contact.

124 i. Three test tubes were filled with 1.2 L white must and a dialysis membrane
125 (Spectra/por, Spectrum Labs, MWCO 12-14 kDa, diameter 48 mm, length 60 cm)
126 containing 600 mL white must was added to each test tube (total must volume 1.8
127 L). The dialysis membranes were inoculated with 10⁶ cells.mL⁻¹ *L. thermotolerans*
128 from YPD pre-cultures, and the test tubes (external medium) inoculated 24 h later
129 with 10⁶ cells.mL⁻¹ of *S. cerevisiae* from a YPD pre-culture.

130 ii. Three test tubes were filled with 1.8 L white must (no dialysis membrane),
131 inoculated with 10^6 cells.mL⁻¹ *L. thermotolerans*, and then 24 h later with 10^6
132 cells.mL⁻¹ *S. cerevisiae*, both from YPD pre-cultures.

133 All test tubes were incubated at 20°C without agitation.

134

135 4. Flow cytometric analysis

136 4.1. Yeast viability

137 All fluorescent dyes used in this study were purchased from ThermoFisher Scientific,
138 Invitrogen.

139 Yeast viability was monitored during fermentation with propidium iodide (PI) dye (maximum
140 excitation/emission wavelengths 538/617 nm), which binds to DNA when the cell membrane
141 is compromised, triggering its fluorescence. Fermenting yeast (1 mL) was centrifuged at
142 12,000 x g for 5 min at 4°C. The pellet was resuspended in 1 mL PBS buffer (137 mM NaCl,
143 2.7 mM KCl, and 11.9 mM Phosphate, pH 7.2) (Fisher Scientific, Illkirch, France) and serial
144 dilutions prepared. PI (1 µL at 0.1 mg.mL⁻¹ in water) was added to a 100 µL aliquot. Samples
145 were incubated 10 min in the dark and analyzed by flow cytometry.

146

147 4.2. Flow cytometer settings

148 Flow cytometry was performed with a BD Accuri C6 flow cytometer and the data analyzed
149 using BD Accuri C6 software. For each run, 20 µL of sample was analyzed at 34 µL.min⁻¹,
150 with a FSC threshold of 80,000, and SSC-H/FSC-H plots analyzed using logarithmic axes. A
151 488-nm wavelength argon laser was used to excite the cells (autofluorescence) and dye. An
152 FL3-H long-pass filter (675 nm) was used for PI fluorescence.

153

154 5. Amino-acid and oxygen quantification

155 Oxygen consumption during alcoholic fermentation was monitored using Pst3 sensors
156 (Nomacorc[®]) placed at the inner face at the top of the test tubes. The oxygen concentration
157 was read using a NomaSense™ O2 P300 device (Nomacorc[®]), following the manufacturer's
158 instructions. The oxygen concentration was measured in triplicate before stirring the media in
159 the test tubes.

160 Amino acids and ammonium were quantified by HPLC as described previously (Gobert et al.,
161 2017).

162

163 6. Phytosterol quantification by gas chromatography-mass spectrometry (GC-MS)

164 Must samples (50 mL) were taken at T0 and T24 h, corresponding to the must without yeast
165 and 24 h of fermentation by *L. thermotolerans*, respectively. Prior to extraction, 250 μ L of
166 cholesterol (Sigma-Aldrich, Merck, Germany) at 1.0 mg.mL⁻¹ in ethyl acetate (Sigma-
167 Aldrich, Merck, Germany) was added in must samples. Must samples were transferred to a
168 separatory funnel, 25 mL of chloroform (Biosolve Chimie, France) added, the samples well
169 agitated, and the organic lower phase collected. This step was repeated three times and the
170 organic phases were combined. To eliminate remaining water, anhydrous sodium sulfate
171 (Na₂SO₄ powder, Sigma-Aldrich, Merck, Germany) was added. Samples were transferred to
172 250 mL glass balloons and the solvent evaporated with a rotary vacuum evaporator to reduce
173 the volume to approximately 1 mL. This volume was transferred to 1-mL brown-glass vials
174 and completely evaporate under an N₂ flux. A cold saponification was done by adding 900 μ L
175 of absolute ethanol (Carlo Erba, France) and 250 μ L of a saturated potassium hydroxide
176 (KOH, Merck Darmstadt, Germany) aqueous solution into each vial. Samples were purged
177 with nitrogen, and was put into a rotary shaker (Edmund Buhler, Johanna Otta GmbH,
178 Hechingen, Germany) at ambient temperature in the dark overnight (15 h). The solution was
179 transferred in a 100 mL separation funnel and 10 mL of distilled water were added. The
180 unsaponifiable fraction was extracted three time with 2 mL of diethyl ether (Sigma-Aldrich,
181 Merck KGaA, Darmstadt, Germany). The combined organic extracts were removed with a
182 rotary vacuum evaporator and the residue was dried under nitrogen flow. After additional 2
183 mL of diethyl ether, the unsaponifiable residue was carefully transferred to a 2 mL glass test
184 tube and then evaporated to dry matter under nitrogen flow. Then the sterol residue was
185 converted to trimethylsilyl (TMS, Sigma-Aldrich, Merck, Germany) ethers with 100 μ L of
186 pyridine and 100 μ L of N,O-Bis(trimethylsilyl)trifluoroacetamide (BSTFA, Sigma-Aldrich,
187 Merck, Germany) at room temperature in the dark overnight and then, diluted with 800 μ L of
188 isooctane (Sigma-Aldrich, Merck, Germany). One microliter of each sample was injected into
189 the GC-MS system. Phytosterol analyses were performed on a GC-MS device, composed of a
190 Varian STAR 3400 GC instrument equipped with an "on-column" injector coupled to a mass
191 spectrometer (Saturn 2000, Varian, France) with Electronic Impact as an ionization source
192 (EI, ionization energy of 70 eV), working with a mass range from 40 to 600 m/z. Data
193 acquisition and processing were performed with Varian Saturn Work Station 5.11 software
194 using the NIST mass spectral database for compound identification. The separation of each
195 compound was performed with a capillary column Factor Four VF-5ms (stationary phase: 5%

196 phenyl-95% dimethylpolysiloxane, thickness of 0.1 μm , 60 m x 0.25 mm, Varian, France).
197 Initial temperature of the column was 50°C (maintained for 2 min). The column temperature
198 was programmed to reach 105°C at a rate of 7°C.min⁻¹ (maintained for 2 min), then 170°C at
199 a rate of 10°C.min⁻¹ (maintained for 2 min), and finally 320°C at a rate of 7°C.min⁻¹
200 (maintained for 15 min). The injector temperature was set to 50°C and programmed to reach
201 300°C at a rate of 100°C.min⁻¹ and kept at this temperature until the end of analysis. The
202 injected volume was set to 1 μL and was under the control of an automatic injector (8500,
203 Varian, France). The carrier gas was Helium (99,9995%, Air liquid, France) and was set to a
204 flow rate of 1 mL.min⁻¹. Sterols were quantified against cholesterol as an internal standard.

205

206 7. Volatile compound quantification

207 Volatile compounds were quantified by HeadSpace-Solid Phase MicroExtraction-Gas
208 Chromatography/Mass Spectrometry as reported previously (Gobert et al., 2017). Briefly, 2
209 mL of wine was placed in a 10-mL vial fitted with a silicone septum, which was then
210 transferred to a silicon oil bath at 40°C and the sample incubated for 10 min with magnetic
211 stirring (700 rpm). A divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS)
212 fiber (Supelco, Bellefonte, PA, USA) was exposed to the sample headspace for 30 min and
213 then subjected to immediate desorption in the gas chromatograph injector set at 260°C.
214 Volatile compounds were analyzed by gas chromatography coupled to a quadrupolar mass-
215 selective spectrometer. GC-MS analysis was performed in complete scanning mode (SCAN)
216 in the 30–300 mass unit range. Compounds were identified by comparing their mass spectra
217 and retention times with those of standard compounds or with those available in the Wiley 6
218 mass spectrum library or reported in previous publications.

219 8. Metabolomics: Fourier Transform-Ion Cyclotron Resonance-Mass Spectrometry 220 (FT-ICR-MS)

221 8.1. FT-ICR-MS metabolome profiling

222 Direct-infusion FT-ICR mass spectra were acquired with a 12 Tesla Bruker Solarix FT-ICR
223 mass spectrometer (Bruker Daltonics, Bremen, Germany). The samples were diluted 2:100
224 (v/v) in methanol (LC-MS grade, Fluka, Germany). Quality control (QC) samples were
225 prepared by pooling equal amounts of all samples. QC samples were analyzed at the
226 beginning and after every 10 samples to monitor the reproducibility of the measurements. QC
227 spectra showed good repeatability and reproducibility of the method (spectrum profiles were
228 very similar between each QC) with a very low coefficient of variation (supp fig. 1). The

229 diluted samples and QC samples were infused into the electrospray ion source at a flow rate
230 of 2 $\mu\text{L}\cdot\text{min}^{-1}$. Settings for the ion source were: drying gas temperature, 180°C; drying gas
231 flow, 4.0 $\text{L}\cdot\text{min}^{-1}$; capillary voltage, 3600 V. The spectra were acquired with a time-domain of
232 4 megawords and 300 scans were accumulated within a mass range of 92 to 1000 m/z. A
233 resolving power of 400,000 at 300 m/z was achieved. Exported features were assigned to
234 elemental formulae and represented using an H/C vs. O/C van Krevelen diagram, which
235 highlights family compounds, such as carbohydrates (H/C 1.5-2; O/C 0.8-1), fatty acids (H/C
236 1.9-2.1; O/C 0-0.25), amino acids (H/C 1-2; O/C 0.2-0.8), nucleic acids (H/C 1.1-1.4; O/C
237 0.3-1), and anthocyanins (H/C 0.5-1; O/C 0.4-0.8) (fig. 1). The metabolite formulae can then be
238 entered into data bases, such as KEGG, Lipidmap, YMDB, Metlin, or an in-house developed
239 plant and wine database, to annotate them and identify corresponding metabolic pathways.

240

241 8.2. Statistical analysis

242 The MS was first calibrated using arginine ion clusters (57 $\text{nmol}\cdot\text{mL}^{-1}$ in methanol). Next, raw
243 spectra were further internally calibrated using a reference list, including known wine markers
244 and ubiquitous fatty acids, to achieve the best possible mass accuracy and precision among
245 the samples. Raw spectra were post-processed using Compass DataAnalysis 4.2 (Bruker
246 Daltonics, Bremen, Germany) and peaks with a signal-to-noise ratio (S/N) of at least six were
247 exported to mass lists. All exported features were aligned in a matrix containing averaged m/z
248 values (maximum peak alignment window width: ± 1 ppm) and corresponding peak
249 intensities of all analyzed samples. Only m/z features of monoisotopic candidates and those
250 with feasible mass defects were retained in the matrix.

251 All further data processing was performed using Microsoft Excel 2010 and R Statistical
252 Language (version 3.4.1). Only molecular features detected in at least two of the three
253 replicates ($S/N \geq 6$) of one sample group were considered for further data analysis and
254 interpretation. Remaining m/z values were assigned to their unambiguous molecular formulae
255 as already described.

256 Principal component analysis (PCA), hierarchical cluster analysis (HCA), and analysis of
257 variance (ANOVA) were performed using Perseus 1.5.1.6 (Max Planck Institute of
258 Biochemistry, Germany). For HCA, the Euclidean distance and average linkage were chosen
259 and for ANOVA, a threshold *p-value* of 0.05.

260

261 III. Results and discussion

262 1. Fermentation kinetics and yeast viability

263 We carried out sequential fermentations to obtain a better understanding of the interaction
264 between *S. cerevisiae* and *L. thermotolerans*. Must was first inoculated with *L.*
265 *thermotolerans* and 24 h later with *S. cerevisiae* to allow the growth of the non-
266 *Saccharomyces* yeast species before the addition of *S. cerevisiae* (Gobbi et al., 2013; Sadoudi,
267 2014; Balikci et al., 2016). Sequential fermentations were carried out with or without physical
268 contact to study the consequences of cell-cell interactions. Indeed, such a strategy has been
269 used previously to successfully investigate this type of interaction (Nissen and Arneborg,
270 2003; Nissen et al., 2003; Renault et al., 2013; Englezos et al., 2019).

271 1.1. Fermentation kinetics

272 The kinetics of sugar consumption and ethanol production for each fermentation are presented
273 in fig. 2. Pure fermentation with *S. cerevisiae* (SC) resulted in complete alcoholic
274 fermentation in 10 days, reaching 13.3% (v/v) ethanol with no remaining sugars. Pure
275 fermentation with *L. thermotolerans* (LT) was slower, the percentage of ethanol reaching
276 12.2% (v/v), but fermentation was not complete, even after 21 days, with remaining sugar at a
277 concentration of approximately 5.7 g.L⁻¹. Sequential fermentations with (SF+) or without (SF-
278) physical contact (fig. 2) were both complete after 16 days, with approximately 13.2% (v/v)
279 ethanol and no remaining sugars. These slower kinetics, relative to those with SC, are a
280 reflection of negative interactions. Thus, yeast viability was monitored to explain this
281 behavior.

282 1.2. Yeast viability

283 We used a modified *S. cerevisiae* strain expressing green fluorescent protein (GFP) to
284 separate the *S. cerevisiae* from the non-*Saccharomyces* population, allowing the use of flow
285 cytometry to follow cell viability during pure and sequential fermentations.

286 Cell viability was determined by PI staining (Delobel et al., 2012) and PI-negative cells were
287 considered to be viable. *L. thermotolerans* cells in LT and both SFs (fig. 3A) showed a
288 maximum viable population of approximately 1.50 x 10⁷ cells.mL⁻¹ after 24 h of alcoholic
289 fermentation, which remained stable until day 5, with no significant difference between the
290 three conditions (t-test, $p < 0.05$). However, there was a rapid decrease of the viable
291 population in both SFs when the percentage of ethanol reached approximately 10% (v/v),
292 whereas no decrease occurred in LT. This result shows that *S. cerevisiae* had no impact on the
293 growth of *L. thermotolerans* but suggests that *L. thermotolerans* has difficulties in adapting to

294 the faster fermentation kinetics imposed by *S. cerevisiae* in SF, confirming previous results
295 (Nissen et al., 2003; Kapsopoulou et al., 2006; García et al., 2017).

296 We observed the largest viable population (1.42×10^8 cells.mL⁻¹) for *S. cerevisiae* cells in SC
297 (fig. 3B), whereas the lowest was found for the two SFs: 6.57×10^7 cells.mL⁻¹ for SF- and
298 only 9.37×10^6 cells.mL⁻¹ for SF+, representing a decrease of 54.0 and 93.4% of the viable
299 population, respectively. These results confirm the negative impact of *L. thermotolerans* on *S.*
300 *cerevisiae* in both SFs, with a lower population of *S. cerevisiae*. This decrease was greater in
301 SF+ than SF-, reflecting a cell-cell contact-dependent mechanism, confirming previous
302 reports (Nissen et al., 2003; Renault et al., 2013; Lopez et al., 2014; Rossouw et al., 2018).
303 However, despite the difference in viable populations between the two SFs, the fermentation
304 kinetics were exactly the same (fig. 2). The lower fermentation activity in SF- could be
305 explained by higher competition for nutrients, since a higher biomass was present than in
306 SF+. Nutrient depletion could explain the reduced fermentation activity, as previously
307 described (Bely et al., 1990; Carrau et al., 2008; Barrajon et al., 2011).

308

309 2. Competition for nutrients

310 Yeast under fermentation conditions are subjected to very low concentration of dissolved
311 oxygen, which could affect their growth rate because they require it for unsaturated fatty-acid
312 (UFA) and sterol synthesis, in particular ergosterol, both involved in yeast membrane
313 formation (Salmon et al., 1998; Deytieux et al., 2005). Indeed, under conditions of aerobiosis,
314 yeast are able to synthesize UFA and sterols with a succession of reactions involving oxygen-
315 dependent enzymes (Tehlivets et al., 2007). However, these enzymes are not active in the
316 absence of oxygen. Under such conditions, yeast use fatty acids and sterols present in the
317 medium, *i.e.* fatty acids and phytosterols present in the must in our case. The impact of
318 dissolved oxygen on non-*Saccharomyces/S. cerevisiae* interactions is only poorly
319 documented (Hansen et al., 2001; Englezos et al., 2018). These authors showed that the
320 addition of oxygen to co-fermentations of *S. bacillaris* and *S. cerevisiae* promoted the
321 persistence of *S. bacillaris*. Based on these results, the competition for oxygen could, in part,
322 explain the observed interaction. Thus, we monitored the consumption of dissolved oxygen
323 during both SFs and quantified phytosterols in the must before inoculation with *S. cerevisiae*
324 (24 h of fermentation by *L. thermotolerans* alone) to determine whether *L. thermotolerans*
325 consumes these nutrients before *S. cerevisiae* inoculation. We measured dissolved-oxygen
326 levels 3 h (time 0.125 days) after *L. thermotolerans* inoculation and every day thereafter,

327 before the other analyses, to determine the kinetics of dissolved-oxygen consumption (figure
328 4). The consumption of dissolved oxygen by *L. thermotolerans* was very rapid, with a drop
329 from 4.1 mg.L⁻¹ at T0 to 0.21 mg.L⁻¹ after 3 h and down to 0 mg.L⁻¹ for all fermentations until
330 day 18, with an increase for SF- by day 21. These results highlight the absence of dissolved
331 oxygen in both SFs. The very low concentration of oxygen present when the fermentation was
332 inoculated with *S. cerevisiae* could explain the lower biomass observed under SF conditions.
333 Indeed, oxygen availability has been shown to affect *S. cerevisiae* biomass production and
334 viability during alcoholic fermentation (Blateyron and Sablayrolles, 2001; Fornairon-
335 Bonnefond and Salmon, 2003). Moreover, it has been recently reported that oxygen
336 availability strongly influences the viability of non-*Saccharomyces* species (Varela et al.,
337 2012; Shekhawat et al., 2017; Englezos et al., 2018). Thus, early consumption of oxygen by
338 *L. thermotolerans* could partially explain the decreased biomass and viability of the yeast
339 during both SFs.

340 We also monitored phytosterol uptake by *L. thermotolerans* during the first 24 h, before
341 inoculation with *S. cerevisiae*, in parallel to oxygen consumption. The major phytosterol
342 present in the must was β -sitosterol (Table 1), as reported in the literature (Luparia et al.,
343 2004; Rollero et al., 2016), at a concentration of approximately 29 mg.L⁻¹, with the two other
344 phytosterols present at lower concentrations, approximately 1.9 mg.L⁻¹ for campesterol and
345 1.6 mg.L⁻¹ for stigmasterol. *L. thermotolerans* consumed approximately 68% of the β -
346 sitosterol, 14% of the stigmasterol, and all the campesterol in only 24 h of fermentation. Thus,
347 only a low concentration of the remaining phytosterols were available for *S. cerevisiae* growth
348 under anaerobiosis. Both oxygen and phytosterol uptake by *L. thermotolerans* may explain, in
349 part, the negative observed interaction, which led to a decrease in biomass, viability, and
350 consequently fermentative capacity. Indeed, the stress encountered by *S. cerevisiae* may be
351 explained, in part, by the direct anaerobiosis at the time of inoculation, blocking ergosterol
352 and UFA synthesis, as well as the absence of phytosterols available to replace ergosterol in
353 the membrane, which can affect growth and fermentative activity, as shown by Luparia *et al.*
354 (2004), Deytieux *et al.* (2005) and Salmon, 2006. This is the first time that competition for
355 oxygen and phytosterol has been reported in a yeast-yeast interaction study. Hansen *et al.*
356 (2001) and Englezos *et al.* (2018) previously showed that low oxygen levels affect non-
357 *Saccharomyces* species and phytosterol uptake has only been studied in *S. cerevisiae* strains
358 (Luparia et al., 2004; Rollero et al., 2016).

359

360 3. Quantification of YAN and volatile compounds during alcoholic fermentations

361 3.1. YAN quantification

362 The nitrogen content of grape must is a key factor for yeast growth and a sufficient quantity is
363 required to avoid stuck/sluggish fermentatio (Wang et al., 2003; Bell and Henschke, 2005;
364 Gobert et al., 2017). An important part of YAN comes from ammonium, which must be in
365 sufficient amounts for the growth of non-*Saccharomyces* and *S. cerevisiae*. Certain amino
366 acids can be used as YAN sources and aromatic amino acids are precursors for volatile
367 compound production, increasing wine complexity (Kemsawasd et al., 2015; González et al.,
368 2018). Thus, we analyzed the consumption of YAN sources during the first day of
369 fermentation by *L. thermotolerans* to assess the remaining YAN sources at the time of *S.*
370 *cerevisiae* inoculation (Table 2). There was considerable uptake of the various nitrogen
371 sources by *L. thermotolerans*. Among them, Arg, Asn, Gln, Ile, and Ser have been shown to
372 be preferred nitrogen sources for *S. cerevisiae* (Godard et al., 2007; Kemsawasd et al., 2015),
373 meaning that under our conditions, only non-preferential or intermediate sources were
374 available at the time of *S. cerevisiae* inoculation. Thus, consumption of the preferential
375 nitrogen sources by *L. thermotolerans* (> 79%) could explain, in part, the lower biomass and
376 viability of *S. cerevisiae* under both SF conditions than that of pure fermentation, for which
377 all nitrogen sources are available.

378 Indeed, there must be a synergistic effect between oxygen, phytosterol, and YAN uptake by *L.*
379 *thermotolerans* that negatively affects the growth of *S. cerevisiae*. It is highly likely that the
380 lower biomass of *S. cerevisiae* under SF conditions is triggered by limited nutrient availability
381 (oxygen, phytosterols, and nitrogen sources).

382 Aside from the effect on yeast biomass and yeast viability, oxygen, phytosterol, and YAN
383 influence the volatile composition of wine (Hirst and Richter, 2016). Consumption of these
384 nutrients by *L. thermotolerans* could thus affect the volatile composition of wine.

385

386 3.2. Volatile-compound quantification

387 We quantified volatile compounds by HS-SPME-GC/MS for all fermentations to assess the
388 impact of *L. thermotolerans* on SF. This method identified 40 volatile compounds in our
389 fermentations (Table 3). We thus performed PCA analysis based on these 40 volatile
390 compounds. PCA analysis clearly distinguished the three different modalities (fig. 5). Wine
391 produced by SC could be separated from that produced by LT on the basis of higher alcohol
392 content. Wines produced by both yeast species form a distinct group, but there were

393 observable differences between the SF+ and SF- modalities, which reflect the impact of cell-
394 cell contact on volatile compound production. This is the first time that volatile compounds
395 have been analyzed under SF conditions, with or without cell-cell contact, although several
396 other studies of co-fermentation with *L. thermotolerans* and *S. cerevisiae* have been
397 performed, but not with physical separation, as discussed above.

398 A detailed examination of volatile compound composition (Table 3) shows that LT resulted in
399 the highest concentration of total alcohols, with approximately 622.7 mg.L⁻¹ versus 475.7,
400 455.2, and 494.3 mg.L⁻¹ for SC, SF+, and SF-, respectively. Thus, the presence of *S.*
401 *cerevisiae* limits the production of higher alcohols by *L. thermotolerans*. These results could
402 reflect a decrease in the viability *L. thermotolerans* after eight days of SF, leading to lower
403 concentrations of higher alcohols than with LT. In contrast, the total concentration of
404 medium-chain fatty acids for LT was approximately 3.6 mg.L⁻¹, lower than for SC (12.2
405 mg.L⁻¹). Each SF condition resulted in a specific concentration of these compounds, 7.03
406 mg.L⁻¹ for SF+, representing an intermediate concentration between that of LT and SC, and
407 14.3 mg.L⁻¹ for SF-. Our results show that the cell-cell contact modulates the production of
408 medium-chain fatty acids, with an almost two-fold lower concentration for SF+ than SC. Our
409 results contradict those of previous studies, which did not find any differences in medium-
410 chain fatty acid production between co-fermentation of *S. cerevisiae* and *L. thermotolerans* and
411 *S. cerevisiae* pure fermentation (Benito et al., 2015, 2016; Balikci et al., 2016). There were no
412 differences in aldehyde, ketone, or lactone levels between LT, SC, and SF+ but they were
413 slightly lower for SF- than SC and SF+. There were also no significant differences for terpene
414 compounds between conditions. The last compound family that we analyzed was esters. The
415 total concentration of these compounds in LT was 559 µg.L⁻¹, the lowest for all conditions.
416 Indeed, the total concentration was similar for SC and SF-, 1,725.0 and 1,873.1 µg.L⁻¹,
417 respectively, whereas SF+ showed an intermediate concentration of 1401.8 µg.L⁻¹. Globally,
418 the presence of *L. thermotolerans* appears to decrease the concentration of some esters more
419 in SF+ than SF-, which was also observed in the study conducted by Balikci *et al.* (2016) in a
420 24-h sequential fermentation. These results demonstrate a negative impact of *L.*
421 *thermotolerans* on *S. cerevisiae* for the production of most esters, showing an effect of cell-
422 cell contact on ester production, whereas these compounds are desirable in wine because of
423 their sweet, floral, or fruity aromas (Beckner Whitener et al., 2015). Studies conducted by
424 Gobbi *et al.* (2013) and Benito *et al.* (2015) on volatile compounds in SF with *L.*
425 *thermotolerans* and *S. cerevisiae* have shown an increase of ethyl lactate, ethyl hexanoate, and

426 isoamyl acetate concentrations, with a decrease of those of ethyl octanoate and phenylethyl
427 acetate, as found in our study.

428 Several studies on nitrogen sources and volatile compounds have attempted to elucidate the
429 relation between amino acids and volatile compounds, two important families for yeast in
430 enological conditions. Indeed, amino-acid precursor and volatile-compound synthesis are
431 linked by regulation of the Ehrlich pathway, which explains the conversion of some amino
432 acids to aromatic volatile compounds (Hazelwood et al., 2008). Thus, the decrease in
433 phenylethyl acetate concentrations, which gives wine a floral aroma, under both conditions of
434 SF can be easily explained by the depletion of phenylalanine by *L. thermotolerans* before
435 inoculation with *S. cerevisiae*. However, recent studies conducted by Crépin *et al.* (2017) and
436 Rollero *et al.* (2017) show that the link between amino acids and volatile compounds is not so
437 simple, even if the influence of YAN on volatile compound production has been confirmed,
438 although it is not fully understood. Their results show that even if phenylalanine is absent for
439 phenylethyl acetate production, the intermediate phenylethyl alcohol is present at the same
440 concentration in all fermentations, meaning that other metabolic pathways may be involved in
441 phenylethyl acetate production. We made a similar observation for isoamyl acetate (banana
442 aroma), which increased under both SF conditions, whereas its precursor, leucine, was also
443 depleted by *L. thermotolerans*.

444

445 4. Metabolomic analysis by FT-ICR-MS

446 We analysed the exo-metabolome at the end of alcoholic fermentation for each fermentation
447 to better understand the nature of the interaction between yeast species, since it has been
448 shown previously that this approach can successfully unravel interaction mechanisms (Liu et
449 al., 2016).

450 FT-ICR-MS analyses of SC, LT, SF+, and SF- were performed at the end of alcoholic
451 fermentation. We performed a PCA that included all fermentation conditions (fig. 6A). PCA
452 showed that SC, LT, and SF- present different exo-metabolomes, with good separation
453 between SF- *versus* SC, LT, and SF+, according to axis 1 (36.8% of the variability) and a
454 separation between LT *versus* SF-, SC, and SF+, according to axis 2 (20.8% of the variability)
455 (fig. 6A). Based on PCA, SC and SF+ appear to have a more similar exo-metabolome than
456 that of SC *versus* LT or SF-. These results show that a must fermented by a non-
457 *Saccharomyces* yeast, here *L. thermotolerans*, has an exo-metabolome distinct from that of
458 the same must fermented by *S. cerevisiae*. Nevertheless, the association of the two yeast

459 species by physical contact (SF+) shows the dominance of *S. cerevisiae* over *L.*
460 *thermotolerans*, whereas physical separation (SF-) led to the modification of both exo-
461 metabolomes, resulting in a new exo-metabolome different from that of SC or LT. We then
462 analyzed the metabolite composition of each fermentation. A Venn diagram (fig. 6B)
463 highlights the difference in composition between SC, LT, SF+ and SF-. First, there were
464 qualitative differences. For example, only two masses were unique to SC, four to SF+, 28 to
465 SF-, and 24 to LT, whereas 15 masses were unique to SF+ and SC, as well as 91 to SC, SF+
466 and SF-. Each yeast species clearly produced unique metabolites in pure fermentation not
467 found in the others. Moreover, the presence of 28 unique metabolites in SF- show that cell-
468 cell contact modifies the metabolism of *S. cerevisiae* or *L. thermotolerans* as 28 metabolites
469 were unique to SF- and only four to SF+. This diagram also shows that 1,247 masses (66.8%
470 of the total composition) were common to all fermentations, but the concentrations varied,
471 depending on the conditions of fermentation (SC, LT, SF+ and SF-). We performed an
472 ANOVA (threshold *p-value* < 0.05) to find markers that can discriminate between
473 fermentations. Specific markers are represented in a hierarchical cluster analysis (HCA) and
474 in Van Krevelen diagrams, highlighting compound families, coupled to histograms of
475 elemental formula composition (fig 7). HCA (fig. 7A) confirmed the four groups found above
476 by PCA, with a greater distance between LT and the other conditions, as well as a very high
477 similarity between SC and SF+ conditions. Thus, for example, SC markers (fig. 7B, SC) are
478 composed of CHO (blue), CHOS (green), and CHON (orange) in similar quantities (between
479 10 and 15), but CHNOS (red) compounds are present in only low quantities (approximately
480 5). These compounds can be associated mostly with carbohydrate, anthocyanin, and amino-
481 acid families. Each fermentation gave a specific profile in which carbohydrate, amino-acid,
482 nucleic-acid, and anthocyanin families were found in all (fig. 7B). However, the intensity of
483 the compounds in each family differed greatly, depending on the fermentation condition,
484 showing that each yeast species has a different metabolism. This metabolism is modified
485 when both yeast species are put together, with a different response, depending on whether the
486 cells are physically separated or not. Moreover, the quantity of compounds was greater for LT
487 and SF-, with approximately 170 for LT (mostly CHO and CHON compounds) and 320 for
488 SF- (mostly CHO and CHON compounds), than for SC (approximately 50) and SF+
489 (approximately 105), meaning that a higher diversity of compounds was found in LT and SF-.
490 Each yeast clearly has its own impact on SF+ and SF- in terms of chemical composition and
491 these results show that interactions between a non-*Saccharomyces* yeast and *S. cerevisiae*

492 affect not only the volatile compound profile but also the metabolite profile, with specific
493 exo-metabolomes for SC, LT, SF+, and SF- fermentations.

494 We carried the analysis further by making pairwise comparisons for all conditions, *i.e.* LT/SC,
495 LT/SF+, SC/SF+, LT/SF-, and SC/SF-, to highlight markers for each pair (fig. 8). First, PCAs
496 were performed for each pair to assess the separation of each fermentation according to axis
497 1, representing 86.2% of the variability for LT/SC, 89.7% for LT/SF-, 61.6% for SF+/SF-,
498 46.9% for LT/SF+, and 38.1% for SC/SF+, again highlighting the difference between LT and
499 both SF conditions, as well as the similarity between the SC and SF+ conditions (fig. 8, left).
500 Second, we used ANOVA (threshold *p-value* < 0.05), to find markers for each pair of
501 conditions, represented in H/C vs. O/C van Krevelen diagrams coupled to histograms of
502 elemental formula composition (fig. 8, middle and right). Carbohydrate, amino-acid, nucleic-
503 acid, and anthocyanin families were mostly represented in all comparisons. The next step
504 consisted of annotating these biomarkers and correlating them with the metabolic pathways
505 involved. We sought biomarkers in several databases (KEGG, Lipidmap, YMDB, Metlin and
506 an in-house plant and wine database) and generated a Search and color KEGG visualization
507 (supp. fig. 2) (Kanehisa and Goto, 2000; Kanehisa et al., 2012). Based on the 76 identified
508 metabolic pathways, most LT and SF- biomarkers are involved in carbohydrate, carbon
509 fixation, and amino-acid metabolism. We were unable to identify any biomarkers for SC and
510 only two for SF+, which are involved in carbohydrate and nucleotide metabolism (supp. fig.
511 2). This highlights the complex metabolite composition of wine, which is still poorly
512 understood, as reported previously by studies using FT-ICR-MS on wine (Roullier-Gall et al.,
513 2014a, 2014b; Roullier-Gall et al., 2015).

514 These results show that metabolites produced by yeast under our different conditions result
515 mainly from sugar and nitrogen source metabolism (also shown in supp. fig. 3), which
516 represents 20 of the 76 identified metabolic pathways. This is not surprising because of the
517 lack of these nutrients at the end of alcoholic fermentation.

518 These results provide new insights for the further study of interactions between non-
519 *Saccharomyces* and *S. cerevisiae* by comparing specific exo-metabolomes (composed of
520 specific markers) of each fermentation, which may reflect the impact of interactions on
521 metabolite production.

522 Thus, the biomarkers found in our fermentations may be useful given the high intensity of
523 metabolites specific for fermentations performed with *L. thermotolerans*, *S. cerevisiae*, and
524 SF of both species, with or without cell-cell contact.

525

526 5. Conclusion

527 Here, we aimed to investigate the interactions that occur between *S. cerevisiae* and *L.*
528 *thermotolerans* during alcoholic fermentation of grape must. The results obtained from all
529 analyses performed in this study highlight a negative interaction between the two species to
530 the detriment of *S. cerevisiae*, due to a cell-cell contact mechanism (SF+ fermentation) and
531 the consumption of essential nutrients by *L. thermotolerans* during both SF conditions.
532 However, *L. thermotolerans* was also negatively affected by the presence of *S. cerevisiae*
533 under both SF conditions, even if the quantification of volatile compounds showed that *L.*
534 *thermotolerans* is able to modulate aroma complexity without differences between the two SF
535 conditions. Moreover, this study provides, a comparison of the exo-metabolomes of *L.*
536 *thermotolerans* and *S. cerevisiae* pure fermentations, as well as SF with both species, with or
537 without cell-cell contact. This comparison showed that interactions also affect metabolite
538 production by *L. thermotolerans* and *S. cerevisiae* during alcoholic fermentation in a different
539 manner as a function of the condition.

540 In conclusion, this study provides new insights concerning the interaction of *L.*
541 *thermotolerans* and *S. cerevisiae* during the alcoholic fermentation of grape must. However,
542 further study of the cell-cell contact mechanism and further identification of the metabolites
543 needs to be carried out to better understand the interactions between these two yeast species
544 and to investigate if these interactions between the two species are strain dependent.

545

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550

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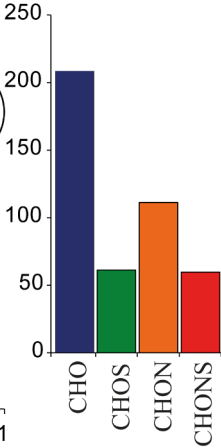
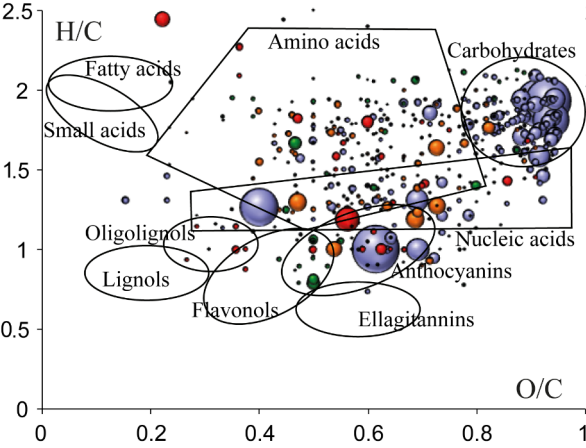
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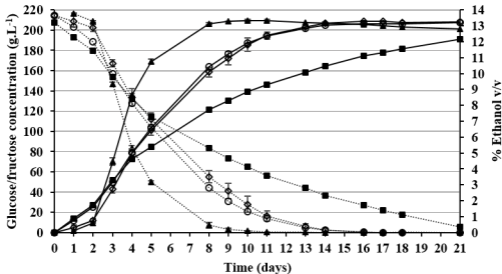
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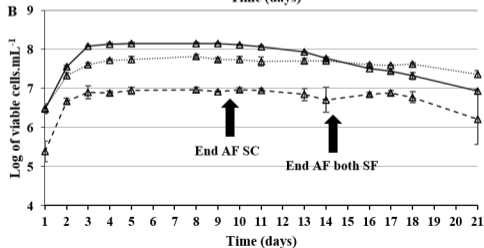
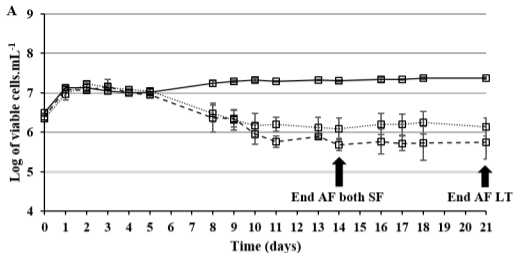
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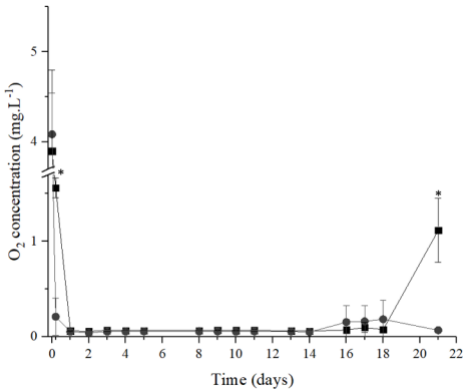
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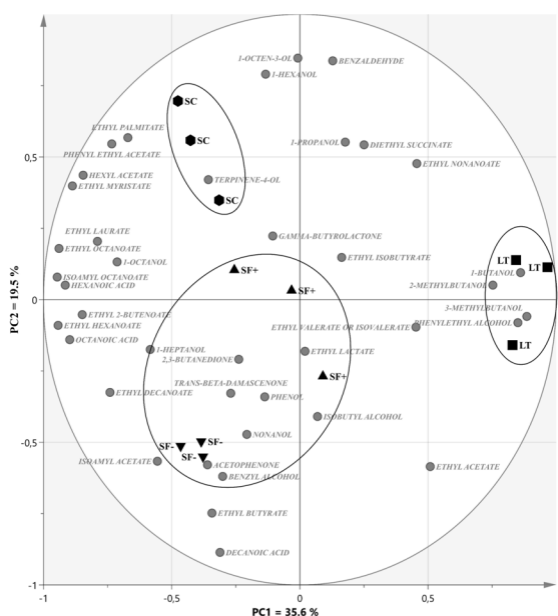
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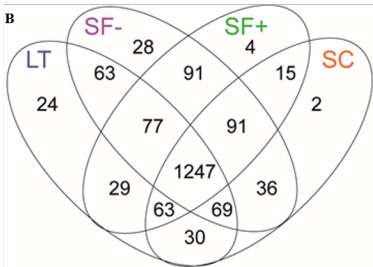
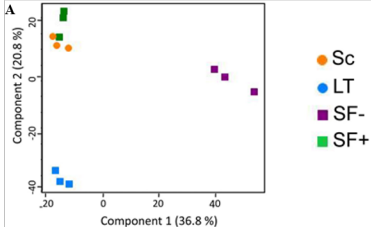


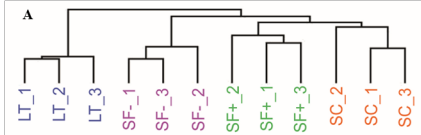




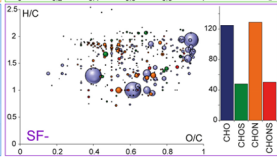
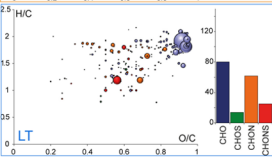
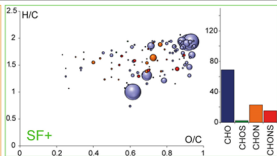
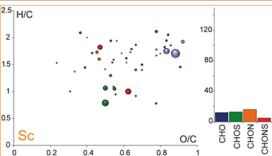








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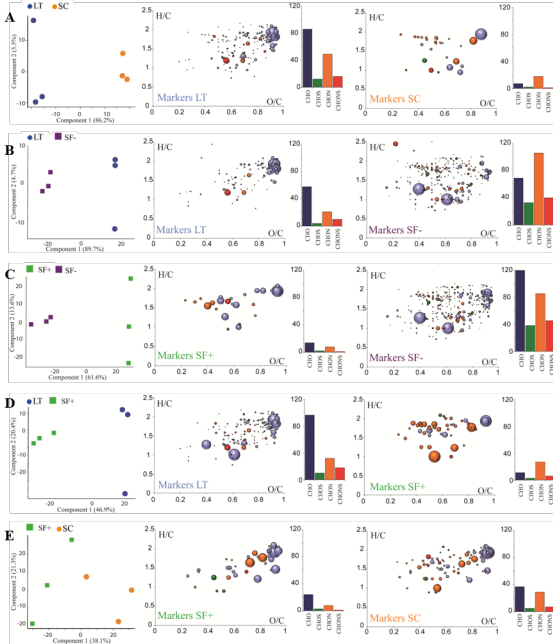


Figure 1: (Left) H/C vs. O/C van Krevelen diagram of common masses found in all fermentations (LT, SC, SF+ and SF-) with the region where metabolite families are represented and (right) histograms representing the abundance of metabolites composed with CHO, CHOS, CHON or CHONS atoms.

Figure 2: Fermentation kinetics with sugar consumptions (glucose and fructose) and ethanol production by yeasts in pure and sequential fermentations with and without dialysis membrane in white must at 20°C: ••▲•• Glucose/fructose and -▲- % Ethanol v/v *S. cerevisiae* pure fermentation (SC); ••■•• Glucose/fructose and -■- % Ethanol v/v *L. thermotolerans* pure fermentation (LT); ••◆•• Glucose/Fructose and -◆- % Ethanol v/v sequential fermentation without contact (SF-); ••○•• Glucose/fructose and -○- % Ethanol v/v sequential fermentation with contact (SF+). Error bars represent the standard deviation of the results.

Figure 3: Curves representing the concentration of PI negative cells (viable cells, log representation) in pure and sequential fermentations in white must at 20°C:

(A) □ *L. thermotolerans* pure fermentation (LT); - - □ - - *L. thermotolerans* SF+; ••□•• *L. thermotolerans* SF-.

(B) ▲ *S. cerevisiae* pure fermentation (SC); - - ▲ - - *S. cerevisiae* SF+; ••▲•• *S. cerevisiae* SF-. Error bars represent the standard deviation of the results.

Figure 4: Consumption of dissolve oxygen during alcoholic fermentation of both sequential fermentations with (SF+) or without (SF-) contact: -●- SF+; -■- SF-. * significant difference (T-test. *p-value* < 0.05) between both fermentations and error bars represent the standard deviation of the results.

Figure 5: Biplot of the principal component analysis (PC1 vs. PC2) for volatile compounds found in each fermentations. Ellipses represent clusters obtained from HCA.

SF+: sequential fermentation with contact

SF-: sequential fermentation without contact (both compartments)

SC: *S. cerevisiae* pure fermentation

LT: *L. thermotolerans* pure fermentation

Figure 6: (A) Principal component analysis (PC1 vs. PC2) of metabolite profiles for each fermentation condition. (B) Venn diagram representing metabolites found exclusively in each fermentation as well as those found in two or more fermentations, with 1247 metabolites that are common to all fermentations.

Figure 7: (A) Hierarchical cluster analysis (HCA) obtained after ANOVA (threshold *p-value* < 0.05).

(B) H/C vs. O/C Van Krevelen diagrams representing masses from the 1247 common masses with a higher intensity in SC, LT, SF+ or SF- after ANOVA (*p-value* < 0.05) with histograms representing their composition and number of elemental formula (CHO, CHOS, CHON or CHONS compounds). Bubble sizes indicate relative intensities of corresponding peaks in the spectra.

Figure 8: (A, B, C, D and E, left) Principal component analysis (PC1 vs. PC2) of both fermentations for each couple of conditions and (A, B, C, D and E, middle and right) H/C vs. O/C Van Krevelen diagrams representing specific metabolites found in both fermentations of each couple described as

markers coupled to histograms of elemental formula composition. Bubble sizes indicate relative intensities of corresponding peaks in the spectra.

Table 1: Concentration of phytosterols (mg.L^{-1}) in the must and after 24 h of alcoholic fermentation by *L. thermotolerans* before *S. cerevisiae* inoculation.

nd, non determined.

	Sterol concentration (mg.L^{-1})		
	Must (T0)	T24 h before <i>S. cerevisiae</i> inoculation	% of decrease
ergosterol	nd	nd	nd
campesterol	1.9 ± 0.1	nd	nd
stigmasterol	1.6 ± 0.3	1.4 ± 0.04	14.0 ± 12.0
β-sitosterol	28.8 ± 1.2	9.2 ± 1.4	68.0 ± 3.8

Table 2: Concentration of amino acids and ammonium (mg of N.L⁻¹) in the must and after the first 24 hours of fermentation with *L. thermotolerans* in LT, as well as the percentage of decrease representing the consumption of each compound.

* Indicates aromatic amino acids.

YAN	Concentration (mg N.L ⁻¹)		% of decrease
	Must (T0)	T24h	
Alanine	22.78 ± 0.44	11.36 ± 3.18	50.2 ± 5.0
Arginine	315.03 ± 2.38	39.08 ± 34.28	87.6 ± 1.5
Asparagine	2.70 ± 0.12	0.05 ± 0.40	98.2 ± 3.2
Aspartic acid	3.99 ± 0.12	0.54 ± 0.52	86.4 ± 0.6
Cysteine	0.47 ± 0.41	0.02 ± 0.19	87.3 ± 18.3
GABA	4.53 ± 0.09	4.41 ± 0.18	2.6 ± 6.8
Glutamic acid	5.71 ± 0.12	1.93 ± 0.94	66.2 ± 3.4
Glutamine	41.35 ± 0.89	3.44 ± 4.01	91.7 ± 1.1
Glycine	1.96 ± 0.11	0.90 ± 0.13	54.0 ± 8.4
Histidine*	13.52 ± 0.37	0.01 ± 1.75	99.9 ± 0.2
Isoleucine	1.62 ± 0.14	0.11 ± 0.25	93.0 ± 3.4
Leucine	2.33 ± 0.03	0.16 ± 0.17	92.9 ± 7.0
Lysine	0.04 ± 0.07	0.04 ± 0.22	/
Methionine	0.96 ± 0.24	0.21 ± 0.12	75.4 ± 20.6
NH4+	242 ± 2	163 ± 18	32.6 ± 9.1
Phenylalanine*	10.15 ± 0.06	0.03 ± 0.92	99.7 ± 0.2
Proline	83.86 ± 1.33	83.66 ± 7.13	0.11 ± 13.0
Serine	19.23 ± 0.24	2.76 ± 2.51	85.7 ± 2.7
Threonine	15.81 ± 0.65	0.80 ± 1.55	94.9 ± 1.1
Tyrosine*	3.87 ± 0.01	0.57 ± 0.43	85.2 ± 3.3
Valine	3.83 ± 0.08	0.80 ± 0.44	79.2 ± 3.1

Table 3: Concentration of volatile compounds at the end of AF for each fermentation (SC, LT, SF+ and SF-). Values with the same letters a, b, c or d were not significantly different in Tukey's test (95%); nd stands for non determined. Aroma descriptors inspired by Beckner Whitener et al. (2015).

Volatile compounds ($\mu\text{g.L}^{-1}$)	Aroma descriptors	LT	SC	SF+	SF-
<i>Alcohols</i>					
1-PROPANOL	weak fusel	2636.9 \pm 609.1 ^a	2619.5 \pm 552.5 ^a	2938.3 \pm 680.9 ^a	1989.4 \pm 600.6 ^a
ISOBUTYL ALCOHOL		14957.5 \pm 1546.9 ^a	13593.4 \pm 3444.2 ^a	16923.5 \pm 2000.3 ^a	15962.0 \pm 2956.7 ^a
1-BUTANOL		1595.1 \pm 588.0 ^a	407.4 \pm 357.1 ^b	287.4 \pm 173.0 ^b	422.8 \pm 216.6 ^b
3-METHYLBUTANOL		64147.9 \pm 10420.4 ^a	36481.7 \pm 8337.5 ^b	37475.9 \pm 6178.1 ^b	41503.1 \pm 5689.8 ^b
2-METHYLBUTANOL		351686.0 \pm 27545.4 ^a	289206.6 \pm 41799.3 ^b	272122.5 \pm 27895.6 ^b	295422.7 \pm 30539.1 ^b
1-HEXANOL	green	3555.6 \pm 633.8 ^{ab}	4412.8 \pm 499.4 ^a	4119.5 \pm 1006.1 ^a	2932.8 \pm 461.5 ^b
1-OCTEN-3-OL	mushroom	46.0 \pm 10.4 ^{ab}	56.6 \pm 12.7 ^a	41.7 \pm 11.6 ^{ab}	35.0 \pm 10.7 ^b
1-HEPTANOL	leafy	146.6 \pm 35.3 ^b	493.5 \pm 58.6 ^a	555.9 \pm 284.4 ^a	592.9 \pm 278.2 ^a
1-OCTANOL	waxy	109.0 \pm 26.8 ^b	244.4 \pm 15.4 ^a	99.3 \pm 20.4 ^b	228.9 \pm 43.5 ^a
NONANOL	fruity	227.2 \pm 43.6 ^a	217.5 \pm 46.4 ^a	304.1 \pm 61.7 ^a	295.7 \pm 88.0 ^a
BENZYL ALCOHOL	fruity	1.0 \pm 1.0 ^b	0.9 \pm 0.6 ^b	1.0 \pm 1.1 ^b	7.2 \pm 5.9 ^a
PHENYLETHYL ALCOHOL	rose	183182.9 \pm 18256.1 ^a	127596.2 \pm 9981.1 ^b	119724.4 \pm 17352.4 ^b	134448.6 \pm 8110.8 ^b
PHENOL		395.9 \pm 134.2 ^a	384.8 \pm 220.5 ^a	589.1 \pm 257.1 ^a	486.4 \pm 97.6 ^a
Total		622687.6 \pm 41831.0 ^a	475715.5 \pm 54330.3 ^b	455182.5 \pm 48966.9 ^b	494327.2 \pm 24460.0 ^b
<i>Medium chain fatty acids</i>					
HEXANOIC ACID	sour	1050.2 \pm 93.1 ^c	4051.7 \pm 217.7 ^a	2109.2 \pm 300.2 ^b	3938.8 \pm 406.4 ^a
OCTANOIC ACID	rancid	1739.9 \pm 188.8 ^d	7605.2 \pm 824.2 ^b	3984.0 \pm 335.5 ^c	8679.0 \pm 573.7 ^a
DECANOIC ACID	unpleasant	785.1 \pm 116.9 ^d	531.2 \pm 73.4 ^c	941.0 \pm 142.3 ^c	1651.6 \pm 207.4 ^a
Total		3575.1 \pm 367.3 ^d	12188.2 \pm 965.3 ^b	7034.2 \pm 719.8 ^c	14269.4 \pm 896.3 ^a
<i>Aldehydes, ketones and lactones</i>					
BENZALDEHYDE	almond	4012.7 \pm 376.1 ^a	4469.8 \pm 362.2 ^a	4396.9 \pm 635.7 ^a	2850.2 \pm 889.8 ^b
ACETOPHENONE	almond, sweet, floral	103.6 \pm 12.5 ^b	89.0 \pm 13.2 ^b	249.1 \pm 67.6 ^{ab}	332.1 \pm 188.7 ^a
2,3-BUTANEDIONE		529.8 \pm 326.4 ^a	608.1 \pm 232.7 ^a	568.9 \pm 296.6 ^a	723.1 \pm 247.9 ^a
GAMMA-BUTYROLACTONE		0.3 \pm 0.05 ^a	0.3 \pm 0.09 ^a	0.3 \pm 0.05 ^a	0.3 \pm 0.07 ^a
Total		4646.4 \pm 415.5 ^{ab}	5167.3 \pm 529.1 ^a	5215.3 \pm 639.4 ^a	3905.6 \pm 456.4 ^b
<i>Terpenes</i>					
TERPINENE-4-OL	spicy, mentol	0.7 \pm 0.5 ^a	5.3 \pm 6.5 ^a	0.5 \pm 0.2 ^a	2.3 \pm 2.6 ^a
TRANS-BETA-DAMASCENONE	rose	0.5 \pm 0.3 ^a	0.5 \pm 0.1 ^a	0.6 \pm 0.2 ^a	0.6 \pm 0.2 ^a
Total		1.2 \pm 0.6 ^a	5.8 \pm 6.5 ^a	1.0 \pm 0.3 ^a	2.8 \pm 2.2 ^a
<i>Esters</i>					
ETHYL ACETATE	fruity or ascendent	102.5 \pm 14.3 ^a	35.9 \pm 8.3 ^b	102.0 \pm 10.3 ^a	91.1 \pm 18.1 ^a
ETHYL ISOBUTYRATE		2.0 \pm 0.5 ^a	1.7 \pm 1.0 ^a	2.3 \pm 1.0 ^a	1.7 \pm 0.7 ^a
ETHYL BUTYRATE	fruity	1.5 \pm 1.0 ^b	1.3 \pm 0.2 ^b	1.6 \pm 0.5 ^{ab}	2.5 \pm 0.7 ^a
ETHYL VALERATE OR ISOVALERATE	fruity	0.2 \pm 0.03 ^a	0.2 \pm 0.04 ^{ab}	0.2 \pm 0.04 ^b	0.20 \pm 0.04 ^{ab}
ISOAMYL ACETATE	banana	19.0 \pm 3.0 ^b	23.7 \pm 2.7 ^b	52.5 \pm 21.8 ^a	65.2 \pm 8.8a
ETHYL 2-BUTENOATE	nd	nd	0.2 \pm 0.1 ^a	0.1 \pm 0.08 ^a	0.2 \pm 0.1 ^a
ETHYL HEXANOATE	fruity	324.6 \pm 50.3 ^c	996.4 \pm 78.0 ^a	719.6 \pm 84.1 ^b	1126.0 \pm 134.4 ^a
HEXYL ACETATE	banana	1.7 \pm 0.2 ^c	26.9 \pm 3.1 ^a	13.8 \pm 10.5 ^b	16.2 \pm 2.3 ^b
ETHYL LACTATE		46.6 \pm 7.2 ^b	nd	194.9 \pm 57.1 ^a	46.4 \pm 15.1 ^b
ETHYL OCTANOATE	apricot	45.5 \pm 8.4 ^d	576.8 \pm 58.2 ^a	272.6 \pm 40.4 ^c	479.5 \pm 89.3 ^a
ETHYL NONANOATE		4.0 \pm 0.5 ^a	3.5 \pm 0.4 ^{ab}	3.6 \pm 1.0 ^{ab}	2.8 \pm 0.6 ^b
ETHYL DECANOATE	sweet	5.2 \pm 0.4 ^c	12.5 \pm 1.2 ^b	20.9 \pm 3.5 ^a	18.6 \pm 1.6 ^a
ISOAMYL OCTANOATE	sweet	0.2 \pm 0.05 ^c	0.6 \pm 0.1 ^a	0.3 \pm 0.06 ^b	0.6 \pm 0.05 ^a
DIETHYL SUCCINATE	fruity	0.8 \pm 0.6 ^a	0.8 \pm 0.3 ^a	0.8 \pm 0.2 ^a	0.6 \pm 0.1 ^a
PHENYL ETHYL ACETATE	floral	2.1 \pm 0.2 ^d	34.2 \pm 1.7 ^a	8.0 \pm 3.4 ^c	15.1 \pm 1.5 ^b
ETHYL LAURATE		0.8 \pm 0.3 ^b	1.4 \pm 0.4 ^a	1.4 \pm 0.3 ^a	1.2 \pm 0.3 ^{ab}
ETHYL MYRISTATE		1.3 \pm 0.4 ^c	5.3 \pm 0.7 ^a	4.0 \pm 1.1 ^b	3.6 \pm 0.9 ^b
ETHYL PALMITATE		1.1 \pm 0.2 ^c	3.7 \pm 1.0 ^a	3.3 \pm 1.3 ^{ab}	1.9 \pm 0.6 ^{bc}
Total		559.0 \pm 78.6 ^c	1725.0 \pm 125.0 ^a	1401.8 \pm 218.5 ^b	1873.1 \pm 167.8 ^a