Carbohydrate composition of red wines during early aging and incidence on spoilage by Brettanomyces bruxellensis

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1 # Carbohydrate composition of red wines during early aging
2 # and incidence on spoilage by *Brettanomyces bruxellensis*

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19 **Abstract**

20 Wine is generally considered as hostile medium in which spoilage microbes have to manage with
21 many abiotic factors among which low nutrient content. Wines elaborated in 8 wineries were
22 sampled during the first summer of aging over two consecutive vintages, and analysed for
23 carbohydrate composition. This revealed the systematic presence of many carbohydrates including
24 those useful for the spoilage yeast *Brettanomyces bruxellensis*. However, during the first summer of
25 aging, the changes in wine carbohydrate composition were low and it was difficult to assess how
26 much carbohydrate composition contributed to wine spoilage by *B. bruxellensis*. Subsequent
27 laboratory experiments in inoculated wines showed that the sugars preferentially consumed in wine
28 by the spoilage yeast are D-glucose, D-fructose, and trehalose, whatever the yeast strain considered.
29 The addition of these sugars to red wines accelerates the yeast growth and the volatile phenols
30 formation. Although probably not the only promoting factor, the presence of high amounts of
31 metabolisable sugars thus really increases the risk of "brett" spoilage.

32

33 Keywords: wine, carbohydrates, aging, *Brettanomyces bruxellensis,* spoilage.

34

35

36 ## 1. Introduction

37 Aging and especially the first summer of aging is described as particularly favourable towards
38 *Brettanomyces bruxellensis* development in Bordeaux vineyards (Chatonnet et al., 1992; Cibrario et
39 al. 2019a, b). *B. bruxellensis* can be found on grapes, in musts and wines and very often in wineries.
40 Indeed, wine environment is one of its favourite ecological niche (Oro et al., 2019). In this context, it
41 is considered a spoilage microorganism, because it converts the hydroxycinnamic acids extracted
42 from grapes (mainly p-coumaric and ferulic acids) into volatile phenols (VP): 4-vinylphenol (4-VP), 4-
43 vinylguaiacol (4-VG), 4-ethylphenol (4-EP) and 4-ethylguaiacol (4-EG) (Chatonnet et al., 1992;
44 Rozpedowska et al., 2011). These molecules confer unpleasant aromatic notes to the wine and
45 constitute one of the main defects of red wines nowadays (Romano et al., 2009; Schumaker et al.,
46 2017).

47 To produce significant and detectable concentrations of these undesired molecules, the spoilage
48 yeasts should first grow and become numerous enough (Gerbaux et al, 2002; Barata et al, 2008;
49 Cibrario et al, 2019b). Recently, we showed that the genetic group of the strain(s) present and the
50 cellar temperature were key factors modulating the yeast growth rate and thus the risk of spoilage.
51 Nevertheless, the main factor was the wine itself, some being much more permissive to *B.*
52 *bruxellensis* development than others (Cibrario et al, 2019a,b). Though the species is described to
53 display low nutritional requirements, one of the keys that could promote its rapid development in
54 many wines is its ability to use many carbon sources as growth substrates (Dias et al, 2003; Conterno
55 et al., 2006; Crauwels et al., 2017; Smith and Divol, 2018; Cibrario et al., 2019a; Da Silva et al., 2019).
56 The wine carbohydrate content could thus contribute to increase its “permissiveness”. Indeed,
57 different studies suggest that the wine carbohydrate composition could strongly differ from one
58 domain to the other depending on the grape variety and oenological practices (Triquet-Pissard, 1979;
59 Pellerin and Cabanis, 1998; Del Alamo et al., 2000; Ayestaran et al., 2004; La Torre et al., 2008; Ruiz
60 Matute et al., 2009; Rovio et al., 2011; Conde et al., 2015Gougeon et al., 2019). The wine
61 carbohydrate composition may also differ due to the activity of the active microorganisms during

62 fermentations (Pellerin and Cabanis, 1998, Dols-Lafargue et al., 2007). In addition, during aging in
63 barrels, the composition of the wine may also change due to the diffusion of wood carbohydrates, or
64 due to the metabolism of the microorganisms present (Del Alamo et al., 2000).

65 We hypothesised that wines differ by their small neutral carbohydrate content and this may
66 modify the risk of spoilage by *B. bruxellensis*. We thus measured the low molecular weight
67 carbohydrate concentrations in many red wines, during two consecutive vintages. We then examined
68 whether a link exists between the concentration and the nature of the carbohydrates present and
69 the spoilage yeast growth or the volatile phenols formation in the barrels examined. Then, at
70 laboratory scale, yeast growth and carbohydrate and volatile phenols concentrations were followed
71 in wines, artificially enriched in carbohydrates or not, and inoculated with *B. bruxellensis.* The strains
72 used were chosen to be representative of the recently highlighted genetic diversity of the species in
73 wine (Avramova et al, 2018; Cibrario et al 2019c).

74

75 ## 2. Material and Methods

76 ### 2.1. Yeast strains

77 Eight *B. bruxellensis* strains were used in this study: L0424, L14190, AWRI1499 (all belonging to
78 the AWRI1499 like genetic group, triploid strains), L0422, and AWRI1608 (belonging to the AWRI1608
79 like genetic group, triploid strains) and 11AVB4, L0611, and CBS2499 (in the CBS2499 like genetic
80 group, gathering diploid strains). Their origin and genetic group are indicated in supplemental Table
81 1.

82 ### 2.2. Experiments in wine at laboratory scale

83 Three red wines (2016 vintage, Bordeaux area) were used for experiments at laboratory scale:
84 wine A had a pH of 3.48 and contained 14.30 %vol ethanol, wine D had a pH of 3.63 and contained
85 13.49 %vol ethanol and wine M had a pH of 3.56 and titrated 13.19 %vol ethanol (Table 1). In
86 addition, the wine M was enriched in alcohol to reach 14.19 %vol (to produce wine M14). All were
87 treated with H_2O_2 to eliminate the total SO_2, and then pasteurized for 30 min at 80 °C. For

88 experiments in enriched wines, glucose, fructose or trehalose solutions were prepared in a
89 concentrated form in water (50 g/l), sterilized (at 121°C, 15 min, 1 bar) and aseptically added to the
90 pasteurized wine one by one (to reach a final concentration of 150 mg/l) or as two-by-two mixtures
91 (75 mg/l each) or altogether (50 mg/l each).

92 Two-hundred milliliters of wine (with no added carbohydrates or with added fructose,
93 trehalose or glucose or mixtures of these carbohydrates) were then inoculated with *B. bruxellensis*,
94 to reach an initial population of 5.10^3 CFU/mL (see specification in the text), with various yeast strains
95 previously adapted to the wine (Cibrario et al, 2019b). The inoculated wines were then distributed
96 into 13 mL tubes filled at their maximum to limit headspace. Then, the tubes were incubated without
97 any agitation for non-aerated conditions. For aerated/agitated conditions, a 2-cm high head space
98 was let and tubes were agitated daily. For all tested media and conditions, a tube was removed from
99 the device at each sampling between 0 and 60 days. All the cultures were made at 20 °C, in duplicate.

100 **2.3. Wine sampling in the cellars**

101 Aging wines of the 2014 and 2015 vintages were sampled in barrels during the summers of 2015
102 and 2016 respectively. Shortly, after the end of the MLF, the different wine lots obtained in a domain
103 are generally mixed to form assembled wines, depending on the choice of the winemaker. We thus
104 studied assembled wines in 8 domains (named A to H) around Bordeaux. These were either single
105 varietal or blends of distinct varieties (Merlot and Cabernet Sauvignon mainly). They were made of
106 distinct wines that had all completed MLF separately before being assembled and stored in barrels.
107 Three barrels were selected for each studied wine. Regularly, racking and transfer to a clean barrel is
108 performed in the cellar in the domains studied. We took advantage from the two summer racking
109 operations to withdrawn samples: the first sample (S0) was collected just after the racking of June or
110 early July, when wine was put in the clean barrel and the second one (S1) was just before the
111 subsequent racking at the end of summer in August or September. The sampling was performed at
112 half height in the barrels. The duration between the two samplings varied from 51 to 170 days
113 depending on the batch and the domain considered, but did not vary between the barrels of the

114 same batch. A total of 51 barrels representing 17 wine lots (A4, A5, A6, B4, B5, B6… H4) were
115 selected, and 49 were analyzed and named: A4a, A4b, A4c, A5a, A5b…. H4c).

116 **2.4. Cultivable cells counts**

117 *B. bruxellensis* cultivable populations were measured in the wine samples by serial dilutions and
118 plate counts. YPD solid medium (Yeast extract 10 $g.L^{-1}$ L, peptone 20 $g.L^{-1}$, glucose 20 $g.L^{-1}$, agar 20 $g.L^{-1}$
119 and pH adjusted to 5.0 with orthophosphoric acid before sterilization at 121°C, 15 min , 1 bar) was
120 used for the analyse of *B. bruxellensis* cultivable populations in wines followed in the laboratory
121 (additional experiments). For wines sampled in the cellars, 0.1 $g.L^{-1}$ L chloramphenicol, 0.15 $g.L^{-1}$
122 biphenyl and 0.5 $g.L^{-1}$ cycloheximide were added to inhibit the growth of microbes others than *B.*
123 *bruxellensis*.

124 **2.5. Viable cells counts**

125 *B. bruxellensis* viable populations were measured in the wine samples collected in the châteaux
126 by qPCR. DNA extraction and amplification were carried out by using the kit VINEO Brettanomytest
127 and the iCycler IQ5 system (Bio-Rad). Standard curves, DNA extraction and amplification were
128 performed according to the manufacturers' instructions. Results were analysed using Bio-Rad CFX
129 Manager® software.

130 **2.6. Carbohydrate analysis**

131 The reference method (OIV-MA-AS311-06, 2006 described by Triquet-Pissard, 1979) was
132 optimized, in order to quantify a larger number of carbohydrates with reduced time of analysis. Ten
133 microliters of penta-erythritol (30 $g.L^{-1}$ diluted in water, internal standard) were added to 1 mL of
134 sample in 13 mL Pyrex tubes, sealed with parafilm, let from 12 to 24 hours at -20 °C and then freeze-
135 dried. The tubes were then closed with a cap bearing a PTFE/ethylene propylene membrane.
136 Reagents were then added in the following order: 0.2 mL of pyridine (solvent), 0.7 mL of
137 hexamethyldizilasane HMS (silylating agent), 0.1 mL of trifluoroacetic acid (catalyst). Dissolution was
138 carried out for 5 min in an Ultrasonic cleaner (VWR). The samples were heated for 3 h at 80 °C, and
139 then cooled down to room temperature before injection. The derivation of too many samples at a

140 time led to long delays and sample deterioration before injection. In order to limit this, silylation was
141 performed by batches of up to 8 samples, directly injected after derivatization. GC-FID analysis was
142 carried-out as described by Triquet-Pissard (1979), with a HP 6890 (Agilent Tech) chromatograph,
143 equipped with an automatic 7683B Series Injector auto-sampler (Agilent Tech) and coupled to a
144 flame ionization detector. The column used was CP-Sil 5 CB, 50 m x 0.32 mm, 0.1 µm film thickness
145 (Agilent Tech.) and the carrier gas was hydrogen (30 mL/min). The following parameters were used:
146 injection in splitless mode, volume: 1µL, purge time: 0.50 min. The temperature of the column
147 initially set at 120 °C was increased by 1° $C.min^{-1}$ until 165 °C, by 12° $C.min^{-1}$ until 217°C, by 3°$C.min^{-1}$
148 until 265°C, and eventually by 10° $C.min^{-1}$ until 295°C, temperature at which the column was finally
149 maintained for 5 min.

150 For each carbohydrate analysed, repeatability, linearity and detection limits were controlled in
151 several red wines in order to verify that the concentrations obtained in the samples were not subject
152 to variation according to the wine studied.

153 **2.7. Volatile phenols determination**

154 Volatile phenols (4-VP, 4-EP, 4-VG and 4-EG) were quantified by GC-MS coupled with solid-phase
155 micro-extraction (SPME) on polyacrylate fibers by the method described by Romano et al. (6).
156 Deuterated 4-ethylphenol (100 $µg.L^{-1}$) was added as an internal standard. In the present paper, the
157 term volatile phenols will refer to the sum of the four molecules examined. In all the wines and
158 media examined the vinyl forms represented less than 5% of the total phenols produced.

159 **2.8. Statistical analysis**

160 A non-parametric Kruskal-Wallis test was used at α=5% to identify the means that were
161 significantly different. Principal Component Analysis (PCA) and Spearman correlation tests were
162 performed using the R program (Dray and Dufour, 2007).

163 ## 3. Results and discussion

164 We first worked to improve the method for separating sylillated carbohydrates, in order to
165 quantify a larger number of molecules. The modified method made it possible to separate and

166 quantify L-arabinose, D-ribose, D-xylose, L-rhamnose, D-mannose, D-galactose, trehalose, cellobiose,
167 maltose, lactose, raffinose, D-mannitol and D-sorbitol. It was not possible with this method to
168 distinguish between D-glucose and D-fructose and these two compounds were therefore analysed
169 together (D-glucose + D-fructose). Ruiz-Matute et al (2009) mentioned the same problems of co-
170 elution of sylillated compounds. However, the number of carbohydrate examined simultaneously in a
171 single run with our method is similar to that quantified by Rovio et al (2011) with capillary
172 electrophoresis but higher than that quantified by the parent method (Triquet Pissart, 1979) or by
173 Ruiz-Matute et al (2009) by CPG, La Torre et al. (2008) by HPLC or Gougeon et al. (2019) by ^{1}H NMR.

174 ### 3.1. Carbohydrate composition and evolution at aging stage

175 Red wines were sampled in barrels at the aging stage, in distinct wineries of Bordeaux area.
176 Wines were sampled at the beginning and then at the end of summer, during the first year in barrels.

177 The minimum, average and maximum carbohydrate concentrations found at each stage of
178 sampling are presented in Table 1. The main carbohydrates found were D-glucose+ D-fructose (176
179 $mg.L^{-1}$on average), L-arabinose (110 $mg.L^{-1}$), D-sorbitol (120 $mg.L^{-1}$L), L-Rhamnose (107 $mg.L^{-1}$),
180 trehalose, (94 $mg.L^{-1}$) and mannitol (83 $mg.L^{-1}$). Cellobiose, galactose, lactose, maltose, melibiose
181 ribose and xylose displayed lower mean concentrations ($\leq$40 $mg.L^{-1}$). Mannose and raffinose were
182 absent from the wines studied (concentrations below the detection threshold). Overall, the average
183 concentrations observed were in the ranges previously described for red wines (Dubernet, 1974;
184 Pellerin and Cabanis 1998; del Alamo et al., 2000; Rovio et al., 2011). Actually, a high content in
185 arabinose was recently identified as a specific trait of Bordeaux red wines (Gougeon et al., 2019) and
186 Rovio et al. (2011) also noticed the absence of mannose in some of the red wines they examined.

187 Each wine appeared as singular regarding the relative proportions between different
188 carbohydrates. Using a PCA, we investigated whether the domain, the vintage and/or the sampling
189 stage could explain the differences observed between samples (Figure 1). The samples in domain A
190 and G were clearly separated from those in the other domains (Figure 1B). In these two domains, the
191 samples were also separated according to the vintage (Figure 1D). Levels of grapes carbohydrates

192 (rhamnose, sorbitol, cellobiose, maltose, lactose, melibiose xylose and arabinose) were the most
193 affected by the vintage parameter. Indeed, each year climate (sunshine, rainfall or water stress) can
194 significantly influence the development of the grape berry, modulate the harvest quality and
195 therefore the composition of the juices (Triquet Pissart 1979; Conde et al., 2015; Geana et al., 2016).
196 In addition, the singularity of certain domains as regards arabinose could be the consequence of
197 singular practices in the vineyard and the domain. Actually pre- and post-fermentation macerations
198 and enzyme addition to the musts were shown to lead to wine enrichment in arabinose, and
199 oligosaccharides and polysaccharides rich in arabinose and galactose (Ayestaran et al., 2004, Doco et
200 al., 2007; Ducasse et al., 2011; Apolinar-Valiente et al., 2013, 2014).

201 The changes in mean carbohydrate concentrations were very low between the two sampling
202 stage (S0 and S1, Table 1). In addition, figure 1C shows that, except for domain C, the samples did not
203 separate according to their sampling stage, S0 or S1.

204 Nevertheless, we examined the carbohydrate concentrations changes, barrel by barrel, and
205 molecule by molecule (Figure 2). No general pattern of carbohydrate concentrations evolution could
206 be drawn, even for a studied wine. Some barrels (33 out of 49) presented a total carbohydrate
207 concentration increase (from 2 to 308 $mg.L^{-1}$), while a decrease was observed in the others (from 5 to
208 318 $mg.L^{-1}$). Some carbohydrate release occurred, probably because of wine polysaccharide
209 degradation (Doco et al., 2007; Martinez-Lapuente et al., 2018), and this superimposed with
210 disappearance phenomena leading to the decrease of other carbohydrate concentrations. Because of
211 the superposition of these phenomena, the variations measured are certainly underestimated. L-
212 rhamnose and L-arabinose were the carbohydrates whose concentration increases the most and/or
213 most frequently (respectively in 21 and 37 carrels out of 49), during the first summer of aging. The
214 quantities released varied between 4 and 160 $mg.L^{-1}$. A significant D-glucose+D-fructose
215 concentration increase (up to 130 $mg.L^{-1}$) was also observed in certain barrels (barrels A4b and c ,
216 A6b and c, and C4c). Cellobiose and melibiose concentration also increased in certain barrels.

217 Conversely, in 16 out of 49 barrels, the total carbohydrate concentration decreased from 5 to 318
218 $mg.L^{-1}$. The carbohydrates contributing to this global decrease varied depending on the barrel
219 considered, but D-glucose + D-fructose, trehalose and rhamnose were mainly concerned by this
220 phenomenon. Their concentrations decreased in 39, 42, and 28 barrels respectively and the
221 variations ranged from a few to 130, 48, and 187 $mg.L^{-1}$ respectively.

222 ### 3.2. Carbohydrate composition as a diagnosis tool?

223 We recently show that, in model growth media, most of the *B. bruxellensis* strains found in wine
224 were able to use glucose, fructose, mannose, ribose, galactose, trehalose, cellobiose, or maltose as
225 single growth substrate in laboratory culture media (Cibrario et al., 2019a). This group of
226 carbohydrate will be referred to as carbohydrates useful for *B. bruxellensis* throughout the paper.

227 All the wines sampled in the cellars contained several of these carbohydrates useful for *B.*
228 *bruxellensis.* We therefore examined whether the analysis of carbohydrate composition of the wine
229 could tell us about the risk of alteration by *B. bruxellensis* and contribute to refine the diagnosis. Of
230 the 49 barrels analyzed, 39 were contaminated, that is to say positive for the presence of detectable
231 populations of *B. bruxellensis* either from S0 (32 barrels) or only at S1 (7 barrels), with viable
232 populations (at half barrel) ranging from 10 $cell.mL^{-1}$ (detection threshold for qPCR) to 3.10^{3} $cell.mL^{-1}$.
233 The cultivable populations found ($CFU.mL^{-1}$) were of the same order of magnitude. This confirmed
234 that the period examined is associated with the presence of *B. bruxellensis* in barrels. Furthermore,
235 of the 39 contaminated barrels, 35 displayed a volatile phenols concentration increase (from 12 to
236 251 $\mu g.L^{-1}$) between S0 and S1, confirming that this period is also associated with alteration (Figure
237 2).

238 Through a Spearman correlation test we then examined if there was a link between the
239 volatile phenols production observed between S0 and S1 and:

240 - the nature and concentration of the carbohydrates present in the S0 sample (beginning of the
241 period),
242 - the nature and concentration of carbohydrates released between S0 and S1,

243 • the nature and concentration of carbohydrates disappeared between S0 and S1.

244 No link could be established. This work with cellar samples suggests that many carbohydrate
245 compositions may be convenient for *B. bruxellensis* development in wine. However, due to low levels
246 of populations and probably to the presence of others microorganisms, the data obtained did not
247 enable to visualise *B. bruxellensis* preferences. In addition, compounds other than carbohydrate may
248 have been used by the microbes present in the wines examined (Shifferdecker et al., 2014; Crauwels
249 et al., 2015; Smith and Divol, 2016).

250 **3.3. Nature of carbohydrates consumed by *B. bruxellensis* in inoculated wines**

251 Additional experiments were conducted at laboratory scale. Three wines A, D (coming from domains
252 A, and D) and M, displaying different by pH, alcohol content or carbohydrate composition (Table 2)
253 were sterilized, inoculated with known strains of *B. bruxellensis* and placed several weeks at 20°C.
254 We selected 8 strains in the 3 main genetic groups found in wine (Avramova et al., 2018, Cibrario et
255 al., 2019). All these strains were able to produce volatile phenols and all were able to grow on the
256 carbohydrates useful for *B. bruxellensis*.

257 The kinetics of strain growth and production of volatile phenols is shown in Figure 3. In wine M, 3
258 strains were studied and exhibited similar behaviour and the volatile phenols olfactory detection
259 threshold (around 400 $\mu g.L^{-1}$) was reached between 5 and 6 weeks of experimentation (Figure 3 A
260 and D). Wine M can be qualified as permissive (Cibrario et al, 2019b; Krizanovic et al., 2019). In wine
261 D, the growth of the triploid strains L0424, AWRI1499 L14190, L0422 and AWRI1608 was slightly
262 faster than that of the diploid strains CBS2499, 11AVB4 and L0611. The volatile phenols
263 concentrations produced by the triploid strains exceeded the detection threshold between 4 and 5
264 weeks of experimentation (Figure 3 B and E). The appearance of detectable concentrations of volatile
265 phenols occurred later with the diploid strains. And, after 6 weeks of experimentation, the volatile
266 phenols concentrations were 2 to 5 times lower than those produced by the triploid strains and, with
267 the exception of CBS2499, these quantities remained below the olfactory rejection threshold. Wine D
268 thus appeared as less permissive than wine M with the diploid strains. In wine A, yeast adaptation

269 seemed even more difficult. Nevertheless, all triploid strains showed an increase in their cultivable
270 population up to 10^6 CFU.mL^{-1} within 6 weeks. Their growth patterns were quite similar and the
271 strains L0424, AWRI1499, L14190 and AWRI1608 did not produce more than 400 µg.L^{-1} volatile
272 phenols in 6 weeks (Figure 3 C and F). In this wine, the cultivable populations of the diploid strains
273 maintained but did not increase, and these strains did not produce any detectable quantity of
274 volatile phenols. The triploid strain L0422 (AWRI1608-like) exhibited an intermediate behaviour
275 (significant growth even if efficient than that of the other triploid strains) but low production of
276 volatile phenols: 41 ± 2 µg.L^{-1} after 6 weeks). Wine A was the less permissive among the three
277 studied. Many abiotic elements may be responsible for this low permissiveness: pH, alcohol content,
278 polyphenols and tannins (Dias et al, 2003, Barata et al, 2008, Comitini et al, 2019)… The tight link
279 between high yeast populations and volatile phenol production, previously mentioned by Gerbaux
280 (2002), Barata et al (2008) and Cibrario et al (2019a, b) is underlined once more in this experiment.

281 All the carbohydrates quantified by the method were present in these 3 wines, with the
282 exception of D-mannose and raffinose, for which the levels were below the detection limits of the
283 method (<1.5 mg.L^{-1}) (Table 2). Each wine had a specific initial carbohydrate composition. But overall,
284 wine A was more concentrated in carbohydrates useful for *B. bruxellensis* than wine D, itself richer
285 than wine M (676, 324 and 168 µg.L^{-1}respectively).

286 Residual concentrations of carbohydrate were measured at the end of the experiment and the
287 decrease of each carbohydrate concentration is indicated strain by strain and wine by wine in tables
288 3 to 5. This revealed a decrease of total carbohydrate concentration (from 99 to 492 mg.L^{-1}), in wines
289 where a significant growth was observed. None of the carbohydrate examined displayed any
290 significant concentration increase in pasteurized wines stored in glass tubes. In the permissive wines
291 D and M, the consumption of between 91 and 173 mg.L^{-1} was sufficient to reach 10^6 CFU.mL^{-1}. In
292 wine A, less permissive but also displaying more carbohydrates, more than 450 mg.L^{-1} were
293 consumed to reach the same level of population. Moreover, in this wine, carbohydrate consumption
294 was observed in the absence of growth: strains L0611 and 11AVB4 respectively consumed 34 and 82

295 mg.L^{-1}, in spite of a stable cultivable population, suggesting that carbohydrates were dissipated for
296 cell maintenance. In the same time, the strain CBS2499 did not consume any significant amount of
297 carbohydrate. The final population of these 3 diploid strains was very low compared with that
298 obtained with the other strains in the same wine or the same strains in the other wines.

299 In all cases, there was no significant change in the concentrations of D-xylose, L-arabinose, L-
300 rhamnose, melibiose and polyols. To simplify, their concentration variation is presented in table 3A
301 and omitted in tables 3B and 3C. Conversely, D-glucose + D-fructose, trehalose, D-galactose and D-
302 ribose were consumed by all strains in wines D and M (table 3A and 3B). In wine A, the yeasts also
303 degraded cellobiose, maltose and lactose (table 3C). But in the 3 wines, whatever the strain
304 considered, D-glucose + D-fructose + trehalose represented between 50 and 91 % of the
305 carbohydrates consumed.

306 *B. bruxellensis* therefore uses many carbohydrates during its growth in wine and shows a preference
307 for D-glucose+D-fructose and trehalose.

308 3.4. Does the presence of carbohydrates "useful for" *B. bruxellensis* aggravate the risk of spoilage?

309 In order to determine whether, in wines with equivalent abiotic constraints, the
310 carbohydrate content may aggravate the risk of spoilage, additional experiments were performed.
311 The wines A, M and M14 were enriched or not with carbohydrates (glucose or fructose or trehalose
312 or combination of these carbohydrates) and inoculated with three different strains of *B. bruxellensis*.
313 Furthermore, the tests were conducted either under unstirred and poorly oxygenated conditions or
314 under increased aeration, to mimic the different conditions of aeration that can be encountered by
315 the wine during aging, depending on the position in the barrel. The cultivable population and the
316 volatile phenols obtained after 4 weeks of experiments are shown in Figure 4.

317 Regardless of the strain, in unstirred conditions and without carbohydrates addition, wine M
318 was more permissive than M14, itself more permissive than wine A. The alcohol content contributed
319 to slowing the growth of *B. bruxellensis* and, in particular, that of the diploid strain L0611 (Figure 4A,
320 wines M and M14). Stirring and increased oxygen supply partly masked the difficulties of this strain in

321 the M14 wine. Oxygen supply was already mentioned as a growth and phenol production promoting
322 factor (Rozpedowska et al., 2011; Tubia et al, 2018). In addition, whatever the wine, the strain or
323 oxygenation conditions considered, the addition of low amounts of carbohydrate systematically
324 resulted in growth stimulation and in an increase in volatile phenols after 4 weeks (Figure 4A, B and
325 C). Identical results were obtained when glucose, fructose or trehalose were added alone or as two-
326 by-two mixtures instead of altogether (not shown). The presence of higher concentrations of
327 carbohydrates preferentially metabolized by *B. bruxellensis* really increases the risk of spoilage.

328 ## 4. Conclusions

329 We clearly show that, in wine, the nature of *B. bruxellensis* preferred carbohydrates is similar to
330 that it consumes in model media: mainly D-glucose + D-fructose and trehalose and, to a less extend,
331 cellobiose, galactose, ribose, maltose and lactose. Furthermore, when wines are artificially enriched
332 with low amounts of some of these carbohydrates, the growth is stimulated and volatile phenols
333 accumulate faster. We also show that these carbohydrates are present in Bordeaux red wines at
334 aging stage. As *B. bruxellensis* is present in most of the cellar examined, these carbohydrates may
335 promote *B. bruxellensis* development in barrels (Chatonnet et al, 1992). High carbohydrate content,
336 especially in D-glucose + D-fructose and trehalose, is thus a clear factor of spoilage aggravation, even
337 though it is probably not the only one. Winemakers should thus limit the presence of residual sugars,
338 and particularly glucose fructose and trehalose, in their finished wines, through a close management
339 of alcoholic and malolactic fermentations.

340

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344 00002990).

345

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458

459

460

461 **Table 1. Carbohydrate concentrations (mg/L) in the 49 red wines samples studied during aging.**

462

	S0 (Aging)			S1 (Aging)		
	Mean	**Min**	**Max**	**Mean**	**Min**	**Max**
L-Arabinose	110 ±128	25	524	118 ± 136	27	522
Cellobiose*	40 ± 11	25	86	40 ± 13	26	104
D-Galactose*	19 ± 6	12	38	19 ± 6	12	40
D-Glucose + D- Fructose*	176 ± 72	87	415	152 ± 85	82	489
Lactose	36 ± 7	22	52	35 ± 7	21	47
Maltose*	7 ± 4	0	21	6 ± 4	0	18
D-Mannitol	83 ± 27	28	123	85 ± 27	45	130
D-Mannose*	ND	ND	ND	ND	ND	ND
Melibiose	22 ± 4	17	32	22 ± 4	17	36
Raffinose	ND	ND	ND	ND	ND	ND
L-Rhamnose	107 ± 43	63	280	102 ± 43	44	242
D-Ribose*	15 ± 6	5	31	14 ± 7	4	33
D-Sorbitol	120 ± 25	50	212	118 ± 21	74	165
Trehalose*	94 ± 73	6	250	86 ± 68	5	240
D-Xylose	14 ± 8	4	39	14 ± 7	4	41
Total	851 ± 243	540	1675	823 ± 257	533	1652

463 ND: not detected.

464 ** Carbohydrates which can support B. bruxellensis growth according to Cibrario et al (2019).*

465

466

467 **Table 2: Description of the wine used for laboratory experiments**

Carbohydrate (mg/L)	Wine A	Wine D	Wine M
L-arabinose	519±12	63±3	72±5
Cellobiose*	68±10	38±2	52±4
D-Galactose*	37±5	21±0	24±1
D-Glucose + D-Fructose*	440±21	195±9	9±0
Lactose	40±1	25±3	41±8
Maltose*	13±0	3±2	11±3
D-Mannitol	41±2	98±14	48±5
Melibiose	22±2	21±0	21±0
L-Rhamnose	109±12	77±4	75±12
D-Ribose*	27±0	23±1	19±4
D-Sorbitol	86±5	99±4	61±6
Trehalose*	91±8	44±2	53±2
D-Xylose	35±3	10±0	10±0
Total carbohydrates « useful » for *B. bruxellensis* * (mg/L)	676±31	324±25	168±15
Total (mg/L)	1528±42	717±38	496±25
pH,	3.48	3.63	3.56
TAV (% vol)	14.30	13.49	13.19
Vintage	2016		
Grape variety	Merlot/Cabernet Sauvignon		

468 ** Carbohydrates which can support B. bruxellensis growth according to Cibrario et al (2019).*

469

470

471 **Table 3.** **Carbohydrates consumed (mg.L^{-1}) in 8 weeks by 3 strains of *B. bruxellensis* grown in wine M.**

	L0424	AWRI1608	L0611
L-Arabinose	-2±1	-3±1	0±0
Cellobiose	23±3	27±2	25±3
D-Galactose	5±2	19±1	7±1
D-Glucose + D-Fructose	5±2	3±2	1±0
Lactose	8±3	6±0	1±0
Melibiose	-1±1	0±0	0±0
Maltose	9±1	9±1	8±0
D-Mannitol	0±0	-1±0	0±0
L-Rhamnose	-2±2	-1±1	0±0
D-Ribose	10±2	8±1	9±0
D-Sorbitol	0±0	0±0	0±0
Trehalose	52±1	51±0	50±3
D-Xylose	-1±1	-1±0	0±0
Total (mg.L^{-1})*	112±13	123±7	101±7

472 *n=2.*
473 **sum of the carbohydrate disappeared (the negative variations are not taken into account).*
474

475 **Table 4.** **Carbohydrates consumed (mg.L^{-1}) in 6 weeks by 7 strains of *B. bruxellensis* grown in wine D.**

	L0424	AWRI1499	L14190	AWRI1608	L0422	CBS2499	L0611
D-Glucose + D-Fructose	105	104 ± 3	102 ± 5	105 ± 0	111 ± 4	88 ± 4	60 ± 13
Trehalose	40	40 ± 0	31 ± 5	38 ± 0	40 ± 1	1 ±1	18 ± 4
D-Galactose	9	7± 1	14 ± 2	12 ± 2	17 ± 0	0 ± 1	4 ± 2
D-Ribose	4	5 ± 1	8 ± 1	8 ± 0	4 ± 2	2 ± 0	3 ± 2
Cellobiose	- 5	- 6 ± 1	-1 ± 1	0 ± 1	- 2 ± 2	- 11 ± 6	6 ± 6
Maltose	- 1	1 ± 1	0 ± 0	1 ± 0	0 ± 1	- 1 ± 1	1 ± 1
Lactose	- 5	- 6 ± 4	- 9 ± 0	- 7 ± 1	- 1 ± 0	- 5 ± 3	2 ± 1
Total (mg.L^{-1})*	158	157 ± 6	155 ± 13	164 ± 3	172 ± 7	91 ± 6	94 ± 29

476 *n=2, except for strain L0424 (single assay).*
477 **sum of the carbohydrate disappeared (the negative variations are not taken into account).The carbohydrates*
478 *that never presented any negative variation of concentration are not presented in this table.*
479

480 **Table 5.** **Carbohydrates consumed (mg.L^{-1}) in 6 weeks by 8 strains of *B. bruxellensis* grown in wine A.**

	L0424	AWRI1499	L14190	AWRI1608	L0422	CBS2499	L0611	11AVB4
D-Glucose + D-Fructose	338 ± 1	338 ± 3	342 ± 5	352 ± 1	348 ± 6	-13 ± 10	26 ± 10	56 ± 0
Trehalose	79 ± 1	79 ± 4	66 ± 1	83 ± 0	70 ± 3	-1 ± 5	-2 ± 3	3 ± 3
D-Galactose	5 ± 2	4 ± 2	12 ± 0	19 ± 1	19 ± 3	-3 ± 3	-1 ± 1	0 ± 0
D-Ribose	10 ± 2	9 ± 1	7 ± 1	8 ± 1	8 ± 0	0 ± 0	2 ± 0	1 ± 1
Cellobiose	15 ± 3	15 ± 1	11 ± 3	15 ± 0	13 ± 2	3 ± 1	5 ± 3	13 ± 0
Maltose	9 ± 1	8 ± 0	7 ± 1	9 ± 1	9 ± 0	0 ± 0	1 ± 0	2 ± 0
Lactose	8± 3	7 ± 1	8 ± 1	6 ± 1	10 ± 1	-1 ± 4	0 ± 2	7 ± 4
Total (**mg.L^{-1}**)*	464 ± 12	460 ± 12	453 ± 11	492 ± 5	477 ± 18	3 ± 1	34 ± 15	82 ± 8

481 *n=2.*
482 **sum of the carbohydrate disappeared (the negative variations are not taken into account). The carbohydrates*
483 *that never presented any negative variation of concentration are not presented in this table.*
484

485 ## Figure captions

486 **Figure 1. : PCA analysis of the carbohydrate composition of wine sampled during the first summer of aging.**

487 A. Correlation circle. The samples represented come from 6 domains (A, B, C, E, F, G) sampled in 2014
488 and 2015 vintages at stages S0 and S1. The domains D and H were not included because of a too low
489 number of samples.

490 B. The samples are grouped according to domain (ellipses at 68% confidence interval).

491 C. The samples are grouped according to the domain and the sampling stage (S0 or S1),

492 D. The samples are grouped according to the domain and the vintage

493

494

495 **Figure 2: Carbohydrate analysis in wines sampled in barrels at the aging stage.**

496 For each sugar, the difference between final concentration and initial concentration (mg.L^{-1}) is represented
497 (cumulative bar).

498 The stars indicate the level of volatile phenol production during the period examined: no star <12 µg.L^{-1}, one
499 star <50 µg.L^{-1}L, two stars <100 µg.L^{-1}, three stars <251 µg.L^{-1}.

500 The 49 barrels (17 wine lots) were followed in one the 8 domains (A to H). Barrels with number 4 and 5 were
501 sampled in 2015 (2014 vintage) and barrels with number 6 were sampled in 2016 (2015 vintage).

502

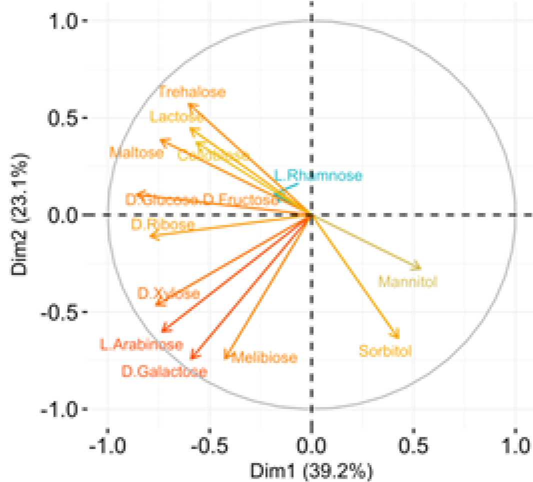
503 **Figure 3.** Kinetics of growth and volatile phenols production by a selection of *B. bruxellensis* strains of
504 inoculated in different wines. Each point represents the mean of two experiments. Legend: diploid strains of
505 the CBS2499 genetic group: • CBS2499, •L0611, •11AVB4. Triploid strains of the AWRI1608 genetic group: ▲
506 AWRI1608; ▲ L0422. Triploid strains of the AWRI1499 genetic group: ■ AWRI1499; ■ L0424; ■ L14190.

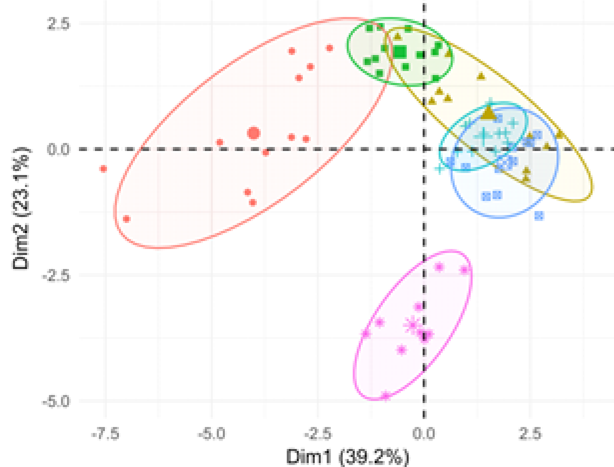
507

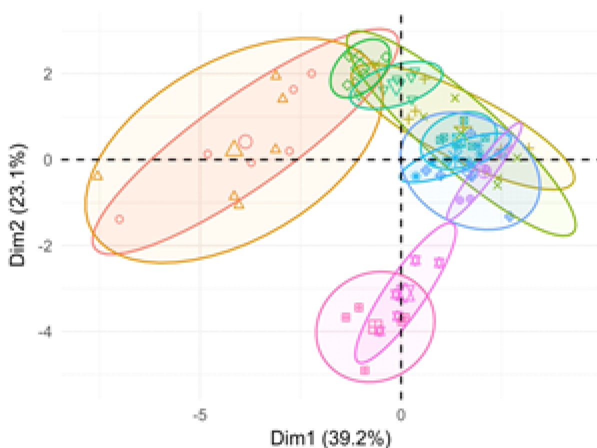
508

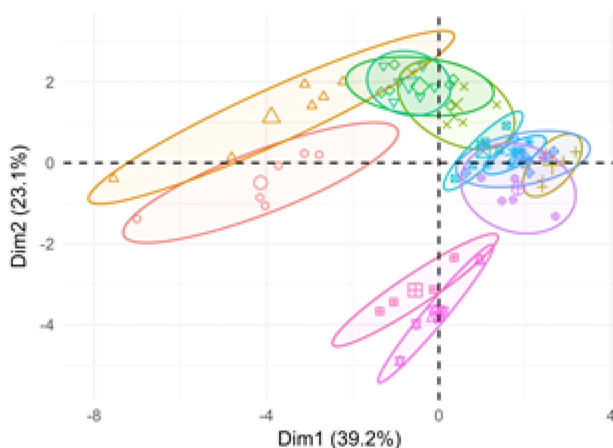
509 **Figure 4.** Growth (circles) and volatile phenol production (bars) after 4 weeks in different wines supplemented
510 (gray) or not (white) with glucose + fructose + trehalose (250 mg.L^{-1} each) and incubated at 20 °C in different
511 conditions of agitation. The experiments were made in duplicate.

512


A: Correlation circle
Trehalose
Lactose
Maltose
L.Rhamnose
D.Glucose
D.Fructose
D.Ribose
Mannitol
D.Xylose
L.Arabinose
D.Galactose
Melibiose
Sorbitol
Dim1 (39.2%)
Dim2 (23.1%)

Contribution
2.5 5.0 7.5 10.0

B: Domain
Dim1 (39.2%)
Dim2 (23.1%)

A C F
B E G

C: Domain - Stage
Dim1 (39.2%)
Dim2 (23.1%)

A-S0 B-S1 E-S0 F-S1
A-S1 C-S0 E-S1 G-S0
B-S0 C-S1 F-S0 G-S1

D: Domain - vintage
Dim1 (39.2%)
Dim2 (23.1%)

A-2014 B-2015 E-2014 F-2015
A-2015 C-2014 E-2015 G-2014
B-2014 C-2015 F-2014 G-2015

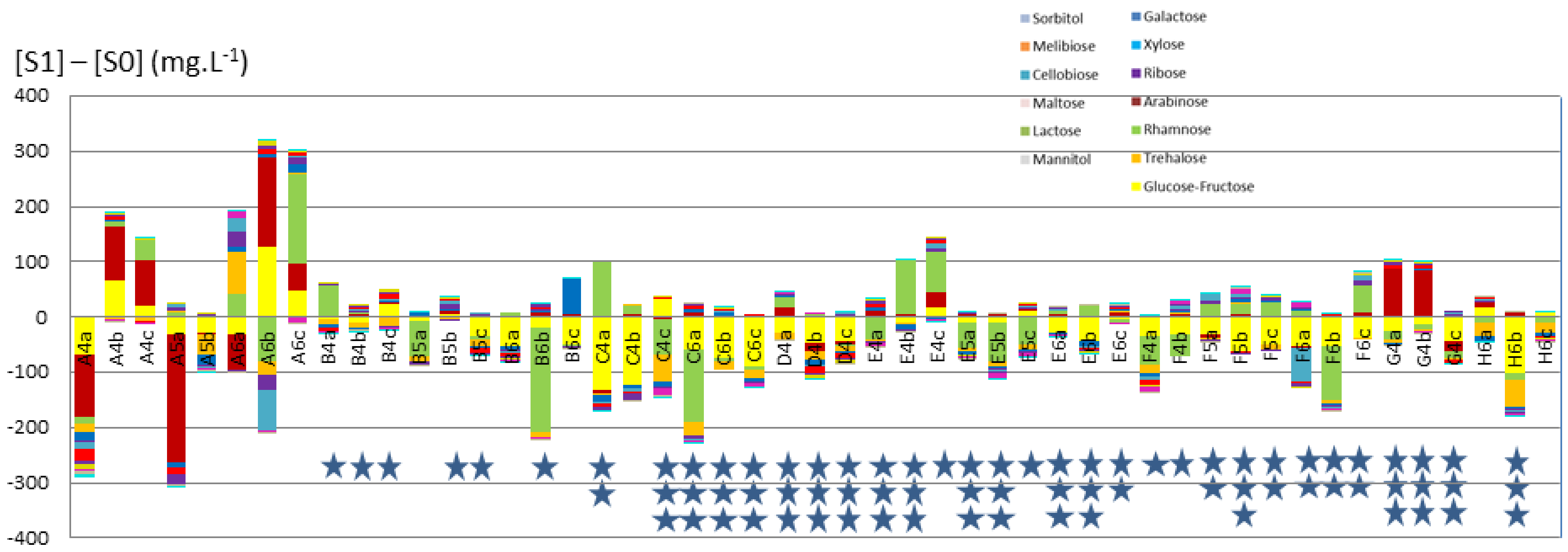


Figure 2

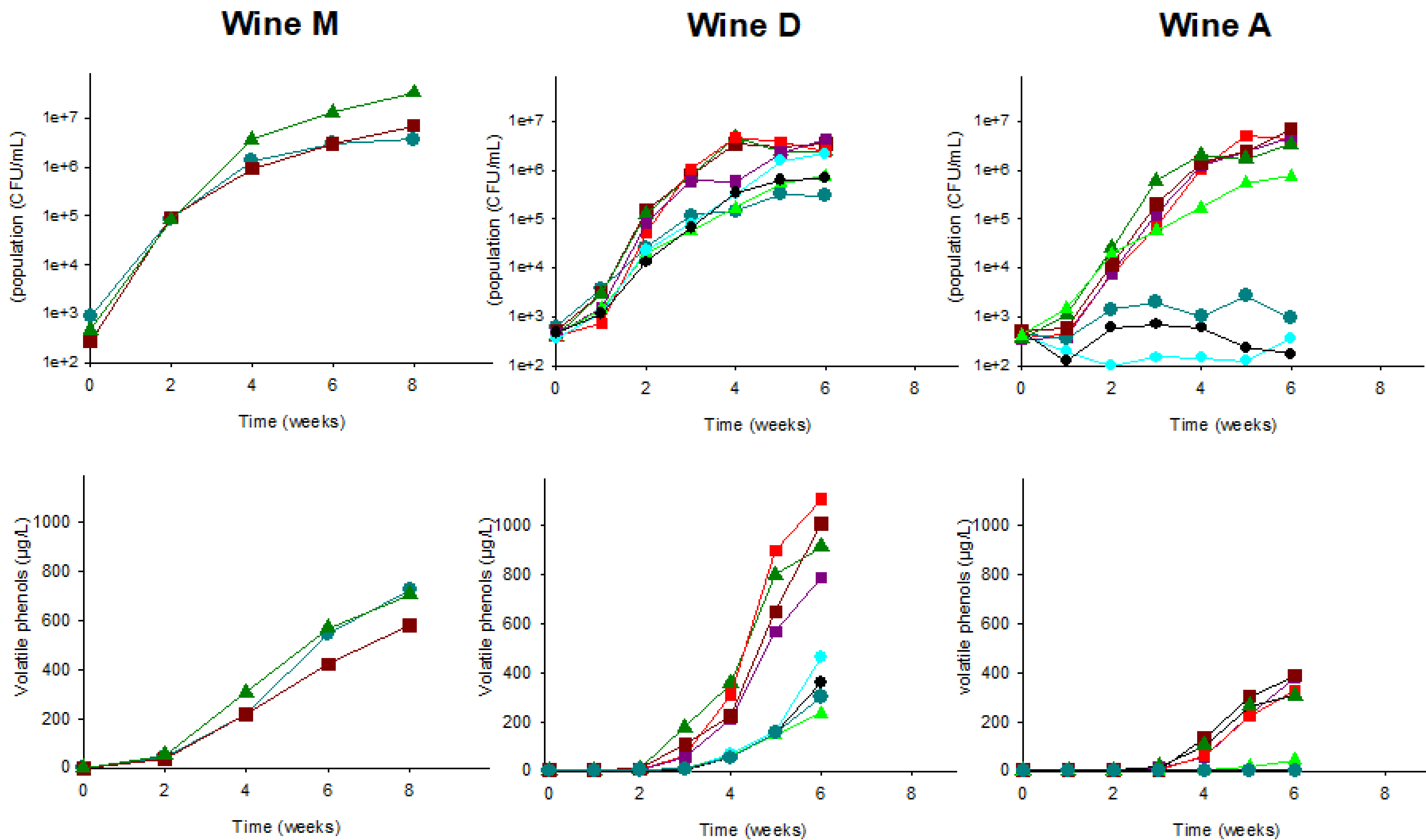
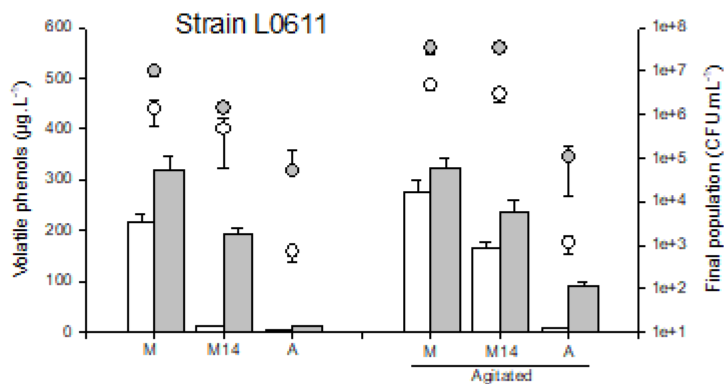
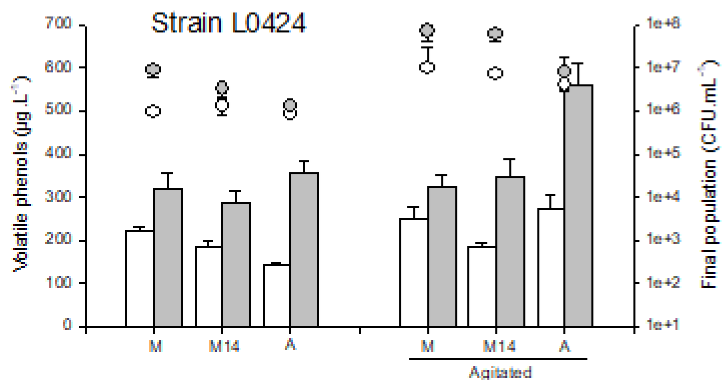


Figure 3





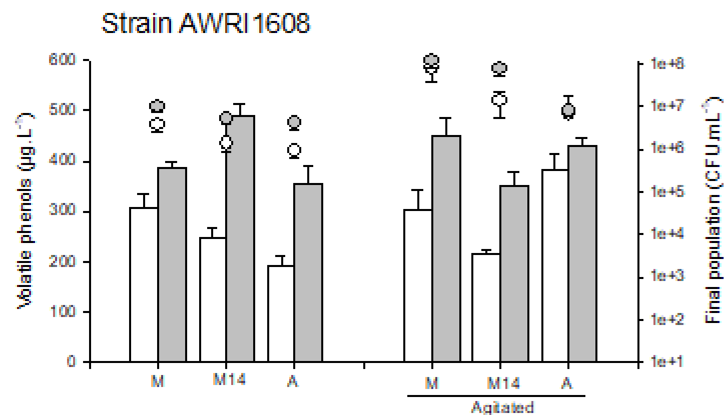


Figure 4