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Alice Cibrario, Marie Claire Perello, Cécile Miot-Sertier, Laurent Riquier, Gilles de Revel, Patricia Ballestra & Marguerite Dols-Lafargue*

Univ. Bordeaux, ISVV, Unité de recherche Œnologie EA 4577, USC 1366 INRAE, Bordeaux INP, F-33882 Villenave d'Ornon, France.

* Corresponding author: marguerite.dols@enscbp.fr Phone +33 (0)5 57 57 58 32;
Fax +33 (0)5 57 57 58 13
ORCID: 0000-0002-7273-1561.

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Abstract

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

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

1. Introduction

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

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

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

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

2. Material and Methods

2.1. Yeast strains

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

2.2. Experiments in wine at laboratory scale

Three red wines (2016 vintage, Bordeaux area) were used for experiments at laboratory scale: wine A had a pH of 3.48 and contained 14.30 %vol ethanol, wine D had a pH of 3.63 and contained 13.49 %vol ethanol and wine M had a pH of 3.56 and titrated 13.19 %vol ethanol (Table 1). In addition, the wine M was enriched in alcohol to reach 14.19 %vol (to produce wine M14). All were 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

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

2.4. Cultivable cells counts

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

2.5. Viable cells counts

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

2.6. Carbohydrate analysis

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

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

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

2.7. Volatile phenols determination

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

2.8. Statistical analysis

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

3. Results and discussion

We first worked to improve the method for separating sylillated carbohydrates, in order to 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

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

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

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

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

3.2. Carbohydrate composition as a diagnosis tool?

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

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

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

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

- the nature and concentration of carbohydrates disappeared between S0 and S1.

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

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

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

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

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

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

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

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

4. Conclusions

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

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

	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

ND: not detected.

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

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

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

n=2.

**sum of the carbohydrate disappeared (the negative variations are not taken into account).*

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

n=2, except for strain L0424 (single assay).

**sum of the carbohydrate disappeared (the negative variations are not taken into account).The carbohydrates that never presented any negative variation of concentration are not presented in this table.*

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

n=2.

**sum of the carbohydrate disappeared (the negative variations are not taken into account). The carbohydrates that never presented any negative variation of concentration are not presented in this table.*

Figure captions

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

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

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

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

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

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

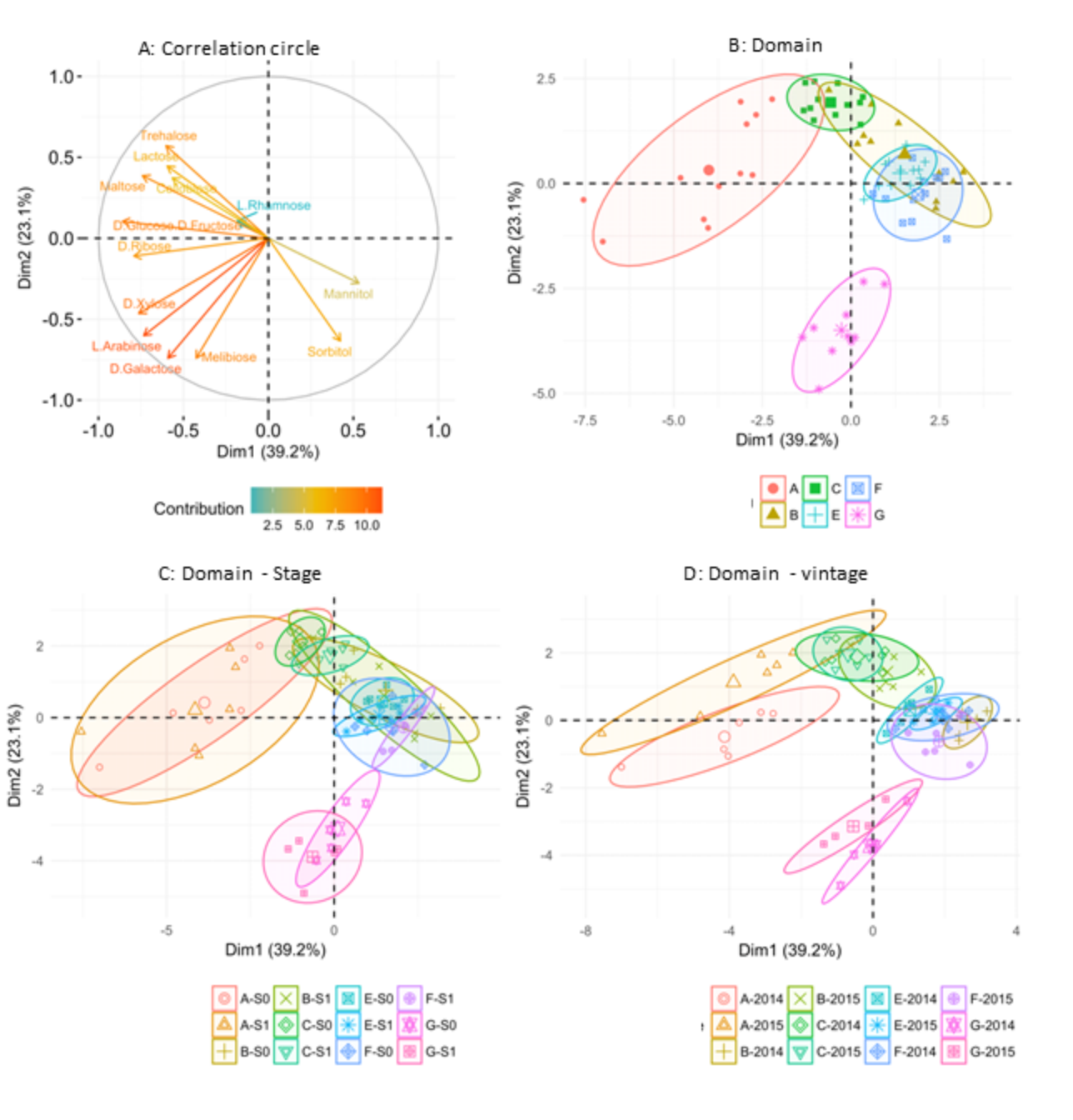
For each sugar, the difference between final concentration and initial concentration ($mg.L^{-1}$) is represented (cumulative bar).

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

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

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

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


A: Correlation circle
Trehalose
Lactose
Maltose
Cellobiose
L.Rhamnose
D.Glucose
D.Fructose
D.Ribose
D.Xylose
L.Arabinose
D.Galactose
Melibiose
Mannitol
Sorbitol
Dim1 (39.2%)
Dim2 (23.1%)
Contribution
2.5 5.0 7.5 10.0
B: Domain
A C F
B E G
C: Domain - Stage
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
A-2014 B-2015 E-2014 F-2015
A-2015 C-2014 E-2015 G-2014
B-2014 C-2015 F-2014 G-2015

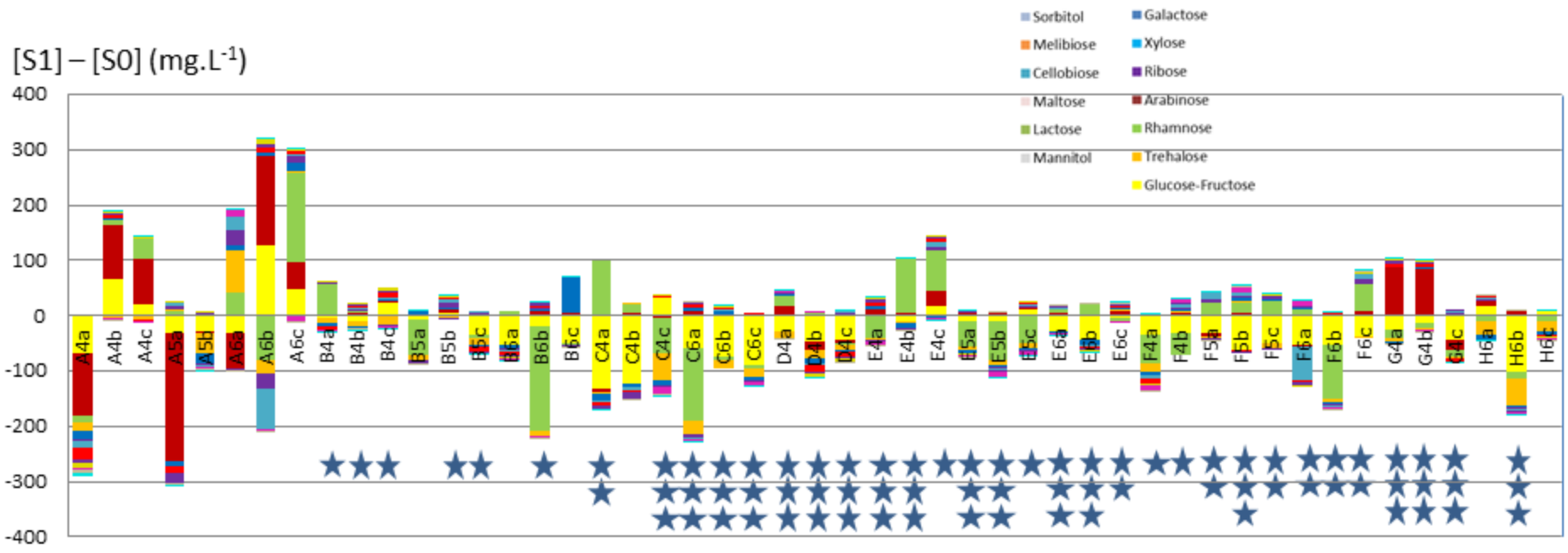

[S1] – [S0] (mg.L-1)
400
300
200
100
0
-100
-200
-300
-400
Sorbitol
Galactose
Melibiose
Xylose
Cellobiose
Ribose
Maltose
Arabinose
Lactose
Rhamnose
Mannitol
Trehalose
Glucose-Fructose
A4a
A4b
A4c
A5a
A5b
A5c
A6a
A6b
A6c
B4a
B4b
B4d
B5a
B5b
B5c
B6a
B6b
B6c
C4a
C4b
C4c
C6a
C6b
C6c
D4a
D4b
D4c
E4a
E4b
E4c
E5a
E5b
E5c
E6a
E6b
E6c
F4a
F4b
F5a
F5b
F5c
F6a
F6b
F6c
G4a
G4b
G4c
H6a
H6b
H6c

Figure 2

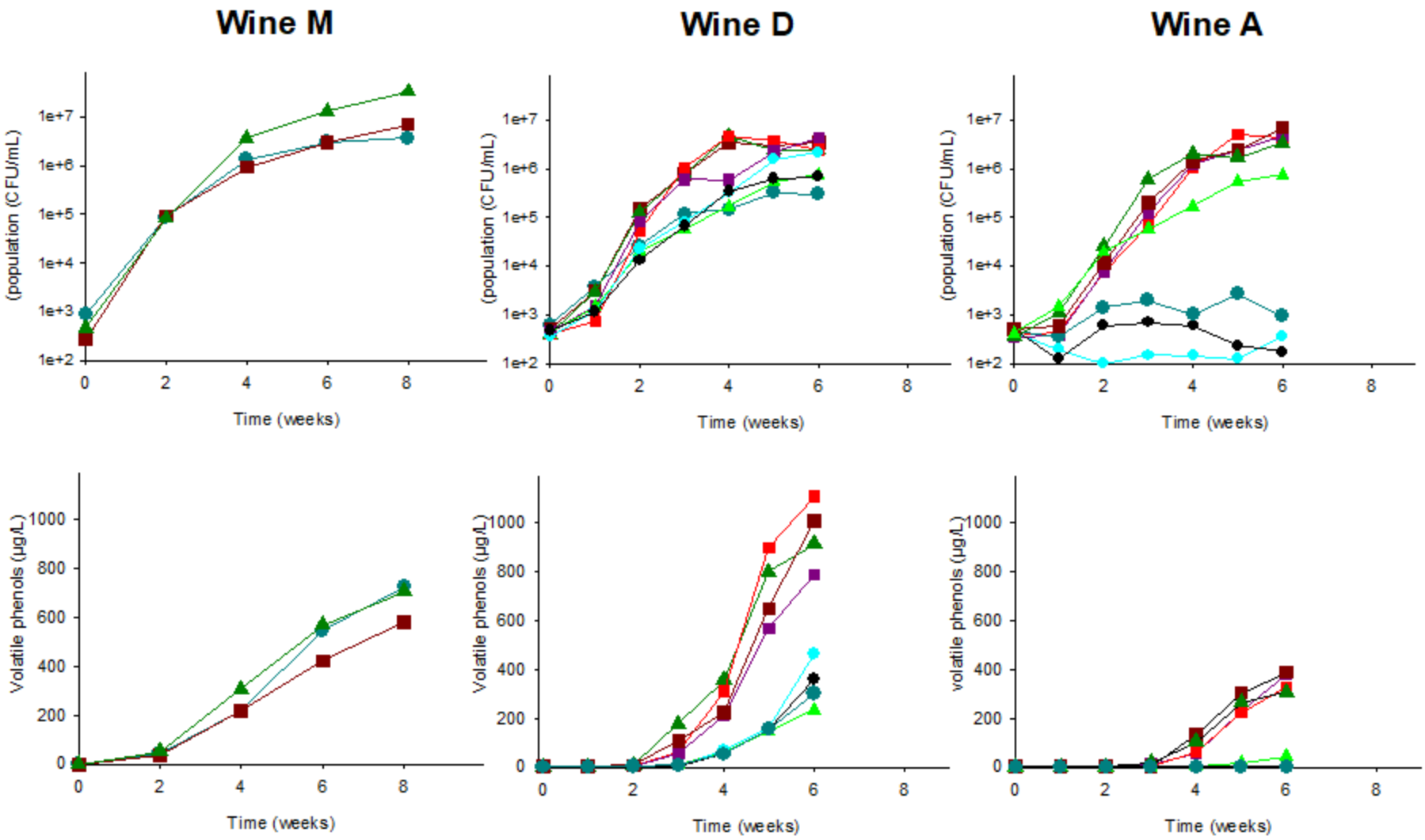
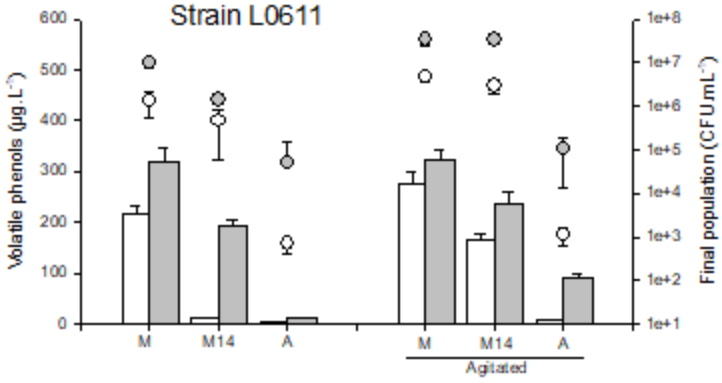
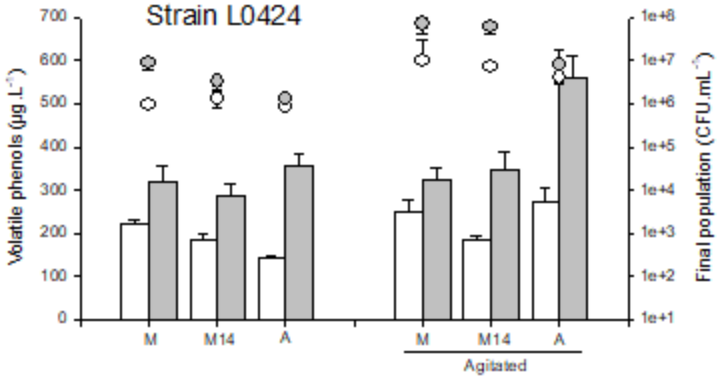

Wine M
Wine D
Wine A
(population (CFU/mL)
1e+2
1e+3
1e+4
1e+5
1e+6
1e+7
0
2
4
6
8
Time (weeks)
Volatile phenols (µg/L)
0
200
400
600
800
1000
volatile phenols (µg/L)

Figure 3


Strain L0611
Volatile phenols (µg.L-1)
Final population (CFU.mL-1)
600
500
400
300
200
100
0
1e+8
1e+7
1e+6
1e+5
1e+4
1e+3
1e+2
1e+1
M
M14
A
M
M14
A
Agitated


Strain L0424
Volatile phenols (µg.L-1)
Final population (CFU.mL-1)
700
600
500
400
300
200
100
0
1e+8
1e+7
1e+6
1e+5
1e+4
1e+3
1e+2
1e+1
M
M14
A
M
M14
A
Agitated

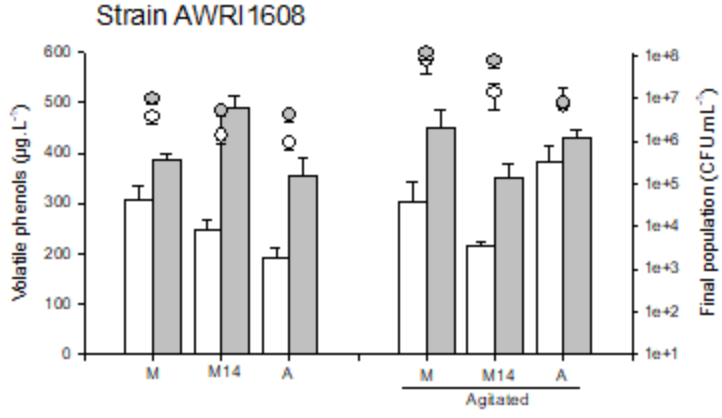

Strain AWRI1608
Volatile phenols (µg.L-1)
Final population (CFU.mL-1)
600
500
400
300
200
100
0
1e+8
1e+7
1e+6
1e+5
1e+4
1e+3
1e+2
1e+1
M
M14
A
M
M14
A
Agitated

Figure 4