Bordeaux red wines display a high diversity in their ability to support *Brettanomyces bruxellensis* growth

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Abstract

Red wines constitute a major production in Bordeaux vineyards. To ensure the quality of these wines, winemakers make every effort to limit the development of *Brettanomyces bruxellensis*, a yeast responsible for wine alteration and feared all over the world. A lot of research work was performed in recent years to explore the genetic diversity of the species and to connect it with phenotypic variation, often in model environments. Few assays in wine suggest that not all wines are equals regarding the ability to support *B. bruxellensis* growth. We therefore examined the growth of five representative strains of *B. bruxellensis* in 53 Bordeaux red wines. Thanks to a notation method and to unsupervised classification analysis, the wines were classified according to their "permissiveness". The impact of distinct factors such as ethanol content, pH, strain present, wine origin or composition (^{1}H-NMR analysis of 45 compounds) on wine permissiveness was then examined.

Keywords: *B. bruxellensis*, wine, permissiveness, ethanol

1. Introduction

One of the major issues in red wine elaboration is spoilage by *Brettanomyces bruxellensis*. This species was first identified in 1904 and the beers where this specific yeast developed were associated with "an English character" (Claussen, 1904). Later *B. bruxellensis* was isolated in wine, where it was found to produce aromas described as "animal", "horse sweat", "burnt plastic",… due to volatile phenols (VP) production (Chatonnet et al., 1992). VP spoiled wines lead to an important economic loss estimated to about 1.4 million $ (Boulton et al., 1996; Fugelsang & Edwards, 2007). The main VP found in wines are 4-vinylphenol, 4-vinylguaiacol, 4-ethylphenol and 4-ethylguaiacol (Chatonnet et al., 1992; Heresztyn, 1986; Rozpędowska et al., 2011). They result from the conversion of the hydroxycinnamic acids naturally brought by grapes. If many wine microbes are able to produce the vinyl forms, *B. bruxellensis* is one of the very few microbial species able to produce the ethyl forms (Heresztyn, 1986; Barata et al., 2006). Several studies suggested that some *B. bruxellensis* strains were more efficient producers

than others; however, all the *B. bruxellensis* strains studied appeared to have the intrinsic ability to produce VP (Conterno et al., 2006; Vigentini et al., 2008; Cibrario, Miot-Sertier, et al., 2019, 2020).

Once the wine is spoiled, the options for the winemakers are limited. Filtration combined with reverse osmosis can be used to successfully remove VP, but this can induce the loss of aromatic compounds such as methyl and ethyl vanillate (Ugarte et al., 2005). Polymers (PVPP) and charcoal are also used in the wine industry, but their main objective is to remove odors linked to other defaults in wines (Suárez et al., 2007). The best option is to avoid wine spoilage by preventing contamination by *B. bruxellensis*. Globally, improvements regarding the cellar hygiene provided positive results. Barrels or material sanitization can be performed by washing with high-pressure hot water or ozonated gas (Cantacuzene, 2003; Pinto et al., 2020). However, despite rigorous cleaning and attentive care, contaminations are still observed, which suggests that the species is a natural resident of vineyards and cellars (Schifferdecker et al., 2014; Agnolucci et al., 2017; Le Montagner et al., 2023). In the wines contaminated by *B. bruxellensis*, VP formation can be observed from the moment when the population is sufficient, i.e. from 10^4 to 10^5 CFU/ml depending on the wine (Fugelsang & Zoecklein, 2003; Cibrario, Miot-Sertier, et al., 2019). Beyond the bioconversion abilities of the strain present, the ability to spoil a wine is thus mainly due to the ability of the strain to survive and reach high populations, i.e. to withstand combinations of stresses such as low pH, alcohol or low nutrient content (Smith & Divol, 2016; Agnolucci et al., 2017; Avramova et al., 2018; Cibrario, Perello, et al., 2020).

To prevent the accumulation of high yeast concentrations, winemakers regularly remove lees by racking the wine. This contributes to reduce the global microbiological population in the tanks or barrels (Ribéreau-Gayon, 2017). Furthermore, antiseptic molecules such as sulfur dioxide (SO_2), dimethyl dicarbonate (DMDC) or chitosan can be used (Delfini et al., 2002; Gómez-Rivas et al., 2004; du Toit et al., 2005). However, strains tolerant to SO_2 or chitosan exist, which limits the efficiency of such treatments (Avramova et al., 2018; Paulin et al., 2020). For these reasons and because the wine industry tries to reduce inputs, physical methods including sterile filtration, flash pasteurization, or pulsed-light were experimented. Some successful results were obtained, but they are not perfectly in line with high quality wine elaboration (Boulton et al., 1996; Benito et al., 2009; Lisanti et al., 2019; Harrouard et al., 2023).

Recent research suggested that the solution could rely in the wine itself, as some wines appeared much more reluctant to support the spoilage yeasts growth (Cibrario, Miot-Sertier, et al., 2020; Paulin et al., 2020). Many researchers examined what could promote or prevent *B. bruxellensis* growth in wine. The influence of the strain present, the carbohydrate content or the stress factors such as SO_2, low pH, ethanol, and temperature has been studied (Steensels et al., 2015), but none of these parameters

efficiently explained the differences observed in *B. bruxellensis* growth in the studied wines (Gerbaux et al., 2000; Cibrario, Miot-Sertier, et al., 2020; Cibrario, Perello, et al., 2020).

This study aims to examine the question: Bordeaux red wines are they really different regarding their permissiveness towards *B. bruxellensis* growth? To give a robust answer to this question, 53 sulfite-free wines from 3 different grape varieties were sampled in different domains and different appellations in the Bordeaux region. To reflect distinct situations of contamination, 5 strains representative of the genetic and phenotypic diversity of the species within the Bordeaux region were selected for inoculation in wine (Cibrario, Avramova, et al., 2019). The analysis of the growth curves obtained allowed us to rate the wines according to their degree of permissiveness using 2 methods: a manual classification and a Hierarchical Agglomerative Clustering (HAC). The influence of easily accessible wine parameters such as pH, TAV, variety was examined. Then, in a second step, the growth analysis and the notation were repeated in 36 "standardized" wines in order to study the wine permissiveness independently of wine pH and ethanol content. The links between standardized wines permissiveness and composition were then studied using ^{1}H-NMR-based metabolomics (Le Mao et al., 2023).

2. Materials and methods

2.1. Wines

For this study, 53 monovarietal wines (28 Merlot, 23 Cabernet-Sauvignon and 2 Cabernet-Franc) from 2020 and 2021 vintages were analyzed. All of them came from five wineries located in the Bordeaux area and were sampled immediately after the end of malolactic fermentation and before barreling. Their pH ranged between 3.41 and 3.98, and the alcohol content between 12.25 and 15.60% vol. ethanol (ABV). For 32 of them, half of the volume treated was standardized at 14% vol. by ethanol addition and pH 3.5 with either H_3PO_4 or KOH. These 14% vol. adjusted wines were associated with 6 wines already displaying a pH equal to 3.5 and an ABV very close to 14% vol. to constitute a list of 38 "standardized" wines (Supplementary Information, Table S1).

To prepare the wines for chemical and microbiological analysis, finning with egg white (Ribéreau-Gayon, 2017) and pasteurization (80°C for 30 min) were performed. The absence of residual indigenous microbes was confirmed by plate count analysis (see method below).

2.2. Strains and culture conditions

Five strains of *B. bruxellensis* were selected, all isolated from wines and representative of the three genetic groups found in Bordeaux (Cibrario, Avramova, et al., 2019): the strains AWRI 1499 and CRBO L0424 are part of the 1st Wine 3N group. This group gathers triploids strains that can grow easily in different wines. CBS 2499 and CRBO L0611 belong to the Wine 2N group, which contains diploids strains; previous studies showed that they have more difficulties than their counterparts to develop in every wine. Finally CRBO L0422 who represented the Wine/Beer 3N group is a triploid strain that showed intermediate growth in wine compared with the others ones (Avramova et al., 2018; Cibrario, Miot-Sertier, et al., 2020).

These strains were gradually adapted to all 53 wines at 25°C before inoculation at 10^2 CFU/ml according to previous work (Cibrario, Miot-Sertier, et al., 2019) (figure 1.). The wines were then "aliquoted" into as many 5 ml tubes as necessary. The tubes were filled to their maximum capacity in order to limit the head space and oxygen input and then, incubated without any agitation at 20°C. A tube was removed from the device at each sampling (every week over a two to three months period).

2.3. Counting methods

Cultivable cell concentrations were determined by colony counts on solid medium using serial dilutions plating on YPG medium (yeast extract 10 g/l, peptone 10 g/l, glucose 20 g/l, agar 20 g/l). The pH was adjusted to 4.8 before sterilization (20 min at 121°C and 1 bar). At least two dilutions and three counts per sample were performed. Results were expressed as CFU/ml based on weighted mean calculations (AFNOR, 1998) and a detection limit of 33 CFU/ml could be achieved.

Flow cytometry was also used to determine the concentration of *B. bruxellensis* in the adaptation wines to determine the inoculation volume necessary to ensure 10^2 CFU/ml in the inoculated wines. Cells were stained with propidium iodide and carboxyfluorescein diacetate succinimidyl ester (cFDA) and then incubated for 10 min at 37°C. Flow cytometry analysis was carried out using CytoFLEX (Beckman Coulter, Fullerton, Californie).

2.4. Growth analysis

Based on the growth curve obtained for each wine, four values were extracted: lag phase, maximal growth rate, maximum population reached, and duration of experiment.

The lag phase was defined as the time between inoculation of the wine and growth beginning. For the strains that showed no growth (under the detection limit of 10^2 CFU/ml), a fixed value of 200 days was attributed for the lag phase.

The growth rate at time t was calculated using the following equation:

$$\text{Growth rate (t)} = \left(\frac{1}{(Day_{t+2} - Day_{t-1})}\right) \times \ln\left(\frac{Population_{t+1}}{Population_{t-1}}\right)$$

The maximal growth rate value obtained over the whole experiment was then searched.

We considered that the stationary growth phase was reached when three consecutive samples displayed the same microbial population. The value for the maximal population was set to the mean of these three populations values, and the experiment duration corresponded to the third week showing the highest population.

2.5. Statistical analysis

All statistical analyses were performed using RStudio (version 1.4.1717, RStudio Team, Boston, MA, USA) with a significance level of 5%. Assessment of the factor's significance was measured using ANOVA, the normality was checked using normality and Levene's test. A HAC was realized using the package ggdendro (R package version 0.1.23). Multivariate analysis was performed with the packages ade4 (R package version 1.7.18) and ggpubr (R package version 0.4.0).

2.6. ^{1}H-NMR analysis

^{1}H-NMR experiments were performed on an Avance III NMR spectrometer (Bruker, France) operating at 600.27 MHz, equipped with a 5 mm TXI probe with Z-gradient coils. All measurements were performed at 293 K, using Topspin 4.0.8 software (Bruker, France). Three magnetic pulse sequences were used: zg30 to determine the resonance frequency of the water signal; zgpr and noesygpps1d for the suppression of the water, and water/ethanol signals with 8 and 32 ns (number of scans), respectively. Regarding data acquisition parameters, free induction decay (FID) was collected in a time domain (TD) of 64K data points, with a spectral width (SW) of 16 ppm, an acquisition time (AQ) of 3.40 s, and a relaxation time (RD) of 5 s per scan.

The FID was multiplied by an exponential function corresponding to a line broadening factor of 0.3 Hz before Fourier transformation. Manual phase and baseline correction was applied to the resulting spectrum, which was then manually phased and zero aligned using the TMSP signal.

Forty five compounds were quantified based on previous studies (Le Mao et al., 2021), including 12 organic acids, 4 esters, ketones, and aldehydes, 5 sugars, 5 phenols and polyphenols, 8 alcohols and polyols, and 11 amino acids. The full list is presented in Table S2. The compounds were quantified by spectral deconvolution using MestReNova 12.0 software (Mestrelab Research, Spain), then mean-centered per compound and scaled to unit variance.

3. Results and discussion

3.1. Wine permissiveness evaluation

3.1.1. Growth profile

The five strains were adapted and inoculated into 53 wines and yeast cultivable concentration was followed as long as necessary (from 35 to 120 days of experiment). Different growth curves were observed, and their comparison enabled to distinguish into four distinct profiles (figure 2.).

In wines displaying profile 1, the growth of the five strains started immediately with no lag phase, and the maximal population was reached very quickly (less than 2 months). The five strains reached a population above 10^6 CFU/ml. The wines in this group were defined as very permissive ones. This group gathered most of the wines, i.e. 29 out of 53 (55%). Profile 2 gathered wines where at least one of the five strains showed some difficulties to grow, with a lag phase before growth. This, associated most often with a slower growth rate for the diploid strains, lengthened the whole experiment. On the other hand, the maximal population was not affected when compared with wines exhibiting profile 1. This profile concerned 11 wines (21%). In the wines with profile 3, no growth was observed for the diploid strains, while the triploid ones first showed a significant lag phase and then grew to a maximal population as high as in profiles 1 and 2. Few wines formed part of this group, only 6 out 53 (11%). For wines classified into profile 4, no growth was observed, or only for one strain, and a drop of the cultivable population under the detection threshold (10^2 CFU/ml) could often be observed after the first week of experiment. A fifth profile was observed, in which four out of five strains never managed to survive the adaptation process. Because it only concerned four wines out of the 53 studied, we decided to merge profiles 5 and 4. These were defined as non-permissive wines. This group gathered the remaining 7 wines (13%).

Each wine profile is indicated in table S1 and all growth curves are represented in figure S1. The five-growth profiles examination enables a first classification of the wines and clearly shows that the Bordeaux red wines examined are not equal regarding their ability to support *B. bruxellensis* growth. This also confirms that some *B. bruxellensis* strains (i.e. the triploid ones) are better suited to the constraints encountered in Bordeaux wines. From a practical point of view, the differences observed are very important. Considering the volatile phenol production rates described in the literature, wines with profile 1 could be spoiled by phenol concentrations above the perception thresholds in 3 to 4 weeks. It would take 40 to 75 days in profile 2 wines and 75 to more than 120 days in profile 3 wines. The phenomenon would not occur in 120 days in profile 4 wines.

3.1.2. Notation of wine permissiveness towards *B. bruxellensis*

To produce a finer classification of the wines, a notation system based on growth parameters was created. The score was designed to be very low for non-permissive wines and it increased with the wine ability to support *B. bruxellensis* growth (as the risk for the winemaker increases). Four growth parameters were considered: the lag phase (=Lag), the maximal growth rate (=Rate), the maximal population reached (=Pop) and the time it took for the strains to achieve it (=Time). A correlation matrix was built on those parameters (figure S2), and, as the time and lag phase were heavily correlated (correlation value of 0.99), the time was removed from further analysis. Lower correlation was observed for the lag phase and growth rate (correlation value = -0.83), but maximal population was not correlated with any other parameters (correlation value lower than 0.8).

To create the notation, each growth curve (one strain in one wine) was examined separately: a total of 53 x 5 curves was thus considered. Each parameter was represented on a separate histogram, and sub-groups deserving the same note could be made by visual similarity. Every histogram is given in supplementary data (figure S3). Every subgroup was attributed a note representing its effect on permissiveness: 0 for the group with the longer lag phase, minimal population, and lowest growth rate, and 3 for the groups with the absence of lag phase, highest population, and growth rate. All parameters were divided into 4 groups (note 0 to 3), except for population for which only 3 groups could be obtained (note 0 to 2, figure S3). The resulting notation grid is given in table 1.

Table 1. Notation grid

Note	Lag phase (days)	Maximal growth rate ($days^{-1}$)	Maximal population (CFU/ml)
0	200	0	100
1	43 < & < 200	< 0.4	10^2 < & < 10^6
2	0 < & < 43	0.4 < & < 0.6	>10^6
3	0	> 0.6	

A high note indicates that *B. bruxellensis* growth is easy and efficient in the wine considered. Four levels are considered for the lag phase and the maximal growth rate and three only for the maximal population.

After every parameter value was noted from 0 to 2 or 3, a sum was done to obtain a note for the curve (= Sum 1, figure 3.). Another sum was calculated by combining the “Sums 1” of the five strains in a given wine, to produce the score for this wine (= Sum 2, figure 3.). The 53 wine scores ranged from 0 to 36: the most permissive one did not achieve the maximal score possible of 40, because surprisingly, in the studied wines where growth was immediate and rapid, the maximal population did not reach

10^7 CFU/ml. On the contrary, 4 wines received the minimal possible score of 0. However, most of the wines could be considered as permissive (table S1).

Independently of the score construction, a hierarchical clustering (HAC) of the growth curves obtained was also done. The best separation was a partition into 3 groups (figure 4. and figure S4). These groups were then confronted with the wine profile and the score described above. When compared, the first HAC group contained the most permissive wines since it gathered the ones with profile type 1 or type 2, and their scores were above 22 except for W_20 which had a score of 20. On the contrary, group 3 gathered the less permissive wines with score below 9 and profile 4 except for W_12 (profile 3 where two out of three triploid strains managed to grow after a lag phase of about 40 days). The group 2 was composed of intermediate wines with a score between 13 and 21 that had profile 3, with one exception, W_21 (profile 4 where no strain exceeded a population of 10^4 CFU/ml).

This three-group classification method and the manually obtained score thus appear to be consistent and validate the hypothesis that Bordeaux red wines are not equal regarding *B. bruxellensis* permissiveness.

3.1.3. Alcohol, pH and wine permissiveness

Alcohol content variation could be observed between the wines, especially between the two vintages: the average ABV was 13.86% vol. for 2020 and 12.83% vol. for 2021. As previous authors mentioned it before, the ethanol impacted the overall growth of *B. bruxellensis*. Wines showing profile 1 or 2 tended to have lower alcohol content than the ones with type 3 or 4 profile (figure 5A.). However, ABV was not sufficient to explain the permissiveness: the wine with the highest ABV observed during this work (15.6% vol., wine 21, vintage 2021) was not the less permissive one and belonged to the intermediate HAC group (figure 5A. and table S1.). The pH of wines was not significantly different between wine HAC groups (figure 5B.).

To measure the impact of those two factors an ANOVA was performed on the three growth parameters (Lag, Rate and Pop). The results are shown for three of the studied strains, one in each genetic group considered in this study (figure 6.). As previously shown in figure 5, the alcohol had a bigger impact than the pH, and its most visible effect was on the lag phase. The effect of ABV was less significant on growth rate and above all on the maximal population observed. The CRBO L0611 was the strain most sensitive and 75% of the differences observed for the lag phase were due to the ABV. The two other strains were slightly less sensitive to the ABV as previously mentioned by Cibrario et al (2020).

To focus on factors others than ABV or pH such as vintage, winery, grape variety or wine composition, inoculation of the same five strains of *B. bruxellensis* was done in standardized wines, i.e. wines whose pH and TAV values have been reduced to the same value.

3.2. Standardized wines permissiveness evaluation

3.2.1. Determination of standardized conditions

For the 2020 wines, the pH was adjusted to a median value of 3.5 and the ethanol concentration was adjusted to 15% vol. since one of the wines displayed an ethanol concentration as high as 15.6% vol. This high standardized ABV completely modified the wine classification: among the non-standardized wines, 29 displayed profile 1, 11 profile 2, 6 profile 3 and 7 showed profile 4. After ABV standardization to 15% vol., only 6 vintage-2020 wines out of 23 supported *B. bruxellensis* growth, and for three of them, the maximal population reached was lower than in their non-standardized counterpart. An ABV of 15% vol. considerably reduced the risk of alteration and completely smoothed the wine permissiveness. It made the analysis of the effects of other factors impossible. Moreover, such a high ABV is contrary to societal expectations and was not observed in vintage 2021 studied wines. Therefore, the vintage 2021 wines ABV and pH were standardized at 14% vol. and 3.5 respectively. The 2020 non-standardized wines with ABV 14 ± 0.2 % vol. were also included in the "standardized" wine panel for further study.

3.2.2. Standardized wines profile and notation

The standardized wines were inoculated and, after growth monitoring, profile evaluation, manual scoring and HAC were performed. The classification criteria were the same as those used for non-standardized wines.

Generally speaking, the raise of wine ABV to 14% vol. induced a lag phase or slowed down the growth, at least for the two diploid strains studied (CRBO L0611 and CBS 2499). The alcohol content clearly exerted a high pressure on these diploid strains and as a result, it decreased the risk of alteration, as none of the wines remained very permissive, i.e., no wine displayed profile 1 anymore. Standardization did not induce any profile change for 7 out of 36 wines; 13 wines displayed a +1 change, 14 wines a +2 change, and finally, yet importantly, two wines went from profile 1 to profile 4 (table 2 and figure 7A.). The wine score was also modified by standardization and it decreased for most of the wines (figure 7B).

Table 2. Notation of the standardized wines (ABV = 14% vol. and pH = 3.5)

Wine_ID	Score S	HAC groups	Profile S	Winery	Variety	Vintage
W_36	0	3	4	C	Me	2021
W_19	5	3	4	G	Me	2020
W_50	5	3	4	H	Cs	2021
W_52	6	3	4	H	Me	2021
W_27	12	2	3	A	Cs	2021
W_32	12	2	3	C	Cs	2021
W_51	12	2	3	H	Me	2021
W_34	13	2	3	C	Me	2021
W_48	13	2	3	G	Me	2021
W_47	14	2	3	G	Cs	2021
W_53	14	2	3	H	Me	2021
W_11	15	2	3	D	Cs	2020
W_29	15	2	3	A	Cs	2021
W_33	15	2	3	C	Cs	2021
W_44	15	2	3	G	Cf	2021
W_08	16	2	3	A	Me	2020
W_49	16	2	3	H	Cf	2021
W_41	17	2	3	D	Me	2021
W_26	18	2	3	A	Cs	2021
W_35	18	2	3	C	Me	2021
W_38	18	2	3	D	Cs	2021
W_39	18	2	3	D	Cs	2021
W_42	18	2	3	D	Me	2021
W_37	19	2	3	D	Cs	2021
W_40	19	2	3	D	Me	2021
W_43	19	2	3	D	Me	2021
W_28	20	2	3	A	Cs	2021
W_24	21	2	3	A	Cs	2021
W_23	25	1	2	H	Me	2020
W_25	25	1	2	A	Cs	2021
W_46	26	1	2	G	Me	2021
W_30	27	1	2	A	Me	2021
W_31	28	1	2	A	Me	2021
W_45	28	1	2	G	Me	2021
W_06	30	1	2	A	Me	2020
W_05	31	1	2	A	Me	2020

However, it had mostly no impact on the triploid strains, which shows again that these are tolerant to many wine matrices and more dangerous. As the AWRI1499-like *Brettanomyces* strains represent about 50% of the strains found in Bordeaux vineyard, the level of alcohol is not sufficient to predict the global risk of "*Brett*" spoilage. From the 36 standardized wines examined, 3 HAC groups were still

observed: one group gathering the 8 most permissive standardized wines (score 25 to 31), one group gathering 24 intermediate wines and one group of 4 non permissive wines (score equal to or lower than 6). Other factors than ABV and pH may thus modulate *B. bruxellensis* growth, either inhibitors or elements promoting yeast growth.

3.2.3. Influence of factors other than ABV and pH on wine permissiveness

ANOVAs were performed on the three growth parameters previously studied and on the score value obtained after doing the first addition ("Sum 1", figure 8A.) or on the global score (figure 8B.).

Most factors had a significant impact on the growth parameters studied. At first sight, the growth of *B. bruxellensis* in a wine (sum 1) seemed to depend for 1/3 on the strain, 1/3 on the wine and 1/3 on other factors not studied here. The influence of the yeast strain present was particularly striking on the lag phase. The wine itself, (i.e. the combination of the vintage, the winery, the variety and the batch), was also an important parameter, as it explained more or less the same part as the strain of the differences observed on the rate, the pop or the sum 1. However, a significant proportion of differences (about one third) was not explained by the examined factors, especially when considering the maximal population for which up to 46% of the information was due to the residuals. Indeed, the maximal population displayed a sort of step variation (see profile examination, section 3.1.1) and the ANOVA performed did not allow to identify what induced the shift from 10^7 CFU/ml to 10^6 CFU/ml or to the detection limit.

Inside the wine factor, the winery and the batch seemed more discriminative than the variety and the vintage, suggesting that "terroir" or winemaking practices may modulate the risk of *B. bruxellensis* development (figure 8A.). This is also the case when considering what affects the global score for a given wine (figure 8B.). Nevertheless, one of the five wineries studied (winery G) was chosen for being completely "immune" to *B. bruxellensis* according to previous work (Cibrario, unpublished). This tendency was confirmed in the 2020 wines (wines 16 and 17) but not in 2021, as wines sampled in this domain proved to be among the most permissive (wines 44, 45 and 46, table S1). Even after raising the alcohol level, those wines were still permissive or became intermediate.

3.3. Quantification of common compounds by ^{1}H NMR

In order to better understand what could make a wine permissive, the wine composition was examined. Forty-five compounds regularly found in wine were quantified by ^{1}H NMR-based metabolomics. After scaling, the concentration of each compound present in permissive and less permissive wines was compared (figure S5), the wines considered as intermediate were not included. To evaluate the differences, a Wilcox test was performed on those data. Out of the 45 compounds, 43

displayed concentrations higher than the quantification threshold, and only one showed a p-value under 0.05: ethyl lactate. This compound is generally assumed to come from the esterification of lactic acid by yeast or bacteria and is described to be more abundant in wines that conducted co-inoculation (Virdis et al., 2021) and probably in wines where malolactic fermentation begins early, before the end of the alcoholic fermentation. The ethyl lactate seems to be more present in non-permissive wines (mean = 128.5 mg/l) than in permissive ones (mean = 91.2 mg/l). Although not significant (p-value 0.11), lactic acid also appears to be more abundant in the less permissive wines, which goes in the direction of a link between effective malolactic fermentation and low permissiveness. However, malic acid could not be quantified by ^{1}H-NMR to support this hypothesis, because being under the limit of quantification. Nevertheless, these results are coherent with what observed in the wineries, where co-inoculation of yeasts and bacteria starters can speed up the implantation of bacteria, thus leaving less microbiological space for *B. bruxellensis* to develop. A PCA was also performed on the 43 quantifiable compounds, and connected with the score observed in the standardized wines (figure 9A.). No permissiveness classes could be separated. Surprisingly, the ethyl acetate did not participate in the separation between samples on the PCA (figure 9B.). These analyses (figure S5 and figure 9.) also confirm that the carbohydrates present (glucose, fructose or arabinose) do not enable to predict the wine permissiveness, as previously suggested by (Cibrario, Perello, et al., 2020). The wine effect may therefore be due to other components than those analyzed by this method.

4. Conclusion

This study shows that diversity regarding wine ability to promote *B. bruxellensis* growth does exist among Bordeaux red wines and wineries. Indeed, depending on the wine and the strain present, the time before wine spoilage becomes noticeable ("*Brett* smell") can vary from one to more than 4 months. This study has showed that the differences were not predictable by any compound currently quantified by ^{1}H-NMR. And, if the pH, in the range that was currently reached in Bordeaux wines in the recent years, has no major effect on the yeast growth, the ABV above 14% vol. clearly decreases the wine permissiveness. The wine effect must now be explored by going further with the wine composition analysis by quantifying more chemical compounds. A deeper analysis of the winemaking process and what differs among the wineries would be also interesting to discover other factors that could promote or demote the yeast growth.

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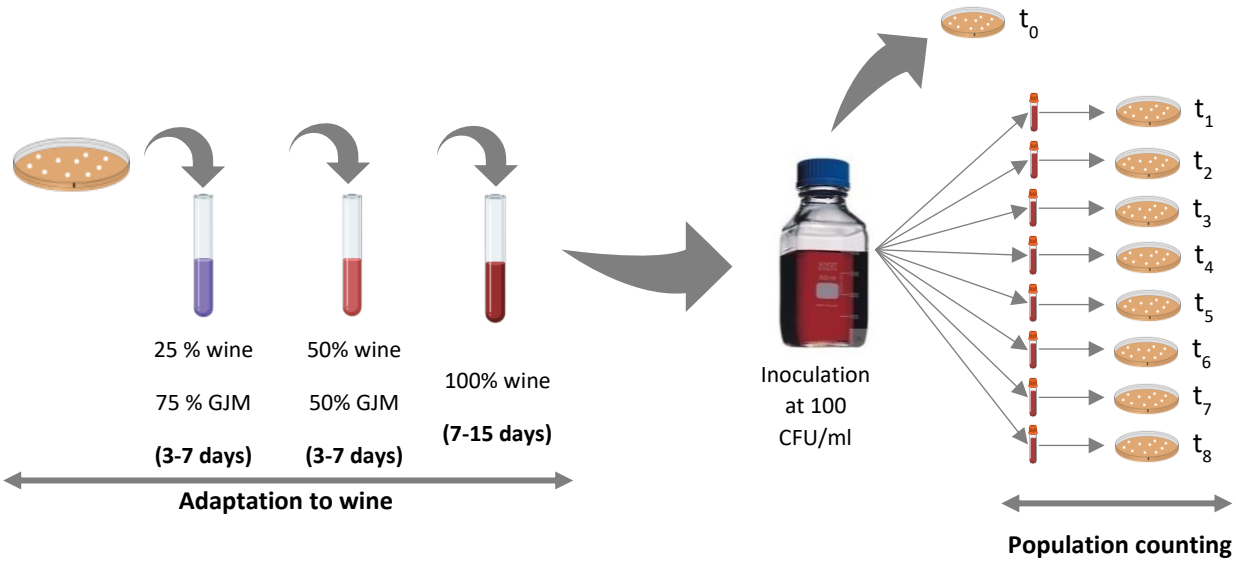

Figure 1. Protocol for strain adaptation to the wine and growth monitoring. GJM = Grape Juice Medium.

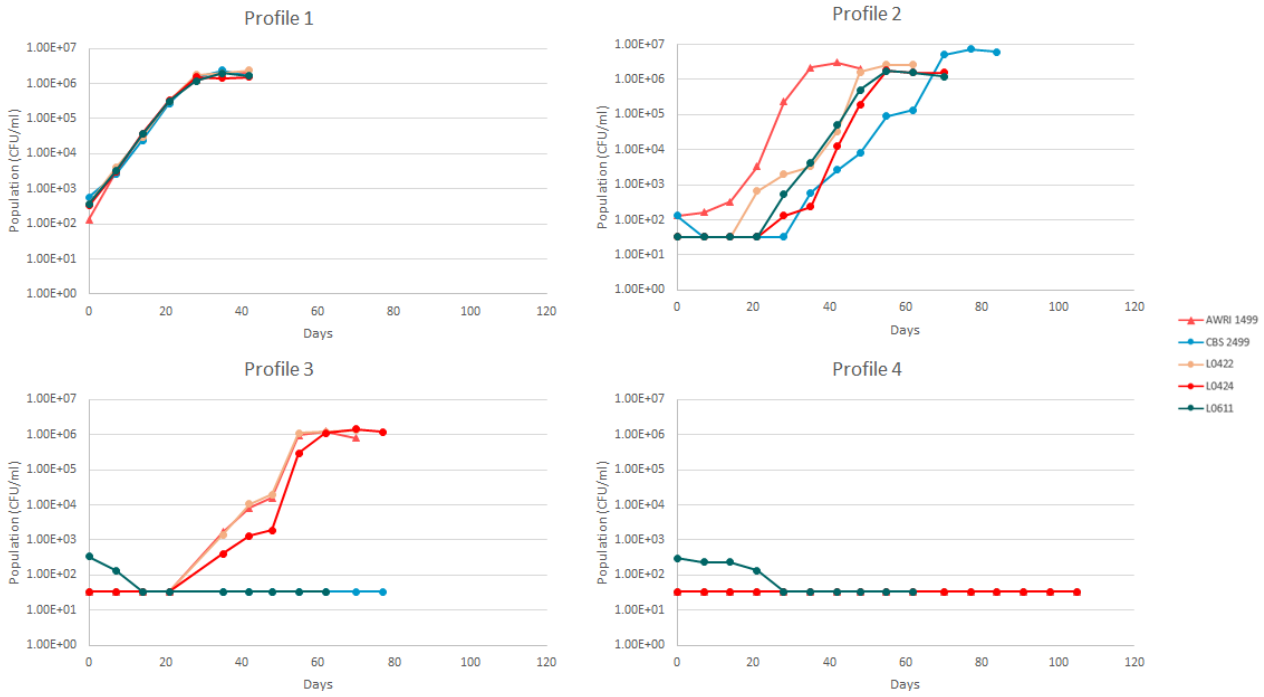

Figure 2. Distinct growth profiles observed. For each profile, the results obtained with one representative wine is shown. In profiles 1 and 4, all the strains display the same behavior, while they distinguish in wines with profile 2 and 3. (AWRI 1499 = light red (triangle), CBS 2499 = cyan, CRBO L0422 = orange, CRBO L0424 = rouge, CRBO L0611 = olive green).

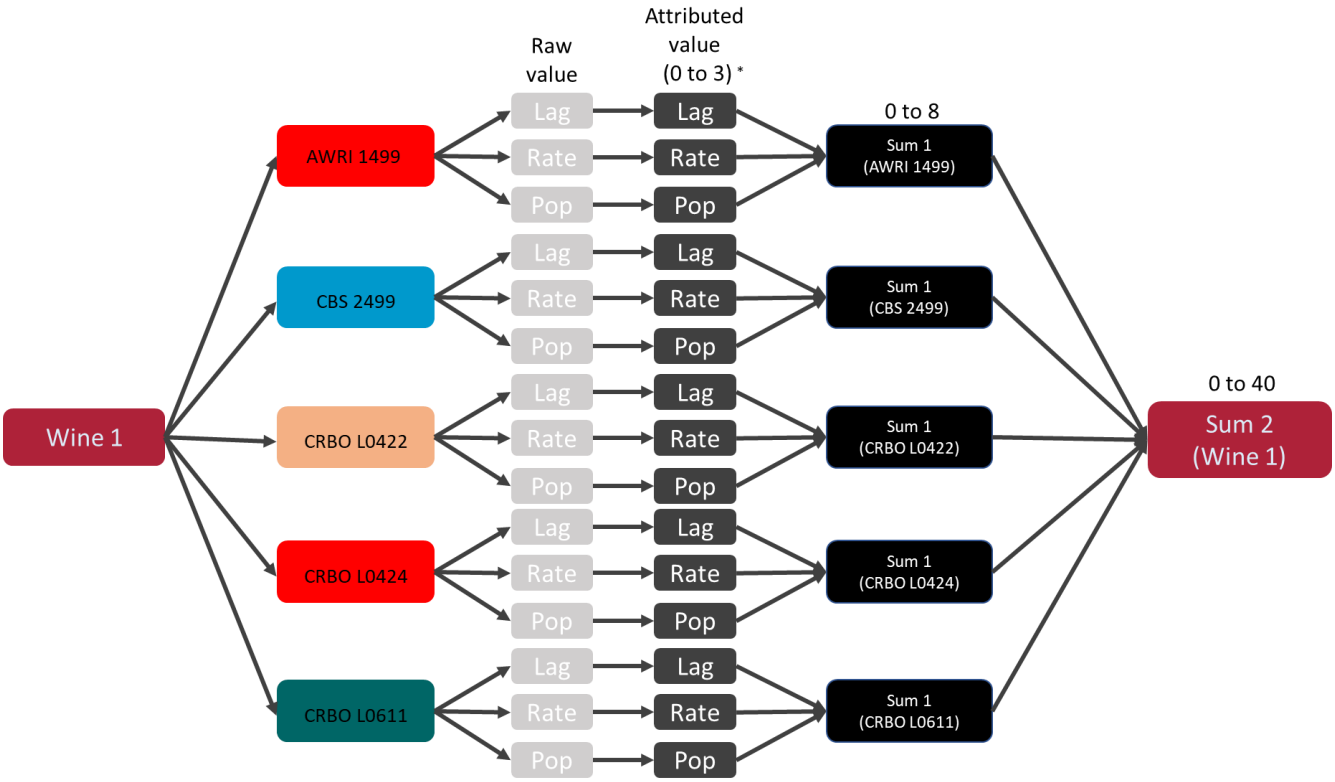

Figure 3. Notation process for one wine, based on five *B. bruxellensis* strains growth curves.
*Except for maximal population which is noted from 0 to 2.

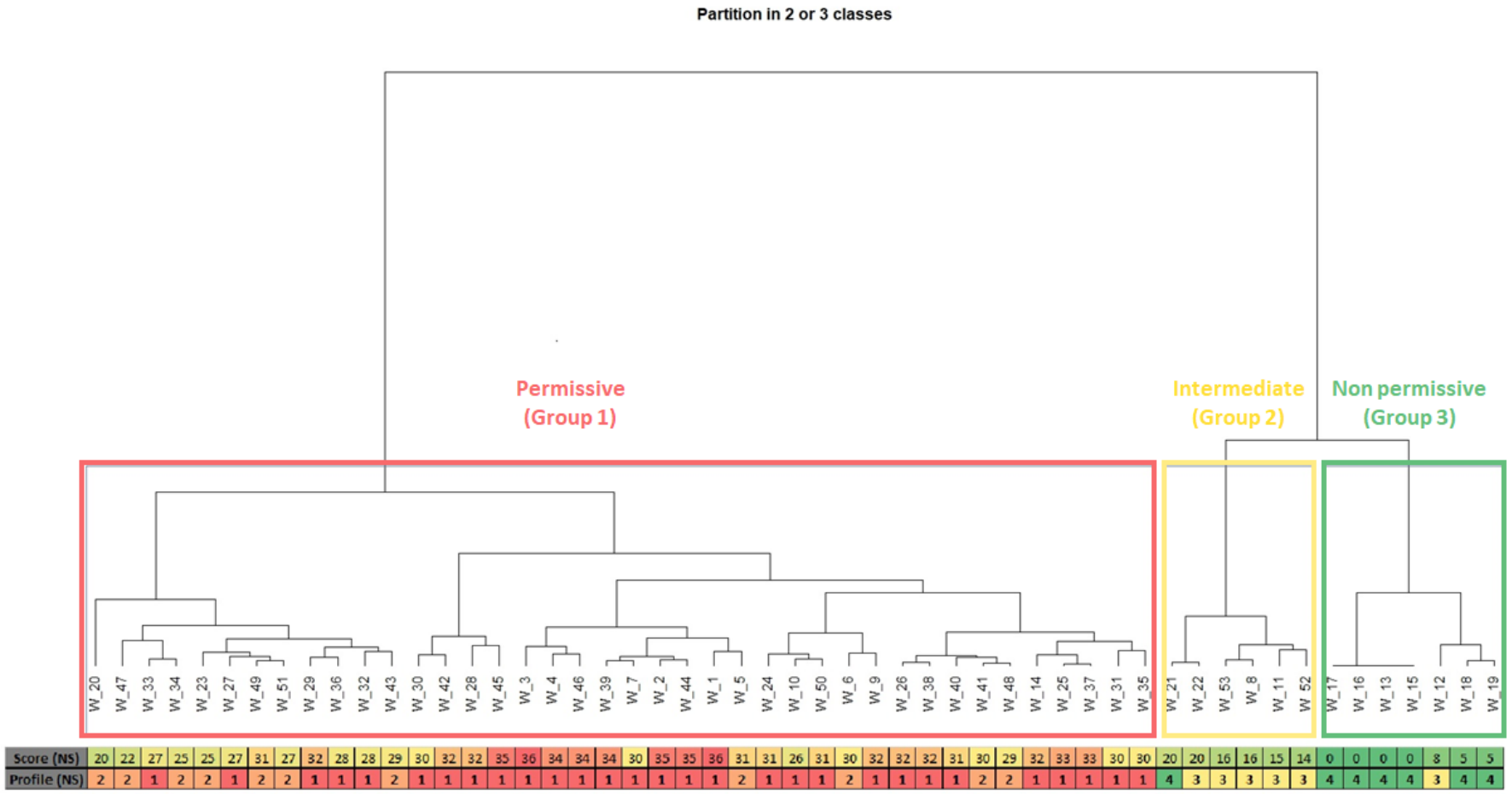

Figure 4. Classification obtained with HAC divided into 3 classes based on the growth parameters of non-standardized wines. The manual score value and the profile are indicated under each wine.

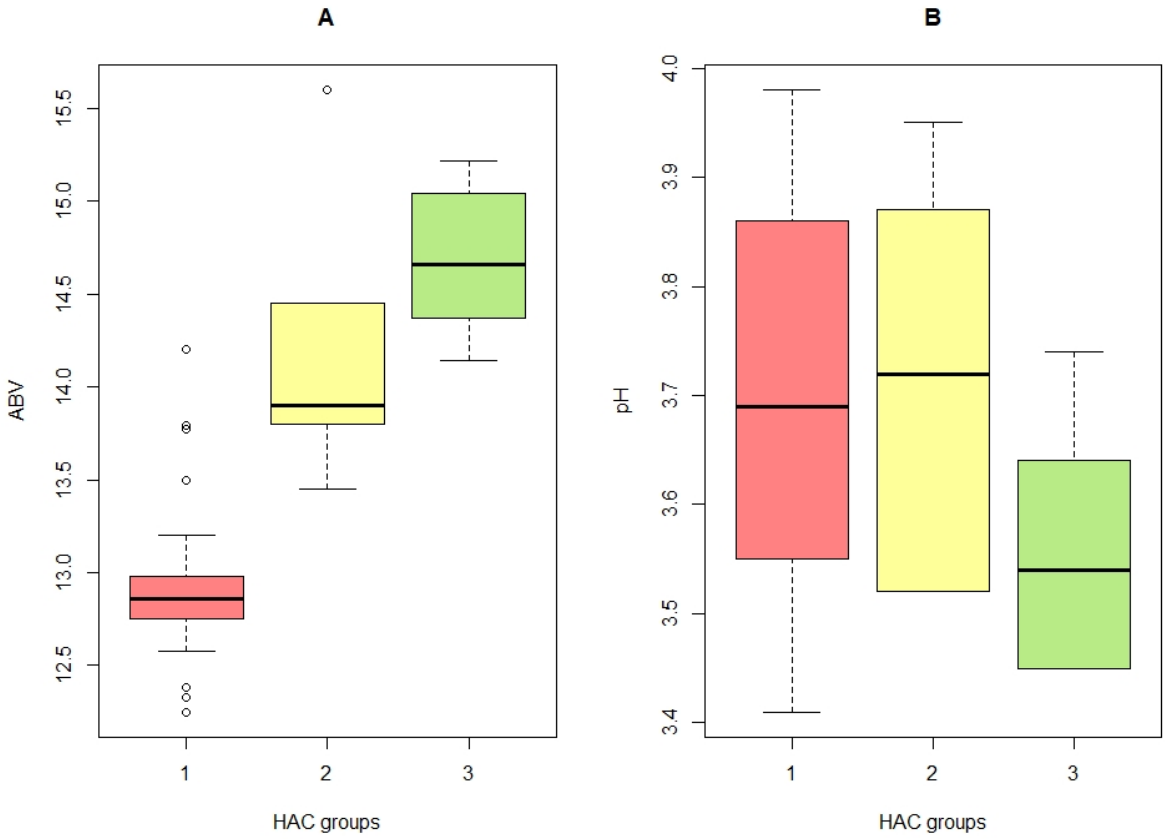

Figure 5. ABV (A) and pH (B) dispersion for each wine subgroup (group 1 = red (permissive), group 2 = yellow (intermediate), group 3 = green (non permissive)).

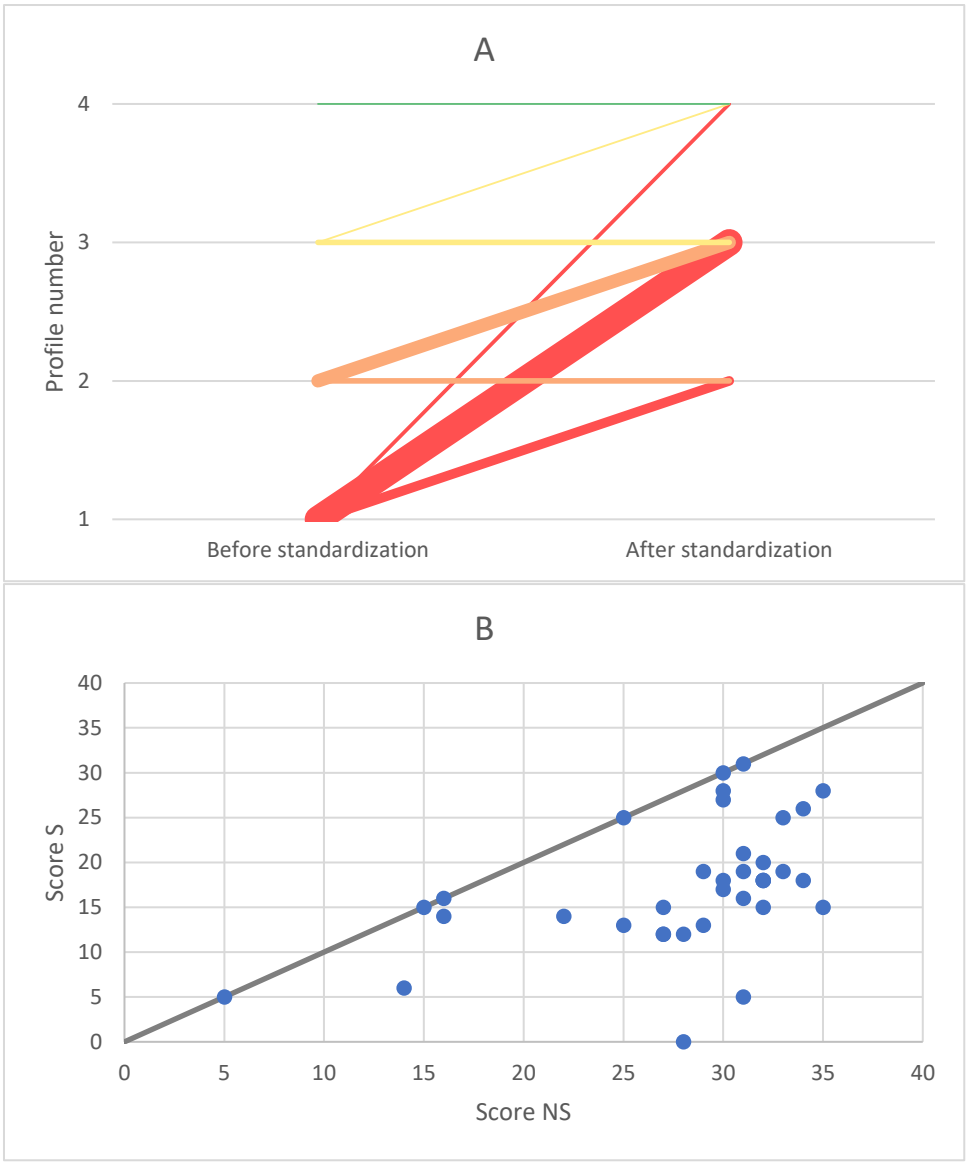

Figure 6. Changes induced by standardization of ABV to 14% vol. and pH to 3.5.

A. Growth profile: the profile observed in the non-standardized wines (1 to 4) is linked to that observed for the same wine after standardization (2 to 4, no profile 1 observed anymore). The line width is proportional to the number of wines concerned by the profile change.
B. Correspondence between the scores in the non-standardized (NS) wines and their standardized (S) counterpart. The line represents the point theoretical position for wines with no score change.

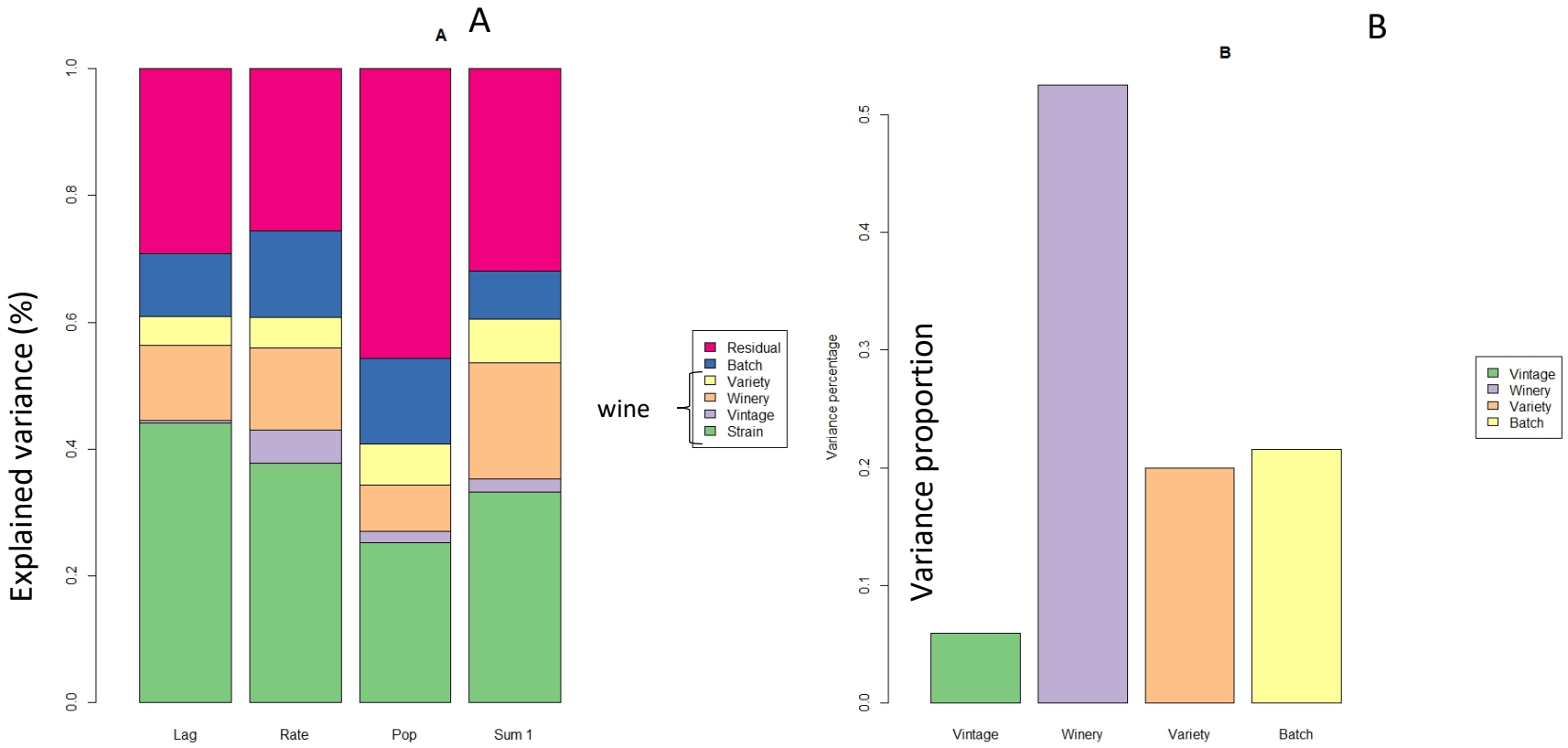

Figure 7. A. ANOVA to discriminate the factors modulating *B. bruxellensis* growth parameters in standardized wines (ABV 14% vol., pH 3.5). B. ANOVA to evaluate the impact of each factor on the score. Since each wine had only one value, the residuals were less than 0.1% and are not represented.

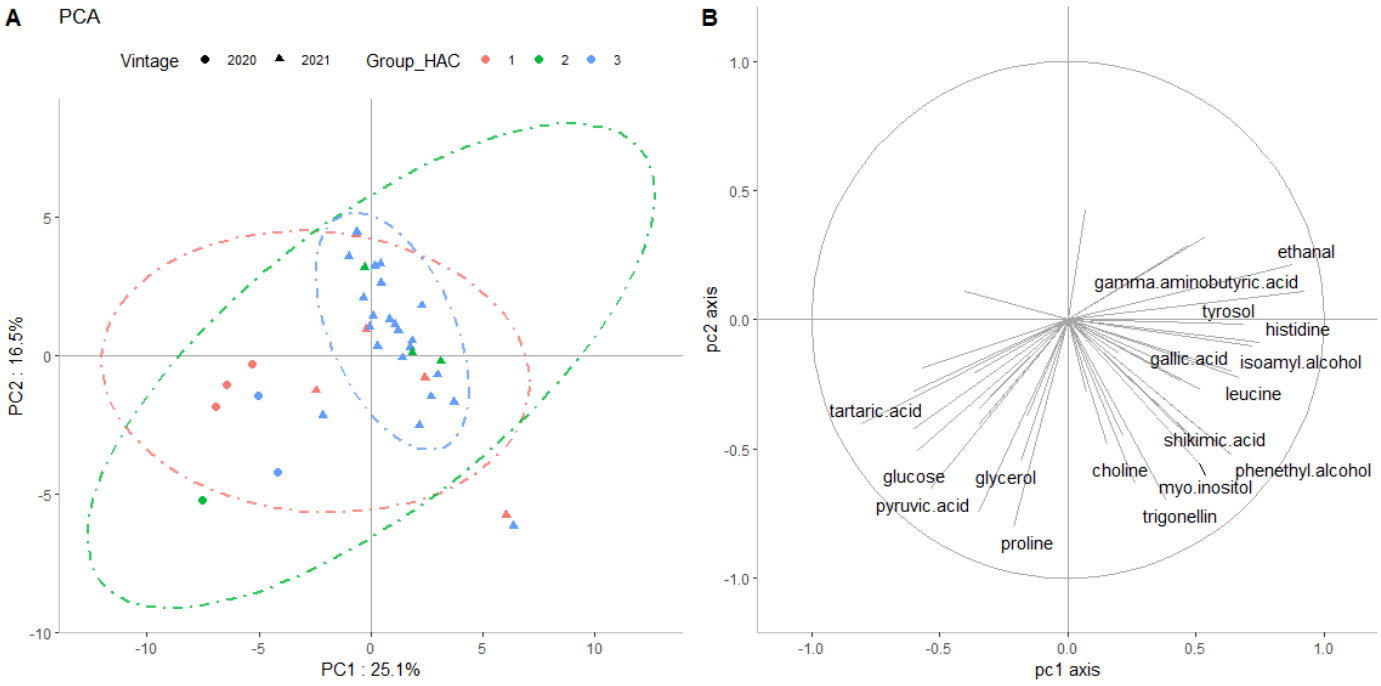

Figure 8. A. Principal Component Analysis (PCA) on 43 compounds quantified by ^{1}H-NMR. Each point corresponds to a wine colored according to its HAC group and shaped according to the vintage. B. Loading plot where the compounds contributing to more than 80% of the distribution are shown.