Effect of alternative fungicides and inoculation strategy on yeast biodiversity and dynamics from the vineyard to the winery

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Abstract

Fungi and oomycetes found in vineyards cause diseases such as powdery and downy mildew. Consequently, conventional and alternative agronomical practices are widely used prior to harvest to protect grapes. Alternative products are considered more eco-friendly and environmentally sustainable in comparison to conventional chemical products. However, the effect of these alternative products on yeast ecology, from the vineyard to the winery, is poorly understood. This study compared the effect of alternative and conventional chemical antifungal compounds (copper and sulphur based) on grapes' mycobiota in the vineyard and during subsequent fermentation in winery using culture-dependent and -independent approaches. Culture-dependent data indicated a treatment-dependent effect on the load and diversity of yeast populations on grapes. It was found that the population of *Hanseniaspora uvarum* was higher on grapes previously treated with laminarin and copper, compared to the other levels registered on grapes previously treated with the rest of antifungal products tested in this study (including the untreated and conventional treatment controls). Concerning, wine quality, the chemical composition was not correlated to the application of antifungal treatment in the vineyard. Understanding the effect of different antifungal products on grape and wine microbial communities may help in setting up guidelines for wine grape production. These guidelines, can be used to guarantee quality in the pursuit of a sustainable competitive advantage in the market.

Keywords: Antifungal compounds, Grapes, Wine, Mycobiota, Fermentation, Sustainability

1. Introduction

Fungal and oomycete diseases, including grey mould (caused by *Botrytis cinerea*), black rot (caused by *Guignardia bedwelli*), downy (caused by *Plasmopara viticola*) and powdery (caused by *Uncinula necator*) mildew are considered the most serious grapevine diseases worldwide, able to cause a significant reduction in crop yields and poor quality grapes (Reynier, 2012). Chemical compounds, such as copper and sulphur-based products are commonly used in conventional and organic vineyard to control fungal infection (Gadoury et al., 2012). However, the use of several pesticides in the field has been associated with health (respiratory, neurological, carcinogenic effects etc.) and environmental (environment and water pollution) issues (Nicolopoulou-Stamati, Maipas, Kotampasi, Stamatis, & Hens, 2016). Thus, the European Parliament and the council of the European Commission in 2009 reduced the number of pesticides that can be applied in the field (Directive 2009/128/EC).

BIOcontrol products containing natural substances of mineral, plant or microbial origin (such as laminarin, chitosan, potassium bicarbonate and calcium oxide), and resistance inducers able to activate plant's own defence mechanism (such as acibenzolar-S-methyl, fosetyl-Al, potassium phosphonate) are being considered against powdery and downy mildews by an increasing number of farmers (Lukas, Innerebner, Kelderer, Finckh, & Hohmann, 2016; Trouvelot et al., 2014; Romanazzi et al., 2016; Pugliese, Monchiero, Gullino, & Garibaldi, 2018).The effect of biofungicides, resistance inducers and biostimulants (Gadoury et al., 2012; Gutiérrez-Gamboa, Romanazzi, Garde-Cerdan, & Perez-Alvarez, 2019) have recently been tested for their efficacy towards fungal diseases in the vineyard (Rantsiou et al., 2020). Interestingly, potassium bicarbonate reduced powdery mildew on Nebbiolo grapes at harvest with an efficiency similar to conventional chemical compounds (Rantsiou et al., 2020). Nevertheless, it is important to investigate the effect of such alternatives on grape mycobiota at harvest.

Indigenous yeasts naturally occurring on the grape surface have an impact on wine fermentation, either spontaneous or inoculated, especially in the first stages. Yeast population can reach levels up to 10^8 colony forming units (CFU/mL) at the beginning of the fermentation and their growth dynamics depend on the grape health and fermentation conditions (Barata, Malfeito-Ferreira & Loureiro, 2012; Fleet, Prakitchaiwattana, Beh, & Heard, 2002). As a consequence of the variable nature of the grape's microbiota, an inconstant amount of biomass is produced during the fermentation process, as a consequence of the variable nature of grape's microbiota and composition (Stefanini & Cavalieri, 2018). The diversity and composition of the yeast community on the grape berries and musts is crucial to

97 produce distinctive and quality wines (Morrison-Whittle & Goddard, 2018; Liu, Zhang, Chen, & Howell,
98 2019).
99 Yeast ecology contributes largely to define regional wine characteristics which are important
100 factors for consumer acceptability (Belda, Zarraonaindia, Perisin, Palacios, & Acedo, 2017; Bokulich et
101 al., 2016; Morrison-Whittle & Goddard, 2018). Importantly, yeast biodiversity is influenced by
102 geographical location, climatic conditions of the area and health status of the grape berries, as well as by
103 the agronomical practices used during the annual cycle of the vineyard (Bokulich, Thorngate,
104 Richardson, & Mills, 2014; Gilbert, van der Lelie, & Zarraonaindia, 2014; Knight, Karon, & Goddard,
105 2020; Zarraonaindia et al., 2015). The microbial changes observed when applying conventional chemical
106 compounds in vineyards on grape berries and during subsequent fermentation has been extensively
107 investigated (Barata et al., 2012; Cordero-Bueso et al., 2011; Grangeteau et al., 2011; Milanovic,
108 Comitini, & Ciani, 2013; Schmid, Moser, Muller, & Berg, 2011). However, the ability of alternative
109 compounds to modulate the mycobiota during wine fermentations is poorly understood.
110 The goal of this study was to compare the mycobiota diversity, oenological parameters and
111 volatile organic compounds (VOCs) of grape berries and resultant wines following alternative and
112 conventional chemical compounds (copper and sulphur) treatments in the vineyard. Furthermore, two
113 fermentation series (spontaneous and inoculated with *Saccharomyces cerevisiae*) were performed in
114 winery conditions. Data and knowledge acquired may contribute in the informed decisions that should
115 be made to accommodate environmentally friendly vineyard protection against fungal disease and wine
116 quality.
117
118 **2. Materials and Methods**
119
120 *2.1 Field trials and sample sites*
121 Grape berries were collected from a vineyard located in Piobesi d'Alba (North-West Italy, GPS:
122 44.731760, 7.988324, hill area) during fall 2018. The vineyard was cultivated with *Vitis vinifera* cv.
123 Nebbiolo vines using a vertical shoot positioning training system. The distance among vines was 0.90 x
124 2.5 m. Vineyard management was uniform and in line with regional agricultural practices. In total twelve
125 experimental sites in quadruplicate were selected from the top, middle and bottom of the vineyard,
126 covering different topological profiles of the vineyard. Each experimental site included four randomized
127 blocks *per* treatment, each containing eight plants. The vines were sprayed with eleven different
128 treatments as reported in Table 1, while a non-treated vine was used as untreated control. Alternative and

conventional chemical treatments were applied using commercial formulations and sprayed with a hand-pulled 2-stroke engine sprayer to ensure total coverage of the bunches, following manufacturer's instructions and as previously reported by Rantsiou et al. (2020). Active ingredients as well as the dose used for the preparation of the treatments are shown in Table 1.

2.2 Samples collection

Grape berries were aseptically and randomly collected with the pedicel attached at maturation stage for each experimental site and block. For each block, 200 grape berries (800 in total for each treatment) were chosen from different grape bunches and immediately placed in sterile stomacher bags and transported to the laboratory at 4 °C. Once in laboratory, 50 g of single grape berries from each block were placed in a sterile stomacher bag and, after manual crushing, the resulting juice was subjected to microbiological analysis. Aliquots of 1 mL were collected, centrifuged for 10 min at 6000 rpm and the resulting pellet was placed in sterile Eppendorf tubes and stored at – 20 °C for molecular analysis.

2.3 Fermentation trials

Grapes for downstream analysis were chosen from the treatments that showed the lowest percentage of berries affected by powdery and downy mildew (this selection was based on the results reported by Rantsiou et al. (2020) and the quality of the grapes at harvest). Briefly, five alternative chemical products, namely T02, T05, T06, T07, and T10 and one conventional chemical product, namely T08 followed two different fermentation protocols, a) spontaneous fermentation; and b) inoculated fermentation with *Saccharomyces cerevisiae* BRL97 (Lallemand Inc. Montreal, Canada). In total, twenty-four fermentations (2 inoculation protocols x 6 treatments x 2 independent biological replicates) were performed in micro-scale condition in the experimental winery scale at the University of Turin. Samples were aseptically collected with sterile serological pipettes at different stages of the alcoholic fermentation (immediately after grape crushing and inoculation and after 2, 4, 7, and 14 days) for microbiological culture-dependent analysis and chemical analysis. Samples for culture-independent analysis were collected immediately after grape crushing and inoculum addition and at the end of the monitored period (14 days), placed on ice and immediately frozen at -20 °C for further DNA extraction. At the end of the fermentation, wines were analysed for basic oenological parameters, and volatile compounds.

2.3.1 Winery micro-scale fermentations

About 12 kg of grapes from each of the six abovementioned selected grapes were crushed together and distributed in 15 L glass fermenters. Two sets of fermentations were performed (spontaneous and inoculated). Inoculated fermentations were performed by using *S. cerevisiae* BRL 97 at 1.0 x 10^6 cells/mL as active dry yeast (ADY), previously rehydrated in sterile glucose solution (5 %) for 20 min at 37 ºC. Ferments were kept at 25 ºC until the end of the fermentation. The cap was punched down twice a day and racking was performed when residual sugars levels were less than 2 g/L. Afterwards, wines were clarified, supplemented with 50 mg/L of total SO2, and then bottled and subjected to chemical analysis.

2.4 Microbiological analyses

At each sampling point, samples were serially diluted in sterile peptone water solution (0.1 %) and the number of colony-forming units *per* milliliter (CFU/mL) was determined by plating aliquots of appropriate serial decimal dilutions. The non-selective Wallerstein laboratory nutrient medium agar (WLN, Biogenetics, Milan, Italy) for the enumeration of fungi and the selective medium Lysine medium agar (Oxoid, Milan, Italy) for the enumeration of non-*Saccharomyces* yeasts were used. Plates were incubated at 28 ºC for 5 days. Results were expressed as means of Log CFU/mL from two independent determinations. Yeast colonies present on WLN were counted based on their color and morphology (Cravero et al., 2016). Ten isolates from each colony morphotype were selected, purified by streaking and maintained in Yeast extract-peptone-dextrose (YPD) broth (1% yeast extract, 2% peptone and 2% dextrose, all from Biogenetics) with 25 % glycerol at −20 °C.

2.5 Molecular analysis

2.5.1 Molecular identification of the isolates

Overnight cultures of the isolates in YPD broth were centrifuged at 14000 rpm for 10 min and the resulting pellet was subjected to DNA extraction, as previously described by Cravero et al. (2016). Isolates were identified by Restriction Fragment Length Polymorphism (RFLP) analysis of the 5.8S ITS rDNA region, by using the restriction endonucleases *HinfI, HaeIII* and *CfoI* (Promega, Milan, Italy) using the protocols reported by Esteve-Zarzoso, Belloch, Uruburu, & Querol, (1999). Identification at species level was further confirmed by sequencing the D1-D2 loop of the 26S rRNA gene (Kurtzman & Robnett, 1997).

2.5.2 Molecular characterization of S. cerevisiae *isolates*

Putative colonies of *S. cerevisiae* were isolated from each sampling point during spontaneous and inoculated fermentation to verify the presence and dominance of *S. cerevisiae* BRL 97 in the inoculated trials and uncover the molecular fingerprinting of the indigenous *S. cerevisiae* strains in spontaneous fermentation. In total 580 putative *S. cerevisiae* colonies were isolated and then characterized using the primers delta12 and delta 21, following the protocols described by Legras &Karst (2003).

2.5.3 DNA extraction, sequencing and bioinformatics

The total DNA of grape must at the beginning and end of the fermentation was extracted from 1 mL of the first decimal dilution using the MasterPure Complete DNA & RNA Purification kit (Illumina Inc, San Diego, CA) following the manufacturer's instructions. Mycobiota was analysed by amplification of the D1 domain of 26S rRNA gene using the primers and conditions described elsewhere (Mota-Gutierrez et al., 2019). Briefly, PCR was carried out using a PCR mixture prepared with 12.5 μL of the 2X Kapa HiFi HotStart ReadyMix Taq (Roche, Milan, Italy), 1 μM each primer, 2.5 μL of DNA template, and PCR-grade water. Each PCR assay was performed according to the following amplification conditions: thirty cycles of 30 s of denaturation (95 °C), 30 s of primer annealing (55 °C), and 30 s of primer extension (72 °C), followed by a final extension step (72 °C) of 10 min. The PCR products were purified twice using the Agencourt AMPure kit (Beckman Coulter, Milan, Italy). Library preparation and sequencing was performed according to the Illumina guidelines. Sequencing was performed using a MiSeq instrument (Illumina).

2.5.3.1 Microbial community and dynamics

After sequencing, raw reads were analyzed by using the Quantitative Insights into Microbial Ecology QIIME2 (Bolyen et al., 2019). Primers and adapters were first trimmed by using Cutadapter and then quality filtered using the DADA2 package (version 1.10.1; Callahan et al., 2017), removing low-quality bases, chimeric sequences, and sequences shorter than 300 bp by using the dada2 denoise-paired plug in of QIIME2. Amplicon Sequence Variants (ASVs) generated by DADA2 were mapped against the constructed 26S database for fungi (Mota-Gutierrez, Ferrocino, Rantsiou, & Cocolin, 2019) by means of the RDP Classifier. To avoid biases due to the different sequencing depth, ASVs tables were rarefied to the lowest number of sequences per sample. The ASVs table displays the higher taxonomy resolution that was reached; when the taxonomy assignment was not able to reach species level the genus was displayed. Only ASVs with relative frequency above 1 % in at least two samples are reported.

225 *2.6 Chemical analyses*

226 Main oenological parameters (glucose, fructose, glycerol, organic acids expressed as g/L and
227 ethanol expressed as %v/v) were measured during and at the end of the fermentation process, using a
228 high-performance liquid chromatography (HPLC) apparatus (Rolle et al., 2018). Total acidity of wines
229 (expressed as g/L of tartaric acid) was determined by titration, following the OIV-MA-AS313-01:R2015
230 official method (OIV, 2015). The pH was measured using an InoLab 730 pH meter (WTW, Weilheim,
231 DE). Volatile organic compounds (VOCs) in wines at the end of the monitored fermentations were
232 identified using a headspace solid-phase microextraction coupled to gas chromatography-mass
233 spectrometry (HS-SPME/GC-MS) following the protocols reported by Englezos et al. (2019a).
234
235 *2.7 Statistical analyses*

236 The plate count data of the yeast populations present on grapes surface at harvest and during
237 fermentation were subjected to one-way Analysis of Variance (ANOVA), using the IBM SPSS Statistics
238 software package. When a significant difference was revealed, the Tukey-HSD post-hoc test for test
239 comparison was performed to identify statistical differences between trials. Alpha and beta diversity
240 calculations of metataxonomic data were performed through the qiime2 diversity script. Bray Curtis
241 distance matrix generated through qiime2 was used to performed Principal coordinate analyses (PCoA)
242 as well as PERMANOVA as a function of fermentation time, type or fungicide treatment. Shannon index
243 was analysed by Kruskal-Wallis test as a function of fermentation time, type or fungicide treatment. In
244 order to see if the different fungicides can affect the mycobiota composition in grapes and at the
245 beginning or at the end of the fermentations we performed differential abundance analysis by using the
246 non-parametric Kruskal-Wallis test on ASVs table in R environment. P value was adjusted by the
247 Bonferroni's method for multiple comparison. Evolution of the mycobiota during the fermentation was
248 performed through (ANOVA). When a significant difference was revealed, the Tukey-HSD post-hoc test
249 for test comparison was performed to identify statistical differences across time. Kruskal-Wallis test for
250 differential abundance on ASVs table was performed in order to see differences in the mycobiota
251 composition at the end of the fermentation process (T14) under spontaneous or inoculated fermentation.
252 Spearman's rank correlation coefficient was obtained as a measure of the association between the fungal
253 ASVs that occurred in at least 2 samples and the chemical variables through the *psych* function and
254 plotted through the *corrplot* package of R. Principal Component Analysis (PCA) of the main oenological
255 parameters and VOCs were performed to differentiate wines.
256

Accession number. The 26S rRNA gene sequences are available in the NCBI Sequence Read Archive (BioProject accession number PRJNA631884).

3. Results

3.1 Yeasts count and biodiversity on grape berries surface

The average total yeast population in grape berries after harvest ranged from 4.56 ± 0.20 to 6.25 ± 0.36 Log CFU/mL. Classical plate count revealed a significant difference in the mycobiota load of the treated grape berries (Fig. 1, Panel A, ANOVA, P = 0.038). In detail, significantly lower yeast populations were found for grape berries treated with T04 (Potassium phosphonate + Sulphur + Copper hydroxide) when compared to the controls (untreated grapes (T01) and grapes treated with Sulphur + Metiram + Copper hydroxide (T08), commonly used as conventional treatment in viticulture) (ANOVA, P = 0.0037).

PCR-RFLP analysis of the ITS1-5.8S-ITS region and further sequencing of the D1/D2 loop of the 26S rRNA encoding gene of the isolates identified a total of eight species in all grape berries previously treated with different antifungal compounds (Fig. 1, Panel B, Table S1 in Supplementary Material). *Aureobasidium pullulans*, *Cladosporium cladosporioides*, *Filobasidium magnus*, *Hanseniaspora uvarum* and *Metchnikowia pulcherrima* were present in all control and treated trials. The predominant yeast species in all grape berries were *A. pullulans* and *C. cladosporioides.* No significant differences in the relative abundance (differential counting of all colonies) of each species detected in grape samples was observed as a function of treatment by one-way ANOVA analysis. However, *Rhodotorula glutinis* was not isolated in the conventional control treatment T08 and conventional treatment T12 (Metiram + Copper hydroxide), and one alternative chemical treatment T09 (Calcium oxide). *Pichia kluyveri* and *Starmerella bacillaris* were not isolated in alternative and conventional chemical treatments T05 (Laminarin + Metiram and Laminarin + Copper hydroxide) and T12, respectively.

Concerning the metataxonomic data of grape berries, the average of Good's coverage for fungal communities indicated a satisfactory coverage (99%). Overall, the grape berries mycobiota did not show any significant difference in terms of alpha diversity index or taxa frequency as a function of fungicide treatment, between the controls (T01 and T08) and the rest trials. The metataxonomic approach identified *A. pullulans*, *C. cladosporioides* and *H. uvarum* as the most abundant ASVs with a relative frequency of

more than 50 % (Table 2). It should be pointed out that *S. cerevisiae* and *B. cinerea* were found in all samples with a relative frequency less than 1 % (Table 2).

3.2 Yeast diversity and winery fermentation kinetics by culture-dependent and independent approaches

Yeast diversity (Fig. 2, panel A), total yeast population (Fig. 2, panel B) and fermentation kinetics (Fig. 2, panel C) of grape berries pre-treated with one conventional control treatment (T08) and five antifungal compounds (T02, T05, T06, T07 and T12) at the different stages of spontaneous and inoculated fermentations conducted winery scale conditions are shown in Fig. 2. These yeasts were identified using PCR-RFLP analysis of the rRNA operon ITS region and sequencing of the partial 26S rRNA gene. Regarding species heterogeneity, in all trials, *S. cerevisiae* dominated the fermentation process, and was the only species isolated after 14 days, except for trial WS8 were the presence of *Starm. bacillaris* and *H. uvarum* was observed at the end of the fermentation (Fig. 2, panel A).

Total yeast population reached the highest values (ANOVA, P = 0.019), after the fifth day in spontaneous trials with the only exception of WS6 and WS10 that reached the maximum levels after 2 and 7 days, respectively (Fig. 2, panel B). On the contrary, the total yeast population reached the maximum levels (ANOVA, P = 0.021), after the second day in inoculated trials (Fig. 2, panel B). Overall, we observed that the starter culture induced a faster sugar consumption rate compared to the respective fermentations performed with indigenous strains (ANOVA, P = 0.010), however, this rate varies between the fermentation of the grapes treated with six different antifungal compounds. In the case of inoculated fermentation, after 5 days, we observed that grapes previously treated with the treatment T2 (WI2) contained more sugars (about 142 g/L of sugars) if compared to the other inoculated fermentations (about 100 g/L of sugars, ANOVA, P = 0.010). Regarding spontaneous fermentation, sugar consumption was slower in WS2 and WS5. These fermentations contained more than 100 g/L of residual sugars after 7 days, while the other trials contained sugars ranging from 49 g/L to 94 g/L (Fig. 2, panel C, ANOVA, P = 0.004).

3.2.1 Mycobiota composition of Nebbiolo fermentations

A total of 5.669.920 high-quality reads were used for the downstream analysis with an average of 59.061 reads/sample. Shannon index increased when T0 was compared with T7 (P=0.043) and was highest in spontaneous fermentation if compared with inoculated once (P=0.002). No differences were observed as function of the fungicide's treatments. Beta diversity calculation based on Bray Curtis distance matrix showed a significant separation of samples according to fermentation type (spontaneous

vs. inoculated) or according to fermentation time (Fig. 3, PERMANOVA p=0.001), while no effect of the fungicide was observed.

Regarding mycobiota composition and evolution during fermentation, in spontaneous fermentations we observed that *H. uvarum* increased from 0.7% of the relative frequency at T0 till 6% after 14th days (P = 0.001, Fig. 4), *Pichia kudriavzevii* from 25% of the relative frequency at T0 to 0% after 14th days (P = 0.001), while *S. cerevisiae* increased from 27% to 48% from T0 to T14 (P = 0.003, Fig. 4). During inoculated winery fermentations we observed that *S. cerevisiae* (90% of the relative frequency at T0) was reduced till 59% at the end of the fermentations (P = 0.001) while *Starm. bacillaris* increased from 0.1 to 17% during the fermentation (P = 0.001, Fig. 4). Spontaneous winery fermentations were associated with the predominance of *Hanseniaspora*, *Hanseniaspora uvarum* and *Pichia kudriavzevii* while inoculated winery fermentations were associated with *Saccharomyces cerevisiae* (P < 0.001).

3.3 Oenological parameters and volatilome profile

Oenological parameters and VOCs data were used to build a Principal Component Analysis (PCA) to visualize the differences among wines produced in the winery scale conditions (Fig. 5, Panel A and B). The PC1 explained 35.3 % and the PC2 21.0 % of the variation on the data set. Wines produced from grape berries treated with the alternative treatment T05 (Laminarin + Metiram) regardless of inoculation protocol used (S or I) were characterized by high ethanol, acetic acid and ethyl acetate values. Noteworthy, a lesser variability between wines produced from grape berries treated with T02, T06 and T07, regardless of inoculation protocol, was observed compared to T05, T08 and T10.

3.4 Correlation between mycobiota and oenological parameters

Significant correlations between oenological parameters and frequency of mycobiota taxa were obtained (exact *P*-values and R coefficients are reported in Table S2 in Supplementary Material).

Concerning correlation patterns in wine produced with spontaneous fermentation, positive correlations were observed between *P. kluyveri* and glycerol (Fig. 6, $P < 0.05$). Interestingly, in inoculated fermentations, we observed negative correlations between *Starm. bacillaris* and acetic acid, ethanol and total acidity, while positive correlations were observed between *S. cerevisiae* and glycerol, total acidity and pH and *H. uvarum* and ethanol (Fig. 6, $P < 0.05$).

3.5 Grape-mycobiota contribution to wine aroma

352 Significant correlations between VOCs and ASVs were obtained (exact *P*-values and R
353 coefficients are reported in Table S3 in Supplementary Material).
354 By plotting the Spearman's correlation between metabolites and fungal ASVs in wine obtained
355 under spontaneous winery fermentation we observed positive correlations between *S. cerevisiae* with
356 1,3-benzothiazole and *H. uvarum* with 1-nonanol, and ethyl-2-hexenoate, while negative correlations
357 were observed between *B. cinerea* with ethyl decanoate, ethyl hexadecanoate and isopentyl hexanoate;
358 *C. cladosporoides* with benzoic acid, benzyl alcohol, decanoic acid and methyl octanoate and *P.*
359 *kudriavzevii* with 2-phenyl ethanol, 2-phenylethyl acetate, 4-methylpentanol, ethyl octanoate, octanoic
360 acid and linalool (Fig. 6, $P < 0.05$). In inoculatedfermentations positive correlations were observed
361 between *S. cerevisiae* with geraniol, *P. kluyveri* with linalool (Fig. 6, $P < 0.05$). Concerning negative
362 correlations, *H. uvarum* was negatively correlated with isoamyl alcohol, *S. cerevisiae* with 1-octanol,
363 and *B. cinerea* with 1-butanol, ethyl decanoate and isopentyl hexanoate (Fig. 6, $P < 0.05$).
364
365 **4. Discussion**
366
367 The effect of alternative and conventional chemical antifungal compounds on mycobiota of
368 "Nebbiolo" grapes and corresponding wines, oenological parameters and volatilome profile were
369 investigated. The total yeast population on wine grapes at harvest time ranged from 4.5 to 6.5 Log
370 CFU/mL, in accordance with those reported in the literature for mature grapes (Alessandria et al., 2015).
371 Similar yeast load among grape samples treated with both alternative and conventional chemical
372 treatments, compared to untreated Control (T01) and the control conventional treatment (T08) was
373 observed, suggesting that both alternative and conventional chemical-based antifungal compounds do
374 not affect significantly the colonization by indigenous yeasts. A significant variation was only observed
375 when applying the alternative treatment T04 (potassium phosphonate and sulphur + copper hydroxide)
376 on grape berries, that led to a reduction of the overall yeast population (about 4.5 Log CFU/mL). The
377 high antifungal activity of sulphur and copper-based treatments against yeasts of oenological interest has
378 been already suggested by Milanović et al. (2013). However, the reduction observed in sample T04 could
379 be attributed to the synergistic effect of potassium phosphonate with sulphur + copper hydroxide, since
380 the last two active ingredients are also present in the control conventional treatment T08.
381 Concerning yeast diversity on grape samples, culture-dependent approach highlight a dominance
382 and colonization of non-fermenting microorganisms, mainly *A. pullulans* and *C. cladosporioides* and
383 reduction of the population of fermenting yeasts such as *H. uvarum* and *Starm. bacillaris*, when

grapevines were treated with all alternative (T02-T07, T09) and two conventional treatments (T11 and T12) independently of the active ingredient used if compared to the untreated (T01) and the treated conventional control product (T08). The ability of these compounds to increase the presence of *A. pullulans* on grapes could be of great interest since this yeast-like fungus has bioprotective antagonistic features against yeasts and moulds and may influence the overall grape ecology, as previously reported by Bozoudi & Tsaltas (2008). On the other hand, culture-independent approach did not show any significant difference in terms of alpha diversity index or taxa frequency as a function of treatment, indicating the importance of applying a multiphasic approach to uncover yeast communities associated with grapes (Alessandria et al., 2015). Also, it is worth noticing that *S. cerevisiae* was detected on all the grape samples using the metataxonomic approach, independently of the treatment applied. This evidence is in line with recent literature and underlines the power of sequencing approach to provide a more sensitive and comprehensive overview of complex microbial communities (Bokulich & Mills, 2012), since culture-based approaches may miss about 95 % of the fungal community (Taylor, Tsai, Anfang, Ross, & Goddard, 2019).

The initial mycobiota composition of each fermentation encompassing both non-*Saccharomyces* and *S. cerevisiae* yeast was further affected in the winery as revealed by both plate counts and metataxonomic analyses. Non-*Saccharomyces* species (*Starm. bacillaris* and *H. uvarum*) have been shown to contribute to the overall chemical and sensorial profile of wines by producing metabolites associated with wine quality (Englezos et al., 2019ab, Jolly, Varela, &Pretorius, 2014). In the present study, *Starm. bacillaris* was identified by culture-dependent method until the middle of the fermentation period in all spontaneous fermentations, except grapes treated with alternative chemical compounds T05. This suggests that this yeasts species might contribute to the wine composition since is correlated with an increase in glycerol and total acidity.

In the same context, metataxonomic analyses at the of the monitored period revealed that mycobiota was greatly influenced by the addition of the starter culture. This is the case of *H. uvarum* and *P. kluyveri*, since were greatly associated with spontaneous fermentations. Concerning, inoculated fermentations, these were mainly associated with *P. kluyveri* and *Starm. bacillaris*, together with *S. cerevisiae*. All these findings, highlight the contribution of the indigenous yeast species and strains within species to overall yeast ecology, in accordance with Morrison & Goddard (2018). Regarding *S. cerevisiae*, this species was dominant in all fermentations regardless of the type of treatment applied, inoculation protocol, confirming the high ability of this yeast to tolerate adverse conditions during the fermentation process (Knight, Klaere, Fedrizzi, & Goddard, 2015). The dominance of the starter culture

was confirmed in inoculated fermentation using interdelta-PCR fingerprinting analysis, while indigenous strains were identified on spontaneous fermentation (data not shown).

The application of copper to the vine has an important role in ensuring a successful must fermentation; however high concentrations of this compound in must could have a negative impact on yeasts growth, fermentation kinetics and the performance of starter cultures during fermentation (Capece, Romaniello, Scrano, Siesto, & Romano, 2018). In the present study we showed that the concentrations of cooper as active ingredient from the alternative treatments (T05, T06 and T10) increased the yeast counts of *S. cerevisiae*, indicating the ability of this species to dominate the must environment during fermentation and reduce the proportion of non-*Saccharomyces* yeasts in the short term. This finding is of particular interest, since several non-*Saccharomyces* species, mainly *H. uvarum* are associated with negative attributes (Belda et al., 2017). However, further studies based on the quantification of the amount of residual copper in the grape must are necessary in order to better investigate this correlation.

Metataxonomic analyses are now commonly used for ecological analysis, however, relatively few studies have employed such methods to characterize the microbial ecology of wine fermentations using grapes previously treated in the winery using alternative fungicide treatments. In this study, no effect of the fungicide treatments was observed in the yeast communities. Especially, in inoculated fermentations in which *S. cerevisiae* strain was inoculated in the medium in all ferments.. These results are in disagreement with those reported by Agarbati et al. (2019), that demonstrated conventional and organic based vineyard treatments can influence yeast communities and therefore wine quality.

Correlations between wine mycobiota and main oenological parameters and VOCs have been extensively investigated (Bokulich et al., 2016; Cravero et al., 2016; Tufariello et al., 2021). In the present study, wines appear to be differentiated by the inoculation protocol, since spontaneously fermented wines contained higher levels of glycerol and total acidity, compared to the respective inoculated fermentations, independently of the treatment applied in the vineyard. The higher levels of glycerol could be explained by the relatively high presence of non-*Saccharomyces* yeasts, mainly *Starm. bacillaris* during fermentation, as previously reported by Englezos et al. (2019b). While, the higher levels of total acidity could be explained by the ability of the non-*Saccharomyces* yeasts to produce higher levels of organic acids, compared to *S. cerevisiae* (Jolly et al., 2014).

5. Conclusion

This study has demonstrated that the application of antifungal compounds against powdery and downy mildew has an impact on the mycobiota present on grapes and in fermenting musts and suggests

the absence of a link between principal active compounds, yeast biodiversity and wine composition. It is, important to underline that only one vineyard from one geographical region and one time point were taken in consideration in this study, therefore further studies are necessary to confirm the findings of this preliminary work. Since, the effect of vineyard management on overall microbial biodiversity differs between organisms and across time and space, as previously demonstrated by Giraldo-Perez, Raw, Greven, & Goddard (2021). Increasing our knowledge of the response of indigenous mycobiota inhabiting grapes and during the fermentation process to the application of different antifungal compounds with low environmental impact serves as a foundation to develop new grape management procedures and guarantee wine quality and fulfilling consumer demands for sustainable wines.

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TABLE LEGENDS

Table 1 Description of the fungicide treatments used, including active ingredients and dosages. For this study we considered T08 as the control conventional treatment as it is the one commonly employed for vineyard protection against fungal diseases.

Table 2 Frequency of mycobiota taxa of Nebbiolo grapes treated with alternative and conventional treatments based on the relative frequency of the amplicon sequence variants (ASVs). T01: untreated control; T08: conventional control.

730 **Table 1**

#	Category	Active ingredients	Dose (g/ha)
T01	Untreated contol	Untreated control	-
T02	Alternative chemical treatment	Acibenzolar-S-methyl	100
		Sulphur + Copper hydroxide	3200+600
T03	Alternative chemical treatment	Fosetyl-Al	3200
		Sulphur + Copper hydroxide	3200+600
T04	Alternative chemical treatment	Potassium phosphonate	3020
		Sulphur + Copper hydroxide	3200+600
T05	Alternative chemical treatment	Laminarin + Metiram	90 + 1400
		Laminarin + Copper hydroxide	90 + 600
T06	Alternative chemical treatment	Chito-oligosaccharides and oligogalacturonides + Metiram	31.25+1400
		Chito-oligosaccharides and oligogalacturonides + Copper hydroxide	31.25+600
T07	Alternative chemical treatment	Potassium bicarbonate+Metiram	4250+1400
		Potassium bicarbonate + Copper hydroxide	4250+600
T08	Conventional control chemical treatment	Sulphur + Metiram	3200+1400
		Sulphur + Copper hydroxide	3200+600
T09	Alternative chemical treatment	Calcium oxide	884
T10	Alternative chemical treatment	Calcium oxide + Metiram	884 + 1400
		Calcium oxide + Copper hydroxide	884 + 1400
T11	Conventional chemical treatment	Sulphur	3200
T12	Conventional chemical treatment	Metiram	1400
		Copper hydroxide	600

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732 **Table 2**

	Untreated		Alternative treatments																Conventional treatments					
	T01		T02		T03		T04		T05		T06		T07		T09		T10		T08		T11		T12	
	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD	mean	SD
Alternaria	3.12	1.93	3.22	3.29	3.61	2.13	1.7	0.3	0.97	0.57	1.39	1.21	2.61	1.82	4.91	5.13	3.42	2.91	1.09	0.99	1.65	0.49	0.95	0.1
Aspergillus niger	0.01	0.01	0	0.01	0.03	0.05	0	0.01	0.01	0.02	0	0	0	0	0	0	0	0	0	0	0.22	0.38	0	0
Aureobasidium pullulans	27.2	7.39	49.52	23.41	48.79	4.61	41.24	20.05	59.43	12.9	36.04	23.47	53.52	23.39	35.3	18.08	47.04	36.93	28.99	4.23	38.75	11.52	72.87	13.04
Botrytis cinerea	0.22	0.07	0.17	0.16	0.28	0.26	0.42	0.27	0.14	0.16	0.16	0.15	0.31	0.3	1.12	1.69	0.16	0.13	0.37	0.54	0.55	0.7	0.06	0.07
Cladosporium cladosporoides	37.02	19.8	26.91	23.35	27.88	15.37	29.16	16.15	27.51	10.16	44.86	38.61	15.82	12.54	39.09	23.28	35.6	35	24.3	21.85	39.75	26.22	10.49	2.5
Erysiphe necator	0.08	0.05	0.26	0.36	0.31	0.5	0.2	0.27	0.18	0.3	0.44	0.59	0.52	0.89	0.27	0.44	0.18	0.15	0.34	0.51	0.3	0.27	0	0
Filobasidium	0.12	0.09	0.61	0.84	0.12	0.06	0.09	0.08	0.14	0.17	0.16	0.2	0.02	0.02	0.05	0.06	0.02	0.02	0	0.01	0.03	0.03	0.01	0.01
Filobasidium magnus	6.27	2.39	5.06	3.13	4.49	4.91	9.47	5.47	2.95	2.61	4.95	5.94	3.3	3.2	6.67	7.26	3.39	2.57	0.97	0.04	5.63	6.18	2.05	2.53
Fusarium	0.47	0.28	0.63	0.48	0.47	0.28	1.21	1.11	0.25	0.29	0.26	0.32	0.22	0.23	0.76	0.62	0.37	0.61	0.19	0.32	0.38	0.42	0.46	0.72
Hanseniaspora	0.22	0.37	0.04	0.03	0.05	0.05	0.15	0.19	0.02	0.02	0.09	0.06	0.56	0.71	0.26	0.23	0.02	0.01	0.11	0.08	0.13	0.17	0.23	0.2
Hanseniaspora uvarum	11.74	20.23	0.53	0.72	1.17	1.73	1.78	3.08	0.13	0.11	1.03	1.76	14.48	24.68	0.68	1.09	0.95	1.17	30.22	32.96	1.27	2.19	7.46	12.86
Pichia kluyveri	0	0	0.02	0.04	0	0	0.02	0.03	0	0	0	0	0.02	0.03	0.01	0.02	0.02	0.03	0.02	0.04	0	0	0.48	0.83
Pichia occidentalis	0	0	0	0	0.04	0.05	0	0	0.03	0.03	0.34	0.58	0.18	0.31	0.14	0.16	0.08	0.07	0	0.01	0	0.01	0.18	0.32
Pichia terricola	0	0	0	0	0	0	0.09	0.15	0	0	0.02	0.04	0.07	0.08	0	0.01	0	0	0.08	0.13	0	0	0	0
Rhodotorula glutinis	0.37	0.38	0.36	0.33	0.26	0.25	0.47	0.41	0.36	0.34	1.02	1.49	0.46	0.42	1.21	1.02	0.94	1.04	0.16	0.14	1.22	1.32	0.59	0.85
Saccharomyces cerevisiae	0.01	0.01	0.11	0.15	0.08	0.12	0.01	0.01	0	0.01	0.02	0.04	0.58	0.99	0.29	0.24	0.04	0.07	0.02	0.02	0.27	0.42	0.07	0.12
Sporidiobolus pararoseus	0.14	0.05	0.58	0.24	0.4	0.19	3.34	5.08	0.22	0.19	0.59	0.61	0.13	0.11	0.34	0.31	0.36	0.41	0.09	0.1	0.31	0.27	0.14	0.24
Sporobolomyces roseus	0.46	0.21	0.37	0.04	0.26	0.24	0.21	0.18	0.2	0.17	0.35	0.4	0.12	0.11	0.35	0.33	0.31	0.2	0.04	0.05	0.44	0.4	0.08	0.13
Starmerella bacillaris	0.01	0.01	0.04	0.06	0.01	0.01	0.04	0.07	0.35	0.58	0.33	0.54	0.08	0.14	0.02	0.02	0.07	0.12	7.75	13.4	0.02	0.03	0.08	0.15
Symmetrospora	0.56	0.11	1.49	1.53	2.07	1.87	1.15	1.15	0.65	0.57	0.85	0.83	0.64	0.87	0.54	0.52	0.17	0.19	0.07	0.08	0.59	0.63	0.1	0.17
Symmetrospora oryzicola	2.29	1.74	1.17	0.27	1	0.9	1	0.81	0.93	0.73	0.55	0.51	0.51	0.56	1.08	0.66	0.69	0.6	0.14	0.2	0.92	0.8	0.54	0.68
Tilletiopsis washingtonensis	0.22	0.25	0.4	0.55	0.09	0.08	0.06	0.06	0.04	0.05	0.09	0.05	0.06	0.1	0.16	0.14	0.9	1.54	0.04	0.03	0.06	0.1	0	0
Vishniacozyma carnescens	2.3	0.64	3.52	2.7	3.82	3.54	3.74	2.83	2.8	2.44	3.4	3.47	1.56	1.35	2.24	2.29	3.38	3.24	1.16	0.62	3.5	3.19	1.37	2.35
Vishniacozyma victoriae	1.38	0.52	1.51	0.56	2.11	1.2	1.47	1.51	0.96	0.75	1.04	1.02	1.17	0.7	1.43	1.54	0.72	0.18	0.26	0.25	0.93	0.83	0.17	0.29
Wickerhamomyces anomalus	0	0	0.12	0.19	0.05	0.06	0.39	0.65	0.01	0.01	0.02	0.03	1.54	2.66	0.74	1.15	0.08	0.09	0.66	1.15	0.54	0.87	0.77	1.33

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FIGURE LEGENDS

Fig. 1 Total yeast population (Panel A) and yeast species biodiversity (Panel B), registered on Nebbiolo grapes after harvest, as determined by plate counts on WLN medium. Data are expressed as mean ± standard deviation of four biological replicates. Different letters in each column, mean significant differences according to Tukey-HSD test ($P < 0.001$). Fungicide treatment descriptors are reported in Table 1. T01: untreated control; T08: conventional control.

Fig. 2 Yeast diversity (Panel A), total yeast population (Panel B) and evolution of metabolites during the alcoholic fermentation (Panel C) of spontaneous musts previously spayed with alternative chemical fungicides (WS2, WST5, WS6, WS7, and WS10) and conventional chemical fungicide (WST8) and inoculated musts previously spayed with alternative chemical fungicides (WI2, WI5, WI6, WI7, and WI10) and conventional chemical fungicide (WIT8) performed in winery conditions are described. Yeast diversity and total yeast population were determined by plate counts on WLN medium. Panel C displays sugar (black circle), ethanol (white square) and glycerol (white diamond) concentrations. **Abbreviations:** Winery, W; Laboratory, L; Spontaneous, S; Inoculated, I, T; treatment previously applied on grapes.

Fig. 3 Principal coordinate analysis (PCoA) of Bray–Curtis distance with each sample represented as a circle and color code according to fermentation time, type or fungicide treatmen

Fig. 4 Boxplot showing the development of fungal taxa during the fermentation time. According to the inoculum protocol of the fermentation conditions (laboratory fermentation or winery fermentation). Only Fugal taxa that display a significant development are shown $p<0.05$; $**p<0.01$).

Fig. 5 Score plot (A) and loading plots (B) of standard chemical compounds and volatile organic compounds determined in Nebbiolo wines at the end of winery scale fermentations. [1] Acetic acid; [2] Glycerol; [3] Glucose; [4] Fructose; [5] Ethanol; [6] Residual sugars; [7] Total acidity; [8] pH; [9] Yglycerol; [10] Yethanol; [11] Ethyl acetate; [12] Ethyl butanoate; [13] Isobutanol; [14] Isoamyl acetate; [15] 1-butanol; [16] Isoamyl alcohol; [17] Ethyl hexanoate; [18] Hexyl acetate; [19] 1-hexanol; [20] Methyl octanoate; [21] Ethyl octanoate; [22] Ethyl nonanoate; [23] Linalool; [24] 1-octanol; [25] Ethyl decanoate; [26] Diethyl succinate; [27] Methionol; [28] Cintronellol; [29] 2-Phenylethy; ethylacetate; [30] β-damascenon; [31] Ethyl dodecanoate; [32] Geraniol; [33] 2-phenyl ethanol; [34] Ethyl myristate; [35] Ethyl heptanoate* ; [36] 4-methylpentanol*; [37] (S)-3-methylpentanol*; [38] Ethyl-2-hexenoate*; [39] Isopentyl

hexanoate*; [40] 2-ethyl hexanol*; [41] (S)-3-ethyl-4-methylpentanol*; [42] Butyrolactone*; [43] Ethyl-3-methylbutyloctanoate*; [44] 1-nonanol*; [45] 3-methylbutyrate*; [46] Ethyl-9-decenoate*; [47] Ethyl undecanoate*; [48] Ethyl phenylacetate*; [49] Hexanoic acid*; [50] Nerolidol 2*; [51] Benzyl alcohol*; [52] 1,3-benzothiazole*; [53] Ethyl tetradecanoate*; [54] Octanoic acid*; [55] Cadalene*; [56] Ethyl hexadecanoate*; [57] Decanoic acid*; [58] Benzoic acid*.

Fig. 6 Correlation plot showing Spearman's correlation between the fungal ASVs and oenological parameters observed with an incidence above > 1% in at least 2 samples during spontaneous and inoculated winery fermentation. Only significant associations between the relative frequency of ASVs and the concentration of metabolites are shown ($P < 0.05$). The intensity of the colors represents the degree of correlation between the fungal ASVs and oenological parameters, as measured by Spearman's correlation, where the blue color represents a positive degree of correlation and red a negative correlation between the oenological parameters and fungal OTUs.

Figures

Fig. 1

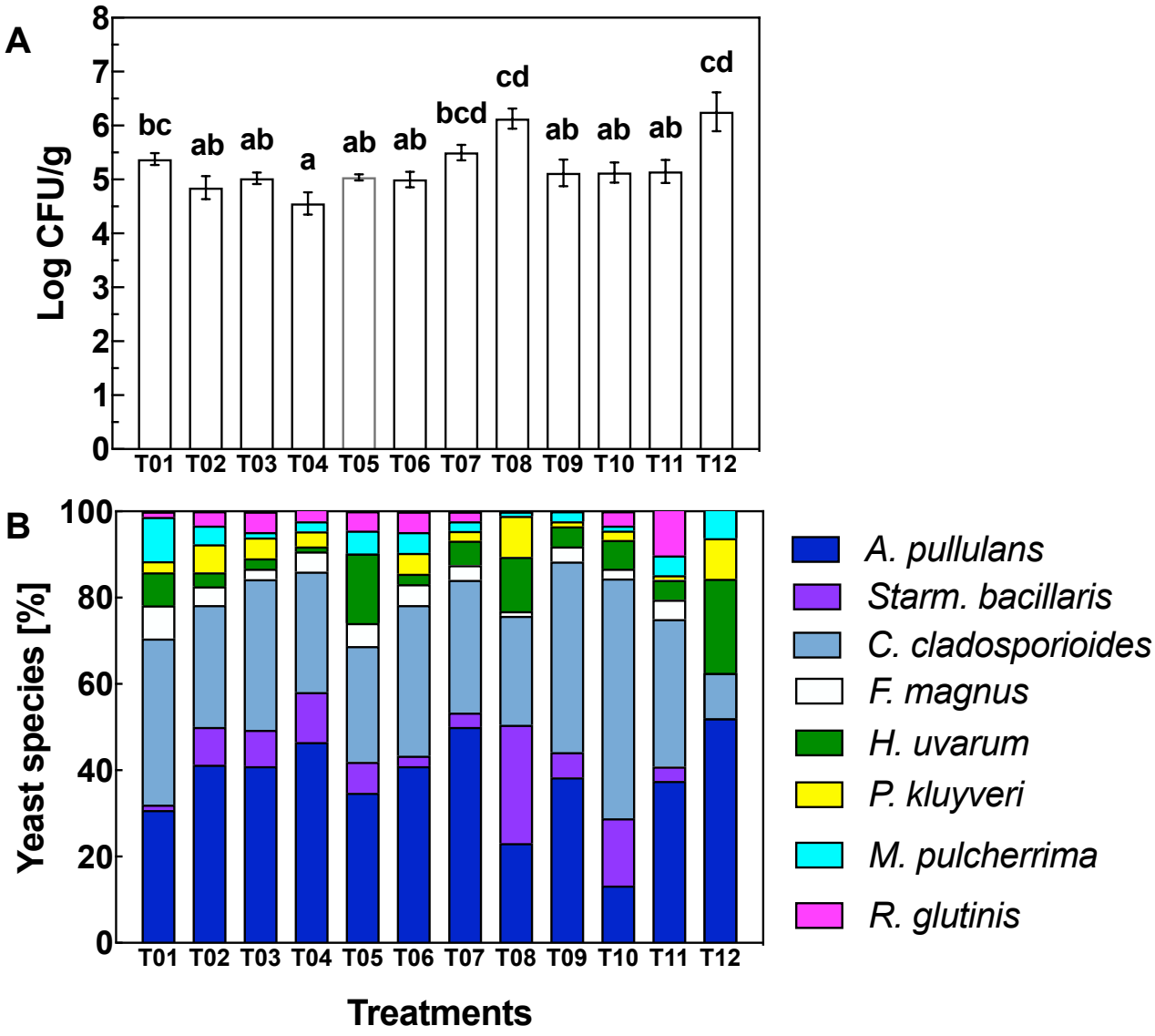


Fig. 2

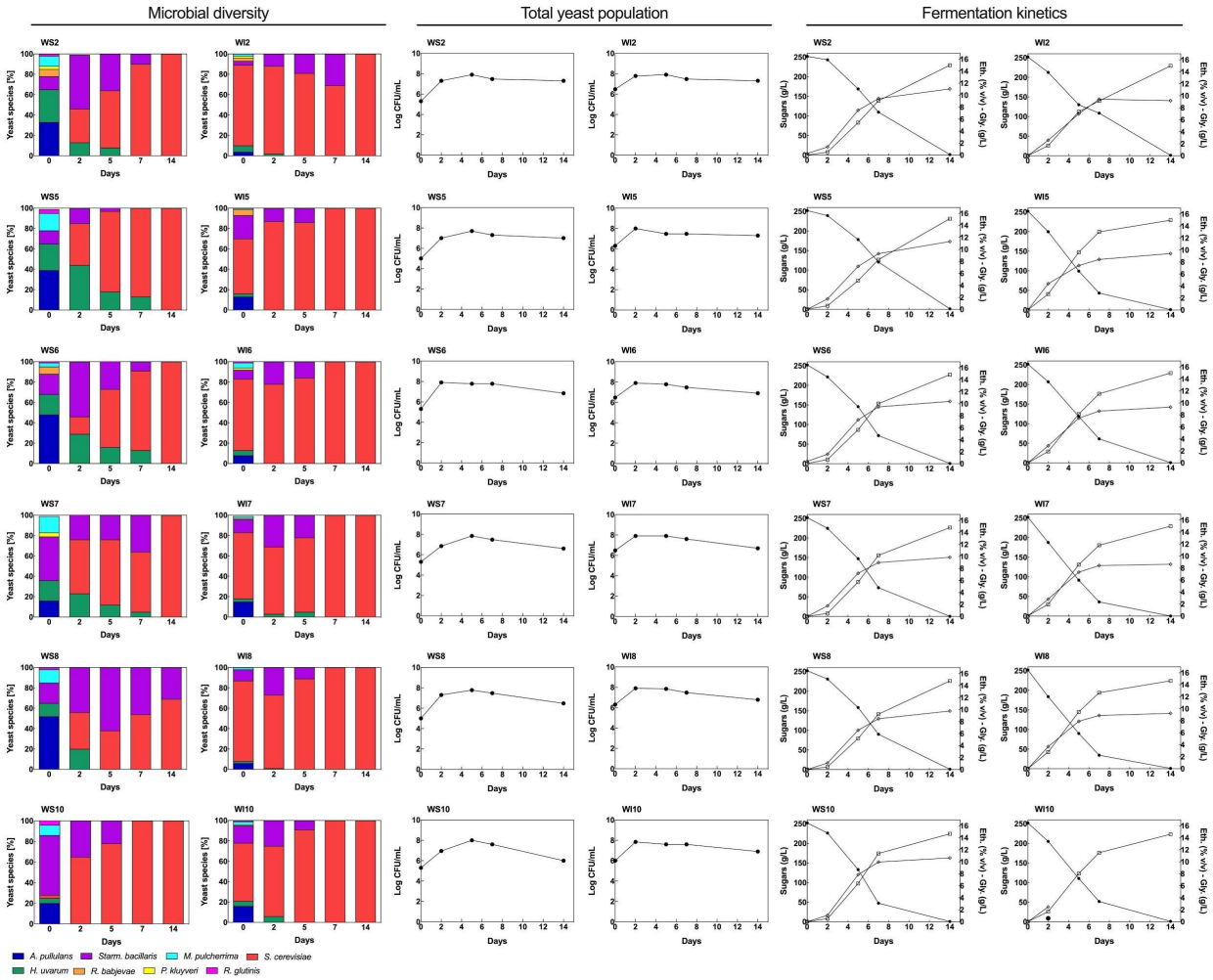

Microbial diversity
Total yeast population
Fermentation kinetics
WS2
WI2
WS5
WI5
WS6
WI6
WS7
WI7
WS8
WI8
WS10
WI10
Yeast species [%]
Log CFU/mL
Sugars (g/L)
Eth. (% v/v) - Gly. (g/L)
Days
A. pullulans
Starm. bacillaris
M. pulcherrima
S. cerevisiae
H. uvarum
R. babjevae
P. kluyveri
R. glutinis

Fig. 3

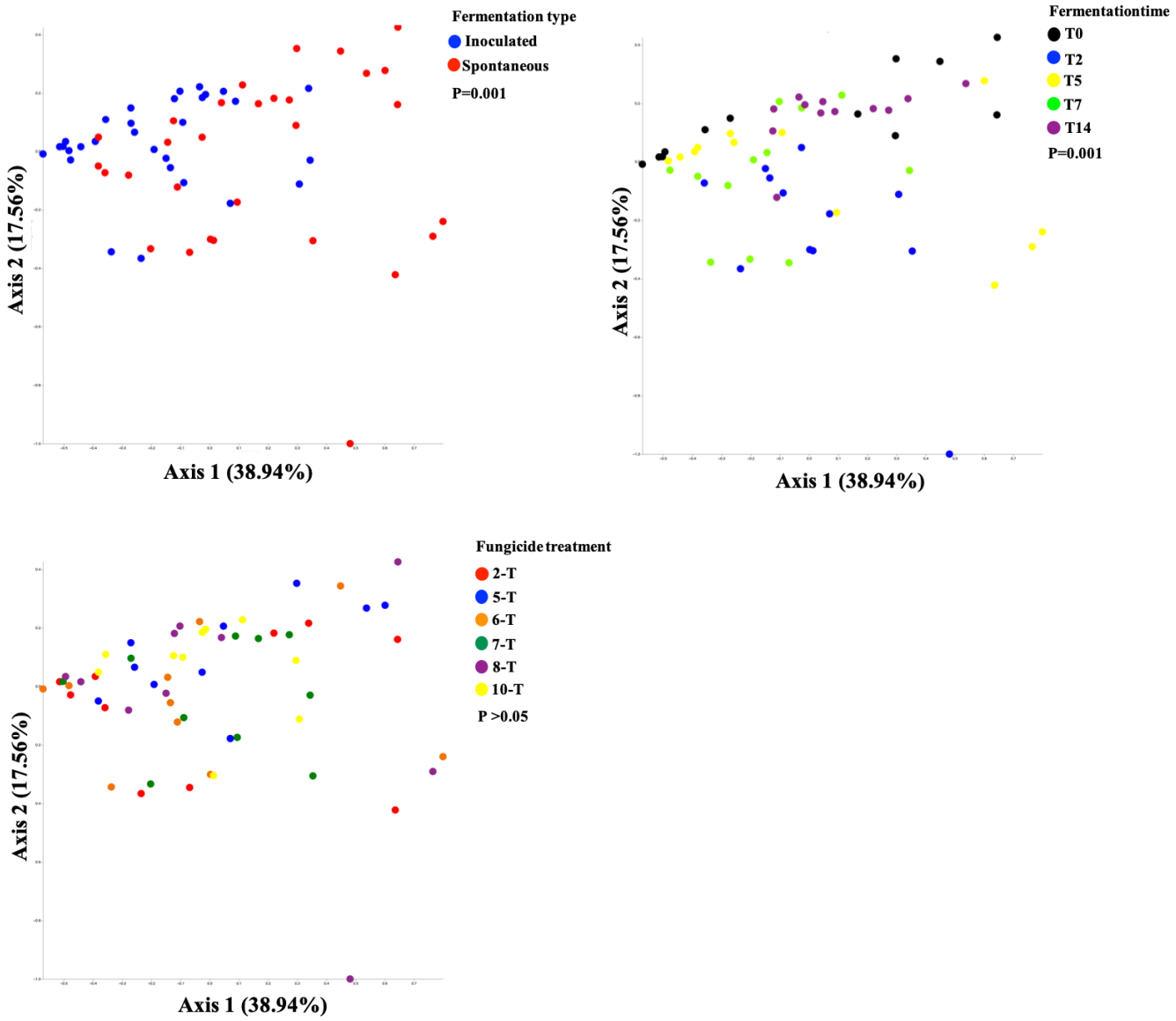

Fermentation type
Inoculated
Spontaneous
P=0.001
Axis 2 (17.56%)
Axis 1 (38.94%)
Fermentationtime
T0
T2
T5
T7
T14
P=0.001
Axis 2 (17.56%)
Axis 1 (38.94%)
Fungicide treatment
2-T
5-T
6-T
7-T
8-T
10-T
P >0.05
Axis 2 (17.56%)
Axis 1 (38.94%)

Fig. 4

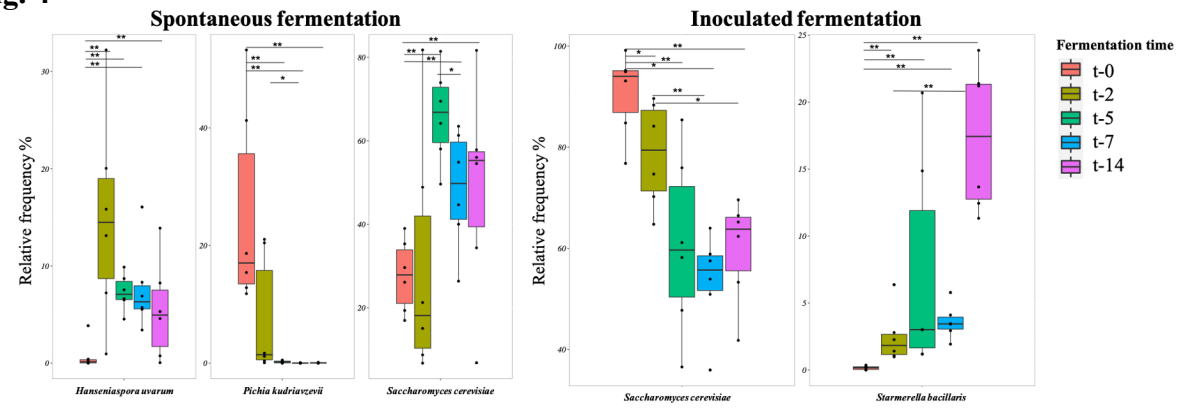

Spontaneous fermentation
Inoculated fermentation
Relative frequency %
Hanseniaspora uvarum
Pichia kudriavzevii
Saccharomyces cerevisiae
Saccharomyces cerevisiae
Starmerella bacillaris
Fermentation time
t-0
t-2
t-5
t-7
t-14

Fig. 5

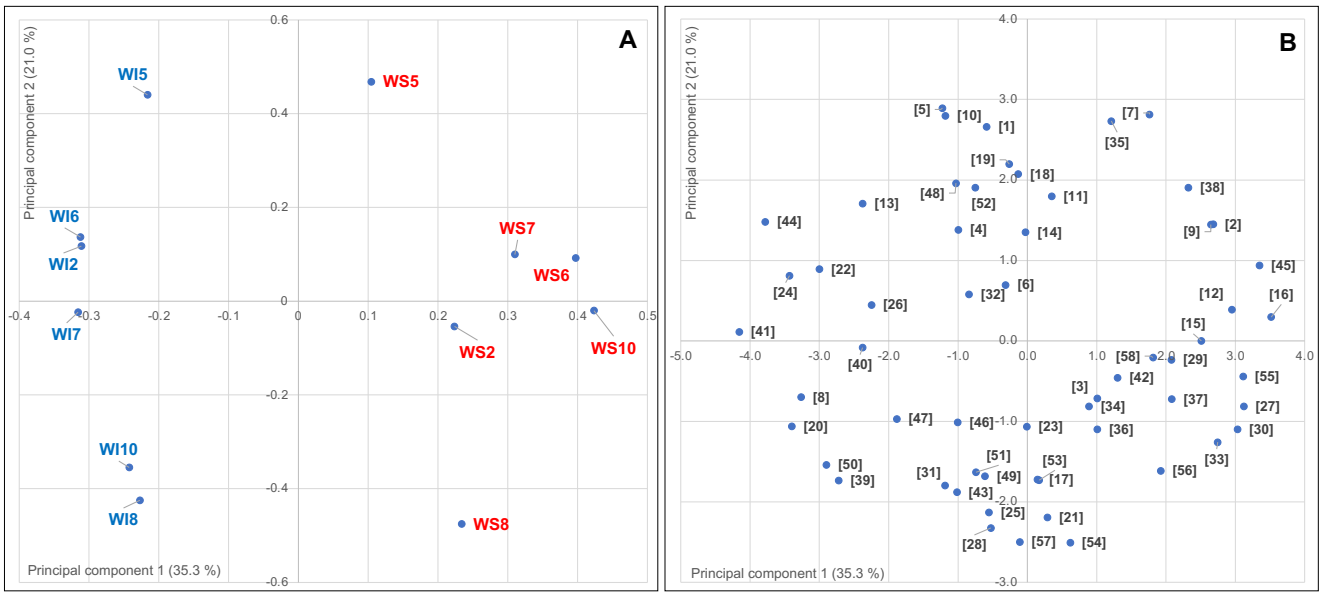
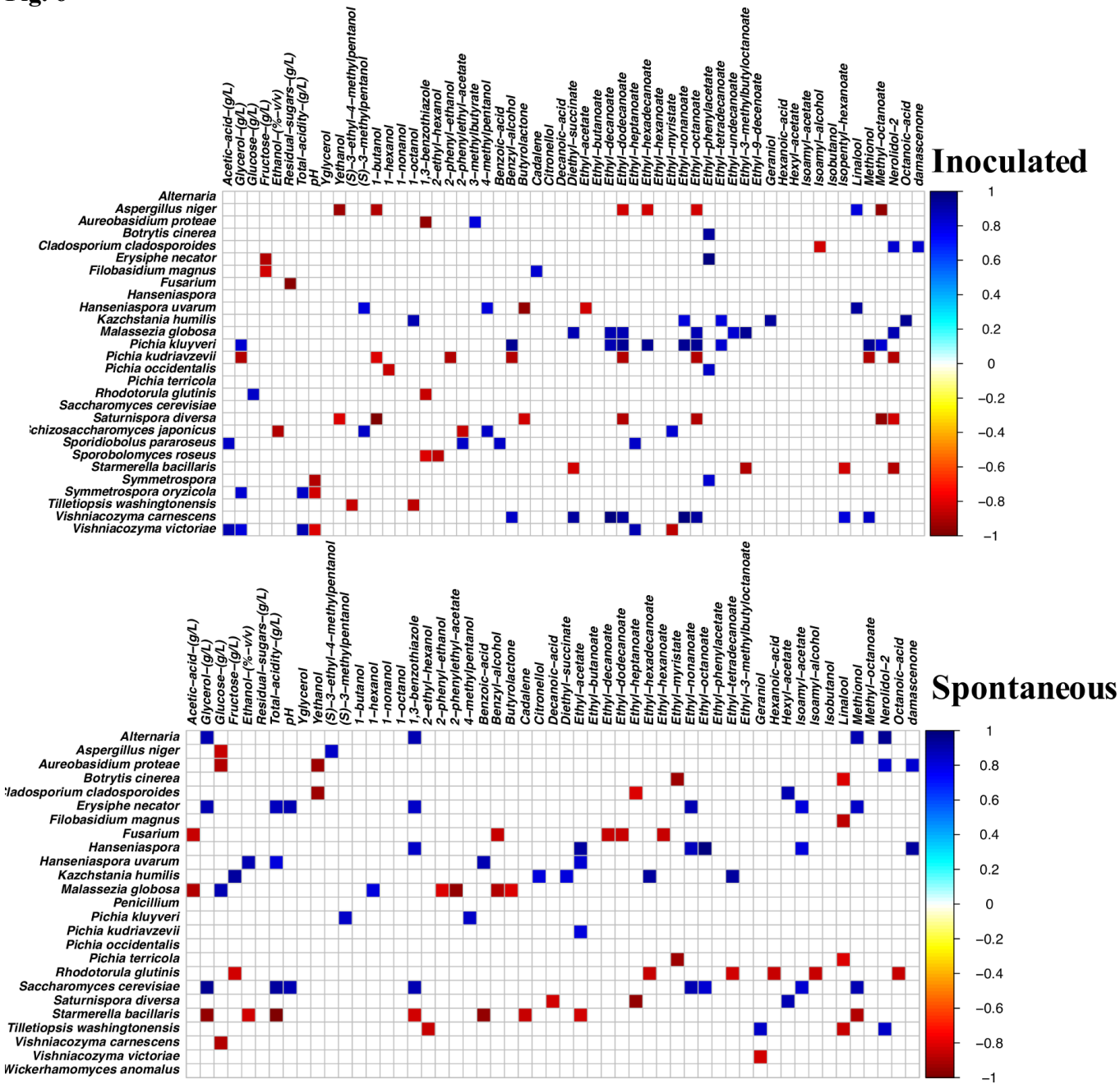

A
Principal component 2 (21.0 %)
Principal component 1 (35.3 %)
WI5
WS5
WI6
WI2
WS7
WS6
WI7
WS2
WS10
WI10
WI8
WS8
B
Principal component 2 (21.0 %)
Principal component 1 (35.3 %)

Fig. 6


Inoculated
Spontaneous
Acetic−acid−(g/L)
Glycerol−(g/L)
Glucose−(g/L)
Fructose−(g/L)
Ethanol−(%−v/v)
Residual−sugars−(g/L)
Total−acidity−(g/L)
pH
Yglycerol
Yethanol
(S)−3−ethyl−4−methylpentanol
(S)−3−methylpentanol
1−butanol
1−hexanol
1−nonanol
1−octanol
1,3−benzothiazole
2−ethyl−hexanol
2−phenyl−ethanol
2−phenylethyl−acetate
3−methylbutyrate
4−methylpentanol
Benzoic−acid
Benzyl−alcohol
Butyrolactone
Cadalene
Citronellol
Decanoic−acid
Diethyl−succinate
Ethyl−acetate
Ethyl−butanoate
Ethyl−decanoate
Ethyl−dodecanoate
Ethyl−heptanoate
Ethyl−hexadecanoate
Ethyl−hexanoate
Ethyl−myristate
Ethyl−nonanoate
Ethyl−octanoate
Ethyl−phenylacetate
Ethyl−tetradecanoate
Ethyl−undecanoate
Ethyl−3−methylbutyloctanoate
Ethyl−9−decenoate
Geraniol
Hexanoic−acid
Hexyl−acetate
Isoamyl−acetate
Isoamyl−alcohol
Isobutanol
Isopentyl−hexanoate
Linalool
Methionol
Methyl−octanoate
Nerolidol−2
Octanoic−acid
damascenone
Alternaria
Aspergillus niger
Aureobasidium proteae
Botrytis cinerea
Cladosporium cladosporoides
Erysiphe necator
Filobasidium magnus
Fusarium
Hanseniaspora
Hanseniaspora uvarum
Kazchstania humilis
Malassezia globosa
Pichia kluyveri
Pichia kudriavzevii
Pichia occidentalis
Pichia terricola
Rhodotorula glutinis
Saccharomyces cerevisiae
Saturnispora diversa
chizosaccharomyces japonicus
Sporidiobolus pararoseus
Sporobolomyces roseus
Starmerella bacillaris
Symmetrospora
Symmetrospora oryzicola
Tilletiopsis washingtonensis
Vishniacozyma carnescens
Vishniacozyma victoriae
ladosporium cladosporoides
Penicillium
Wickerhamomyces anomalus
1
0.8
0.6
0.4
0.2
0
−0.2
−0.4
−0.6
−0.8
−1