Bioprotective Effect of Pichia kluyveri and Lactiplantibacillus plantarum in Winemaking Conditions

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Abstract: Consumer demand for wines free of or with reduced levels of preservatives provide new challenges for innovation in winemaking. The addition of microorganisms as bioprotective agents to avoid or reduce the sulphur dioxide (SO_2) addition during winemaking is a possible intervention strategy that could be of interest to winemakers. To this regard, a strain of *Pichia kluyveri* and one of *Lactiplantibacillus plantarum* were inoculated in a Nebbiolo red grape must, previously inoculated with a mix of microorganisms to mimic a grape must environment. The synergistic effect of the above-mentioned strains with no, low (1 g/hL) or moderate (3 g/hL) addition of total SO_2 was explored in two vintages (2019 and 2020). Wine fermentations were monitored for microbiological (yeasts, lactic and acetic acid bacteria populations) and physicochemical attributes (standard chemical parameters, chromatic characteristics, phenolic and aroma compounds). Microbiological analyses showed a decrease of undesired microorganisms, namely *Acetobacter aceti* and *Hanseniaspora uvarum*, independently from the strain used, while this decrease was faster when starter cultures were combined with SO_2. Chemical analyses revealed a decrease of acetic acid and ethyl acetate in the bio-protected wines. The bioprotective ability of the starter cultures was confirmed in both vintages. This study reveals new knowledge about the use of *P. kluyveri* and *L. plantarum* as bioprotective agents in winemaking and could assist to reduce the use of SO_2 in the first steps of wine production.

Key words: Bioprotection, Fermentation dynamics, *Lactiplantibacillus plantarum*, *Pichia kluyveri*, Wine composition, Sulfur dioxide

Introduction

Within the wine production system, alcoholic fermentation is considered a critical phase, since yeasts and bacteria interact and determine the final quality of this beverage. However, the presence of undesirable microbiota on grapes/grape musts is often an unpredictable factor and represents a risk for the subsequent steps in wine production and for the final product (Escribano-Viana et al. 2018). The early addition of sulfur dioxide (SO_2) helps to reduce the population levels of microorganisms, mainly those with negative impact on wine quality, like bacteria and some non-*Saccharomyces* yeasts with low SO_2 tolerance (Ubeda et al. 2020). However, diverse compounds present in the must are able to bind SO_2, thus reducing the percentage of free SO_2 form and, hence, its activity (Lisanti et al., 2019). As a result, higher levels are needed to enhance its effectiveness as antimicrobial and antioxidant agent (Liu et al. 2017). At the same time, the resulting sulfites pose a risk for human health, particularly for sensitive individuals (Giacosa et al. 2019). For these reasons, the use of this additive is strictly controlled by European Union legislation (European Commission, 2009).

Nowadays, the increasing concern about the negative impact of preservatives (including SO_2) in addition to the legal restrictions, pushes for alternative solutions, which can be seen as new opportunities for innovation and adaptation to a changing market (Deroover et al., 2021). Thus, there is an increasing interest in the research of alternative technologies able to replace or reduce SO_2 levels in the early stages of the fermentation (Lisanti et al. 2019). Among them, physical methods such as ultrasound, ultraviolet radiation, pulsed electric field (PEF), electrolyzed water (EW) and high hydrostatic pressure (HHP) or the addition of phenolic compounds, chitosan and lysozyme have been studied (Guerrero and Cantos-Villar, 2015). Despite the promising results of these studies, up to now there are no evidence that the abovementioned methods are able to replace completely the use of SO_2 (Giacosa et al. 2019).

Another strategy investigated to replace chemical preservatives in foods is the use of bioprotective microorganisms and/or their antimicrobial compounds during the production chain or after to extend their shelf-life (Arena et al. 2016). While this practice is widely diffuse in some foods like fruits and dairy products and bioprotective cultures are already available on the market (Leyva Salas et al. 2017, Mukherjee et al. 2020), in winemaking, this strategy is still under study. Since, grape must is a complex ecosystem where different microorganisms interact, so the addition of a species could inhibit some species/strains and have neutral or positive influence on others. Furthermore, the complete substitution of SO_2 results difficult because this compound presents also, among others, an antioxidant activity.

In oenology, the term "bioprotection" designates the use of viable antagonist microorganisms (bacteria, yeasts, or a mixture of them) and/or their antimicrobial products added to the grape must and/or fermenting wine in order to protect it by eliminating or minimizing SO_2 dose. Microorganisms inoculated in fermented foods may be divided in two categories: starter or protective cultures (Singh, 2018). In wine production, the first group indicates yeasts and bacteria inoculation to control alcoholic and/or malolactic fermentations and modulate the chemical composition of the wines. Conversely, a protective culture has the task to limit the survival and growth of undesired microorganisms. Rapid growth and effective use of nutrients at low concentrations as well as the production of antimicrobial compounds have been described as means of microbial antagonism (Gramsci et al. 2018). The selection of microorganisms that possess a combination of both abilities (starter and bioprotective) is generally preferred (Singh, 2018) to obtain a 'self-protected' wine, with the phenotypic characteristics of the starter culture.

Among wine microorganisms, non-*Saccharomyces* yeasts and lactic acid bacteria (LAB) are capable to release antimicrobial compounds, like fatty acids, peptides, proteins, SO_2, carboxylic acids, proteases, glucanases, bacteriocins, bacteriocins-like inhibitory compounds and other inhibitory molecules during the fermentation process, useful for antagonistic purpose against spoilage

microorganisms (Liu et al. 2017, Bartle et al. 2019). Generally, the use of these microorganisms is associated with several target metabolites of oenological interest and with the improvement of wine aroma complexity (Padilla et al. 2016). However, the antagonistic strategies exerted by these microorganisms during fermentation are poorly understood.

In the last years, few studies investigated the application of non-*Saccharomyces* and LAB as bio-protective cultures in wine (Oro et al. 2014, Rubio-Bretón et al, 2018, Simonin et al. 2018, Çorbacı and Uçar, 2018). However, the increasing interest of winemakers in improving the quality of wines, limiting possible health-related problems associated to SO_2, requires further effort into understanding the metabolic and interaction profiles of specific non-*Saccharomyces* yeasts and LAB and grape must microbiota (Guerrero and Cantos-Villar, 2015).

To get an insight into the contribution of these species as bioprotective agents (BPAs), the aim of this study was to assess the biocontrol effectiveness of two selected strains of *Pichia kluyveri* and *L. plantarum* in preventing the development of undesirable microorganisms, and to reduce or replace the SO_2 addition, before the beginning of the fermentation, in two different vintages. In addition, in the second vintage the synergic effect of the starter cultures together with low and moderate levels of SO_2 was also investigated. Furthermore, their antioxidant properties and the impact on wine composition was investigated.

Materials and Methods

Microorganisms. A *Pichia kluyveri* Viniflora® FrootZen® and a *L. plantarum* Viniflora® NoVA™ Protect (Hansen Holding A/S, Hørsholm, Denmark), were used in this study. *L. plantarum* was commercially available as pure freeze-dried culture, while *P. kluyveri* was inoculated from a frozen liquid yeast. The yeasts and bacteria used to mimic a spontaneous grape must environment were *Hanseniaspora*

uvarum Y1, *Metschnikowia pulcherrima* W1, *Starmerella bacillaris* FC54, *Saccharomyces cerevisiae* FF18 and *Acetobacter aceti* Sc10, all belonging to the culture collection of Turin University Culture Collection (Englezos et al. 2019a).

Must preparation: *Vitis vinifera* L. cv. Nebbiolo red grapes were manually harvested during two consecutive years (2019 and 2020), destemmed and crushed. Whole must, including pulp and skins, was heated at 60 ºC for 1 h to inactivate yeasts and bacteria already present and to promote color extraction (Boulton et al. 1996). The grape juice was then separated from pulp and skins using a sterile stainless-steel sieve and stored at −20 ºC until further processing. Initial components concentration in the 2019 must was: sugars 236 g/L, yeast assimilable nitrogen (YAN) 175 mg/L, L-malic acid 3.33 g/L, pH 3.46 and total acidity 5.60 g/L (expressed as tartaric acid). In 2020 the final must was composed of sugars 236 g/L, YAN 182 mg/L, malic acid 3.30 g/L, pH 3.48 and total acidity 7.03 g/L (expressed as tartaric acid). In the second vintage malic acid and pH were adjusted to those registered in the first vintage by adding L- malic acid and 1 N NaOH (Sigma-Aldrich, Milan, Italy). The absence of microorganisms prior to inoculation was checked by plate count using specific media (see section Enumeration of microbial populations).

Inoculation procedure. For each yeast strain used to mimic a spontaneous grape must environment, pre-cultures were grown by inoculating a single fresh colony into 5 mL of YPD (10 g/L yeast extract, 20 g/L peptone, and 20 g/L glucose, all from Biogenetics, Milan, Italy), following incubation at 25 °C for 24 h. Instead, *A. aceti* was propagated by introducing a fresh colony into 5 mL of YPGD broth (5 g/L yeast extract, 5 g/L peptone, 5 g/L glucose and 5 g/L glycerol, all from Biogenetics) and incubated at 25 °C for 4 days. After incubation, the activated cells were pre-adapted by inoculating them in 90 mL of fresh pasteurized must and then incubated at 25 °C for 24 h (except for non-*Saccharomyces* and *A. aceti* that were grown for 48 h). In order to calculate the inoculum size, yeast cells were stained with a cell

viability marker (methylene blue) and counted using a Thoma hemocytometer chamber under the microscope. Finally, the commercial starters were inoculated according to the manufacturer's instructions.

Experimental design. Four and ten fermentations, each one in triplicate trials, were performed in laboratory conditions during the 2019 (first) and 2020 (second) vintage, respectively (according to the experimental plan reported in Figure1). Each fermentation was performed in 500 mL Erlenmeyer sterile flasks containing 350 mL of pasteurized must. In order to mimic a typical grape must environment, all trials were inoculated with five pre-adapted microorganisms (Figure 1). When necessary, potassium metabisulphite was added to obtain the different SO_2 amounts chosen.

In 2019, the following inoculation protocols were used: a) Control1: no further additions, b) Control2: 5 g/hL of SO_2 addition; c) AFPK: *P. kluyveri* Viniflora® FrootZen® inoculation; d) AFLP: *L. plantarum* Viniflora® NoVA™ inoculation. In 2020, a) Control21: no further additions. b) Controls 22, 23 and 24: addition of three different concentrations of SO_2 (1, 3 and 5 g/hL, respectively), c) AFPK1-3 trials were inoculated with *P. kluyveri* without SO_2 (AFPK1), with 1 g/hL (AFPK2) and 3 g/hL (AFPK3) of SO_2, respectively. d) AFLP1-3 trials were performed inoculating *L. plantarum* strain in the must, without SO_2 addition (AFLP1), with 1 g/hL (AFLP2) and 3 g/hL (AFLP3) of SO_2, respectively. Each flask was fitted with sterile air-locks to ensure sterile conditions during fermentation and kept at 25 °C in static conditions until the end of the monitored period. When residual sugars were less than 2.0 g/L, the fermentations were considered finished, and samples were taken from each flask to evaluate the chemical and volatile profiles of the resulting wines.

Enumeration of microbial populations. Viable yeasts and bacteria populations during fermentation were determined by plate count (colony forming units, CFU/mL). For all samples, aliquots of 1 mL were taken, serially diluted ten-fold in sterile Ringer solution (Biogenetics, Milan, Italy) and spread on culture media for the enumeration of each microorganism. Yeasts populations were determined

by plating on WLN agar medium (Biogenetics, Ponte San Nicolò, Italy) and incubating aerobically at 28°C for 5 days. Yeast species identification was based on the morphological characteristics (pigmentation and shape) of each microorganism on this medium (Pallmann et al. 2001). *L. plantarum* populations were determined on MRS agar plates (Oxoid, Milan, Italy) following aerobic incubation at 28°C for 3-5 days. *A. aceti* population was followed using ethanol agar medium (10 g/L yeast extract (Biogenetics), 20 g/L $CaCO_3$ (Sigma-Aldrich, Milan, Italy), 20 g/L agar (Biogenetics), 20 mL/L ethanol (99.9 % v/v, Sigma-Aldrich) and incubation at 28°C for 5 days. Both MRS and ethanol agar media were supplemented with 25 mg/mL of Delvocid (DSM Specialties, Heerlen, The Netherlands) to avoid yeast growth. After colony counting, means and standard deviations were calculated.

Chemical analyses. Sugars (glucose and fructose, g/L), acetic acid, (g/L), glycerol (g/L), and ethanol (% v/v) concentrations were determined during and at the end of the fermentation process, using an Agilent 1260 HPLC-UV-RI system (Agilent Technologies, Santa Clara, CA) following the chromatographic conditions reported by Rolle et al. (2018). L-malic acid, and D- and L-lactic acid (g/L) were analyzed using enzymatic kits provided by Megazyme International (Bray, Ireland) according to the manufacturer's instructions. Furthermore, also YAN concentration (primary amino acids and ammonium, mg/L) was monitored daily by using two enzymatic kits according to the manufacturer's protocol (Megazyme International). Titratable acidity (expressed as g/L of tartaric acid) was determined according to the official methods of the International Organization of Vine and Wine (OIV, 2019). pH values were registered using an InoLab 730 pH meter (WTW, Weilheim, DE). Phenolic composition and chromatic characteristics of the wines were determined by spectrophotometric indices and the protocols were described by Rolle et al. (2018) and OIV-MA-AS2-11 method (OIV, 2019), respectively. Volatile compounds in the final wines were identified and quantified by headspace solid-phase microextraction coupled to gas chromatography-mass spectrometry (HS-SPME/GC-MS), according to the protocols

reported by Englezos et al. (2018).

Statistical analyses. Statistical analysis was performed using the software IBM SPSS Statistics (version 25.0, IBM Corp., Armonk, NY, USA). Tukey-HSD post hoc test at $p < 0.05$ was applied to establish significant differences by one-way analysis of variance (ANOVA). Principal Component Analysis (PCA) was used to evaluate the fermentation performance in terms of chemical composition.

Results

2019 vintage

Population dynamics during fermentation. The growth dynamics of each microorganism was followed during fermentation (Figure 2). In AFPK (*P. kluyveri*) and AFLP (*L. plantarum*) samples, the initial populations remained stable for 4 (AFPK) and 5 (AFLP) days. Afterwards the cell counts of the above-mentioned microorganisms started to decline and became undetectable after 6 days of fermentation. *S. cerevisiae* reached its maximum population in the presence of SO_2 (Control2) (8.3 Log CFU/mL) after 3 days and remained stable until the end of the fermentation. On the contrary, in the other trials its population reached the highest levels between day 4 and 6, with values ranging from 7.7 Log CFU/mL (Control1 and AFLP) to 7.9 Log CFU/mL (AFPK). Regarding the population kinetics of the non-*Saccharomyces* yeasts initially inoculated in the must, the results showed a major inhibition in Control2. In all samples, *S. bacillaris* behavior was similar in all analyzed samples, in fact it reached the maximum population in 2-3 days, after remained stable and then started to decline from day 6 until about 5.0 Log CFU/mL at the end of the monitored period. However, differences were found in the maximum population number reached. In fact, it grew until 7.5 Log CFU/mL (Control1), 7.9 Log CFU/mL (AFPK) and 7.7 Log CFU/mL (AFLP), while, in Control2 it reached 6.5 Log CFU/mL.

In Control2 *M. pulcherrima* reduced its population from the day 1 to undetectable levels (< 10

CFU/mL) at 2 days, while in the other fermentations after an initial increase it became undetectable after 3 days for Control1 and 4 days for AFPK and AFLP.

The efficacy of the different inoculation protocols to reduce the populations of *A. aceti* and *H. uvarum*, during the first 6 days of fermentation is reported in Figure S1. In Control2, *H. uvarum* and *A. aceti* populations reached the lowest values and became undetectable in shorter time compared to the other trials. Conversely, in AFPK and AFLP, the viable count of the abovementioned microorganisms was significantly lower than the Control1, independently of the day of fermentation and starter culture used. *A. aceti* population reached the highest value after 1 day in Control1 (4.7 Log CFU/mL), while in the other trials it reached the maximum number of 3.2 Log CFU/mL (AFLP) and 3.7 Log CFU/mL (AFPK). The highest reduction in AFPK and AFLP was achieved after 5 days (population reduction down to 2.8 Log CFU/mL) and became undetectable after 6 days. In Control1, similar results were obtained after 10 days. In the same way, *H. uvarum* population was significantly reduced by the starter cultures (AFPK-AFLP) from day 1 onwards. Interestingly, no significant differences were observed between inoculated trials with only exceptions at days 5 and 6, in which wines fermented with *P. kluyveri* contained a lower density of *H. uvarum* (about 0.6 ± 0.1 Log CFU/mL less) when compared to the respective trial with *L. plantarum*.

Principal Component Analysis (PCA). Wines obtained were analyzed for their physico-chemical components (standard chemical parameters, color, phenolic and aroma compounds) (Table S1) and results were subjected to a Principal Component Analysis (PCA) (Figure 3). The first principal component (PC1) explained 47.8% and the second (PC2) 33.5%, together 81.3% of the variation in the data set. Bio-protected wines were positioned on the lower part of the plot and well separated from Control wines by Component 2. The PC1 was strongly positively correlated with hexanoic acid, ethyl dodecanoate, ethyl decanoate, ethyl octanoate, but negatively associated with pH, total anthocyanins, 2-ethyl hezanol, isoamyl alcohol, ethyl acetate and isobutanol. The PC2 was positively correlated mainly with hexanoic

acid, pH, ethyl acetate, L-lactic acid, while negatively corelated to ethanol, isoamyl alcohol, linalool and isoamyl acetate. Control2 wines were positioned on the upper right part of the PCA plot, mainly due to the relative abundance in ethyl esters of fatty acids (ethyl dodecanoate, ethyl decanoate, ethyl octanoate and ethyl hexanoate), 2-phenylethanol, high total acidity, and consequently low pH. Wines AFPK presented the highest amount of acetate esters of higher alcohols namely (E/Z) - ethyl 2-hexenoate, hexyl acetate (fruit and herb), isoamyl acetate (banana) and 2-phenylethyl acetate. AFLP were the only wines that underwent malolactic fermentation, consequently the samples had the lowest levels of malic acid and the highest amount of lactic acid. As result of Lpb. plantarum metabolism, only in these samples, ethyl lactate was identified. Control1 wines were distinguished for a high concentration of acetate esters of higher alcohols like 2- ethyl hexenol isoamyl alcohol, ethyl acetate, butanol, hexanol and isobutanol. AFLP and AFPK wines were differentiated, mainly due to the relatively high levels of 2-phenylethyl acetate and low levels of acetic acid, ethyl acetate and methionol (Table S1).

2020 vintage

Population dynamics. In the second vintage, the combined effect of the bioprotective cultures and SO_2 addition was explored. As for the 2019 vintage, the growth kinetics of each microorganism were followed by plate counts as reported in Figs. 4 and 5. *P. kluyveri* showed comparable behavior over the first 6 days of fermentation, independently the SO_2 addition (trials AFPK1-3). Indeed, yeasts number remained stable until days 6 with values of about 7.0 Log CFU/mL. Afterwards, the viable population exhibited drop to undetectable levels (< 10 CFU/mL) at day 8. On the contrary, *L. plantarum* strain showed slight differences in population dynamics due to SO_2 addition (trials AFLP1-3). While in AFLP3 the initial population remained stable for 2 days, afterwards the cell count started to decrease and became

undetectable after 6 days, in fermentations AFLP1 and AFLP2, the same behavior was observed with 2 days of delay.

Regarding the population dynamics the artificial microbiota, the results showed that the different inoculation strategies applied, influenced the growth dynamics of the added microbiota. In Control23, Control24, AFPK3 and AFLP3 trials *M. pulcherrima* population dropped from day 1 to undetectable values at day 3, while *S. bacillaris* was detected until the end of the monitored period (about 5 Log CFU/mL) (Figs. 4 and 5). The addition of moderate and high SO_2 concentrations in Control23 and 24, respectively, promoted the dominance of *S. cerevisiae* compared to the other microorganisms; since it reached its maximum population, of about 8.3 Log CFU/ml, after 3 (Control23) and 4 days (Control24), respectively, and remained stable until the end of the monitored period. In fermentations performed with the addition of *P. kluyveri* and *L. plantarum*, *S. cerevisiae* population achieved the highest counts only when 3 g/hL of SO_2 were added in the must (AFPK3-AFLP3). Concerning the two non-*Saccharomyces* yeast species *S. bacillaris* and *M. pulcherrima*, the latter was greatly affected by SO_2 addition in a dose-dependent way, since its population reduced immediately to undetectable levels (< 10 CFU/mL) after 4 days in Control22 and after 3 and 2 days in Control23 and 24 trials, respectively. In fermentations performed in presence of *P. kluyveri* (AFPK1) and *L. plantarum* (AFLP1 and AFLP2), *M. pulcherrima* cells remained at higher levels and for longer period, compared to the respective controls with and without SO_2 addition. *S. bacillaris* achieved maximum populations in Control21-22, AFPK1-2 and AFLP1-2 trials (about 7.1 Log CFU/mL) and the lowest in Control23 and 24 (about 6.5 Log CFU/mL), AFPK3 and AFLP3 (about 6.0 Log CFU/mL).

The efficacy of the different inoculation protocols to reduce the populations of the unwanted microorganisms is presented in Fig S2 and S3. Viable *A. aceti* and *H. uvarum* counts were significantly lower in Control23-24, AFPK1-3, AFLP1-3 compared to Control21 and Control 22, independently from

the day of fermentation, starter culture used and SO_2 addition. For both microorganisms, the reduction was always higher in Control24. However, the use of starter cultures in combination with 1 and 3 g/hL, lead to higher decrease of both *A. aceti* and *H. uvarum*, compared to respective fermentation with only starter culture inoculation (AFPK1 and AFLP1). Regarding *A. aceti* population at day 3, AFPK2-3 together with AFLP2-3, showed similar viable counts with those registered in Control23 (about 3.0 Log CFU/mL). It is worth noticing that in fermentations AFPK2-3 and AFLP3 the viable cells of *A. aceti* dropped to undetectable levels after 5 days, while the respective trials with *L. plantarum* AFLP2 contained about 2.0 Log CFU/mL. After 6 days of fermentation in all fermentations the viable cells dropped to undetectable levels, except in Control21. The addition of 3 and 5 g/hL SO_2 (Control23 and 24) reduced at undetectable levels *A. aceti* population after 4 days. In the same way, *H. uvarum* population was significantly reduced by the starter cultures, from day 1 onwards. However, the reduction was higher in wines inoculated with *P. kluyveri* and *L. plantarum* supplemented with 3 g/hL of SO_2, followed by those supplemented with 1 g/hL of SO_2, compared to the respective controls without SO_2 addition. This was more evident from day 3 onwards. Control24 wines presented the lowest cell counts followed by Control23. On the contrary, Control21 and 22 have similar cell values during the first 6 days of the fermentation process. On day 6 *H. uvarum* population dropped to undetectable levels in trials Control24, AFPK2-3 and AFLP3. While in the other trials on day 8, except Control21 (10 days). It is important to note that, from day 6, the only presence of the bioprotective culture (trials AFPK-AFPK1- AFPK2, AFLP-AFLP1-AFLP2) determined a reduction of *H. uvarum* higher than the moderate SO_2 addition (Control3) and that the synergic effect of the combination 3 g/L SO_2/bioprotective culture was observed from day 2 onwards.

Wine main enological parameters. All wines evidenced residual sugars content (glucose and fructose) below 2.0 g/L (Table 1). Ethanol concentration ranged between 14.11 ± 0.02 % v/v (Control23) and 14.24 ± 0.04 % v/v (AFLP1). Concerning glycerol, Control24 presented the lowest levels (9.60 ± 0.07

g/L), while trials AFPK1 (13.55 ± 0.19 g/L) and AFPK2 (13.34 ± 0.04 g/L) the highest. All the inoculated samples gave moderate values of acetic acid (below 0.30 g/L). However, wines inoculated with *P. kluyveri* and *L. plantarum* contained significantly lower levels of acetic acid compared to Control21 and Control22, independently to the SO_2 addition. Interestingly, both AFPK2 and AFPK3 wines contained the lowest levels (0.20 ± 0.01 g/L). Regarding acidity parameters, Control23 and Control24 had the highest values of total acidity (8.16 ± 0.04 g/L and 8.13 ± 0.04 g/L), while Control21, Control22, AFLP1 and AFLP2 had the lowest values ranging from 6.67 ± 0.04 g/L (Control1 and AFLP2) to 6.79 ± 0.04 g/L (AFLP1) as g/L of tartaric acid, and this situation was reflected also on pH values.

Phenolic and color characteristics. For all treatments, the starting point in terms of phenolic content coincided with the pre-fermentative maceration carried out at high temperature (60 °C for 1 h). Therefore, the evaluation of the phenolic and color characteristics of the produced wines included the effects only due to the inoculation protocols, without a concurrent maceration. The total anthocyanin content of the produced wines was not significantly affected by the treatments (Table 1). In terms of phenolic indexes such as total flavonoids and non-anthocyanin flavonoids indexes, AFPK1 and AFLP1 showed a reduced content when compared with Control21. However, when the trials with same SO_2 added content are compared, a significant difference was observed ($p < 0.05$) only between AFPK1 and Control21, with AFLP1 presenting intermediate values. This sustained preservation capability of the phenolic content was found not to be always linear with the added concentration of SO_2 (i.e. Control23-24 differences).

The differences in the phenolic composition resulted in a slightly different color at the end of the experiments (Table 1): while color intensity values were not significantly different ($p > 0.05$) among treatments, an in-depth analysis of color through CIELab color space expression evidenced a slight increase in the L* coordinate (luminosity) when SO_2 was added in highest quantities: indeed, the only

significant difference found was between Control21 and AFPK3-AFLP3. The a* coordinate (red-green) values were distributed in a similar way but with the highest value for Control21, and a decreasing non-significant trend was found for AFPK1-3 trials with respect to all control trials and AFLP1-2. In terms of b* (yellow-blue) coordinate, Control23 presented significantly different values when compared with all samples with a lower added SO_2 content. Chroma (C*) and hue angle (h*) were also calculated from wine color measurements: regarding the latter parameter, all samples are in the portion 24.14-27.84°, and the samples with 3 g/hL of added SO_2 (namely Control23, AFPK3, AFLP3) showed the highest values among those tested. The hue angle of Control24 (5 g/hL of added SO_2) was slightly less influenced by the addition, resulting in non-significant differences among other trials.

Volatile composition. In this study, 23 volatile compounds were identified in the wines (Table 1). Statistical analysis highlighted significant differences among them. By comparing these secondary compounds, Control21 and Control22 wines showed the highest amounts of unpleasant volatiles namely hexanol, isobutanol and ethyl acetate, while Control23 and Control24 wines were characterized by the highest amount of hexanoic acid, methionol and pleasant ethyl esters of fatty acids (ethyl butanoate, ethyl decanoate, ethyl dodecanoate, ethyl hexanoate and ethyl octanoate). It is worth noticing that AFLP1 wines presented the lowest amounts of methionol compared to the rest of wines. Among bioprotected wines, AFPK3 and AFLP3 had the highest concentration of isoamyl alcohol and AFPK1-2, AFLP1-2 wines the highest amounts of 2-phenylethanol, even if the levels of this last compound were higher in Control24. Regarding fermentation derived esters, Control23 and 24 together with AFLP3 wines presented the highest concentrations of diethyl succinate, while Control21-22 AFLP1-2 the lowest. In the same context, Control23-24 contained the highest amounts of ethyl decanoate, ethyl dodecanoate and ethyl octanoate, compared to the other wines. Wines fermented with *P. kluyveri* independently the SO_2 addition were characterized by the highest concentration of acetate esters of higher alcohols like ethyl 2-hexenoate, hexyl

acetate, isoamyl acetate and 2-phenylthyl acetate. Furthermore, all wines inoculated with the putative bioprotective cultures as well as the Control23 and 24 presented a significative lower contain of ethyl acetate (unpleasant nail polish and vinegar aroma) than Control21 and 22. The terpene linalool (floral and fruity) was found only in wines inoculated with the starter cultures, while SO_2 did not influence their concentration. Finally, ethyl lactate was identified in wines that underwent MLF.

Principal Component Analysis. All analyzed chemical-color components (except for C* and h* derived color parameters) were used to perform a PCA to gain further insight in the differences among wines (Figure 6). The first two PC explained 63.0% of the total variance. PC1 (38.1 % of the variance) was highly positively correlated with pH, glycerol, 1-propanol, isobutanol and 2-ethyl hexanol (coefficient $\geq$ 2.00) and strongly negatively correlated to methionol, hexanoic acid, ethyl octanoate, isoamyl alcohol, ethyl decanoate, total acidity, ethyl dodecanoate and diethyl succinate (coefficient $\geq$ -2.00). The PC2 (24.9 % of the variance) was mostly positively correlated to ethyl-2-hexenoate, hexyl acetate, 2-phenylethyl acetate and isoamyl acetate (coefficient $\geq$ 2.00) and negatively correlated to a* color coordinate, hexanol, acetic acid and butanol (coefficient $\geq$ -2.00). AFPK wines were clearly grouped on the upper right part of the plot and could be easily differentiated from the other wines, mainly due to the high levels of ethyl-2-hexenoate, hexyl acetate, 2-phenylethyl acetate and isoamyl acetate. AFPK1-2 wines appeared closely in the upper right part of the panel, and differentiated from AFPK3, mainly due to lower pH values and the lower contents of glycerol and 2-ethyl hexanol in the last sample and the higher diethyl succinate content. AFLP1-2 wines were positioned on lower right part of the plot and grouped closely to Control21 and 22, due to the high levels of pH caused by the partial MLF, 1-propanol and isobutanol. Control21 and 22 presented the highest values of ethyl acetate and isobutanol. On the contrary, AFLP3 wine appeared more closely to Control23 and 24, and differentiated from AFLP1-2 wines for the higher amounts of fatty acids, isoamyl alcohol, methionol and total acidity.

Discussion

Despite the extensive addition of bioprotective cultures in food industry, little is known about their application in the winemaking industry. To this end, two starter cultures, namely *P. kluyveri* and *L. plantarum* were tested for their bioprotective role, as opposed to SO_2 addition during the first steps of the alcoholic fermentation in two consecutive vintages. The synergistic effect of the inoculation protocol and SO_2 addition was also investigated in the second vintage. Known quantities of wine-borne yeasts and bacteria were added to a pasteurized must in order to mimic a spontaneous grape must biodiversity after grape crushing.

In both years, the early addition of low, moderate and high SO_2 levels at the beginning of the fermentation process (case of 2019 vintage: Control2; 2020 vintage Control22, 23 and 24) had a selective effect on the must microbiota by favoring the dominance of *S. cerevisiae* compared to the other microorganisms. Sensitivity of some non-*Saccharomyces* yeasts and spoilage wine bacteria in presence of SO_2 is well known, as well as the high tolerance of *S. cerevisiae* (Pérez-Torrado et al. 2017). However, the time needed to achieve the stationary phase, was strongly affected by SO_2 addition, in a dose-dependent way. Non-*Saccharomyces* and *A. aceti* populations were divergently influenced by the inoculation protocol applied. On the other hand, *S. bacillaris* and *M. pulcherrima* populations were not influenced negatively by the addition of the starter cultures. The only exception was observed when moderate SO_2 levels were added in the must together with the inoculation of the putative bioprotective starter cultures during the second vintage, since *S. bacillaris* reached lower maximum cell values, compared to the respective trials without and low SO_2 addition. The lack of antagonistic effect against these yeasts could be considered a good result due to their positive effect on wine quality (Petruzzi et al. 2017) and their antimicrobial efficacy. For example, it was demonstrated that *M. pulcherrima* strains

produce pulcherrimic acid. This compound depletes iron present in the medium that is not available for undesired wild spoilage yeasts in wine, such as *Brettanomyces*/*Dekkera*, *Hanseniaspora* and *Pichia* genera (Oro et al. 2014, Sipiczki, 2020).

H. uvarum behavior was partially affected by the presence of bioprotective cultures in both years. In fact, even if its population was not at the same day as Controls without SO_2 addition (Control1 and Control21), a reduced maximum population was reached, particularly with *P. kluyveri*. On the contrary, in both years *A. aceti* population became undetectable 4 days earlier than Control without SO_2 addition demonstrating the effect of all tested microorganisms as bioprotective cultures. Again, the decrease of these microorganisms was further reduced in the presence of low and moderate SO_2 levels in the medium during the second vintage. It is worth noticing that the addition of low SO_2 levels in the must together with *P. kluyveri* and *L. plantarum* lead to further decrease of the unwanted microorganisms, compared to the respective control trials, highlighting the key role of starter cultures in reducing these populations. This reduction in cell number during fermentation could reduce the negative impact of *A. aceti* on wine composition, as reported in previous studies (Varela et al. 2017). In a recent study, Next Generation Sequence analysis on a spontaneous fermentation of 'Uva di Troia' must revealed that the inoculum of *L. plantarum* to conduct MLF caused a strong reduction in abundance of *Gluconobacter* and *Acetobacter* (Berbegal et al. 2019). A similar behavior found in presence of *T. delbruekii*, but not when *M. pulcherrima* was added, demonstrates that this inhibition is species- and strain- specific (Simonin et al. 2018, Berbegal et al. 2019). The results of this study suggest that both species could act as bioprotective cultures against spoilage microorganisms without negatively affecting *S. cerevisiae* performances. However, it is important to highlight that SO_2 addition determined a greater reduction of all the populations investigated other than *S. cerevisiae*. This finding is in contrast with Simonin et al. (2018) that found a comparable biodiversity reduction of populations in Aligoté must between SO_2 addition and the inoculum of *T.*

delbrueckii. Probably, this difference could be due to the different conditions and species chosen as bioprotective culture.

To understand the impact of the bioprotective intervention on wine quality, the evolution of the principal metabolites and chemical parameters were evaluated (data not shown). Similar trends were registered in both years for YAN and sugar consumption. In Control21-22 and AFLP1-2 trials (second vintage) the sugar mostly consumed was glucose, compared to the rest of fermentations in which higher levels of the fructophylic yeast *S. bacillaris* were registered during fermentation (Englezos et al. 2019b). Interestingly, YAN consumption was found to be slower in the presence of *P. kluyveri* compared to the respective trials with *L. plantarum*, independently to the SO_2 addition. The slower YAN utilization by *P. kluyveri* supports general observations that this yeast consume YAN at lower levels than *S. cerevisiae* and non-*Saccharomyces* yeasts like *L. thermotolerans* and *T. delbrueckii*-(Gobert et al. 2017, Since this low nitrogen utilization, we can hypothesize that *P. kluyveri* bioprotective action could not be associated to nutrients competition. Mewa-Ngongang et al. (2019a) attributed the antagonistic effect of *P. kluyveri* against fruits spoilage fungi (*Botrytis cinerea*, *Colletotrichum acutatum* and *Rhizopus stolonifer*) and wine yeasts (*Dekkera bruxellensis* and *Dekkera anomala*) to some volatile organic compounds like 2-phenyl ethylacetate (Mewa-Ngongang et al. 2019b). Effectively, wines obtained with the addition of *P. kluyveri* are generally characterized by the increase of amount of this volatile compound.

In wines produced using bioprotective cultures and in Control wines produced without and low SO_2, the presence of higher levels of non-*Saccharomyces* yeasts was clearly reflected in the chemical composition of the wines. Of particular relevance, that these wines presented a high amount of glycerol, as already reported in other studies (Contreras et al. 2014, Tristezza et al. 2016, Rantsiou et al. 2017). Interestingly, in wines produced using starter cultures, the addition of SO_2 negatively influenced the glycerol production in a dose-dependent way, independently of the starter culture used. In fact, these wines

contained the lowest amounts of this metabolite compared to the respective trials with and without SO_2 addition, probably due to the negative impact of moderate SO_2 levels on non-*Saccharomyces* yeasts related to glycerol increase, namely *S. bacillaris* (Englezos et al. 2019b). Our analyses revealed a noticeable impact of the bioprotective starter cultures on acetic acid production in both years. The musts inoculated with the starter cultures either with or without SO_2 addition, produced wines with significantly lower contents of acetic acid compared to the respective controls. The most obvious explanation of the higher acetic acid reduction in these wines is the positive synergistic effect of the putative bioprotective starter cultures and SO_2 addition compared to the simple SO_2 addition in the must, towards the metabolic activity of apiculate yeasts and acetic acid bacteria. As they considered high producers of this metabolite and therefore less attractive for wine production (Padilla et al. 2016). *L. plantarum* NOVA was not able to complete malolactic fermentation in our conditions, while the addition of SO_2 further reduced malic acid consumption. Rubio-Breton et al. (2018) found the same behavior of this strain in Tempranillo.

Regarding the phenolic composition, as previously indicated, a short maceration was performed prior to fermentation and the resulting anthocyanins and flavanols content reflected this initial operation. The increasing trends reported for total flavonoids and non-anthocyanin flavonoids values in presence of added SO_2 (3 g/hL) were probably due to the protective antioxidant action of this compound (Guerrero and Cantos-Villar, 2015). The results of our study demonstrated that the use of *P. kluyveri* as well as *L. plantarum* did not have an antioxidant activity as efficient as that of SO_2. Simonin et al. (2018) found similar results in the use of *T. delbrueckii* as BPA. Regarding color, despite the absence of significant differences in the anthocyanin content, the addition of 3 g/hL SO_2 was found to have the highest values in hue angle color component (h*), and also the lowest chroma (C*) reported for AFPK3 and AFPL3 but not for Control23, which in turn showed the highest values of all samples. In fact, chroma values for Control wines were not following a definite trend in function of SO_2 added dose. This combination of effects on

wine color is probably due to several factors: for instance, a significantly lower pH value was found in wines with 3 g/hL added SO_2 and this parameter has a prominent effect on anthocyanin color (Heredia et al. 1998), while also the combination of SO_2 with anthocyanins may have influenced their presence in a red-colored state in wine (Giacosa et al., 2019). In this last regard, however, Control24 (5 g/hL of added SO_2) surprisingly did not follow this behavior: its hue angle, not significantly different by any other sample, showed a lowering trend with respect to the samples with 3 g/hL of added SO_2. Considering wine color, the differences between two wines can be quantified using CIELab data by means of the ΔE_{ab} color parameter (OIV, 2019). Inside each level of added SO_2 (0, 1, and 3 g/hL), ΔE_{ab} values were calculated for AFPK and AFLP trials with respect to the proper Control. The highest ΔE_{ab} values were found for Control23-AFPK3 and Control23-AFLP3 pairs (2.06 and 2.60 units, respectively), while the differences between AFPK-AFLP pairs for all added SO_2 levels (AFPK1-AFLP1, AFPK2-AFLP2, AFPK3-AFLP3) were below 0.9 units. A value of 3 units for ΔE_{ab} was previously reported as the minimum threshold needed to visually detect a wine color difference between two wines through tasting glasses (Martínez et al. 2001).

Significant differences were found also for the volatile composition of wines. The SO_2 addition determined the highest amount of ethyl esters of fatty acids. Generally, this increase was higher in wines supplemented with moderate and high SO_2 levels, during the two vintages. These compounds are formed during alcoholic fermentation by *S. cerevisiae* and significantly contribute to the fruity aroma of wines (Hu et al. 2018). Wines supplemented with 1 g/hL of SO_2 and without SO_2 were characterized by the highest amount of ethyl acetate. This ester can have favorable effects on wine aroma at concentrations below 100 mg/L. However, its concentration in the wines studied was higher than 174 mg/L (2019 vintage) and 233 mg/L (2020 vintage) and could result in altered sensory properties described as pungent and solvent/nail polish-like (Plata et al. 2003). The significantly higher ethyl acetate contents in these wines

could be explained by the higher population levels of *H. uvarum*, since this unpleasant volatile compound is considered an aromatic marker of this non-*Saccharomyces* yeast species. Furthermore, it is worth noticing that the lowest average amount of ethyl acetate (25 mg/L) was found in wines previously supplemented with 5 g/hL of SO_2, and that no significant differences were evidenced among the two bioprotective inoculation protocols during the 2019 vintage for this compound. This trend was also confirmed on the second vintage, interestingly bioprotective wines contained the lowest amounts of this unpleasant metabolite, together with Control24. Divergently, the presence of *P. kluyveri* determined the highest concentration of acetate esters of higher alcohols in both years. Our results agree with those published by other authors who have found that this yeast was able to produce high quantities of 2-phenylethyl acetate and isoamyl acetate (Padilla et al. 2016, Dutraive et al. 2019, Prior et al. 2019). Further studies are necessary to understand if this high production is correlated with the capacity of non-*Saccharomyces* to produce precursors (Lu et al. 2017), from modifications of the metabolic regulation of *S. cerevisiae* (because of their coexistence) or, presumably, as a sum of both factors (Belda et al. 2017).

Conclusion

In this study, the efficacy of the oenological starters *P. kluyveri* Frootzen and *L. plantarum* NOVA, as bioprotective agents against some wine spoilage microorganisms was investigated during alcoholic fermentation in two consecutive vintages. The synergic effect of these starter cultures with low and moderate SO_2 levels was also explored. Results have shown that, in our experimental conditions, the inoculation of *P. kluyveri* or *L. plantarum* represents a putative strategy to reduce SO_2 content in wine, since it led to a faster decrease of the portion of microorganisms considered negative for wine quality, compared to uninoculated trial. However, the two considered species are not able to totally replace SO_2, because an antioxidant activity was not found. From a chemical point of view, in both years, wines

produced by starters inoculation were associated with a reduced content of both acetic acid and ethyl acetate that could be considered negative for wine quality. In the second year, acetic acid reduction was even more pronounced in wines produced by *P. kluyveri* inoculation and SO_2 addition (1 and 3 g/hL). Since all data presented here were obtained under laboratory scale conditions using a pasteurized natural must and artificial inoculation of indigenous microorganisms, further investigations are necessary to access the bioprotective ability of the inoculated strains in real winemaking conditions.

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Table 1 Chemical parameters of wines produced by the different inoculation protocols (2020 vintage).

Inoculation protocol	Control21	Control22	Control23	Control24	AFPK1	AFPK2	AFPK3	AFLP1	AF
Residual sugars (g/L)	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0	< 2.0	<
Acetic acid (g/L)	0.30 ± 0.01 d	0.28 ± 0.01 d	0.23 ± 0.01 bc	0.25 ± 0.01 c	0.23 ± 0.01 bc	0.20 ± 0.02 a	0.20 ± 0.01 a	0.24 ± 0.01 bc	0.23 ±
Glycerol (g/L)	12.63 ± 0.07 c	12.80 ± 0.10 c	12.14 ± 0.01 b	9.60 ± 0.07 a	13.55 ± 0.19 d	13.34 ± 0.04 d	12.80 ± 0.20 c	12.71 ± 0.05 c	12.69
Ethanol (% v/v)	14.20 ± 0.01 ab	14.18 ± 0.03 ab	14.11 ± 0.02 a	14.21 ± 0.01 b	14.17 ± 0.06 ab	14.20 ± 0.05 ab	14.16 ± 0.02 ab	14.24 ± 0.04 b	14.20 ±
L-malic acid (g/L)	2.99 ± 0.05 d	2.88 ± 0.01 d	2.89 ± 0.03 d	2.90 ± 0.02 d	2.85 ± 0.03 d	2.88 ± 0.01 d	2.84 ± 0.04 d	1.07 ± 0.09 a	2.05 ±
D-lactic acid (g/L)	0.27 ± 0.04 b	0.27 ± 0.01 b	0.26 ± 0.02 b	0.28 ± 0.01 b	0.26 ± 0.01 b	0.28 ± 0.02 b	0.25 ± 0.01 b	0.08 ± 0.01 a	0.10 ±
L-lactic acid (g/L)	0.09 ± 0.01 ab	0.08 ± 0.02 ab	0.13 ± 0.01 b	0.08 ± 0.01 a	0.09 ± 0.01 ab	0.09 ± 0.01 ab	0.11 ± 0.01 ab	1.80 ± 0.03 e	0.97 ±
Glycerol yield[a]	0.052 ± 0.001 c	0.053 ± 0.002 c	0.05 ± 0.001 b	0.04 ± 0.001 a	0.056 ± 0.001 d	0.055 ± 0.001 d	0.053 ± 0.001 c	0.052 ± 0.001 c	0.052 ±
Ethanol yield[b]	0.058 ± 0.001 ab	0.058 ± 0.001 ab	0.058 ± 0.001 a	0.058 ± 0.001 b	0.058 ± 0.002 ab	0.058 ± 0.001 ab	0.058 ± 0.001 ab	0.059 ± 0.001 b	0.058 ±
pH	3.52 ± 0.01 cd	3.53 ± 0.01 de	3.43 ± 0.02 a	3.44 ± 0.01 a	3.52 ± 0.01 cde	3.51 ± 0.01 c	3.48 ± 0.01 b	3.54 ± 0.01 e	3.53 ±
Total acidity (g/L as tartaric acid)	6.67 ± 0.04 a	6.75 ± 0.04 a	8.16 ± 0.04 c	8.13 ± 0.04 c	7.18 ± 0.06 b	7.32 ± 0.04 b	7.32 ± 0.07 b	6.79 ± 0.08 a	6.67 ±
Total anthocyanins (mg/L)[c]	42.7 ± 0.5	44.5 ± 0.4	43.4 ± 0.8	42.4 ± 5.1	45.5 ± 1.2	45.5 ± 1.4	42.2 ± 4.7	41.0± 1.5	42.6
Total flavonoids (mg/L)[d]	369 ± 1 bcd	367 ± 12 bcd	403 ± 6 e	384 ± 31 cde	338 ± 9 a	343 ± 5 ab	385 ± 1 de	354 ± 5 abc	352
Non-anthocyanin flavonoids (mg/L)[b]	307 ± 2 bcd	303 ± 13 abcd	340 ± 6 e	322 ± 39 cde	272 ± 9 a	277 ± 3 ab	324 ± 7 de	294 ± 3 abcd	289 ±
L*	72.3 ± 1.5 a	73.2 ± 0.7 ab	73.5 ± 0.3 ab	74.2 ± 2.2 ab	73.6 ± 0.7 ab	74.3 ± 0.5 ab	74.9 ± 0.4 b	73.5 ± 0.5 ab	73.8 ±
a*	31.11 ± 0.31 c	30.65 ± 0.31 bc	30.82 ± 0.28 c	30.25 ± 1.67 abc	30.06 ± 0.20 abc	30.07 ± 0.06 abc	29.48 ± 0.03 ab	30.95 ± 0.13 c	30.52 ±
b*	13.96 ± 0.96 a	13.73 ± 0.24 a	16.02 ± 0.13 b	14.78 ± 1.18 ab	14.26 ± 0.54 a	14.37 ± 0.22 a	15.23 ± 0.47 ab	14.27 ± 0.22 a	13.81
C*	34,11 ± 0,13 bc	33.59 ±0.23 bc	34.73 ±0.31 c	33.69 ±0.99 abc	33.27 ±0.40 ab	33.33 ±0.05 ab	33.18 ±0.23 ab	34.08 ±0.20 bc	33.50 ±0.34 ab
h* [deg]	24.16 ±1.67 a	24.14 ± 0.56 a	27.46 ±0.07 b	26.09 ±3.05 ab	25.38 ±0.72 ab	25.55 ±0.39 ab	27.32 ±0.71 b	24.76 ±0.26 ab	24.35 ±0.17 a
Color intensity (a.u., 10 mm OP)	1.17 ± 0.09	1.12 ± 0.03	1.15 ± 0.02	1.10 ± 0.08	1.11 ± 0.04	1.08 ± 0.02	1.06 ± 0.02	1.12 ± 0.03	1.10
Color hue	0.80 ± 0.03	0.80 ± 0.01	0.83 ± 0.01	0.82 ± 0.04	0.83 ± 0.01	0.82 ± 0.01	0.84 ± 0.01	0.80 ± 0.01	0.80
Butanol	36.14 ± 0.95 bcd	36.87 ± 3.85 cd	29.38 ± 1.86 a	41.19 ± 2.07 d	blq	blq	33.33 ± 2.53 abc	34.24 ± 3.18 abc	36.5 ±
Hexanol#	778.84 ± 7.33 d	746.35 ± 18.16 d	556.1 ± 14.28 bc	521.67 ± 16.17 b	403.59 ± 11.12 a	350.13 ± 39.89 a	355.67 ± 14.85 a	725.22 ± 22.9 d	742.83
Isoamyl alcohol#	127512.67 ± 2660.61 ab	126610.12 ± 670.82 ab	209738.36 ± 4736.12 d	219630.82 ± 11791.97 d	145234.27 ± 3296.03 bc	137490.16 ± 10380.03 abc	162134.64 ± 9697.77 c	115962.08 ± 12095.15 a	120202.11 ±
Isobutanol	688.02 ± 18.85 c	671.57 ± 9.06 c	462.74 ± 22.98 ab	41.19 ± 2.07 a	420.17 ± 17.63 ab	352.27 ± 10.66 a	438.18 ± 38.9 ab	430.57 ± 86.33 ab	457.01 ±
1-propanol	193.74 ± 39.69 ab	217.26 ± 13.91 ab	186.76 ± 14.32 ab	162.43 ± 1.36 a	262.04 ± 38.12 b	264.28 ± 35.41 b	226.6 ± 43.61 ab	232.88 ± 6.87 b	222.35
2-phenyl ethanol#	52098.65 ± 2625.88 b	55258.2 ± 4782.34 b	90661.48 ± 7498.03 d	104681.68 ± 4073.71 e	82318.98 ± 1611.38 c	84774.51 ± 4851.87 c	59564.12 ± 2620.61 b	80904.3 ± 3321.07 d	80551.28 ±
2-ethyl hexanol	14.12 ± 24.46	56.62 ± 8.97	blq	18.39 ± 31.85	51.6 ± 5.41	33.26 ± 28.81	blq	23.67 ± 21.27	19.51
Diethyl succinate#	98.63 ± 0.84a	94.13 ± 5.78 a	403.87 ± 10.84 e	380.9 ± 13.49 e	194.89 ± 12.15 b	235.86 ± 2.16 c	321.81 ± 31.74 d	98.78 ± 8.66 a	94.22
Ethyl acetate#	219480.61 ± 8349.36 c	233105.9 ± 6004.12c	72752.38 ± 4279.26 b	25155.79 ± 3759.72 a	49702.13 ± 13160.96 ab	40375.6 ± 1668.95 b	23243.24 ± 12007.07 a	22495.26 ± 9370.88 a	22778.68
Ethyl butanoate#	blq	blq	162.98 ± 6.84 b	131.38 ± 2.83 a	blq	blq	blq	blq	b
Ethyl decanoate#	6.63 ± 1.12 a	6.91 ± 1.67 a	34.67 ± 0.95 c	42.95 ± 3.25 c	18.08 ± 6.94 b	16.28 ± 2.61 ab	22.09 ± 4.03 b	6.95 ± 1.25 a	7.48 ±
Ethyl dodecanoate#	3.76 ± 1.07 a	3.79 ± 3.47 a	9.36 ± 0.84b c	9.66 ± 0.28 bc	3.73 ± 0.22 a	5.72 ± 1.26 ab	7.33 ± 0.67 abc	3.92 ± 0.3 a	3.35 ±
Ethyl hexanoate#	53.2 ± 0.68	49.61 ± 1.09	205.67 ± 9.05	866.34 ± 1135.43	105.9 ± 4.35	111.93 ± 12.97	134.61 ± 9.34	48.62 ± 3.21	54.2
Ethyl lactate	blq	blq	blq	blq	blq	blq	blq	4100.21 ± 6.23	3000
Ethyl nonanoate#	blq	0.17 ± 0.3	blq	blq	blq	0.3 ± 0.52	0.42 ± 0.38	0.39 ± 0.67	0.51
Ethyl octanoate#	24.78 ± 8.13 a	24.39 ± 8.13 a	141.95 ± 11.11 c	147.31 ± 19.28 c	45.23 ± 2.38 ab	64.63 ± 14.79 b	74.34 ± 9.07 b	18.75 ± 2.92 a	21.59
Ethyl-2-hexenoate	blq	blq	blq	blq	25.77 ± 0.75 b	27.23 ± 1.81 b	19.3 ± 0.23 a	blq	b
Hexyl acetate#	7.1 ± 0.47 a	6.93 ± 0.47 a	3.1 ± 2.68 a	7.7 ± 1.36 a	55.33 ± 6.61 b	64.27 ± 2.31 c	55.72 ± 1.33 b	6.32 ± 0.47 a	6.72 ±
Isoamyl acetate#	279.45 ± 19.76 a	276.29 ± 0.52 a	488.93 ± 37.22 b	721.22 ± 3.99 c	1389.81 ± 33.49 e	1307.1 ± 169.88 e	904.75 ± 60.67 d	272.69 ± 26.95 a	299.9 ±
2-phenylethyl acetate*	139.22 ± 4.65 a	99.29 ± 85.99 a	191.18 ± 3.4 a	307.95 ± 10.9 a	1627.45 ± 111.11 b	1967.75 ± 243.46 c	1716.11 ± 102.72 bc	156.46 ± 31.31 a	136.25
Linalool*	blq	blq	blq	blq	7.83 ± 0.37	8.59 ± 0.23	8.8 ± 0.23	7.71 ± 0.27	2.61
Hexanoic acid	42.25 ± 3.36 ab	32.96 ± 0.25 a	111.13 ± 8.84 d	146.12 ± 9.31 e	49.34 ± 6.54 b	66.77 ± 2.18 c	71.71 ± 1.82 c	27.21 ± 3.99 a	27.86
Methionol#	2335.31 ± 52.47 ab	2427.52 ± 86.78 ab	3676.95 ± 50.83 d	4263.09 ± 70.01 e	2401.47 ± 118.92 ab	2581.9 ± 180.62 bc	2897.5 ± 118.45 c	2153.02 ± 190.31 a	2310.94 ±

Sugars concentration before fermentation: 242 g/L (glucose 120 g/L, fructose 122 g/L). Values are expressed as mean ± standard deviation of three independent replicates.

Sign.: *, *** and NS indicate significance at $p < 0.05$, $p < 0.001$ and not significant, respectively. Different letters in the same column mean significant difference at $p < 0.05$

(Tukey HSD test). [a]Glycerol yield: glycerol production/sugar consumption. [b]Ethanol yield: ethanol production/sugar consumption. [c]: mg/L as malvidin-3-O-glucoside chloride. [d]: mg/L as (+)-catechin; OP: optical path. Aroma compounds in wines are expressed in µg/L as mean ± standard deviation of three independent replicates. blq: below quantitation limit. [#]Concentration of each identified compound was calculated by a calibration with standard solutions analysed under the same conditions as the wine samples. The rest of volatile compounds were semi-quantified in relation to 1-heptanol internal standard.

Figure captions

Figure 1 Schematic representation of the different sets of fermentations performed.

Figure 2 Growth dynamics of the microorganisms during the different inoculation protocols applied in this study (2019 vintage). Left panel: *Acetobacter aceti* and *Hanseniaspora uvarum*; Middle panel: *Metchinikowia pulcherrima* and *Starmerella bacillaris*; Right panel: *Saccharomyces cerevisiae*, *Pichia kluyveri* (AFPK) and *Lactobacillus plantarum* (AFLP). Data are the means ± deviation standard of three independent experiments.

Figure 3 Score plot and loading plots of the first and second principal components after PCA of standard chemical compounds, chromatic characteristics and volatile compounds determined in Nebbiolo wines at the end of fermentation (2019 vintage).

Figure 4 Growth dynamics of the microorganisms during the control fermentations (2020 vintage). Left panel: *Acetobacter aceti* and *Hanseniaspora uvarum*; Middle panel: *Metchinikowia pulcherrima* and *Starmerella bacillaris*; Right panel: *Saccharomyces cerevisiae*. Data are the means ± deviation standard of three independent experiments.

Figure 5 Growth dynamics of the microorganisms during the different inoculation protocols applied in this study (2020 vintage). Left panel: *Acetobacter aceti* and *Hanseniaspora uvarum*; Middle panel: *Metchinikowia pulcherrima* and *Starmerella bacillaris*; Right panel: *Saccharomyces cerevisiae*, *Pichia kluyveri* (AFPK1-3) and *Lactobacillus plantarum* (AFLP1-3). Data are the means ± deviation standard of three independent experiments.

Figure 6 Score plot and loading plots of the first and second principal components after PCA of standard chemical compounds, chromatic characteristics and volatile compounds determined in Nebbiolo wines at the end of fermentation (2020 vintage).

Figures

Figure 1

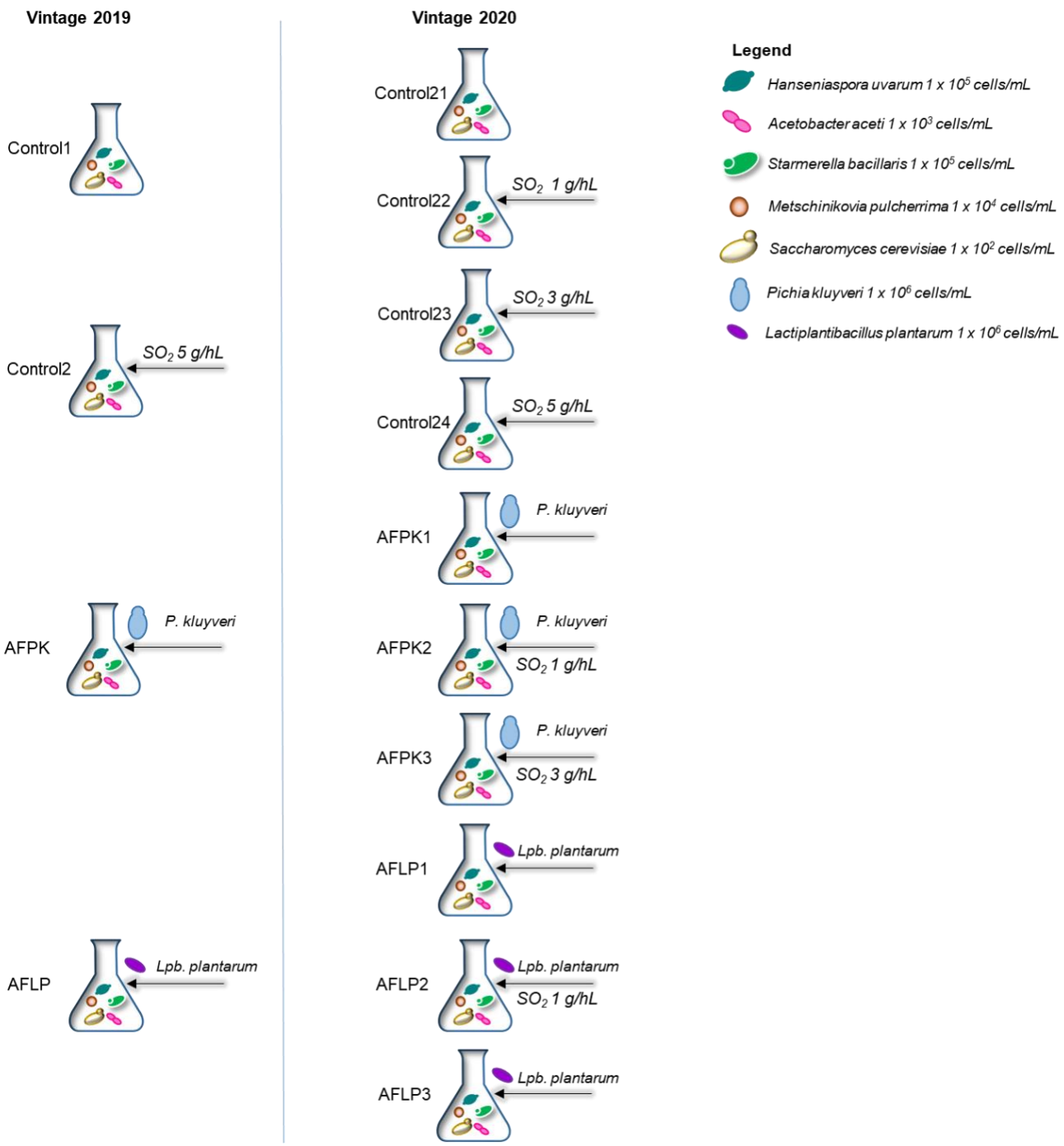


Figure 2

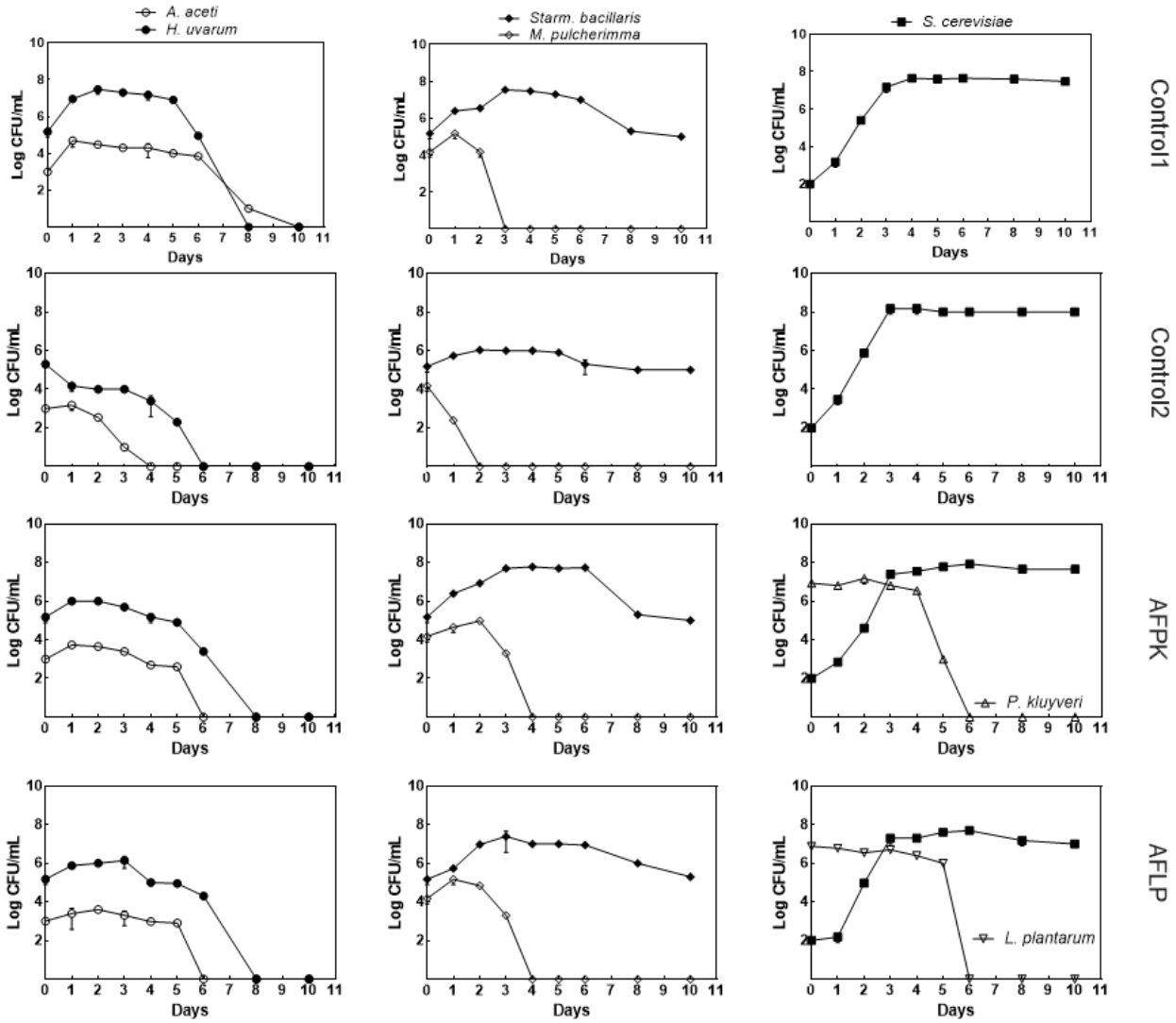

A. aceti
H. uvarum
Starm. bacillaris
M. pulcherimma
S. cerevisiae
P. kluyveri
L. plantarum
Log CFU/mL
Days
Control1
Control2
AFPK
AFLP

Figure 3

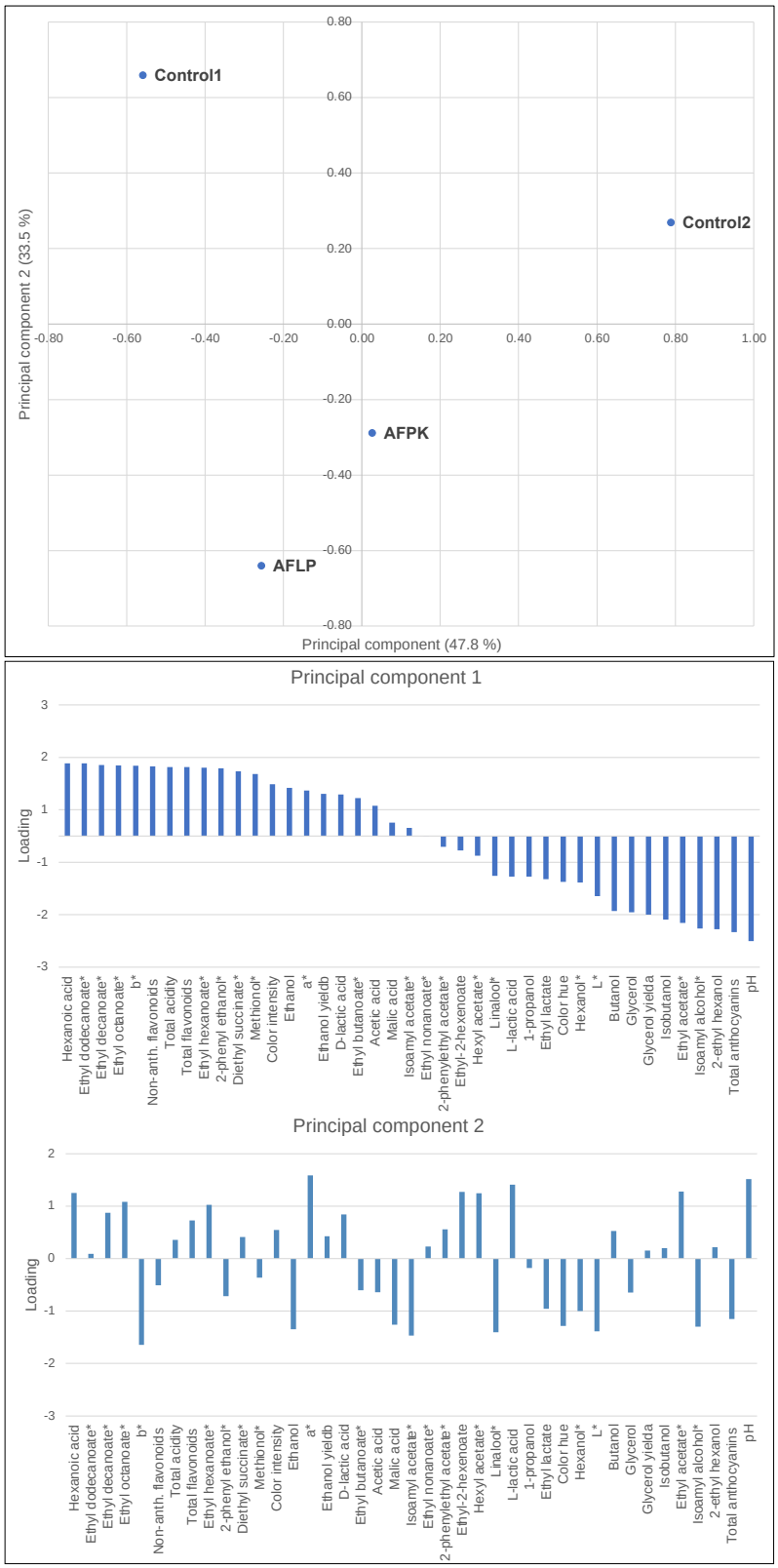

Control1
Control2
AFPK
AFLP
Principal component 2 (33.5 %)
Principal component (47.8 %)
Principal component 1
Loading
Principal component 2
Loading
Hexanoic acid
Ethyl dodecanoate*
Ethyl decanoate*
Ethyl octanoate*
b*
Non-anth. flavonoids
Total acidity
Total flavonoids
Ethyl hexanoate*
2-phenyl ethanol*
Diethyl succinate*
Methionol*
Color intensity
Ethanol
a*
Ethanol yieldb
D-lactic acid
Ethyl butanoate*
Acetic acid
Malic acid
Isoamyl acetate*
Ethyl nonanoate*
2-phenylethyl acetate*
Ethyl-2-hexenoate
Hexyl acetate*
Linalool*
L-lactic acid
1-propanol
Ethyl lactate
Color hue
Hexanol*
L*
Butanol
Glycerol
Glycerol yield a
Isobutanol
Ethyl acetate*
Isoamyl alcohol*
2-ethyl hexanol
Total anthocyanins
pH

Figure 4

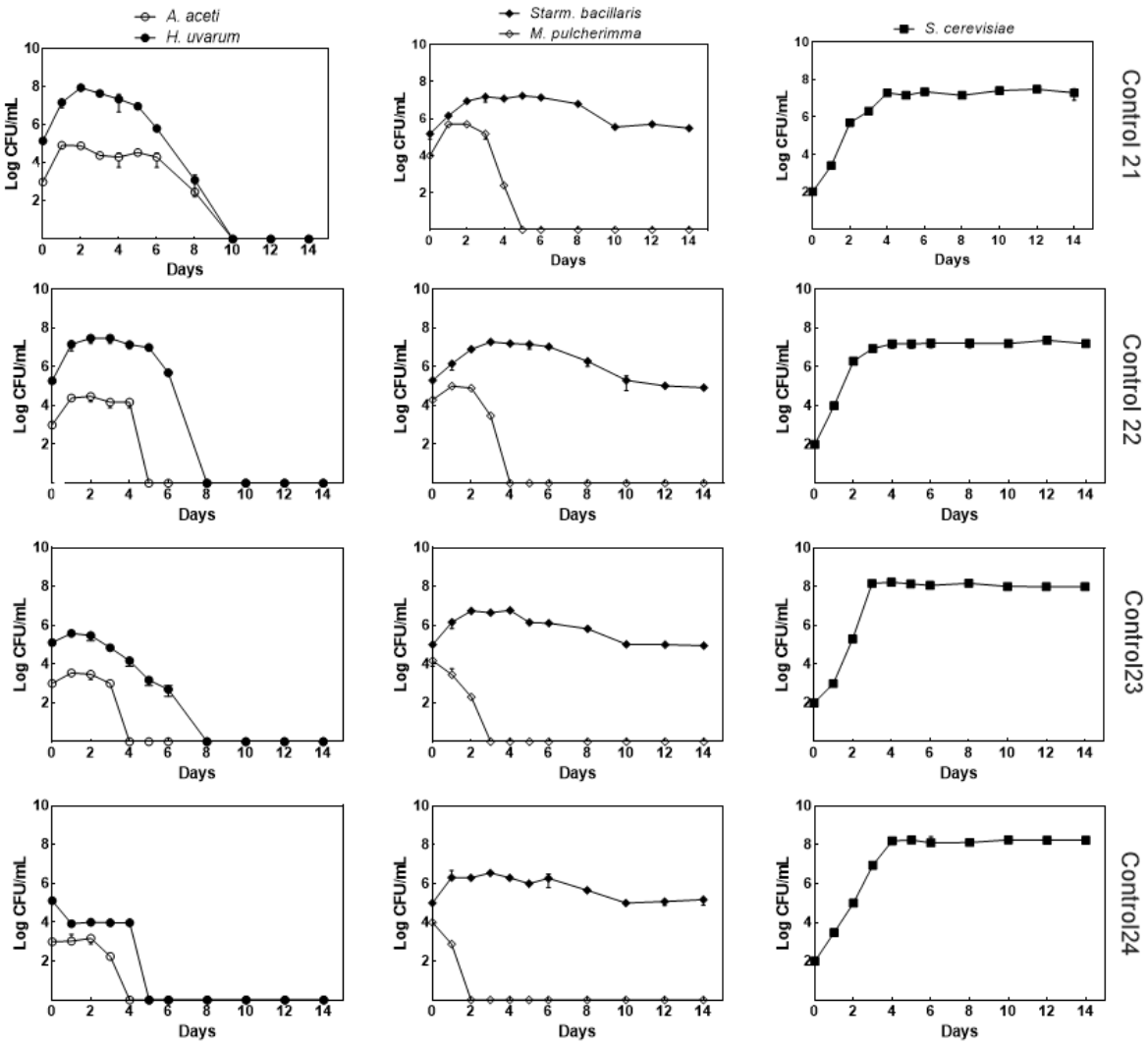

A. aceti
H. uvarum
Starm. bacillaris
M. pulcherimma
S. cerevisiae
Log CFU/mL
Days
Control 21
Control 22
Control23
Control24

Figure 5

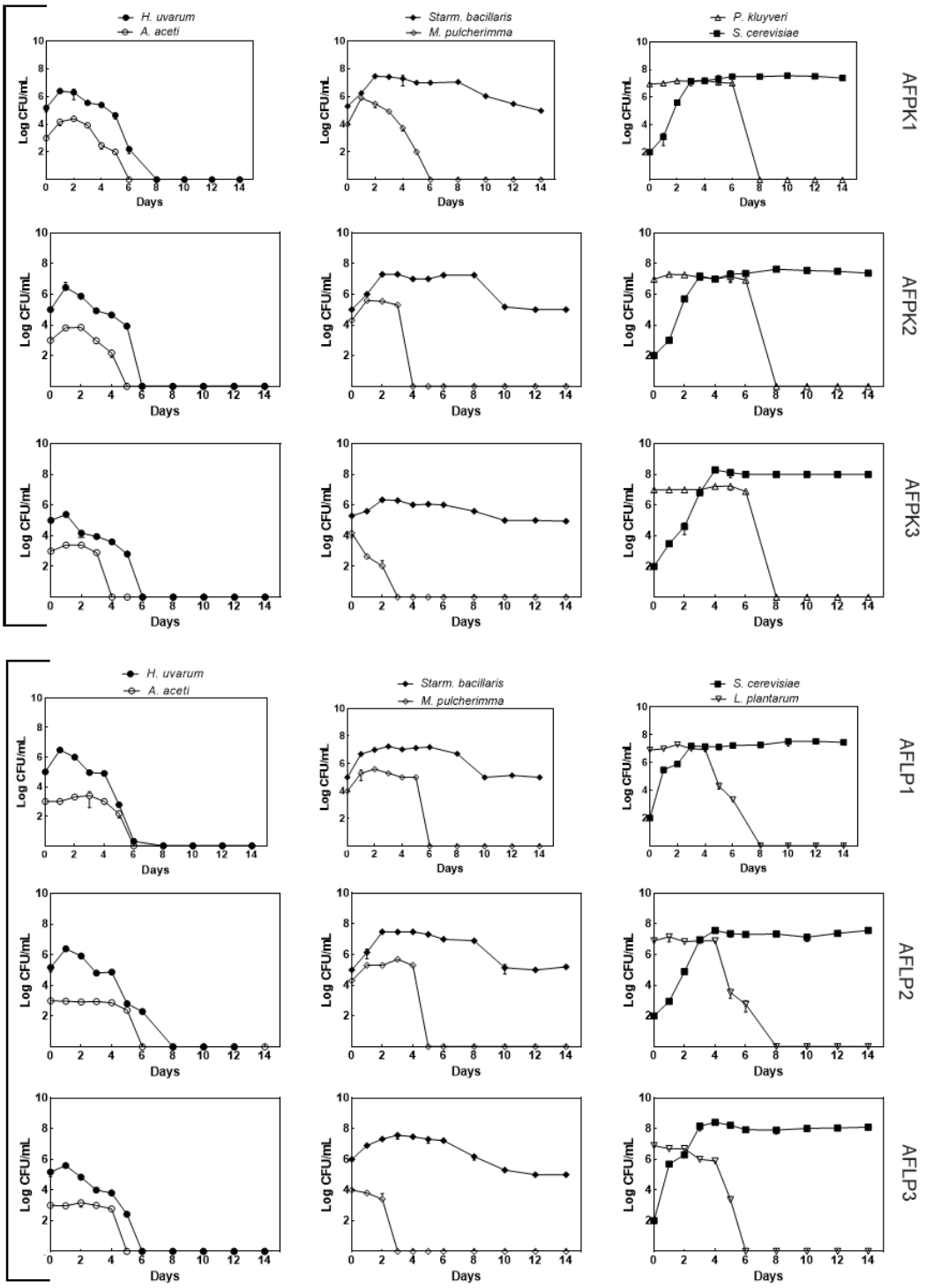
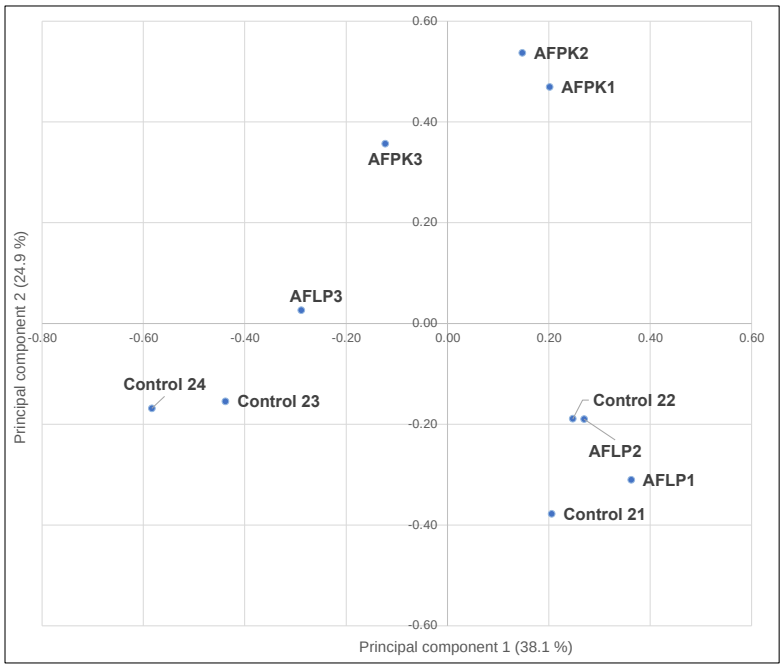
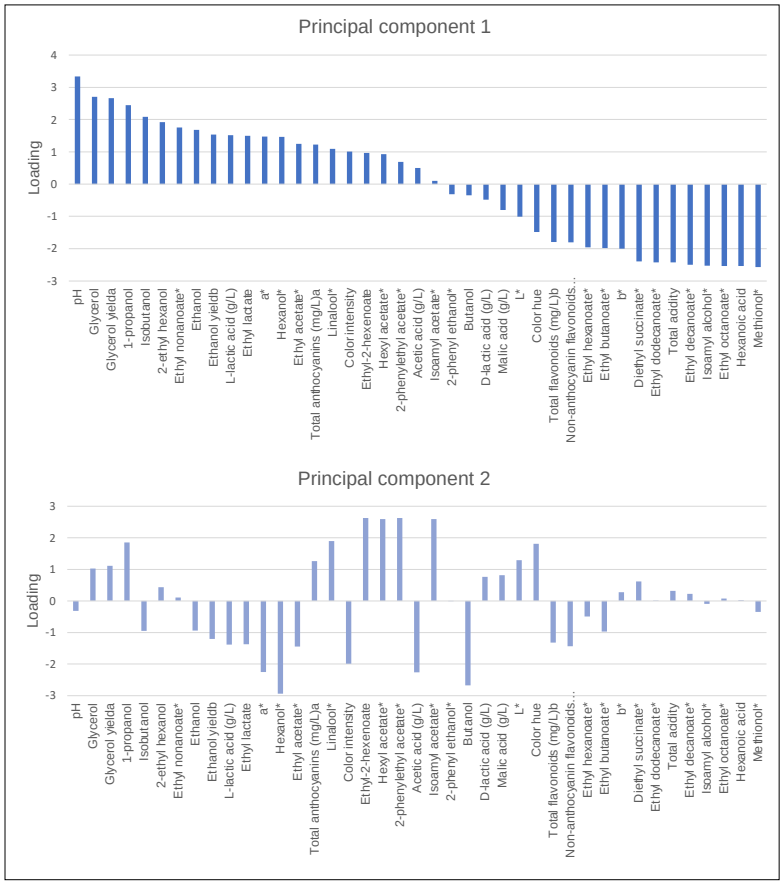

H. uvarum
A. aceti
Starm. bacillaris
M. pulcherimma
P. kluyveri
S. cerevisiae
S. cerevisiae
L. plantarum
AFPK1
AFPK2
AFPK3
AFLP1
AFLP2
AFLP3
Log CFU/mL
Days

Figure 6


AFPK2
AFPK1
AFPK3
AFLP3
Control 24
Control 23
Control 22
AFLP2
AFLP1
Control 21
Principal component 2 (24.9 %)
Principal component 1 (38.1 %)


Principal component 1
Loading
Principal component 2
Loading