Inoculum Strategies and Performances of Malolactic Starter Lactobacillus plantarum M10: Impact on Chemical and Sensorial Characteristics of Fiano Wine

Silvia Jane Lombardi 1, Gianfranco Pannella 1, Massimo Iorizzo 1,*, Bruno Testa 1, Mariantonietta Succi 1, Patrizio Tremonte 1, Elena Sorrentino 1,*, Massimo Di Renzo 2, Daniela Strollo 2 and Raffaele Coppola 1

1 Department of Agricultural, Environmental and Food Sciences, University of Molise, 86100 Campobasso, Italy; silvia.lombardi@unimol.it (S.J.L.); gianfranco.pannella@unimol.it (G.P.); bruno.testa@studenti.unimol.it (B.T.); succi@unimol.it (M.S.); tremonte@unimol.it (P.T.); sorrentino@unimol.it (E.S.); coppola@unimol.it (R.C.)
2 Mastroberardino spa winery, via Manfredi, 75-81, 83042 Atripalda, Avellino, Italy; massimo.direnzo@mastroberardino.com (M.D.R.); daniela.strollo@mastroberardino.com (D.S.)
* Correspondence: iorizzo@unimol.it

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Abstract: Malolactic fermentation (MLF) is a biological process that, in addition to deacidifying, also improves biological stability and changes the chemical and sensorial characteristics of wines. However, multiple biotic and abiotic factors, present in must and wine, make the onset and completion of MLF by indigenous malolactic bacteria or added commercial starters difficult. This work illustrates the metabolic and fermentative dynamics in winemaking Fiano wine, using a commercial starter of Saccharomyces cerevisiae and the selected strain Lactobacillus plantarum M10. In particular, an inoculum of malolactic starter was assessed at the beginning of alcoholic fermentation (early co-inoculum), at half alcoholic fermentation (late co-inoculum), and post alcoholic fermentation (sequential inoculum). The malolactic starter, before its use, was pre-adapted in sub-optimal growth conditions (pH 5.0). In sequential inoculum of the Lb. plantarum M10, even in a wine with high acidity, has confirmed its good technological and enzymatic characteristics, completing the MLF and enriching the wine with desirable volatile compounds.

Keywords: Lactobacillus plantarum; malolactic fermentation; inoculum timing; Fiano wine

1. Introduction

Malolactic fermentation (MLF) does not only represent the enzymatic conversion of L-malic acid to L-lactic acid and CO₂, but it also includes other metabolic activities by lactic acid bacteria (LAB), which influence the aromatic compounds and provide biological stability to wine [1–3]. Generally, MLF takes place at the end of alcoholic fermentation and is mainly carried out by lactic acid bacteria belonging to two species: Oenococcus oeni and Lb. plantarum.

This fermentation may occur spontaneously or be induced by the addition of LAB commercial starters. Recently, their use has been preferred by the winemakers to guarantee more success in wine deacidification, also enhancing the sensorial features of wines. Scientific literature has highlighted that malolactic starters are selected according to very strict criteria and are able to survive in extremely difficult conditions [4–8].

However, despite current scientific and technical knowledge, the malolactic fermentation often remains unpredictable and difficult to manage. This is because the bacterial performances are strongly affected by different biological and chemical-physical variables: nutritional competition with yeasts,
In recent years, apart from the search for new and increasingly effective starters, different inoculum techniques are used: sequential (after AF), co-inoculum (beginning or during alcoholic fermentation) and different forms of pre-adaptation of the malolactic starter [15–17]. Lately, a study has proved that *Lb. plantarum* strains long-term adapted to sub-optimal pH values better guarantee the course of the malolactic fermentation [17]. Moreover, several authors studying *Lb. plantarum* strains have described resistance mechanisms to high concentrations of ethanol and low pH, also evidencing that the stress response is variable and strain-dependent [18–23]. In addition, it has been demonstrated that *Lb. plantarum* strains during malolactic fermentation play an important role in wine quality definition [24,25]. In particular, their β-glycosidase activity release odorous compounds, including monoterpenes, that strongly influence the flavor profile of wine [26–28]. Despite that the influence of malolactic *Lb. plantarum* strains on the quality of wine has been widely investigated [29], our knowledge is still limited. Certain wine, such as Fiano, obtained from white grapes variety grown in Southern Italy, contain low levels of free volatile but are characterized by remarkably high concentrations of aromatic glycosylated precursors that can be transformed into odor-active volatiles during winemaking. In this respect, the β-glycosidase activity of *Lb. plantarum* can promote the release of odorous aglycones that increase the aromatic complexity of the wine [30–34]. So far, due to the hard-environmental condition characterizing Fiano wine, malolactic fermentation does not spontaneously take place and is difficult to manage even using commercial malolactic starters.

Fiano grapes, despite being harvested later than other white grapes, are characterized by pH values of about 3.3, which are sub-lethal conditions for malolactic bacteria.

The present study monitors the evolution of the fermentation processes and the chemical profile of Fiano wines produced using a commercial *Saccharomyces cerevisiae* and selected strain *Lb. plantarum* M10.

In particular, we have investigated the effects of malolactic starter, pre-adapted in sub-optimal growth conditions, and used at the beginning, at half-time, or after alcoholic fermentation.

2. Materials and Methods

2.1. Strains, Media, Growth Conditions and Inoculum Preparation

In this work, the oenological commercial yeast *Saccharomyces cerevisiae* (Fermol Elegance, AEB, Brescia, Italy; indicated below with the abbreviation FE) and the bacterial strain *Lactobacillus plantarum* M10 were used. The M10 strain was taken up by a selection program aimed at selecting strains of *Lactobacillus plantarum* possessing good technological properties for malolactic fermentation, not a biogenic amine producer and possessing β-glucosidase activity [35]. The strain *S. cerevisiae* FE was rehydrated according to the manufacturer’s instructions, while the strain *Lb. plantarum* M10, stored at −80 °C in skim milk (Biolife, Milan, Italy), was propagated twice in de Man, Rogosa, and Sharpe (MRS) medium (Oxoid Ltd., Basingstoke, Hampshire, UK) at 28 °C before being used for inoculum preparation.

In order to obtain pre-adapted cells at sub-optimal pH, *Lb. plantarum* M10 was cultivated at 28 °C in MRS broth acidified at pH 5.0, using a laboratory-scale fermentation system (Biostat Aplus, Sartorius AG, Germany) with a working volume of 4.5 L, mixed at 200 rpm. Non-pre-adapted cells were obtained following the same procedure using MRS at pH 6.5 (Batch C). At the beginning of the stationary phase (approximately 10⁹ CFU/mL), the cells of both cultures were recovered and centrifuged at 10,000 rpm for 10 min at 4 °C. The supernatant was discarded and the pellet was washed twice with 1× of phosphate-buffered saline (PBS). Cells were suspended in filtrate must (with 0.4 μm membrane filter; ZP 130 Dalcin, Italy) and used to inoculate (10⁷ CFU/mL) [15].

2.2. Experimental Design of Winemaking Process

In this study, white grape of *Vitis vinifera* cv. Fiano was used, harvested in the area of the city of Avellino (Italy) during the 2018 vintage. The winemaking process was carried out in a pilot plant in the
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Mastroberardino winery, located in the Campania region (Italy). The grape must show the following chemical composition: pH 3.24 ± 0.03, reducing sugars 216 g/L, titratable acidity 8.64 ± 0.12 (g/L tartaric acid), L-malic acid 3.44 g/L, L-lactic acid 0.04 g/L, acetic acid 0.01 g/L and YAN (yeast assimilable nitrogen) 158 ± 3 mg/L. To maintain an optimal YAN level for yeasts, after 72 h of fermentation, all the batches were added with 25 mg/L of ammonium phosphate. The wine fermentation process was performed according to the usual white wine winemaking. In detail, 50 mg/L of potassium metabisulphite was added to the grape must before fermentation. Then, five batches were set up and inoculated with about $10^7$ CFU/mL of *S. cerevisiae* FE. *Lb. plantarum* M10 ($10^7$ CFU/mL) was added at different stages of the winemaking process, as detailed below.

Batch A: Grape must, was inoculated with *S. cerevisiae* FE and halfway through the alcoholic fermentation (about 5% ethanol) *Lb. plantarum* M10 was added (late co-inoculum); Batch B: *S. cerevisiae* FE and *Lb. plantarum* M10 were inoculated at the beginning of the alcoholic fermentation, after about 24 h, (early co-inoculum); Batch C: Grape must was inoculated with *S. cerevisiae* FE and at the end of the alcoholic fermentation *Lb. plantarum* M10 was added (sequential inoculum non pre-adapted); Batch D: Grape must was inoculated with *S. cerevisiae* FE and at the end of the alcoholic fermentation strain *Lb. plantarum* M10 was added (sequential inoculum pre-adapted); Batch E: Grape must was only inoculated with *S. cerevisiae* FE (control). In Batches C, D and E, at the end of the alcoholic fermentation, 20 g/L of nutrient for MLF (LATTittante, Dal Cin, Milan, Italy) was added, according to the usual practice in winery for malolactic fermentation.

The experiments were performed in triplicates in stainless steel tanks (AISI 304 quality) of a working volume of 1 hl at 20 °C. The alcoholic (AF) and the malolactic (MLF) fermentation were monitored during the winemaking process at regular intervals. The alcoholic fermentation was considered finished when the reducing sugars were less than 2 g/L.

2.3. Microbiological Analyses

Colony counting was conducted in order to follow the growth trend of yeasts and lactic acid bacteria (LAB) during the winemaking processes. In detail, samples of different batches were picked up and at regular intervals (2 days), until the end of winemaking. One mL of sample was serially diluted with a sterile solution of peptone water (0.1%) and inoculated in appropriate media as follows: LAB were counted after 48 ± 2 h of incubation at 28 ± 1 °C in anaerobic conditions (GENbox anaer, bioMérieux, Marcy-l’Etoile, France) on MRS agar supplemented with 40 mg/L of cycloheximide; yeasts were detected on YPD agar (2% yeast extract, 2% w/v peptone and 4% w/v dextrose, 2% w/v agar), adding 30 mg/L of streptomycine, incubated at 28 ± 1 °C for 48 ± 2 h.

2.4. Physico-Chemical Analyses

Physico-chemical analyses, performed on samples at the end of AF and on the 30th day of the winemaking process, were performed according to the corresponding European Community (EC) methods [36]. The malic acid and the lactic acid were determined using the enzymatic kit (Boehringer Mannheim, GmbH, Mannheim, Germany). Volatile compounds (mg/L) were determined by gas chromatography (GC) (Thermoquest Mod. 8000, Rodano, Milan, Italy) and flame ionization detection equipped with a fused capillary column ZB-Wax (30 m × 0.32 mm i.d., 0.50 μm film thickness, Phenomenex, Torrance CA, USA), according to International Organisation of Vine and Wine (OIV) methods [37]. The wines, after the addition of the internal standard (Butan-2-ol, Fluka, Steinheim, Germany; 0.1 mg/mL in water), were injected directly in split mode (1:50); injection port at 250 °C; program of oven from 40 °C (5min) to 240 °C at a rate of 7 °C/min; carrier gas helium with a flow rate of 60 kPa [29,38].
2.5. Sensory Analysis

In order to evaluate the different sensorial characteristics of the wines, at the end of the AF and MLF (Batch D), a sensorial analysis of the samples was performed by a panel of 12 professional testers from the National Organization of Wine Tasters (ONAV, Italy).

A tasting sheet was created in order to measure the intensity of each chosen descriptor using an unstructured intensity scale presented on a wheel. Three replicates of samples of each wine were sensory analyzed. The samples were presented randomly. This test was carried out in accordance with international standards. The sensory attributes assessed were for olfactory descriptors—fruity, herbal, floral, spicy, and for taste descriptors—reduced, acidity, persistence, astringency, softness. Finally, an overall judgment on the wine was expressed. Scores used ranged from 0 (absence of perception) to 9 (maximum perception) [39].

2.6. Statistical Analysis

Data obtained from three independent experiments were analyzed using the software RStudio (v 3.5.0) [40]. Analysis of variance (ANOVA) coupled with the Tukey HDS post-hoc test was used to evaluate differences between batches. Statistical significance was attributed to values of \( p < 0.05 \). Principal components analysis (PCA) was performed on wine volatile compounds to discriminate the volatiles between the different batches.

3. Results and Discussion

3.1. Trend of Cell Biomass, Alcoholic and Malolactic Fermentation

The evolution of the yeasts and LAB, as well as the trend of alcoholic and malolactic fermentation were followed during the winemaking process. In Figure 1, the development of the yeasts and the LAB during the alcoholic and malolactic fermentation is reported in the different batches. No differences in AF trend were noticed between the wines (Figure 1), whether they were inoculated with LAB (co-inoculum) or not (sequential inoculum). The results show that in all the batches, yeast cells increased rapidly after the inoculum, reaching the maximum density (≅8 Log CFU/mL) on the 6th day of fermentation. The cell concentration remained high (≅6 Log CFU/mL) until the 12th day, after which a progressive decay was observed until reaching a concentration of about 2 Log CFU/mL on the 29th day (end of winemaking). The quick beginning of the AF, observed in all trials, most likely indicates a good implantation of the yeasts.

The trend of yeast growth curves that we observed in all batches followed those of sugar consumption and alcohol production (Figure 2), highlighting that alcoholic fermentation was taking place. In fact, between the 9th and 12th day of the winemaking process, the reducing sugars were consumed and the alcohol content reached the maximal final value of about 12.5\% (Figure 2) in all batches. These results highlight that the growth of the yeasts and AF were unaffected by the presence LAB. On the contrary, the authors reported the negative effect of LAB on yeast metabolism when used in co-inoculum [41–43], whilst other studies showed the positive effect on the growth and fermentative activity of yeast when it was co-inoculated with the LAB in grape must [44–46]. Therefore, the choice of the yeast and bacterial strains is crucial to avoid the onset of negative interactions between them.

Relative to LAB trend, several differences were observed between batches in response to the inoculation strategy. As reported in Figure 1, the survival of the bacteria was affected by the time of inoculum as well as the pre-adaption to sub-optimal pH condition. We observed a strong decay of bacteria survival, when non-preadapted \emph{Lb. plantarum} M10 was inoculated according to sequential inoculum strategy (Figure 1c). In fact, at about 16 days (from days 12 to 16) of the winemaking process, the LAB load decreased from 7 to about 4 Log CFU/mL. The reduction of bacteria, when inoculated in the wine at the end of AF, was also evidenced by the failure of MLF. In fact, as evidenced in Figure 3, no difference was observed, in terms of L-malic acid consumption, following the inoculum of \emph{Lb. plantarum} M10 (Batch C) in the wine. The loss of survival of malolactic starter and consequently the
failure of MLF, is a negative event that frequently occurs during the winemaking process because of harsh conditions of wine, such as low pH, high ethanol contents, and low sugar concentration [42,47].

Figure 1. Trend of yeasts and LAB during the alcoholic (AF) and malolactic (MLF) fermentation of the 5 batches of Fiano grape must inoculated with *S. cerevisiae* FE at initial phase of the winemaking process and with *Lb. plantarum* M10 in accordance to different strategies of inoculum. (a) Batch inoculated with the strain M10 halfway through AF, early co-inoculum; (b) batch inoculated with M10 at the beginning of AF, early co-inoculum; (c) batch inoculated with *Lb. plantarum* M10 at the end of AF, sequential inoculum non-pre-adapted; (d) batch inoculated with M10 cells previously pre-adapted to acid and ethanol stress, sequential inoculum pre-adapted M10; (e) batch without inoculum of *Lb. plantarum* M10, control. The arrows indicate the point of M10 inoculum.

Figure 2. Consumption of reducing sugars and production of ethanol during the alcoholic fermentation of Fiano wine in accordance to several strategies of inoculum. Batch A, late co-inoculum strategy; Batch B, early co-inoculum strategy; Batch C, sequential inoculum non-pre-adapted strategy; Batch D, sequential inoculum pre-adapted strategy; Batch E, control.
The worst-case scenario (Figure 1a) was observed when *Lb. plantarum* M10 was added to the grape must halfway through alcoholic fermentation (late co-inoculum). In this case, the microbial load was reduced to about 5 Log CFU/mL in the six days following the inoculation (from days 6 to 12), then reached values near the detection limit (1 Log CFU/mL). Consequently, as shown in Figure 3, the malolactic fermentation was also compromised. It is probable that the prohibitive conditions of the environment, associated with a potential competitive action of the yeast for nutrients, could lead to a major reduction of LAB concentration in late co-inoculum strategy, compared to the sequential inoculum approach. In fact, when the strain M10 was inoculated in the must, according to the late co-inoculum strategy (Figure 1a), it encountered more competitive cells of yeast because they were in the stationary phase. On the contrary, in the sequential inoculum, the yeast cells were in the decline phase at the moment of M10 inoculum and were, therefore, less competitive (Figure 1c). Similar results were carried out in a work conducted by Rosi et al. [48], where the authors reported the antagonist effect of yeast against *Oenococcus oeni*.

Regarding the early co-inoculum method (Figure 1b), the reduction of LAB concentration was also recorded during the winemaking period. However, in this case, the survival curve of bacteria was less pronounced compared to those observed in the late co-inoculum and in the sequential inoculum non-pre-adapted (Figure 1a,c). In detail, after the inoculum *Lb. plantarum* M10 (7 Log CFU/mL), the number of viable cells remained constant until the 9th day of AF; subsequently, it decreased up to about 2 Log CFU/mL (30th day). In this case, a decrease in the L-malic acid concentration (about 0.5 g/L) was also observed (Figure 3, Batch B). In particular, the degradation of L-malic acid was triggered on the 6th day of AF, when the concentration of LAB was of about 7 Log CFU/mL. Following the reduction of bacterial load, under 6 Log CFU/mL (after the 12th day), the concentration of malic acid remained unchanged. These results once again highlight that the L-malic acid degradation is strongly correlated to an appropriate concentration of malolactic starter culture.

This hypothesis was confirmed by the results obtained when *Lb. plantarum* M10 was pre-adapted to sub-optimal pH and subsequently inoculated in the wine at the end of alcoholic fermentation (Figure 1d). In this Batch (D), the microbial load (7.5 Log CFU/mL) was unchanged until the end of winemaking process (30th day), and consequently the MLF was also carried out (Figure 3).

In detail, from the 12th day (inoculum of *Lb. plantarum* M10) to the 30th day (end of winemaking), the L-malic acid was completely degraded, going from values of about 3 to 0.2 g/L. Moreover, with the degradation of malic acid, there was a significant increase in lactic acid and pH, as can be seen from the physico-chemical parameters reported in Table 1.
### Table 1. Physico-chemical analysis of wine at the end of alcoholic fermentation (AF) and at the end of malolactic fermentation (MLF). The wine was obtained with the sequential inoculation pre-adapted (Batch D).

| Physico-Chemical Parameters | AF        | ± | MLF        | ± |
|-----------------------------|-----------|---|-----------|---|
| pH                          | 3.25      | ± | 0.08 *    | 3.68 | ± | 0.09 |
| Total acidity (g/L)         | 8.54      | ± | 0.16 *    | 6.52 | ± | 0.23 |
| Reducing sugars (g/L)       | 1.10      | ± | 0.05 *    | 0.75 | ± | 0.17 |
| Alcohol (% vol)             | 12.58     | ± | 0.17      | 12.55 | ± | 0.13 |
| Volatile acidity (g/L)      | 0.31      | ± | 0.07      | 0.42 | ± | 0.04 |
| L-malic acid (g/L)          | 3.18      | ± | 0.06 *    | 0.25 | ± | 0.11 |
| L-lactic acid (g/L)         | 0.13      | ± | 0.03 *    | 2.23 | ± | 0.07 |
| Free SO₂ (mg/L)             | 11.21     | ± | 0.41      | 11.34 | ± | 0.44 |
| Total SO₂ (mg/L)            | 45.16     | ± | 1.13      | 48.23 | ± | 1.75 |

Mean values of three independent replicate ± standard deviation. * indicates significant differences (p < 0.05) between values obtained in wine after AF and wine at the end of MLF.

This finding shows how the pre-adaptation of LAB strain can positively influence both survival and L-malic acid degradation in the wine. The positive impact of cell pre-adaption to acid stress on microbial survival has also been reported in several studies [6,49,50]. Moreover, the same authors reported that the cells of *Lb. plantarum* pre-adapted to acid stress (pH 5.0); not only were they able to survive better in wine-like medium but showed a greater ability to degrade malic acid compared to non-pre-adapted cells [16].

The success of sequential inoculation compared to co-inoculation may also be due to other factors. In co-inoculation, the antagonistic effect attributed to yeast, linked to nutritional competition or the presence of medium chain fatty acids, can compromise the vitality of malolactic bacteria. On the contrary, in sequential inoculation, the yeast can favor the growth of LAB, releasing vitamins and amino acids after its autolysis. This involves enrichment of nutrients and subsequent stimulation of MLF.

#### 3.2. Wine Chemical Analysis

The analyses of chemical parameters carried out in the wines obtained from Batches A, B, C, and E did not show significant differences after the AF and after 30 days of the winemaking process (data not shown). However, in Batch D after the MLF, the chemical compositions were significantly different from those observed in the other batches. In particular, as highlighted in Table 1, an increase in pH values (from 3.25 to 3.68) and in L-lactic acid levels (from 0.13 to 2.23) and a decrease in total acidity (from 8.54 to 6.52) as well as in L-malic acid (from 3.18 to 0.25) were registered.

A total of 19 volatile compounds were identified in the Fiano wine at the end of the winemaking process (30th day) and their concentration is reported in Table 2. In particular, two main classes of compounds were detected, distinguishable in higher alcohols and terpenes. These compounds, together, contributed to the complexity of the wine flavor.

Higher alcohols represent the major volatiles detected in the samples. Fourteen compounds were identified, and their concentration ranged from 984.18 to 1071.59 mg/L, respectively, in the wines from Batches C and B. Among all identified alcohol, 2,3-butanediol, 3-methyl-1-butanol, and 1-heptanol were the most abundant in all wines, contributing up to 77% of the whole concentration.

2,3-butanediol is a compound related to a fruity note of wine [51–54]; it is obtained from the reduction of diacetyl (2,3-butanedione) by means of diacetyl reductase.

2,3-butanediol can be produced during alcohol fermentation as an intermediate product of amino acids metabolism of yeast, and through malolactic fermentation in acid citric metabolism. Consequently, a major content of 2,3-butanediol could be detected after MLF [51-53,55]. In our study, the concentration of 2,3-butanediol (Table 2) was significantly higher in wine where MLF occurred (Batch D) compared to wines where MLF failed (Batches A and B).
3-methyl-1-butanol (iso-amyl alcohol) was detected in high concentrations in all wines, even if relevant differences were observed among the batches. In particular, we observed a lower significant concentration of 3-methyl-1-butanol in wine where MLF occurred (Batch D), and the highest concentration in the wines where the yeast and bacteria coexisted (Batches A and B). Although, 3-methyl-1-butanol is a metabolite produced by yeast, through the metabolism of amino acids leucine and valine, or by metabolism of pyruvate [56], our results highlight that concentration of iso-amyl alcohol is strongly influenced by yeast-bacteria interaction, according to results reported by Lee et al. [21]. 3-methyl-1-butanol gives a banana and pear-like aroma at optimal concentration, but excessive concentrations are responsible of the nail polish odor of wines [56–58].

The concentrations of 1-octanol were above their aroma detection thresholds in all samples, but more in Batches A and B, indicating a direct impact on the aroma characteristics of these wines. Some authors believe that 2-phenylethanol does not derive de novo from yeasts but originates from the aglycosidically-bound form in the fruit, which is liberated during fermentation [59–61] as a result of enzyme activity. In fact, higher concentration of 2-phenylethanol was observed in Batch D, while the concentration significantly decreased in wines where the MLF was absent (Batches A, B, C, and E), which proved an enzymatic activity was carried out by the malolactic bacteria.

In this study, the concentration of some higher alcohols, 1-propanol, 1-methyl-propanol, 2-pentanol, and methyl-ttyo-propanol, significantly decreased. Maicas et al. [55] reported the important influence of the LAB strain participating in MLF on the evolution of alcohol concentrations, and affirmed that the decreases observed when certain strains were used may have been due to the physical adsorption of the bacteria.

Linalool, 4-terpineol, α-terpineol, and geraniol were the esters quantified in wines from different batches. Wine from Batch D, obtained with pre-adapted inoculum of *Lb. plantarum* M10, showed a significantly higher concentration in linalool (124.50 µg/L) compared to the other batches (Table 2); the levels of 4-terpineol were also higher (18.07 µg/L), which are expression of the varietal aroma characteristics of Fiano wine [33]. Furthermore, terpenes are known to bring desirable floral and citrus aroma, a contribution of this compound to the aroma of young white wines.

Higher concentration of hexanoic acid in Batches A and B, where the co-inoculum was carried out, could be one of the causes of the inhibition malolactic fermentation.

Some researchers reported that short- to medium-chain fatty acids, such as hexanoic, octanoic and decanoic acids, produced during alcoholic fermentation, inhibit the growth of malolactic bacteria [62–64]. Production of these acids varies significantly with yeast species and strain. Though, the nature of the antagonism between yeast and malolactic bacteria remains unclear.
Table 2. Concentration of volatile compounds at the end of winemaking process of Fiano wines produced by different inoculum strategies.

| Compounds                  | Odor Description                        | Batch A | Batch B | Batch C | Batch D | Batch E | Threshold | References |
|----------------------------|-----------------------------------------|---------|---------|---------|---------|---------|-----------|------------|
| Higher alcohols (µg/L)     |                                         |         |         |         |         |         |           |            |
| 1-propanol                 | alcohol, pungent                        | 15.67±  | ±       | ±       | ±       | ±       | ±         | ±          |
| 1-methyl-propanol          |                                        | 26.97±  | ±       | ±       | ±       | ±       | ±         | ±          |
| 3-methyl-1-butanol         | whiskey, malt, burnt                    | 255.87± | ±       | ±       | ±       | ±       | ±         | ±          |
| 2-pentanol                 |                                        | 22.67±  | ±       | ±       | ±       | ±       | ±         | ±          |
| 1-pentanol                 | balsamic                                | 11.17±  | ±       | ±       | ±       | ±       | ±         | ±          |
| 2-methyl-1-butanol         | whiskey                                 | 10.13±  | ±       | ±       | ±       | ±       | ±         | ±          |
| 1-octanol                  | chestnut flowers, mushroomy            | 0.03±   | ±       | ±       | ±       | ±       | ±         | ±          |
| Tyrosol                    |                                        | 58.1±   | ±       | ±       | ±       | ±       | ±         | ±          |
| 1-heptanol                 | lemon, orange, copper                   | 114.6±  | ±       | ±       | ±       | ±       | ±         | ±          |
| Methyl-ttyo-propanol       |                                        | 85.9±   | ±       | ±       | ±       | ±       | ±         | ±          |
| 2,3-butanediol             | butter, creamy                          | 362.5±  | ±       | ±       | ±       | ±       | ±         | ±          |
| 2-nonanol                  | fruity, green                           | 2.2±    | ±       | ±       | ±       | ±       | ±         | ±          |
| 2-phenylethanol            | rose, honey, spice, lilac              | 55.9±   | ±       | ±       | ±       | ±       | ±         | ±          |
| Methanol                   |                                        | 27.8±   | ±       | ±       | ±       | ±       | ±         | ±          |
| TOTAL                      |                                        | 1067.5± | ±       | ±       | ±       | ±       | ±         | ±          |
| Esters (µg/L)              |                                         |         |         |         |         |         |           |            |
| Linalool                   | muscat, flowery, fruity                 | 53.46±  | ±       | ±       | ±       | ±       | ±         | ±          |
| 4-terpinol                 | light aroma, wood, soil                | 8.47±   | ±       | ±       | ±       | ±       | ±         | ±          |
| Alpha-terpinol             | oil, arone, mint                        | 20.78±  | ±       | ±       | ±       | ±       | ±         | ±          |
| Geranial                   | fresh, citrus, floral, green, sweet,    | 4.63±   | ±       | ±       | ±       | ±       | ±         | ±          |
| TOTAL                      |                                        | 96.3±   | ±       | ±       | ±       | ±       | ±         | ±          |
| Others (µg/L)              |                                         |         |         |         |         |         |           |            |
| Hexanoic acid              | cheese, rancid, fruity                  | 120.9±  | ±       | ±       | ±       | ±       | ±         | ±          |
| TOTAL                      |                                        | 121.00± | ±       | ±       | ±       | ±       | ±         | ±          |

The data are the mean of triplicates ± SD; different letters in the row denotes statistically significant differences (p < 0.05).
3.3. Principal Component Analysis

The principal component analysis was applied to volatile compounds detected in the wines of five batches at the end of the winemaking process (30 days). The first two principal components (Dim 1 and Dim 2) displayed about 87% of variance, of which 54% was due to Component 1 (Figure 4). The volatile compounds profile of wines was clearly distinguishable along the coordinates of the two principal components. Batch A and Batch B were divergent from Batch D across the Dim 1; moreover they were also separated from Batches C and E across the Dim 2 (Figure 4). From the bi-plot, it is evident that the presence of Lb. plantarum M10 as well as the timing of inoculations in the winemaking process, could modulate the volatile compounds of wines. Wines located on the upper right-hand side of the bi-plot (Figure 4), obtained by the early inoculum (Batch A) and co-inoculum (Batch B) of strains FE and M10, were mainly characterized by 1-octanol, methanol, and 3-methyl-1-butanol. The sample wines, placed on the lower right quadrant of bi-plot (Figure 4) and achieved by the sequential inoculum (Batch C) of strains FE and M10 or without the inoculum of M10 (Batch E), showed aromatic profiles mainly influenced by the compounds 1-heptanol, 1-propanol, and 2-pentanol, whilst the wine (Batch D) obtained with the pre-adapted sequential inoculum strategy, located on the upper left-hand side of the two principal components, showed an aromatic profile attributable to linalool, 2-phenylethanol, 4-terpineol, geraniol, and 2,3-butanediol.

Figure 4. Principal components analysis (PCA)-biplot of volatile compounds in different batches at the end of the winemaking process. The big circles represent the mean values of three independent experiments.

3.4. Sensory Evaluation

In order to confirm the results obtained with the analysis of volatile compounds, the sensorial analysis was performed only on the sample where malic acid was consumed (Batch D), evaluating the differences of the wine at the end of the alcoholic fermentation (AF) and at the malolactic fermentation performed (MLF). The judges found that the wine at the end of MLF had best overall quality and gave a score around 8 points (Figure 5). Furthermore, comparing sensory variation between wines (AF, MLF), it varied significantly in all attributes except for the astringency and persistence. The aroma of the wine MLF was mainly characterized by fruit and floral notes, while the attribute “reduced” was especially low. The increase in the concentration of some volatile compounds seems to show the determining contribution of the β-glucosidasic activity of lactic acid bacteria to wine flavor [33].
The sensations perceived by the sensorial analysis of the analyzed samples are related to the greater quantity of odorous compounds chemically determined.

![Radar plot of Fiano wine](image)

**Figure 5.** Radar plot of Fiano wine obtained by sequential inoculum strategy with M10 cells pre-adapted to acid stress. The two samples evaluated come from Batch D, at the end of alcoholic fermentation (AF) and at the end of malolactic fermentation (MLF). * indicates significant differences ($p < 0.05$) of attributes between AF and MLF.

4. Conclusions

The improvement of the quality of the wine is linked not only to the quality of the raw material, but also to the winemaking techniques and the starters used.

Malolactic fermentation (MLF) is “secondary” in wine only chronologically but, like alcoholic fermentation (AF), a “primary” process by importance in defining the quality of wine.

In this study, the success of *Lb. plantarum* M10, suitably pre-adapted and inoculated after alcoholic fermentation and used as a malolactic starter, testifies the importance of the inoculation technique and the characteristics of the strain used. Furthermore, metabolic activities are strongly linked to the ability to survive specific stress factors found in wine (mainly high ethanol content and low pH). Stress responses have been correlated with specific phenotypes so that they can be induced in a controllable and reproducible way. Furthermore, the use of sequential inoculation was more favorable, probably due to the lack of adverse interaction between yeast and bacterium. These results indicate that, despite the very low pH of the wine, correct management of the inoculation strategies can determine both the success of the malolactic fermentation and the improvement of the sensorial characteristics of the wines. In this case, the sequential inoculum combined with the pre-adaptation at sub-optimal pH proved to be decisive.

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References

1. De Revel, G.; Martin, N.; Pripis-Nicolau, L.; Lonvaud-Funel, A.; Bertrand, A. Contribution to the knowledge of malolactic fermentation influence on wine aroma. J. Agric. Food Chem. 1999, 47, 4003–4008. [CrossRef] [PubMed]

2. Liu, S.Q. Malolactic fermentation in wine—Beyond deacidification. J. Appl. Microbiol. 2002, 92, 589–601. [CrossRef] [PubMed]

3. Bartowsky, E.J.; Costello, P.J.; Chambers, P.J. Emerging trends in the application of malolactic fermentation. Aust. J. Grape Wine R. 2015, 21, 663–669. [CrossRef]

4. Teixeira, H.; Goncalves, M.G.; Rozes, N.; Ramos, A.; San Romao, M.V. Lactobacillic acid accumulation in the plasma membrane of Oenococcus oeni: A response to ethanol stress? Microbial Ecol. 2002, 43, 146–153. [CrossRef]

5. Bravo-Ferrada, B.M.; Brizuela, N.; Gerbino, E.; Gómez-Zavaglia, A.; Semorile, L.; Tymczyszyn, E.E. Effect of protective agents and previous acclimation on ethanol resistance of frozen and freeze-dried Lactobacillus plantarum strains. Cryobiology 2015, 71, 522–528. [CrossRef]

6. Papadimitriou, K.; Alegría, Á.; Bron, P.A.; De Angelis, M.; Gobbetti, M.; Kleerebezem, M.; Lemos, J.A.; Linares, D.M.; Ross, P.; Stanton, C.; et al. Stress physiology of lactic acid bacteria. Microbiol. Mol. Biol. Rev. 2016, 80, 837–890. [CrossRef]

7. Brizuela, N.; Tymczyszyn, E.E.; Semorile, L.C.; La Hens, D.V.; Delfefederco, L.; Hollmann, A.; Bravo-Ferrada, B. Lactobacillus plantarum as a malolactic starter culture in winemaking: A new (old) player? Electron. J. Biotechnol. 2019, 38, 10–18. [CrossRef]

8. Siciliano, R.A.; Pannella, G.; Lippolis, R.; Ricciardi, A.; Mazzoeo, M.F.; Zotta, T. Impact of aerobic and respiratory life-style on Lactobacillus casei N87’ proteome. Int. J. Food Microbiol. 2019, 298, 51–62. [CrossRef]

9. Sieiro, C.; Cansado, J.; Artés, X.; Velázquez, J.B.; Borriss, R. Isolation and enological characterization of malolactic bacteria from the vineyards of northwestern. Spain. Appl. Environ. Microbiol. 1990, 56, 2936–2938. [CrossRef]

10. Valiante, H.; Formisyn, P.; Gerbana, V. Malolactic fermentation of wine: Study of the influence of some physico-chemical factors by experimental design assays. J. Appl. Bacteriol. 1995, 79, 640–650. [CrossRef]

11. Zapparoli, G.; Moser, M.; Delaglio, F.; Tourdot-Marechal, R.; Guzzo, J. Typical metabolic traits of two Oenococcus oeni strains isolated from Valpolicella wines. Lett. Appl. Microbiol. 2004, 39, 48–54. [CrossRef] [PubMed]

12. Testa, B.; Lombardi, S.J.; Tremont, P.; Succi, M.; Tipaldi, L.; Pannella, G.; Sorrentino, E.; Iorizzo, M.; Coppola, R. Biodiversity of Lactobacillus plantarum from traditional Italian wines. World J. Microbiol. Biotechnol. 2014, 30, 2299–2305. [CrossRef] [PubMed]

13. Lombardi, S.J.; Pannella, G.; Iorizzo, M.; Moreno-Arribas, M.V.; Tremont, P.; Succi, M.; Sorrentino, E.; Macciola, V.; Di Renzo, M.; Coppola, R. Sequential inoculum of Hanseniaspora guilliermondii and Saccharomyces cerevisiae for winemaking Campanino on an industrial scale. World J. Microb. Biol. 2018, 34, 161. [CrossRef] [PubMed]

14. Testa, B.; Lombardi, S.J.; Iorizzo, M.; Letizia, F.; Di Martino, C.; Di Renzo, M.; Strollo, D.; Tremont, P.; Pannella, G.; Ianiro, M.; et al. Use of strain Hanseniaspora guilliermondii BF11 for winemaking process of white grapes Vitis vinifera cv Fiano. Eur. Food Res. Technol. 2020, 246, 549–561. [CrossRef]

15. Zapparoli, G.; Tosi, E.; Azzolini, M.; Vagnoli, P.; Krieger, S. Bacterial inoculation strategies for the achievement of malolactic fermentation in high-alcohol wines. S. Afr. J. Enol. Vitic. 2009, 30, 49–55. [CrossRef]

16. Succi, M.; Pannella, G.; Tremont, P.; Tipaldi, L.; Coppola, R.; Iorizzo, M.; Lombardi, S.J.; Sorrentino, E. Sub-optimal pH preadaptation improves the survival of Lactobacillus plantarum strains and the malic acid consumption in wine-like medium. Front. Microbiol. 2017, 8, 470. [CrossRef]

17. Sumby, K.M.; Bartle, L.; Grbin, P.R.; Jiranek, V. Measures to improve wine malolactic fermentation. Appl. Microbiol. Biotechnol. 2019, 103, 2033–2051. [CrossRef]

18. Alegría, G.E.; López, I.; Ruiz, J.I.; Saenz, J.; Fernández, E.; Zarazaga, M.; Dizy, M.; Torres, C.; Ruiz-Larrea, F. High tolerance of wild Lactobacillus plantarum and Oenococcus oeni strains to lyophilisation and stress environmental conditions of acid pH and ethanol. FEMS Microbiol. Lett. 2004, 230, 53–61. [CrossRef]

19. Du Plessis, H.W.; Dicks, L.M.T.; Pretorius, I.S.; Lambrechts, M.G.; du Toit, M. Identification of lactic acid bacteria isolated from South African brandy base wines. Int. J. Food Microbiol. 2004, 91, 19–29. [CrossRef]
20. Lopez, I.; Lopez, R.; Santamaria, P.; Torres, C.; Ruiz-Larrea, F. Performance of malolactic fermentation by inoculation of selected Lactobacillus plantarum and Oenococcus oeni strains isolated from Rioja red wines. *Vitis* 2008, 47, 123–129.

21. Lee, J.E.; Hong, Y.S.; Lee, C.H. Characterization of fermentative behaviors of lactic acid bacteria in grape wines through 1H NMR-and GC-based metabolic profiling. *J. Agric. Food Chem.* 2009, 57, 4810–4817. [CrossRef] [PubMed]

22. Cho, G.S.; Krauß, S.; Huch, M.; Du Toit, M.; Franzen, C.M. Development of a quantitative PCR for detection of *Lactobacillus plantarum* starters during wine malolactic fermentation. *J. Microbiol. Biotechnol.* 2011, 21, 1260–1266. [CrossRef] [PubMed]

23. Miller, B.; Franz, C.; Cho, G.S.; du Toit, M. Expression of the malolactic enzyme gene from *Lactobacillus plantarum* under winemaking conditions. *Curr. Microbiol.* 2011, 62, 1682–1688. [CrossRef] [PubMed]

24. Du Toit, M.; Engelbrecht, L.; Lerm, E.; Krieger-Weber, S. *Lactobacillus*: The next generation of malolactic fermentation starter cultures—An overview. *Food Bioprocess Technol.* 2011, 4, 876–906. [CrossRef]

25. Lucio, O.; Pardo, I.; Krieger-Weber, S.; Heras, J.M.; Ferrer, S. Selection of *Lactobacillus* strains to induce biological acidification in low acidity wines. *LWT-Food Sci. Technol.* 2016, 73, 334–341. [CrossRef]

26. Matthews, A.; Grimaldi, A.; Walker, M.; Bartowsky, E.; Grbin, P.; Jiranek, V. Lactic acid bacteria as a potential source of enzymes for use in vinification. *Appl. Environ. Microb.* 2004, 70, 5715–5731. [CrossRef]

27. Grimaldi, A.; Bartowsky, E.; Jiranek, V. Screening of *Lactobacillus* spp. and *Pediococcus* spp. for glycosidase activities that are important in oenology. *J. Appl. Microbiol.* 2005, 99, 1061–1069. [CrossRef]

28. Matthews, A.; Grbin, P.R.; Jiranek, V. Biochemical characterisation of the esterase activities of wine lactic acid bacteria. *Appl. Microbiol. Biot.* 2007, 77, 329–337. [CrossRef]

29. Iorizzo, M.; Macciola, V.; Testa, B.; Lombardi, S.J.; De Leonardis, A. Physicochemical and sensory characteristics of red wines from the rediscovered autochthonous Tintilia grapevine grown in the Molise region (Italy). *Eur. Food Res. Technol.* 2014, 238, 1037–1048. [CrossRef]

30. De Leonardis, A.; Testa, B.; Macciola, V.; Lombardi, S.J.; Iorizzo, M. Exploring enzyme and microbial technology for the preparation of green table olives. *Eur. Food Res. Technol.* 2016, 242, 363–370. [CrossRef]

31. Ugliano, M.; Genovese, A.; Moio, L. Hydrolysis of wine aroma precursors during malolactic fermentation with four commercial starter cultures of *Oenococcus oeni*. *J. Agric. Food Chem.* 2003, 51, 5073–5078. [CrossRef] [PubMed]

32. Genovese, A.; Gambuti, A.; Piombino, P.; Moio, L. Sensory properties and aroma compounds of sweet Fiano wine. *Food Chem.* 2007, 103, 1228–1236. [CrossRef]

33. Ugliano, M.; Moio, L. Free and hydrolytically released volatile compounds of *Vitis vinifera* L. cv. Fiano grapes as odour-active constituents of Fiano wine. *Anal. Chim. Acta.* 2008, 621, 79–85. [CrossRef] [PubMed]

34. Calabretti, A.; Volpe, M.G.; Sorrentino, A.; Ionata, E.; Santomauro, F.; La Cara, F. Aglialico and Fiano wines obtained with an autochthonous non- *Saccharomyces cerevisiae* yeast. *Ann. Microbiol.* 2011, 61, 131–136. [CrossRef]

35. Iorizzo, M.; Testa, B.; Lombardi, S.J.; García-Ruiz, A.; Muñoz-González, C.; Bartolomé, B.; Moreno-Arribas, M.V. Selection and technological potential of *Lactobacillus plantarum* bacteria suitable for wine malolactic fermentation and grape aroma release. *LWT-Food Sci. Technol.* 2016, 73, 557–566. [CrossRef]

36. European Community. Methods for the analysis of wines. *Off. J. Eur. Commun. Regul.* 1990, 272, 1–192.

37. OIV. *Compendium of International Methods of Analysis of Spirituous Beverages of Vitivinicultural Origin*; International Organisation of Vine and Wine: Paris, France, 2014.

38. De Leonardis, A.; Macciola, V.; Iorizzo, M.; Lombardi, S.J.; Lopez, F.; Marconi, E. Effective assay for olive oil production from olive oil mill wastewaters. *Food Chem.* 2018, 240, 437–440. [CrossRef]

39. Lombardi, S.J.; De Leonardis, A.; Lustrato, G.; Testa, B.; Iorizzo, M. Yeast autolysis in sparkling wine aging: Use of killer and sensitive *Saccharomyces cerevisiae* strains in co-culture. *Recent Patents Biot.* 2015, 9, 223–230. [CrossRef]

40. R Core Team. *R: A Language and Environment for Statistical Computing; R* Foundation for Statistical Computing: Vienna, Austria, 2018; Available online: [http://www.r-project.org/index.html](http://www.r-project.org/index.html) (accessed on 25 February 2020).

41. Costello, P.J.; Henschke, P.A.; Markides, A.J. Standardised methodology for testing malolactic bacteria and wine yeast compatibility. *Aust. J. Grape Wine R.* 2003, 9, 127–137. [CrossRef]

42. Alexandre, H.; Costello, P.J.; Remize, F.; Guzzo, J.; Guilloux-Benatier, M. *Saccharomyces cerevisiae-Oenococcus oeni* interactions in wine: Current knowledge and perspectives. *Int. J. Food Microbiol.* 2004, 93, 141–154. [CrossRef]

43. Rossouw, D.; Du Toit, M.; Bauer, F.P. The impact of co-inoculation with *Oenococcus oeni* on the transcriptome of *Saccharomyces cerevisiae* and on the flavour-active metabolite profiles during fermentation in synthetic must. *Food Microbiol.* 2012, 29, 121–131. [CrossRef] [PubMed]
44. Jussier, D.; Morneau, A.D.; de Orduna, R.M. Effect of simultaneous inoculation with yeast and bacteria on fermentation kinetics and key wine parameters of cool-climate Chardonnay. Appl. Environ. Microbiol. 2006, 72, 221–227. [CrossRef] [PubMed]

45. Taniasuri, F.; Lee, P.R.; Liu, S.Q. Induction of simultaneous and sequential malolactic fermentation in durian wine. Int. J. Food Microbiol. 2016, 230, 1–9. [CrossRef] [PubMed]

46. Guzzon, R.; Moser, S.; Davide, S.; Villegas, T.R.; Malacarne, M.; Larcher, R.; Nardi, T.; Vagnoli, P.; Krieger-Weber, S. Exploitation of simultaneous alcoholic and malolactic fermentation of Incrocio Manzoni, a traditional Italian white wine. S. Afr. J. Enol. Vitic. 2016, 37, 124–131. [CrossRef]

47. Bauer, R.; Dicks, L.M.T. Control of malolactic fermentation in Wine. A Review. Crit. Rev. Food Sci. Health 1997, 32, 502–509. [CrossRef] [PubMed]

48. Bravo-Ferrada, B.M.; Tymczyszyn, E.E.; Giovannetti, G.; Studer, Y. Influence of diacetyl metabolism and biosynthesis of selected aromatic esters in cool-climate grape wines. Molecules 2018, 23, 2549. [CrossRef]

49. Zhang, S.; Petersen, M.; Liu, J.; Toldam-Andersen, T. Influence of pre-fermentation treatments on wine volatile and sensory profile of the new disease tolerant cultivar Solaris. Molecules 2015, 20, 21609–21625. [CrossRef] [PubMed]

50. Maicas, S.; Gil, J.V.; Pardo, I.; Ferrer, S. Improvement of volatile composition of wines by controlled addition of malolactic bacteria. Food Res. Int. 1999, 32, 491–496. [CrossRef]

51. Swiegers, J.H.; Bartowsky, E.J.; Pretorius, I. Yeast and bacterial modulation of wine aroma and flavour. Aust. J. Grape Wine R. 2005, 11, 139–173. [CrossRef]

52. Samappito, S.; Butkhup, L. Effect of skin contact treatments on the aroma profile and chemical components of mulberry (Morus alba Linn.) wines. Afr. J. Food Sci. 2010, 4, 52–61. [CrossRef]

53. Tao, Y.S.; Li, H. Active volatiles of cabernet sauvignon wine from Changli County. Health 2009, 1, 176. [CrossRef]

54. Pederson, C.S.; Albury, M.N.; Christensen, M.D. The Growth of Yeasts in Grape Juice Stored at Low Temperature: IV. Fungistatic Effects of Organic Acids. Appl. Microbiol. 1961, 9, 162. [CrossRef]

55. Rocha, S.M.; Rodrigues, F.; Coutinho, P.; Delgadillo, I.; Coimbra, M.A. Volatile composition of Baga red wine: Assessment of the identification of the would-be impact odourants. Anal. Chim. Acta 2004, 513, 257–262. [CrossRef]

56. Zalacain, A.; Marín, J.; Alonso, G.L.; Salinas, M.R. Analysis of wine primary aroma compounds by stir bar sorptive extraction. Talanta 2007, 71, 1610–1615. [CrossRef]

57. Woolford, M.K. Microbiological screening of the straight chain fatty acids (C1–C12) as potential silage additives. J. Sci. Food Agric. 1975, 26, 219–228. [CrossRef]

58. Lonzau-Funel, A.; Desens, C.; Joyeux, A. Stimulation de la fermentation malolactique par l’addition au vin d’enveloppes cellulaires de levure et différents adjuvants de nature polysaccharidique et azotée. OENO One 1985, 19, 229–240. [CrossRef]

59. Schwab, W.; Schreier, P. Studies on bound aroma compounds of sour cherry fruit (Prunus cerasus L.). Z. Lebensm.-Unters. und-Forsch. 1990, 190, 228–231. [CrossRef]

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