Together is better. Experience of simultaneous fermentation of yeast and bacteria as a possible strategy to prevent stuck fermentation in *difficult* wines

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Abstract

In the field of winemaking, malolactic fermentation is a key aspect in obtaining high quality wines. Unfortunately, in some oenological contexts effective evolution does not take place because of the occurrence of certain limiting factors for malolactic bacteria in wine. Simultaneous alcoholic and malolactic fermentation in grape must is a promising alternative that promotes the survival of bacteria, due to the absence of certain limiting factors such as ethanol or other toxic substances produced by yeasts in the native grape must. The risk of wine depreciation due to the spoilage activity of malolactic bacteria can be reduced by using selected strains of *Oenococcus oeni*, with proven behaviour in terms of malolactic fermentation occurring in the presence of sugars. In this work we compared the activity of a strain of *Oenococcus oeni* in malolactic fermentation of a Chardonnay grape must, using different winemaking protocols characterised by sequential or simultaneous inoculums of microbial starters. The results are discussed both in terms of fermentative behaviour and the quality of the wines obtained, with careful analysis of the main chemical parameters of the wines and of 47 different volatile compounds, giving an exhaustive overview of the opportunities and the risks related to different wine fermentation strategies.

Introduction

Malolactic fermentation (MLF), which is the biological conversion of the malic acid present in wine into lactic acid, is one of the fundamental bio-transformations occurring during winemaking.[1] Although this process today takes place in almost all red wines, and a significant proportion of white and sparkling wines, MLF raises several concerns among winemakers because to date its evolution cannot be guaranteed.[2-4] The lactic acid bacteria responsible for this process generally work in wine after alcoholic fermentation, an environment not suitable for microbial growth. Wine has numerous chemical factors able to limit bacterial activity, including ethanol, sulphur dioxide, low pH, and the absence of fermentable sugars.[5-9] In addition, some authors highlighted other causes of troubles during MLF, including nutritional imbalance or toxic compounds made by yeast responsible of alcoholic fermentation.[10] The sum of these factors in wine frequently causes stuck MLF, or delays its occurrence, requiring several weeks to complete. This timescale is not suitable for modern winemaking, and in any case exposes wines to the risk of microbial spoilage, due to the absence of the antimicrobial agents (such as sulphur dioxide), necessary in order not limit the action of lactic acid bacteria.

The main solution to MLF problems is, currently, a careful use of selected strains of malolactic bacteria, whose characteristics in terms of resistance to wine limiting factors have already been verified, and proven to be higher than those of wild bacteria.[11-13] However, this is not always enough, especially in years with extreme conditions, when the composition of wines deviates from the range suitable for bacterial activity. Indeed, considerable difficulties in performing MLF have been observed both in warmer and colder years. In the first case, the main problems come from the high concentrations of ethanol, with lactic acid bacteria suffering particularly above level of 13% ethanol in wine, and the low concentrations of malic acid.[14] This second aspect is very significant because malic acid is the main energy source for bacteria in wine,[1,8] therefore in the case of content lower than 2 g/L, the development of the microbial flora can be compromised. Vice-versa, in cold years opposite difficulties prevail. Pronounced acidity, with a pH below 3.3, can lead to stress in bacteria, making even selected bacterial cultures ineffective, if not specifically adapted to extremely acidic conditions.[15] These experiences suggest that alternative approaches are needed for the management of MLF, including the simultaneous fermentation (SW) of yeast and bacteria.

Simultaneous fermentation means the inoculum of selected cultures of bacteria in the grape must, approximately 48 hours after the active dry yeast, or when the yeast culture has begun alcoholic fermentation.[16-18] The reason for this is that the grape must is an environment more suitable for microbial growth in wine, because it does not contain the majority of the limiting factors listed above. The obstacles to microbial activity accumulate gradually during alcoholic fermentation, allowing time for bacterial biomass adaptation and ensuring a greater chance of survival for lactic acid bacteria. Considering that the consumption of sugars and malic acid occurs simultaneously, the bacteria utilised in this approach to wine fermentation must be specifically tailored to avoid spoilage phenomena associated with the consumption of sugars by lactic acid bacteria via eterolactic fermentation. However, targeted selections of wine microorganisms, appropriate winemaking, and careful monitoring of fermentation can reduce the risks, ensuring effective results in different types of wine.[5,19,20] In this work we describe experiments conducted at the Experimental Winery of the Edmund Mach Foundation (Italy), devoted both to monitoring the evolution of fermentation and the impact of final wine features induced by different management of alcoholic fermentation and MLF, to underline the risks and opportunities associated with different timing of bacteria inoculums during winemaking.

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Materials and Methods

Winemaking procedure

The experimental winemaking took place after the 2013 harvest, using a Chardonnay grape must (Table 1) made using grapes from Trentino (northern Italy). The grapes were manually harvested in the 1st ten days of September, then gently crushed using a pneumatic press. Cleaning of the grape must obtained was performed through cold storage (3°C) for 24 hours in stainless steel vats. No sulphur dioxide was added in the first steps of winemaking. Traditional winemaking (TW) was carried out with sequential inoculums of

0.3 g/L of active dry yeast (CY3079 Lalvin; Lallemand Inc., Montreal, Canada) in grape must, and 1 g/L of lactic bacteria (PN4; Lallemand inc.) in wine, after post-alcoholic fermentation. Both for yeast than for bacteria the inoculum rate respects the supplier indication, and it is in the range usually employed in winemaking. Simultaneous fermentation was performed using the same selected microorganisms, but adding the bacteria to the grape must 48 hours before the yeast (according to supplier indications), at the same concentration for both yeast and bacteria. As a reference, the 3rd fermentation protocol did not provide for bacterial inoculums, allowing spontaneous MLF (SMLF). In all cases, fermentation was carried out in 20 L stainless steels vats, with 3 replicates for each protocol. Data were expressed as mean±standard deviation. Fermentation was carried out under nitrogen gas saturation and at a temperature of 22°C. The wines were cold stabilised and bottled after 6 months of ageing on the yeast lees, before proceeding with 2.5 micrometer filtration.

Microbiological analysis and yeast/bacteria rehydration

Yeast/bacteria rehydration and microbiological analysis were carried out according to the OIV method[21] from the Microbiological Laboratory of Edmund Mach Foundation, which is a *Reference Laboratory* recognized by the Italian state, and accredited by Accredia (www.accredia.it). All microbiological media were provided by Oxoid (Basingstoke, UK). Yeast were quantified on WL Agar, while lactic bacteria were enumerated using MRS agar supplemented by 15% v/v of apple juice. Petri plates were incubated at 25°C for 4 (yeast) and 10 (bacteria) days. These last samples were incubated in anaerobic conditions using an Anaerogen Kit (Oxoid).

Chemical analysis

The chemical parameters of the grape must

Table 1. Chemical composition of the Chardonnay grape must utilised in this work.

Parameter	Value
pH	3.40
Total acidity (g/L)	6.90
Sugars (g/L)	218.80
Tartaric acid (g/L)	3.68
Malic acid (g/L)	2.49
Lactic acid (g/L)	<0.2
Promptly available nitrogen (g/L)	220

Data were obtained using Fourier transform infrared spectroscopy.

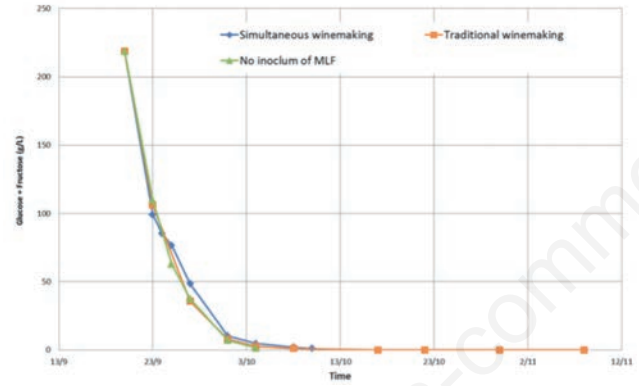

Figure 1. Evolution of sugar consumption with 3 different winemaking protocols (mean data n=3).

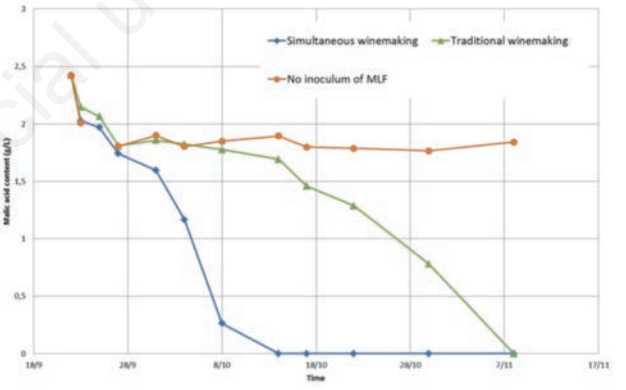

Figure 2. Evolution of malic acid consumption with 3 different winemaking protocols (mean data n=3).

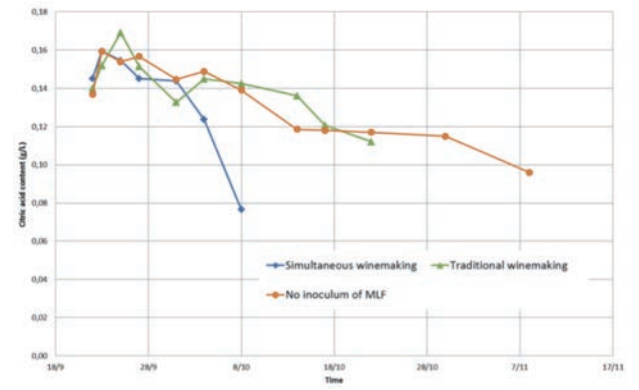

Figure 3. Evolution of citric acid with 3 different winemaking protocols (mean data n=3).).

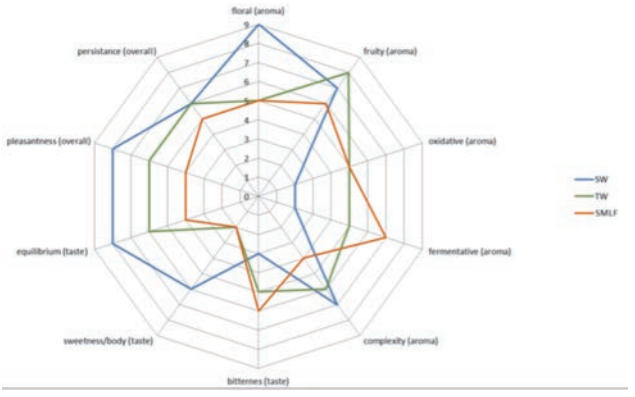

Figure 4. Sensorial characteristics of the wines obtained using different winemaking protocols (SW=simultaneous fermentation; TW=traditional fermentation; SMLF=spontaneous malolactic fermentation).

and wines were monitored using Fourier transform infrared spectroscopy (FT-IR) (Foss, Hillerød, Denmark); from the Chemical Laboratory of Edmund Mach Foundation, which is a *Reference Laboratory* recognized by the Italian state, and accredited by Accredia (www.accredia.it). Malic and lactic acid quantification was carried out in the grape must during fermentation and in the final wines using ion chromatography coupled to a conductometric detector Dionex ICS-5000, according to Masson (2000).[22] The volatile profile of the wines obtained was carried out using gas chromatography, coupled to a mass spectrometer;[23] analysis were performed on proportional mix of the 3 replicates of each thesis. Sensory evaluation of wines was performed by a panel of 5 experts from the Edmund Mach Foundation, employing pre-arranged a card that contained 10 descriptors, and a rating scale from 0 (bad) to 10 (excellent).

Results and Discussion

One of the main risks of simultaneous inoculation of yeast and bacteria in grape must is related to incompatibility between the 2 strains involved in wine fermentation.[13] Careful choice of the yeast and bacteria strains is therefore essential. In our tests we did not observe any significant differences in alcoholic fermentation with the 3 protocols considered (Figure 1). The sugar consumption in the trial containing both lactic acid bacteria and yeast (SW) had the same trend observed in the case of conventional winemaking (TW, SMLF). In contrast, it was possible to observe relevant differences in the evolution of MLF in the 3 experiments. In SW trials, MLF took place along with alcoholic fermentation, with complete consumption of malic acid even before the final degradation of sugars (Figure 2). These evidences agree which those of previous works,[16-18] and confirm that alcoholic and MLFs, performed by specifically selected yeast and lactic acid bacteria, result two independent metabolisms, based on different substrates, without mutual interferences. In the TW trial, MLF took place 10 days after bacterial inoculums, at the end of alcoholic fermentation, and the entire winemaking process took about 2 months to complete, compared to the 3 weeks of the test carried out with simultaneous inoculation. With SMLF, degradation of malic acid by the lactic acid bacteria did not occur in the first two months after alcoholic fermentation, requiring an additional period of 45 days to complete (Figure 2).

Microbial concentration in the different fermenting grape musts and wines was essayed using periodic microbiological counts, as reported in Table 2. The yeast population followed a general trend[24] with an exponential phase from the 3rd day after the inoculums, increasing to 10^7 ufc/mL over 5 days. The bacterial population indeed followed a different trend in the SW and TW trials. In the SW trial we observed a decrease in the concentration of lactic bacteria in the first 7 days after the inoculums, but 11 days after the beginning of the test the lactic acid bacteria reached 1.3×10^7 UFC/mL (18th day), ensuring fast evolution of MLF. In the case of TW, the initial decrease in the bacterial population was more drastic, not being detectable for 8 days; the maximum concentration, obtained 30 days

Table 2. Evolution of microbiota with three different winemaking protocols.

Days after crushing	Trials	Total yeast (10^6 CFU/mL)	Lab (CFU/mL)
0	SW	2.3±0.2	Not detectable
3°	SW	5.0±0.3	2.7±0.4 E+06
5	SW	40.0±2.2	1.6±0.3 E+06
7	SW	41.0±3.4	1.7±0.6 E+06
11	SW	26.0±1.8	3.9±0.4 E+06
14	SW	22.0±0.9	4.4±0.4 E+06
18	SW	3.7±0.8	1.3±0.2 E+07
20	SW	2.4±0.9	8.0±0.3 E+06
0	TW	3.7±0.1	Not detectable
3	TW	47.0±3.4	Not detectable
7	TW	35.0±1.4	Not detectable
5	TW	33.0±3.2	Not detectable
11°	TW	25.0±0.8	Not detectable
14	TW	23.0±1.2	Not detectable
18	TW	3.6±0.8	1.7±0.7 E+06
27	TW	<5	2.7±0.4 E+06
32	TW	<5	6.7±0.6 E+06
40	TW	<5	6.8±0.7 E+06
50	TW	<5	6.2±0.4 E+06
0	SMLF	2.8±0.2	<5
3	SMLF	41.0±8.3	<5
5	SMLF	44.0±2.6	<5
7	SMLF	37.0±4.4	<5
10	SMLF	28.5±3.2	<5
14	SMLF	24.0±1.8	<5
18	SMLF	2.8±0.9	<5
27	SMLF	<5	<5
32	SMLF	<5	1.0E+04±1.1
40	SMLF	<5	1.8E+04±0.4
50	SMLF	<5	8.5E+04±0.3

SW, simultaneous fermentation; TW, traditional fermentation; SMLF, spontaneous malolactic fermentation. °Day of malolactic bacteria inoculum.

Table 3. Final composition of wines.

	Free SO_2 (mg/L)	Tot. SO_2 (mg/L)	Ethanol (% vol)	pH	Total acidity (g/L)	Acetic acid (g/L)	Tartaric acid (g/L)	Malic acid (g/L)	Lactic acid (g/L)
SMLF	29±4	89±7	13.0±0.1	3.48±0.2	3.8±0.1	0.21±0.1	1.45±0.1	0.16±0.5	1.76±0.6
TW	44±6	93±8	13.0±0.2	3.49±0.5	3.6±0.2	0.23±0.1	1.39±0.2	0.04±0.1	1.93±0.2
SW	40±2	93±6	13.0±0.1	3.48±0.3	3.5±0.1	0.22±0.2	1.44±0.1	0.01±0.0	1.91±0.1

SMLF, spontaneous malolactic fermentation; TW, traditional fermentation; SW, simultaneous fermentation. Determination performed using Fourier transform infrared spectroscopy and chromatography (organic acid).

after the bacteria inoculums, was also lower than that of SW, 6.8×10^6 UFC/mL (Table 2). The differences in the behaviour of the bacteria in the SW and TW trials agree with the premises for bacteria inoculums in grape must, related to the lower selective pressure exerted by this environment, as compared to that found in wine.[3,6] The long delay in the occurrence of spontaneous MLF demonstrates that the action of native lactic acid bacteria is also not easily predictable in the case of wines, such as those considered in this work, not characterised by a strong presence of limiting factors.

The simultaneous inoculation of yeast and bacteria did not affect the composition of the wines obtained (Table 3). Apart from the regular consumption of sugars (Table 1), and the absence of differences in the ethanol yield (Table 3), the accumulation of acetic acid did not differ in the 3 trials (Table 3). The consumption of citric acid was found to be proportional to the performance of MLF, without significant differences in terms of residual amounts for the 3 protocols (Figure 3). This result is particularly interesting considering that the pH of the wine was close to 3.5, a value considered to represent the threshold risk for the spoilage activity of *Oenococcus oeni,* recognised as a heterofermentative species, from which the PN4 strain comes.[25] After 3 months' ageing on the lees, characterisation of the volatile compounds in the finished wines was performed (Table 4). Some differences were found, both in compounds having their origin in the grapes, and in molecules generated by the fermentative metabolism. In the first category we should point out the noticeable differences observed in terms of the geraniol, nerol and *trans* geranic acid content. Among compounds originating from the microbial metabolism we found relevant decrease in the SW test, about the 50% of content in wine made from TW, in the concentration of capric acid and its ethyl ester, diethyl succinate, butanoic and 3-methylbutaonic acid. A similar trend is observed comparing the composition of wine made by SW test with that of wine obtained by spontaneous MLF. Conversely, we observed the increase of some fermentative compounds, such as ethyl lactate, isoamyl and *n*-ehxyl acetate, and ß-phenylethyl alcohol. The difference between SW and other thesis is in the order of +20%. In other cases, such as the diethyl succinate, we observed variation in the content of volatile compounds, however these changes are deprived of relevance because under the sensorial threshold.[26] In conclusion, the SW reduces the concentration of compounds having unpleasant smell, such as the volatile fatty acid, and increases the content of ethyl lactate and of certain acetates that could improve the fruity notes of wine.[27]

Tasting of wines conducted by a panel of experts can help to *interpret* the effect of

Table 4. Characterisation of volatile compounds in wines obtained using three different winemaking protocols.

Volatile compounds	SMLF	TW	SW
Methanol (mg/L)	18	19	17
1-propanol (mg/L)	22	22	23
2-methyl propanol (mg/L)	25	25	25
1-butanol (mg/L)	<0.5	<0.5	<0.5
2-methyl butanol (mg/L)	26	26	26
3-methyl butanol (mg/L)	138	135.5	134
Higher alcohols (mg/L)	211	208.5	208
Acetaldehyde (mg/L)	28	29.5	25
Ethyl acetate (mg/L)	43	40	38.5
Isobutyl acetate (mg/L)	0.06	0.07	0.08
N-butyl acetate (mg/L)	0.59	0.6	0.62
Isoamyl acetate(mg/L)	4.37	4.68	5.22
N-hexyl acetate (mg/L)	0.42	0.4	0.51
ß-phenylethyl acetate (mg/L)	0.26	0.31	0.3
Ethyl lactate (mg/L)	30	32.9	36.3
Ethyl butyrate (mg/L)	0.56	0.57	0.55
Ethyl caproate (mg/L)	1.3	1.27	1.26
Ethyl caprylate (mg/L)	1.47	1.35	1.26
Ethyl caprate (mg/L)	0.12	0.12	0.06
Diethyl succinate (mg/L)	1.01	0.67	0.36
Isobutyric acid (mg/L)	1.6	1.73	1.7
Butanoic acid (mg/L)	0.09	0.09	0.05
3-methylbutanoic (mg/L)	0.09	0.09	0.05
Valerianic acid (mg/L)	0.03	0.03	0.03
Capronic acid (mg/L)	8.75	8.72	10
Caprylic acid (mg/L)	23.94	23.36	25.35
Capric acid (mg/L)	7.15	7.37	3.37
1-hexanol (mg/L)	2.51	2.54	2.38
Trans 3-hexen-1-ol (mg/L)	0.02	0.01	0.01
Cis 3-hexen-1-ol (mg/L)	<0.01	<0.01	<0.01
3-methylthio-1-propanol (mg/L)	0.27	0.23	0.22
Benzylic alcohol (mg/L)	0.06	0.07	0.06
ß-phenyl ethyl alcohol (mg/L)	14.9	15.2	18.8
Trans furan linalool oxide (µg/L)	7	8	5
Cis furan linalool oxide (µg/L)	1	<1	1
Linalool (µg/L)	8	4	3
α-terpineol (µg/L)	5	2	4
4-terpineol (µg/L)	<0.5	<0.5	<0.5
Citronellol (µg/L)	<1	<1	<1
Nerol (µg/L)	310	370	430
Geraniol (µg/L)	380	420	390
Trans geranic acid (µg/L)	150	140	<5
Rosa oxide I (µg/L)	2	2	2
Rosa oxide II (µg/L)	3	2	2
Guaiacol (µg/L)	3	2	4
Benzaldehyde (µg/L)	7	6	6
Methyl salicylate (µg/L)	5	3	3

SMLF, spontaneous malolactic fermentation; TW, traditional fermentation; SW, simultaneous fermentation. Data obtained using gas chromatography-mass spectrometry.

changes on the analytical quality of the wines produced. The tasting was conducted 1 month after bottling, in the late spring of 2014 (Figure 4). The SW wine showed outstanding floral notes and the absence of *lactic* characteristics resulting from the uncontrolled activity of lactic acid bacteria.[25,28] The TW wine was characterised by a flavour of ripe fruit, oxidative notes and more bitterness than the SW wine at tasting. Finally, the wine obtained from spontaneous MLF was characterised by a strong fermentative taste and moderate persistence. These differences between the 3 wines are probably due to 2 factors: the suitability of the bacteria to the different fermentation environments, which have a significant impact on the production of secondary compounds, and the different timing of winemaking, which in the case of TW and SMLF trials increased the risk of oxidative deviation of products or, more generally, of a lack of improvement during the ageing of wine, due to the prolonged latency of lactic microflora.

Conclusions

The experience described in this work demonstrates that the SW of yeast and bacteria may be an interesting winemaking strategy, albeit confined to specific contexts. In our case, the chemical composition of the grape must, and particularly the pH value, was higher than that required to ensure safe inoculums of bacteria in the grape must. However, a careful choice of the *Oenococcus oeni* strain involved in this work ensured the absence of spoilage activity. In conclusion, the risks associated with the proliferation of lactic acid bacteria in grapes must not be underestimated, so simultaneous inoculation can be carried out when the composition of the grape minimises the risk of spoilage and prompt analytical control allows early identification of potential problems. If carried out in this context, the SW of yeast and bacteria allows an increase in the success rate for MLF, reducing winemaking times and ensuring more varying wines as compared to production using TW protocols.

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