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Influence of different malolactic fermentation techniques on changes in chemical properties and volatile compounds of cv. Teran red wine (*Vitis vinifera* L.)

Utjecaj različitih načina provedbe jabučno-mliječne fermentacije na promjene kemijskih svojstava i hlapivih komponenti crnog vina Teran (*Vitis vinifera* L.)

Tomislav PLAVŠA[1] (✉), Ana-Marija JAGATIĆ KORENIKA[2], Igor LUKIĆ[1], Marijan BUBOLA[1], Ana JEROMEL[2]

[1] Institute of Agriculture and Tourism, K. Huguesa 8, 52440 Poreč, Croatia

[2] University of Zagreb Faculty of Agriculture, Svetošimunska 25, 10000 Zagreb, Croatia

✉ Corresponding author: tomislav@iptpo.hr

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ABSTRACT

Malolactic fermentation (MLF) is a complex biochemical process playing an important role in the production of red wines. The main reasons for its implementation are the reduction of titratable acidity and the consequent increase of pH, microbiological stability of wine, and changes in aromatic and sensory properties of wine. The aim of this study was to determine the compatibility of yeast and bacteria used by different malolactic fermentation techniques and their influence on the fermentation duration, concentration of individual organic acids, aroma compounds, and on chemical and sensory properties of Teran wines. The experiment included control treatment (without MLF), spontaneous MLF, induced MLF at the beginning of alcoholic fermentation with simultaneous inoculation of yeast and bacteria (co-inoculation) and induced MLF after alcoholic fermentation (sequential MLF). In the co-inoculation treatment MLF had no negative effect on the alcoholic fermentation kinetic. Alcoholic fermentation was complete in all treatments. Co-inoculation resulted in a significantly shorter duration of the MLF process. In all MLF treatments, significant reduction of titratable acidity and the increase of pH values was noted. Wines of the spontaneous MLF treatment obtained the highest concentration of volatile acidity and ethyl acetate. In all MLF treatments a complete consumption of malic acid and a decrease in concentration of citric acid, total amount of higher alcohols, and acetaldehyde were observed. Furthermore, significantly higher concentrations of ethyl esters, diacetyl, acetoin, and 2,3-butanediol were present in wines from all MLF treatments.

Keywords: *Oenococcus oeni*, co-inoculation, aroma compounds, organic acids, duration of malolactic fermentation

SAŽETAK

Jabučno-mliječna fermentacija (JMF) složen je biokemijski proces koji ima važnu ulogu u proizvodnji većine crnih vina. Glavni učinci primjene JMF su snižavanje ukupne kiselosti uz rast pH vrijednosti, mikrobiološka stabilnost vina te promjena aromatskih senzornih svojstava vina. Cilj ovog istraživanja bio je utvrditi utjecaj različitih načina jabučno-mliječne fermentacije na kompatibilnost kvasca i bakterija, duljinu trajanja fermentacije, koncentraciju pojedinačnih organskih kiselina i spojeva arome te senzorna svojstva vina sorte ´Teran´ (*Vitis vinifera* L.). Pokus je obuhvaćao kontrolni tretman (bez JMF), spontanu JMF, induciranu JMF u početku alkoholne fermentacije (koinokulacija) te induciranu JMF po završenoj alkoholnoj fermentaciji. U svim tretmanima alkoholna fermentacija je u potpunosti završila, bez zastoja ili

usporavanja. Koinokulacija je utjecala na značajno kraće trajanje JMF. Svi tretmani s JMF značajno su utjecali na sniženje koncentracije titracijske kiselosti i povećanje pH vrijednosti. Najviše koncentracije hlapive kiselosti i etil acetata utvrđene su u vinima spontane JMF. U svim tretmanima s JMF zabilježena je potpuna razgradnja jabučne kiseline te sniženje koncentracije limunske kiseline, ukupnih viših alkohola i acetaldehida. Nadalje, značajno više koncentracije etilnih estera, diacetila, acetoina i 2,3-butandiola zabilježene su u svim tretmanima s JMF.

Ključne riječi: *Oenococcus oeni*, koinokulacija, spojevi arome, organske kiseline, trajanje jabučno-mliječne fermentacije

INTRODUCTION

Wine production is a complex biochemical process involving alcoholic fermentation (AF), which is carried out by yeasts, although malolactic fermentation (MLF) carried out by lactic acid bacteria is also quite common (Cañas et al., 2014). MLF is a biological deacidification process in which dicarboxylic L-malic acid is converted into monocarboxylic L-lactic acid and carbon dioxide by the action of different bacteria, mostly *Oenococcus oeni*, but also some *Lactobacillus* spp. and *Pediococcus* spp. (Liu, 2002), having significant influence on wine flavor (Lonvaud-Funel, 1999).

Malolactic fermentation can occur as a spontaneous process (usually after alcoholic fermentation (AF) is completed) carried out by lactic acid bacteria of the genera *Lactobacillus*, *Pediococcus* and *Oenococcus*, or as an induced process, using commercial lyophilized starter cultures of *Oenococcus oeni*, added to must or young wine. The time of inoculation with selected bacteria can have significant impact on the malic acid degradation process and on wine quality (Cañas et al., 2014). Temperature, ethanol, pH, and SO_2 are the main factors influencing the occurrence and rate of MLF, while medium-chain fatty acids (caproic, caprylic, capric, and lauric) are according to Lonvaud-Funel et al. (1988) the major inhibitory compounds, secondary products of yeast metabolism, that may affect the growth and reproduction of lactic acid bacteria as well as MLF flow.

There are three possible times for bacterial inoculation: simultaneous inoculation of yeast and bacteria before AF (co-inoculation), inoculation during AF, and inoculation after AF (sequential inoculation). As a possible risk of co-inoculation, Alexandre et al. (2004) pointed out the possible development of unwanted and/or antagonistic interactions between yeast and bacteria (sluggish/stuck AF, formation of potentially negative volatile compounds in wine). In contrast, sequential inoculation avoids unwanted interactions between yeast and bacteria and reduces the risk of acetic acid formation due to lower concentrations of residual sugars. Despite the benefits, there are also risks associated with sequential inoculation, such as the presence of high concentrations of ethanol, SO_2, and other toxic compounds produced by yeast, as well as nutrient deficiencies (Larsen et al., 2003). Positive effects of co-inoculation presented in their work Lasik-Kurdyś et al. (2017) and Jussier et al. (2006) stating that MLF in the presence of fermented sugar does not necessarily lead to increased acetic acid synthesis if AF proceeds rapidly without delay. In the work by Rosi et al. (2003) co-inoculation affected faster degradation of malic and citric acid without negative effects on the course of AF.

Among the most important changes that occur during MLF is the synthesis and/or degradation of compounds carriers of varietal and fermentation aroma of wine (Cappello et al., 2017). According to Knoll et al. (2011) the moment of bacterial inoculation, especially co-inoculation, significantly increased the synthesis of ethyl and acetate esters, carriers of the wine aroma fruity component. On the other hand, Malherbe et al. (2012) stated an increase in ethyl esters in wines in which sequential MLF was conducted. Some species of lactic acid bacteria, especially *Oenococcus oeni*, can catabolize acetaldehyde, a secondary product of AF, significantly reducing the so-called vegetative, green, or grassy aromas of some wines (Liu, 2002). Previous research reported that the MLF influence on the concentration of higher alcohols in wine was negligible (Herjavec et al., 2001), while Jeromel et al. (2008) found a slight increase in their

concentration. Diacetyl is one of the most important aromatic compounds associated with MLF (Swiegers et al., 2005). As a chemically unstable compound, diacetyl can be reduced to acetoin and 2,3-butanediol. In the work by Antalick et al. (2013) the moment of bacterial inoculation had significant effect with co-inoculation generally stood out with higher concentrations of acetoin and 2,3-butanediol, however below their odor detection thresholds. Lasik-Kurdys et al. (2018) also observed an increase in acetoin and 2,3-butanediol after MLF was performed.

Teran (*Vitis vinifera* L.) is a red grape variety mostly grown in the north Adriatic area, including the Croatian Istria viticultural subregion (Maletić et al., 2015; Rusjan et al., 2015; Žulj Mihaljević et al., 2020). It is characterized by high yield and medium level of sugar in grapes, while the typically high titratable acidity of its grape juice and wine, usually ranging from 7 to 10 g/L (Mirošević and Turković, 2003, Plavša et al., 2012, Bubola et al., 2017) makes it suitable for MLF. The aim of this study was to determine the influence of different types of MLF (spontaneous and induced) as well as inoculation time (co-inoculation and sequential inoculation) using two commercial strains of *Oenococcus oeni* (Lalvin 31 and Uvaferm Alpha) on the kinetics of AF and MLF, degradation and synthesis of organic acids and changes in volatile compounds concentrations.

MATERIALS AND METHODS

Microorganisms

Saccaharomyces cerevisiae strain Uvaferm 299 (Lallemand Inc., Canada) and pure freeze-dried cultures commercially available strains of *Oenococcus oeni* (Uvaferm Alpha and Lalvin 31) (Lallemand Inc., Canada) were used in this study.

Wine production

Teran grapes grown at Koreniki in the viticultural subregion of Istria, Croatia were harvested by hand, crushed and destemmed, homogenized and evenly distributed in eighteen 50 L stainless steel containers (six treatments in triplicate), as follows: K (control - without MLF), S (spontaneous MLF), KI31 (co-inoculation with strain Lalvin 31), KIA (co-inoculation with strain Uvaferm Alpha), NI31 (sequential inoculation with strain Lalvin 31) and NIA (sequential inoculation with strain Uvaferm Alpha). $K_2S_2O_5$ was added in the control treatment (K) in the dosage of 80 mg/L to prevent MLF and in the other treatments in the dosage of 10 mg/L. Fermaid E (Lallemand Inc., Canada) was used as a yeast nutrient (20 mg/L). The temperature was maintained at 24 ± 0.5 °C in all treatments until the end of AF and MLF. The course of AF and MLF was monitored every two days until their completion. Maceration was carried out for seven days. Pressing was done at 0.8 bar using an 80L-capacity hydro press and samples were taken for chemical and volatile components analysis. After completion of AF and MLF (residual sugar <1.0 g/L, malic acid <0.1 g/L) the concentration of free SO_2 was adjusted to 30 mg/L, and after two days the wines were raked from the gross lees. After two months the free SO_2 was adjusted to 30 mg/L, the wines were re-racked and filtered with Seitz-Schenk EK grade filter plates and bottled in 0.75 L bottles with cork and stored for organoleptic evaluation.

Must and wine analysis

Chemical analysis

Basic wine parameters including alcohol content (% v/v), reducing sugar, titratable and volatile acidity and pH value were analyzed using the methods proposed by OIV (2016).

Analysis of organic acids

The concentration of citric, malic and lactic acid was determined by a UV-VIS spectrophotometer VarianCarry 50 (Varan Inc., Harbour City, Harbour City, CA, USA) after enzymatic reaction using kits for enzymatic determination (R-Biopharm AG, Darmstadt, Germany) at a wavelength of 340 nm (Mato et al., 2005). Tartaric and succinic acid concentrations were analyzed by high-performance liquid chromatography using an HPLC Varian Pro Star Model500 equipped with a MetaCarb 87H HPLC column (300 × 7.8 mm) and a UV-VIS detector after direct injection of

the filtered and diluted sample (Castellari et al., 2000). Chromatograms were recorded at a wavelength of 214 nm.

Volatile compounds analysis

Esters and medium chain fatty acids were separated from wine samples by solid-phase extraction technique using octadecylsilica(C18) as asorbent and dichloromethane as asolvent (Lukić et al., 2006). Prior to extraction, 3-heptanol was added as an internal standard. The obtained extracts were analyzed by gas chromatography usinga Varian 3350 gas chromatograph equipped with a fused silica capillary column Rtx-Wax (30 m × 0.25 mm I.D. × 0.25 mm d.f.) and a flame ionization detector (FID).

Acetaldehyde, compounds formed during MLF (diacetyl, acetoin, ethyl lactate and 2,3-butanediol) and higher alcohols were determined by gas chromatography on a Varian 3350 gas chromatograph with the same equipment as mentioned before after direct injection of diluted and acidified sample according to the method of Peinado et al. (2004). 1-Pentanol was used as an internal standard.

Volatile compounds were identified by comparing their retention times to those of the pure standards. Calibration curves (relative peak area versus concentration ratio of aroma compound/internal standard) and all quantifications were performed by the internal standard method using Varian Star 4.51 software (Varian Inc., Harbor City, CA). All samples were analyzed in duplicates and mean values were used in further data processing.

Odor activity values (OAV) of volatile compounds were calculated as quotients of concentrations and corresponding odor detection thresholds (ODT) found in literature.

Statistical analysis

One-way analysis of variance (ANOVA) was further analyzed by post-hoc Fisher's comparison of means and principal component analysis (PCA) were carried out using Statistica 13.4 software (TIBCO Software Inc., 2018).

RESULTS AND DISCUSSION

Must composition

After primary processing and unification of crushed grapes, chemical parameters were determined (Table 1). The results showed a high proportion of malic acid in the titratable acidity values, meaning that this must was an ideal candidate for the application of MLF. The sugar content was at the level of approximately 11 vol% of potential alcohol, which could not have a toxic effect on lactic acid bacteria (LAB), according to Larsen et al. (2003).

Alcoholic and malolactic fermentation

The duration of AF and MLF as well as the duration of the LAG phase (time to onset of L-malic acid degradation) of MLF are shown in Table 2. LAG phase (LAB growth Phase I) ends and Phase II begins when cell numbers exceed 10^6 CFU/mL, and L-malic acid degradation begins with L-lactic acid formation (Krieger-Weber and Silvano, 2015). The duration of AF did not differ significantly among the different types of MLF (spontaneous and inoculated), inoculation time (co-inoculation and sequential inoculation) and LAB strains (Uvaferm Alpha, LALVIN31) and control treatments without MLF. Furthermore, the presence of LAB did not adversely affect the viability of the yeast as well as its ability to conduct alcoholic fermentation and complete sugars degradation (Table 3). Such findings correspond to those obtained by Abrahams and Bartowsky (2012), Muñoz et al. (2014) and Tristezza et al. (2016). In contrast, Suriano et al. (2015) observed a delayed onset and longer duration of alcoholic fermentation when applying co-inoculation. The shortest LAG phase duration was found in the co-inoculation (KI) treatment whereas sequential and spontaneous MLF lasted the same. The total duration of MLF was as follows: KI31=KIA<NI31=NIA<S what is in agreement with the results reported by Suriano et al. (2015). The differences in LAG phase duration and total MLF duration between the treatments can be explained by the competitive action of yeast and bacteria towards nutrients such as vitamins and amino acids (Arnink

Table 1. Chemical parameters of Teran grape must

Organic acids (g/L)					
Citric	Tartaric	Malic	Titratable acidity	Sugar (g/L)	pH
0.47[1]±0.03	3.40±0.07	4.45±0.05	8.33±0.11	187±2.0	3.24±0.03

[1] Means ± SD (n=6)

Table 2. Duration of alcoholic fermentation and LAG phase in Teran wines

Duration (days)	Treatments						Sig.
	K	S	KI31	KIA	NI31	NIA	
AF	7±1	8±1	7±0	7±1	7±0	8±1	ns
LAG phase of MLF	NO	81±1a	2±0b	2±0b	8±1a	8±0a	***
MLF	NO	22±2a	10±1c	10±1c	16±1b	16±1b	***

1Means ± SD (n=3) with different letters are differ significantly within treatments (means separation by Fisher's LSD test at P<0.05)

*** and ns indicate significant at $p \leq 0.001$ and not significant, respectively

Abbreviations: K (control, without malolactic fermentation), S (spontaneous malolactic fermentation), KI31 (co-inoculation with Lalvin 31 bacteria strain), KIA (co-inoculation with Uvaferm Alpha bacteria strain), NI31(sequential with Lalvin 31 bacteria strain), NIA (sequential with Uvaferm Alpha bacteria strain.), NO (MLF not performed)

Table 3. Chemical analysis of Teran wines

Parameter		Treatments						Sig.
		K	S	KI31	KIA	NI31	NIA	
Alcohol content	(%v/v)	11.191±0.1	11.22±0.2	11.24±0.1	11.23±0.2	11.24±0.1	11.22±0.1	ns
Titratable acidity	(g/L)	8.65±0.05a	6.83±0.02b	6.81±0.01b	6.71±0.01b	6.84±0.01b	6.87±0.02b	***
Citric acid	(g/L)	0.42±0.09a	0.08±0.02d	0.05±0.02d	0.06±0.01d	0.17±0.04c	0.23±0.01b	***
Tartaric acid	(g/L)	2.81±0.1a	2.73±0.05b	2.75±0.03b	2.44±0.07b	2.38±0.04b	2.54±0.07b	***
Malic acid	(g/L)	4.18±0.1a	0.05±0.01b	0.07±0.01b	0.07±0.01b	0.05±0.01b	0.06±0.01b	***
Lactic acid	(g/L)	n.d.	2.81±0.1a	2.95±0.1a	2.87±0.1a	2.84±0.1a	2.86±0.1a	***
Succinic acid	(g/L)	0.91±0.06a	0.76±0.02b	0.94±0.01a	0.94±0.01a	0.94±0.01a	0.93±0.04a	***
Volatile acidity	(g/L)	0.51±0.1d	0.81±0.1a	0.72±0.05b	0.64±0.02c	0.70±0.01b	0.61±0.05c	***
pH		3.29±0.04c	3.59±0.1a	3.57±0.02a	3.6±0.1a	3.58±0.01a	3.45±0.1b	***
Residual sugar	(g/L)	1.23±0.3	1.43±0.5	1.44±0.4	1.27±0.7	1.15±0.6	1.5±0.3	ns

1Means ± SD (n=3) with different letters are differ significantly within treatments (means separation by Fisher's LSD test at P<0.05)

*** and ns indicate significant at $p \leq 0.001$ and not significant, respectively

Abbreviations: K (control, without malolactic fermentation), S (spontaneous malolactic fermentation), KI31 (co-inoculation with Lalvin 31 bacteria strain), KIA (co-inoculation with Uvaferm Alpha bacteria strain), NI31(sequential with Lalvin 31 bacteria strain), NIA (sequential with Uvaferm Alpha bacteria strain.), NO (MLF not performed)

and Henick-Kling, 2005; Pardo and Ferrer, 2019), the inhibitory effect of alcohol (Sanchez et al., 2019), low pH, and the presence of SO_2 (Muñoz et al., 2014). Although the duration of MLF depends largely on LAB strain (Cañas et al., 2013; Sun et al., 2013), it seems that this was not the case in this study.

Chemical properties of wines

The results of basic chemical analysis of wines are shown in Table 3.

In MLF treatments wines, there was significant decrease in titratable acidity by an average of 1.84±0.06 g/L and an increase in pH by 0.27±0.06. These results are in accordance with typical changes during MLF involving a decrease in titratable acidity in the range of 1.0 to 3.0 g/L and an increase in pH by 0.1 to 0.3 units (Malherbe, 2010). The concentration of malic acid in all MLF treatments compared to the control treatment K was significantly lower as it was completely utilized (<0.2 g/L), which is in line with the results obtained by Cañas et al. (2012, 2014). Tristezza et al. (2016) reported that the choice of yeast/bacteria strain combination, as well as the inoculation time significantly affects the breakdown of malic acid. In our study, observing the inoculation time and the LAB strain used, no significant differences regarding malic acid degradation were found, which is in accordance with the results published by Abrahamse and Bartowsky (2012).

Citric acid concentration was significantly lower in all the MLF treatments compared to control K wines (Table 3) and such results are in accordance with a study by Lerm et al. (2010) who stated that LAB have the ability to degrade citric acid. Pan et al. (2011) also observed faster complete degradation of citric acid in a co-inoculation treatment compared to a sequential inoculation treatment. On the contrary, Cañas et al. (2014) recorded significantly higher concentrations of this acid after co-inoculation treatments. Furthermore, in this study a lower rate of citric acid degradation was found after the sequential inoculation treatment (NIA), which is agreement with the study of Pérez-Martín et al. (2014) who found that LAB strain used could notably affect the degradation of citric acid.

Spontaneous MLF influenced significantly higher concentration of volatile acidity (Table 3), which is in agreement with the study of Lasik-Kurdyś et al. (2017), and its increase can be explained by the uncontrolled growth of LAB species from the genera *Lactobacillus*, *Leuconostoc*, *Streptococcus*, and *Pediococcus* (Pretorius, 2001). Tristezza et al. (2016) presumed that the presence of volatile acids could be dependent on the LAB strain as it was the case in this study, where lower volatile acidity concentrations were found in the wines where LAB strain Uvaferm Alpha was used, regardless of the inoculation time.

Aroma compounds

Modification of secondary metabolites by LAB strains play an important role in the odor (Swiegers et al., 2005) and flavor profile of wine (Lasik-Kurdys et al., 2018). Changes in ethyl and acetate ester concentrations are shown in Table 4. Matthews et al. (2004) pointed out LAB esterase role in the synthesis and hydrolysis of esters that can lead to the changes in their concentration (Swiegers et al., 2005). Although most authors reported an increase in the concentration of esters in wines where MLF was performed (Knoll et al., 2011; Antalick et al., 2012), Gámbaro et al. (2001) recorded a decrease. In our work diethyl succinate and ethyl lactate concentrations significantly increased in induced but also in spontaneous MLF compared to the control treatment. Significantly, higher concentration of diethyl succinate was observed in wine of the spontaneous MLF treatment, probably because of a larger rate of esterification of succinic acid with ethanol.

Comparing the inoculation time, the highest concentrations of diethyl succinate and ethyl lactate, as well as total ethyl esters, were observed in wines of the co-inoculation treatments, especially in wines produced with the Uvaferm Alpha strain (Table 4). In their study, Malherbe (2010) and Knoll et al. (2012) also presented influence of inoculation time and LAB strain

on ethyl esters concentrations. In this study the highest concentrations of ethyl acetate and hexyl acetate, as well as the content of total acetate esters were present in the wines with spontaneous MLF. Herjavec et al. (2001) did not record changes in ethyl acetate concentration between treatments with spontaneous and induced MLF. In contrast, Maicas et al. (1999) and Cañas et al. (2013) found that the bacteria strain can affect changes in acetate ester concentrations. In this study, significantly lower concentrations of these esters were observed in wines from the co-inoculation treatments, especially with the Uvaferm Alpha strain.

Concentrations of individual and total higher alcohols are presented in Table 5. Previously published results regarding the influence of MLF on higher alcohol concentrations are quite different. Particular authors (Knoll et al., 2012, and Celik et al., 2019) reported their increase, while some (Herjavec and Tupajić, 1998, Jeromel et al., 2008) detected no changes in the concentration of total higher alcohols. In this work, a decrease in total higher alcohol concentration was observed in the treatments with MLF compared to control K treatment (without MLF). Regarding the inoculation time of the LAB strains, higher concentrations of total higher alcohols were observed in the sequential inoculation treatments, which corresponds to the results obtained by Versari et al. (2015). A reduction of higher alcohol concentrations by co-inoculation was also found by Versari et al. (2015), while lower concentrations of 2-phenylethanol in wines produced by co-inoculation was recorded by Cañas et al. (2014). As shown in Table 5, concentrations of isoamyl alcohol and 2-phenylethanol were significantly lower in wines of all MLF treatments with respect to control K wine, and both compounds were above the odor detection thresholds in all analyzed wines. Co-inoculation treatments significantly reduced the concentrations of isoamyl alcohol, 2-phenylethanol and 1-propanol, and increased the concentrations of isobutanol and 1-hexanol.

Although the lipolytic activity of LAB has not been investigated in detail, Matthews et al. (2004) stated the possibility that some LAB`s may form lipases in a substrate of non-wine origin. Nevertheless, in numerous studies (Pozo-Bayón et al., 2005; Celik et al., 2019) the changes in volatile fatty acid concentrations by bacterial were recorded. These compounds are responsible for fatty, rancid, and buttery notes in wines. The content of individual and total fatty acids is shown in Table 5, and their concentrations varied not only depending on the type of MLF but also on the LAB used. The highest values of these acids were found in wine of the spontaneous MLF treatment, while the lowest values were found in KI31 treatment wine.

Acetaldehyde, as one of the most important carbonyl compound formed during alcoholic fermentation, can be reduced during MLF. As reported by other authors (Osborne et al., 2000; Ruiz et al., 2012), MLF influenced a decrease in acetaldehyde concentrations (Table 6). Although the differences were not significant, the lowest content of this compound was recorded in the co-inoculation treatment where LAB strain Uvaferm Alpha (KIA) was used. Furthermore, significantly higher concentrations in wines of all the MLF treatments were reported for diacetyl, acetoin, and 2,3-butanediol (Table 6), which agrees with numerous other studies (Cañas et al., 2012; Antalick et al., 2013; Tristezza et al., 2016; Lasik-Kurdys et al., 2018; Celik et al., 2019).

Principal component analysis

Principal component analysis (PCA) was applied on a dataset consisting of all the treatments as cases (Figure 1a) and the concentrations of volatile compounds with OAV>1 as variables (Figure 1b). The first two principal components (PC's) obtained were found to have an eigenvalue greater than 1 and cumulatively explained 63.25% of the variability among the data. Eigenvector analysis showed that isobutanol, acetaldehyde, ethyl hexanoate, and diacetyl had the greatest effect on the first principal component (PC 1) and isoamyl acetate, caproic acid, ethyl octanoate, and isoamyl alcohol on the second principal component (PC 2).

Table 4. Concentrations (mg/L) of ethyl and acetate esters in Teran wines

Compounds	ODT	K		S		KI31		KIA		NI31		NIA		Sig.
	(mg/L)	Mean	OAV	Mean	OAV	Mean	OAV	Mean	OAV	Mean	OAV	Mean	OAV	
Ethyl esters														
Diethyl succinate	200[1]†	0.091±0.02e	0	1.48±0.03a	0	0.21±0.01c	0	0.43±0.01b	0	0.15±0.02d	0	0.17±0.01d	0	***
Ethyl lactate	154.6[1]††	4.27±1.22e	0	69.35±2.77b	0.4	67.30±2.07b	0.4	78.62±1.45a	0.5	47.85±2.12d	0.3	60.2±1.75c	0.4	***
Ethyl hexanoate	0.014[2]†††	0.17±0.01c	12.4	0.21±0.03b	14.8	0.21±0.01b	15	0.25±0.01a	17.9	0.23±0.02ab	16.4	0.22±0.01ab	15.7	**
Ethyl octanoate	0.005[2]†††	0.18±0.01b	35.3	0.14±0.02c	28	0.15±0.01c	30	0.16±0.01bc	32	0.22±0.01a	44.7	0.15±0.01c	30.7	***
Ethyl decanoate	0.2[2]†††	0.04±0.02	0.2	0.06±0.02	0.3	0.06±0.01	0.3	0.07±0.02	0.4	0.06±0.01	0.3	0.04±0.01	0.2	ns
∑ ethyl esters		4.76e		71.24b		67.93b		79.53a		48.51d		60.78c		***
Acetate esters														
Ethyl acetate	12[3]†	55.4±2.79bc	4.6	65.55±0.48a	5.5	48.36±3.0d	4.0	36.29±1.11e	3	56.5±0.41b	4.7	53.17±0.78c	4.4	***
Hexyl acetate	0.67[4]†	0.15±0.03b	0.2	0.2±0.02a	0.3	0.17±0.01b	0.2	0.17±0.01b	0.2	0.17±0.01b	0.2	0.17±0.01b	0.2	*
2-phenyl acetate	0.25[2]†††	0.27±0.02b	1.1	0.28±0.02b	1.1	0.29±0.02ab	1.2	0.32±0.02a	1.3	0.30±0.03ab	1.2	0.26±0.01b	1	*
Isoamyl acetate	0.03[2]†††	0.46±0.02b	15.4	0.43±0.12bc	14.3	0.35±0.02c	11.8	0.45±0.01bc	14.9	0.60±0.02a	19.9	0.50±0.03b	16.5	**
∑ acetate esters		56.28bc		66.47a		49.18d		37.23e		57.57b		54.09c		***

[1] Means ± SD (n=3) with different letters differ significantly within treatments (means separation by Fisher's LSD test at P<0.05)

*, **, *** and ns indicate significant at $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$ and not significant, respectively

Abbreviations: ODT (odor detection threshold), K (control, without malolactic fermentation), S (spontaneous malolactic fermentation), KI31 (co-inoculation with Lalvin 31 bacteria strain), KIA (co-inoculation with Uvaferm Alpha bacteria strain), NI31(sequential with Lalvin 31 bacteria strain), NIA (sequential with Uvaferm Alpha bacteria strain), OAV-odor active values (calculated by dividing the mean concentration of the aromatic compound by the ODT value)

Odor detection threshold in the literature ([1] Bleve et al., 2016, [2] Ferreira et al., 2000, [3] Budić-Leto et al., 2010, [4] Zhao et al., 2017), † -in 10 - 12% water/ethanol mixture, †† - in wine, ††† - in synthetic wine(11% v/v ethanol, 7g/L glycerin, 5g/L tartaric acid, pH adjusted to 3.4 with 1M NaOH)

Table 5. Concentrations (mg/L) of higher alcohols and fatty acids in Teran wines

Compounds	ODT	K		S		KI31		KIA		NI31		NIA		Sig.
	(mg/L)	Mean	OAV	Mean	OAV	Mean	OAV	Mean	OAV	Mean	OAV	Mean	OAV	
Higher alcohols														
1-Butanol	150[1]†	0.601±0.12	0	0.58±0.13	0	0.69±0.24	0	0.58±0.15	0	0.69±0.30	0	0.76±0.21	0	ns
1-Hexanol	8[2]††	1.31±0.05c	0.2	1.24±0.06d	0.2	1.44±0.05b	0.2	1.83±0.01a	0.2	1.40±0.03b	0	1.39±0.02b	0.2	***
1-Propanol	306[3]††	26.91±3.71ab	0.1	31.4±2.44a	0.1	23.84±3.25b	0.1	22.16±1.14c	0.1	31.39±1.61a	0.1	27.90±1.96ab	0.1	**
2-Phenylethanol	14[2]††	70.91±3.09a	5.1	51.75±1.09c	3.7	54.64±1.37bc	3.9	57.48±0.66b	4.1	57.4±2.67b	4.1	68.76±3.16a	4.9	***
Isobutanol	40[2]††	72.14±1.69e	1.8	80.73±1.44bc	2	81.25±1.36b	2	88.30±1.20a	2.2	75.07±0.51d	1.9	78.29±2.01c	2	***
Isoamyl alcohol	30[2]††	380.26±2.42a	12.7	292.53±2.16d	9.8	271.10±1.03e	9	338.63±0.53c	11.3	370.34±0.58b	12.3	381.69±1.11a	12.7	***
∑ higher alcohols		552.133 a		458.223 d		432.966 e		508.973c		536.290 b		558.783 a		***
Fatty acids														
Caproic acid	0.42[2]††	2.09±0.19b	5	2.14±0.19ab	5.1	1.94±0.12b	4.6	2.12±0.05b	5	2.49±0.03a	5.9	2.05±0.05b	4.9	**
Caprylic acid	0.5[2]††	1.65±0.05bc	3.3	1.85±0.04ab	3.7	1.51±0.04c	3	1.96±0.05a	3.9	1.76±0.15b	3.5	1.79±0.08ab	3.6	***
Capric acid	1[2]††	0.27±0.06bcd	0.3	0.46±0.01a	0.5	0.3±0.02bc	0.3	0.34±0.02b	0.3	0.2±0.01d	0.2	0.25±0.02c	0.3	***
Lauric acid	1[4]†††	0.18±0.02a	0.2	0.11±0.01b	0.1	0.03±0.01d	0	0.08±0.01bc	0.1	0.05±0.01cd	0.1	0.03±0.02d	0	***
∑ fatty acids		4.19ab		4.55a		3.78b		4.49a		4.51a		4.12ab		***

[1] Means ± SD (n=3) with different letters differ significantly within treatments (means separation by Fisher's LSD test at P<0.05).

, * and ns indicate significant at $p \leq 0.01$, $p \leq 0.001$ and not significant, respectively.

Abbreviations: ODT (odor detection threshold), K (control, without malolactic fermentation), S (spontaneous malolactic fermentation), KI31 (co-inoculation with Lalvin 31 bacteria strain), KIA (co-inoculation with Uvaferm Alpha bacteria strain), NI31(sequential with Lalvin 31 bacteria strain), NIA (sequential with Uvaferm Alpha bacteria strain), OAV-odor active values (calculated by dividing the mean concentration of the aromatic compound by the ODT value).

Odor detection threshold in the literature([1] Bleve et al., 2016, [2] Ferreira et al., 2000,[3] Celik et al.,2019, [4] Li et al., 2008), † - in 10% (v/v) ethanol–water solution, adjusted to pH 3.5 with tartaric acid, †† - in synthetic wine (11% v/v ethanol, 7g/L glycerin, 5g/L tartaric acid, pH adjusted to 3.4 with 1M NaOH),††† - in12% ethanol/water mixture containing 5 g/L tartaric acid at pH 3.2

Table 6. Concentrations (mg/L) of acetaldehyde, diacetyl, acetoin and 2,3-butanediolin Teran wines

Compounds	ODT	K		S		KI31		KIA		NI31		NIA		Sig.
	(mg/L)	Mean	OAV	Mean	OAV	Mean	OAV	Mean	OAV	Mean	OAV	Mean	OAV	
Acetaldehyde	0.5[1]†	59.261±2.12a	119	9.12±0.19b	18.2	4.48±0.58c	9	3.05±0.16c	6.1	3.93±0.32c	7.9	5.37±0.34c	10.7	***
Diacetyl	0.1[2]††	1.61±0.04c	16.1	7.06±0.07b	70.6	8.73±0.35a	87.3	7.24±0.44b	72.4	8.49±0.28a	84.9	8.45±0.33a	84.5	***
2.3-butanediol	600[3]†††	490.05±9.65e	0.8	686.75±5.6b	1.1	820.55±1.45a	1.4	692.86±5.88b	1.2	564.42±3.11d	0.9	620.64±4.87c	1	***
Acetoin	150[3]†††	4.22±0.18c	0.0	7.0±0.22b	0.0	8.41±0.61ab	0.1	10.2±1.85a	0.1	10.06±0.72a	0.1	10.57±0.47a	0.1	***

[1] Means ± SD (n=3) with different letters differ significantly within treatments (means separation by Fisher's LSD test at P<0.05)

*** indicate significant at $p \leq 0.001$

Abbreviations: ODT (odor detection threshold), K (control, without malolactic fermentation), S (spontaneous malolactic fermentation), KI31 (co-inoculation with Lalvin 31 bacteria strain), KIA (co-inoculation with Uvaferm Alpha bacteria strain), NI31 (sequential with Lalvin 31 bacteria strain), NIA (sequential with Uvaferm Alpha bacteria strain), OAV-odor active values (calculated by dividing the mean concentration of the aromatic compound by the ODT value)

Odor detection threshold in the literature([1] Guth, 1997, [2] Ferreira et al., 2000, [3] Bartowsky and Henschke, 2004),† - in 10 - 12% water/ethanol mixture, †† - in synthetic wine (11% v/v ethanol, 7g/L glycerin, 5g/L tartaric acid, pH adjusted to 3.4 with 1M NaOH),††† - in wine

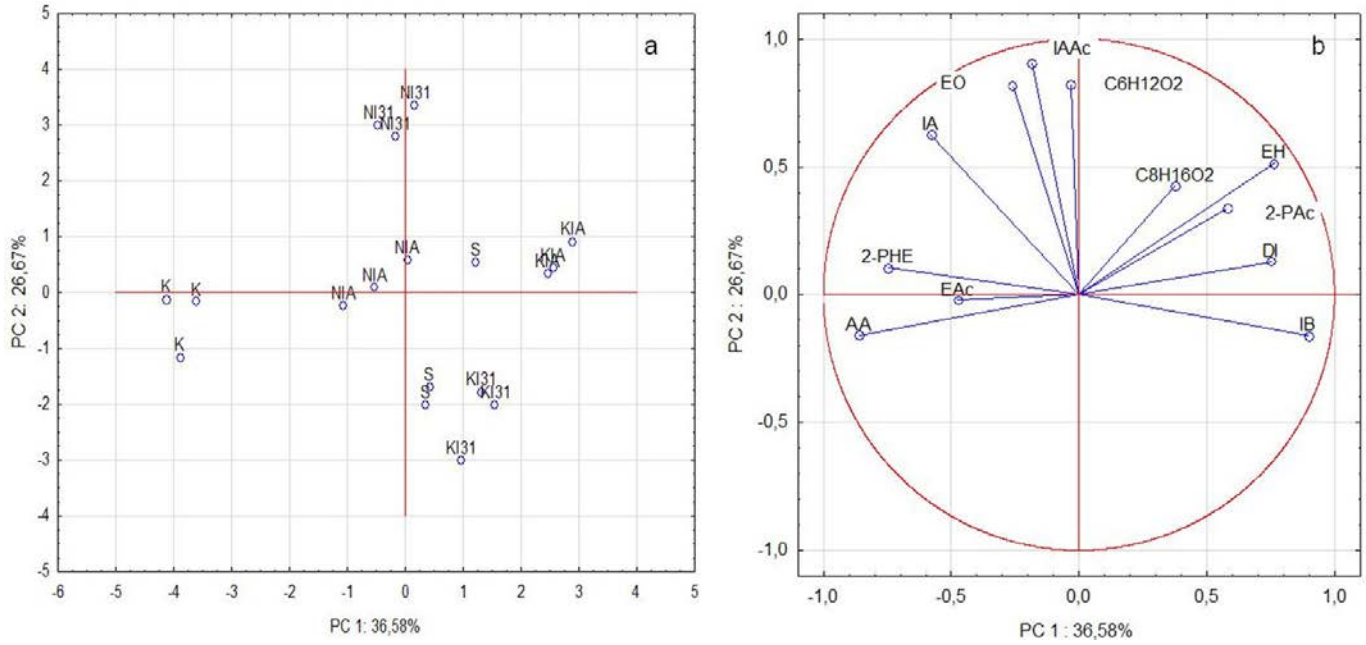

Figure 1. Principal component analysis (PCA) Teran wines: (a) samples (cases); (b) 12 volatile compounds with OAV>1(variables)

Abbreviations: K (control, without malolactic fermentation), S (spontaneous malolactic fermentation), KI31 (co-inoculation with Lalvin 31 bacteria strain), KIA (co-inoculation with Uvaferm Alpha bacteria strain), NI31(sequential with Lalvin 31 bacteria strain), NIA (sequential with Uvaferm Alpha bacteria strain.), AA (acetaldehyde), EAc (ethyl acetate), 2-PHE (2-phenylethanol), IA (isoamylalcohol), EO(ethyl octanoate), IAAc (isoamyl acetate), C6H12O2 (caproic acid), C8H16O2 (caprylic acid), EH (ethyl hexanoate), 2-Pac (2-phenyl acetate), DI (diacetyl), IB (isobutanol)

Figure 1 shows the results for the first two principal components which together explained 63.25% of the total variability (PC1 36.58%, PC2 26.67%). The first principal component (PC1) showed the separation of two groups: group 1 (S, KIA and KI31) and group 2 (NI31, NIA and K). Along the direction of the second principal component (PC2), within group 1, KIA treatment was separated from S and KI31 treatments. Furthermore, separation was also observed within group 2 where treatment K was separated from treatments NIA and NI31. The control treatment (K) located on the left side of the graph was characterized by higher concentration of acetaldehyde and 2-phenylethanol with respect to all the other treatments.

CONCLUSIONS

Malolactic fermentation significantly decreased titratable acidity and increased pH value of Teran wines. Complete degradation of malic acid was observed in all the MLF treatments regardless of inoculation time and LAB strain used. A significant decrease in the concentration of citric acid was found in all wines subjected to MLF, with a significantly higher degradation in the co-inoculation treatment where LALVIN 31 strain was used. The highest concentration of volatile acidity was found in wines in which spontaneous MLF was performed. The moment of LAB inoculation did not affect the concentration of volatile acidity, while lower concentrations were present in wines produced by the Uvaferm Alpha strain. The concentrations of higher alcohols were lower in MLF treatments. The type of MLF, inoculation time, and the LAB strain used significantly affected the concentration of ethyl esters in wines of all the MLF treatments, while a significant increase in ethyl acetate and total acetate esters was observed only in wines obtained by spontaneous MLF. The most abundant volatile fatty acids were caproic and caprylic acid with the concentrations above the sensory threshold regardless of the treatment. Significantly, lower concentrations of acetaldehyde and higher concentrations of diacetyl, acetoin, and 2,3-butanediol were found in wines of all the MLF treatments. The LAG phase and the total duration of MLF were the shortest in the co-inoculation treatments and the presence of LAB did not affect the alcoholic fermentation kinetic.

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