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Original research paper

THE EFFECTS OF MALOLACTIC FERMENTATION AND BENTONITE TREATMENT ON THE AROMA OF WINES FROM AUTOCHTHONOUS KRSTAČ AND ŽIŽAK VARIETIES

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Abstract: The aim of this study was to investigate the influence of two lactic acid bacteria strains (*Lactobacillus plantarum*, *Oenococcus oeni*) and a bentonite treatment on the content of aromatic compounds in wines of the autochthonous grape varieties Krstač and Žižak. Higher alcohols, medium-chain fatty acids (C6, C8, C10), esters and other volatile compounds were detected by GC/FID-MS analysis. The concentration of higher alcohols was lower in the wines from Krstač and Žižak in which malolactic fermentation was performed. The results of this study showed that the content of aromatic compounds depends on the lactic acid bacterial strains. *L. plantarum* yielded a higher content of total higher alcohols and esters compared to *O.oeni*. The content of total esters ranged from 30.28 to 32.70 mg/L for Krstač wines and from 19.35 to 23.21 mg/L for Žižak wines. *O. oeni* and *L. plantarum* had a statistically significant effect on the concentration of most esters. Lactic acid bacteria significantly reduced the content of ethyl butyrate, ethyl hexanoate, ethyl decanoate and isoamyl acetate. Furthermore, the content of ethyl lactate, diethyl hydroxybutanedioate, diethyl succinate and ethyl hidrogen succinate was higher in wines produced with *L. plantarum*. The addition of bentonite in increasing concentrations did not affect the concentration of the higher alcohols in Žižak wines. The lowest content of fatty acids was detected in wines produced with 200 g/hL bentonite.

Key words: *aroma compounds, L. plantarum, O. oeni, bentonite, GC/MS-FID, white wines*

INTRODUCTION

Malolactic fermentation (MLF) is an important process in winemaking in which lactic acid bacteria (LAB) conducted the bioconversion of dicarboxylic L-malic acid to monocarboxylic L-lactic acid and CO_2 (Gil-Sánchez, Bartolomé, Moreno-Arribas & Moreno-Arribas, 2019; Krieger-Weber, Heras & Suarez, 2020). MLF is a desirable process for the production of most red wines (Gil-Sánchez et al., 2019) and some white wines with high acidity. It can

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be carried out by inoculation with commercial LAB starter cultures or spontaneously with autochthonous lactic acid bacteria (Brizuela et al., 2019). The use of spontaneous malolactic fermentation could lead to a significant increase in volatile acidity in wine and the formation of undesirable compounds such as biogenic amines (Brizuela et al., 2019). Therefore, commercial starter cultures are increasingly used to improve the efficiency and reliability of MLF (Bartowsky, Costello & Chambers, 2015), shorten the duration of MLF and reduce the risk of wine spoilage (Brizuela et al., 2019).

MLF leads to a reduction in acidity with an increase in pH (Cappello, Zapparoli, Logrieco & Bartowsky, 2017; Hao et al., 2023) and improves the microbial stability of wine due to removal of malic acid as a possible carbon substrate for lactic acid bacteria (Sereni, Phan, Osborne & Tomasino, 2020). In addition, MLF affects the aroma and improves the complexity of aromas and flavors (Gil-Sánchez et al., 2019) as well as the quality and sensory characteristics of wines (Sereni et al., 2020). MLF increases fruity and buttery aromas, while reducing herbal, green and grassy aromas (Lasik-Kurdyś, Majcher & Nowak, 2018).

Depending on the type of aromatic compounds, their concentration and the physicochemical properties of wine, LAB can increase and decrease various compounds that can have a positive or negative effect on the sensory properties of wine (Sumby, Bartle, Grbin & Jiranek, 2019). Compounds that can have a negative effect on the aroma of wine in higher concentrations include diacetyl, ethyl acetate and acetoin (Sumby et al., 2019). In addition, ethylphenols (4-ethylphenol and 4-ethylguaiacol) are the main aromatic compounds associated with unpleasant odors such as horse sweat, leather and stable odor and are produced by yeasts of the genus *Brettanomyces* from the precursor hydroxycinnamic acid (Sumby et al., 2019; Virdis, Sumby, Bartowsky & Jiranek, 2021). In addition, MLF produces biogenic amines and ethyl carbamate that are harmful to consumer health and are the most important indicators of food safety and quality (Capozzi, Tufariello, De Simone & Fragasso, 2021; Emer, Marques, Colla & Reinehr, 2021). The main limiting factors that inhibit LAB are pH, high concentrations of SO_2 and ethanol, insufficient temperatures and their synergistic effect (Diez-Ozaeta, Lavilla & Amárita, 2020; Sumby et al., 2019; Virdis et al., 2021).

The influence of LAB on the aroma profile depends on the grape variety used for wine production (Jeromel, Herjavec, Orlić, Redžepović & Wondra, 2008). During MLF, lactic acid bacteria can alter aroma and flavor by mo-difying yeast-derived compounds or synthesizing volatile compounds (Knoll et al., 2012; Knoll et al., 2011; Maicas, Gil, Pardo & Ferrer, 1999). In winemaking, the commercial lactic acid bacterium *Oenococcus oeni* is the preferred species, while some strains of *Lactobacillus plantarum* are as effective as *O. oeni* in carrying out MLF (Brizuela et al., 2019). In addition, *L. plantarum* can produce β-glucosidase, esterases, decarboxylases, proteases and is considered to have a greater sensory influence on wine (Engelbrecht & du Toit, 2011; Sumby et al., 2019). Studies have shown that there are significant metabolic differences between the species *O. oeni* and *L. plantarum* (Pozo-Bayón et al., 2005). *O. oeni* is best adapted to survive harsh wine conditions such as low pH, high alcohol concentration and the presence of SO_2 (Brizuela et al., 2019; Costello, Siebert, Solomon & Bartowsky, 2013; Emer et al., 2021; Lerm et al., 2011; Sumby et al., 2019). The development of alternative efficient malolactic starter cultures is very important for scientific research (Bravo-Ferrada et al., 2013; Sumby et al., 2019).

The citric acid metabolic pathway and the amino acid metabolic pathway are two types of metabolic pathways for the biosynthesis of flavour compounds (Wang et al., 2021). Metabolic amino acids include deamination and decarboxylation reactions in lactic acid bacteria (Wang et al., 2021). Ethyl esters are considered the most important compounds that contribute to the complexity of fruit aroma and the quality of wines (Diez-Ozaeta et al., 2020). Studies have shown that esters can be synthesised or hydrolyzed by esterification or ester hydrolysis (Capozzi et al., 2021; Virdis et al., 2021). This leads to an increase or decrease in ester concentration, which influences the aroma profile of wine. The degree of contribution of LAB to the ester profile is specific to the strain used (Virdis et al. 2021). The ester content is the result of the activity of some enzymes, such as lipases, esterases and alcohol acyl transferases (Diez-Ozaeta et al, 2020). In

addition, the ester precursors fatty acids and higher alcohols are important compounds for the production of fruit-flavoured esters (Diez-Ozaeta et al., 2020; Sumby et al., 2013).

The effect of bentonite to remove proteins and turbidity was significantly more effective in wine than in must (Vela, Hernández-Orte, Castro, Ferreira & Lopez, 2017). In addition to protein removal, bentonite also influences the aroma content of wine. The effect of bentonite on aroma content depends on the initial concentration of bentonite, the initial content of aromatic compounds (Lambri, Dordoni, Silva & De Faveri, 2012) and the content and properties of proteins (Lambri, Dordoni, Silva & De Faveri, 2010).

Volatile compounds in wine consist of compounds with different properties (polarity, solubility and volatility) (Lambri, Colangelo, Dordoni, Torchio & De Faveri, 2016; Lambri et al., 2013). Aromatic compounds can be removed in two ways: indirectly (by deproteinization) or by direct adsorption of bentonite (Lambri et al., 2010).

Considering the fact that Krstač and Žižak are white autochthonous varieties that accumulate a lot of protein in dry years, which requires the use of larger amounts of bentonite from year to year to remove it, the idea of this study was to investigate the influence of different concentrations of bentonite on the aromatic complex of wine.

On the other hand, under conditions of global warming, malic acid in grape berries degrades rapidly, so the second aim of this study was to investigate the influence of malolactic fermentation on the wine aroma. It is known that the biological decomposition of malic acid produces not only the basic products - lactic acid and CO_2 - but also other compounds that are important for the aromatic character of wine.

MATERIALS AND METHODS

Chemicals and reagents

Anhydrous sodium sulphate, 4-methyl-1-pentanol, methanol and methylene chloride were used in this study. All chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA), except methylene chloride which was purchased from Merck (Darmstadt, Germany). Methylene chloride and methanol of analytical grade were used. These reagents were purified and then dried with anhydrous sodium sulphate (Madžgalj, Petrović, Tešević, Anđelković & Sofrenić, 2023b).

Plant material and winemaking

Krstač (K) and Žižak (Z) are autochthonous white grape varieties from Montenegro. Both varieties are grown in Ćemovsko polje, 13. Jul Plantaže. Krstač is high-yielding and medium-late variety. The Krstač grape cluster is medium-sized and cross-shaped, from which it takes its name. The berry is large, slightly elliptical and yellow-greenish in colour. The negative characteristic of this variety is that it is sensitive to *Botrytis cinerea* (Savić, 2016).

Žižak is a Montenegrin variety for the production of quality wines. Recently, wine producers have become increasingly interested in this promising grape variety. Žižak is medium-yielding and late variety. The cluster is medium-sized and short. The berry is small, round and greenish-yellow. In contrast to Krstač, this variety is resistant to *Botrytis cinerea*, so it can remain on the vine for a long time and accumulate more sugar (Savić, 2016).

The grapes were healthy and harvested when fully ripe. The wines were produced according to the white wine method. The grapes were crushed, destemmed and sulfited (8 g $K_2S_2O_5$/100 kg grapes). The crushed grapes were then pressed using the Sottovuoto 405 Press (Siprem International spa, Pesaro, Italy) with the addition of 3 g/hL of the enzyme Vinozym Process (Novozymes, Copenhagen, Denmark). The grape juice was clarified by static settling for 24 hours at 11 ºC and racking.

Alcoholic fermentation of Krstač and Žižak grape juices took place at 18 ºC with the addition of 25 g/hL of ICV D47 yeast, Go-Ferm and Opti-White (Lallemand Inc., Montreal, Canada). Fermentation lasted 10 days for Krstač wines and 12 days for Žižak wines.

After fermentation, the young wines were separated from the sediment by racking. In following months, wine care measures were carried out (racking and sulfiting). The results of basic chemical analysis after alcoholic fermentation are presented in Table 1.

Malolactic treatment

This experiment was carried out with the wines from Krstač and Žižak. The lactic acid bacteria (LAB) used for malolactic fermentation (MLF) were VP 41, *Oenococcus oeni* (Lallemand Inc.,

Table 1.
Chemical composition of Krstač and Žižak wines after alcoholic fermentation

Samples	Alcohol (vol %)	Titratable acidity (g/L)	Malic acid (g/L)	Malic acid after MLF (g/L)	pH	Reducing sugar (g/L)
Krstač wines	12.0	6.3	2.3	0.6	3.21	0.75
Žižak wines	12.6	5.4	1.8	0.7	3.44	0.75

Table 2.
Abbreviations for Krstač and Žižak wines

Wines	Malolactic fermentation			Bentonite		
	Control	*O. oeni*	*L. plantarum*	Control	100 g/hL	200 g/hL
Krstač wines	KCTRL	KMFOO	KMFLP	KCTRLB	KB100	KB200
Žižak wines	ZCTRL	ZMFOO	ZMFLP	ZCTRLB	ZB100	ZB200

Montreal, Canada) and ML Prime, *Lactobacillus plantarum* (Lallemand Inc., Montreal, Canada).

The samples of Krstač and Žižak wines were inoculated with lactic acid bacteria (1 g/hL *O. oeni*; 10 g/hL *L. plantarum*). The preparation of the samples with bacteria was carried out at Faculty of Agriculture of the University of Belgrade.

The experiment was divided into three treatments for Krstač and Žižak wines: CTRL – no addition of lactic bacteria; MFOO – with the addition of lactic acid bacteria *O. oeni*; MFLP - with the addition of lactic acid bacteria *L. plantarum* (Table 2). The wine samples were incubated for 21 days at a temperature of 28 ºC, after which a GC/MS-FID analysis was performed.

Bentonite treatment

A bentonite solution was prepared (10% suspension). The experiment was carried out with increasing concentrations of bentonite, Siha Puranit, active Na-Ca bentonite (PUR, Eaton, Langenlonsheim, Germany) for Krstač and Žižak wines: CTRLB – 0 g/hL bentonite; B100 – with 100 g/hL bentonite; B200 – with 200 g/hL bentonite (Table 2). Sedimentation of the bentonite was conducted for 14 days. The samples were then separated from sediment and prepared for GC/MS-FID analysis.

Liquid-liquid extraction

Sample preparation was performed by liquid-liquid extraction (Avram et al., 2014). Five milliliter of methylene chloride and twenty-five milliliter of wine were stirred at 0 ºC for one hour in an ice bath. The mixture was then placed in an ultrasonic bath for five minutes. In this way, the formation of an emulsion was prevented. After separation, the organic phase was dried with anhydrous sodium sulphate and then filtrate (Madžgalj et. al., 2023b). Subsequently, 600 microliters of extract sample were analysed using the GC/FID-MS technique.

GC/FID-MS analysis

The analysis of aromatic compounds was conducted by GC/FID-MS technique using a previously published method with some modifications (Veljović et al., 2019). GC/FID-MS analysis was performed using an Agilent 7890A gas chromatograph (GC) (Santa Clara, CA, USA). An Agilent 19091N-113 HP-INNOWax fused silica capillary column (30 m x 0.32 mm i.d., 0.25 μm film thickness) was used for the separation. The analysis was performed in split mode 3:1 with helium as carrier gas at flow rate of 1.46 mL/min. The injection volume of the sample was 1 μL for all analysis. The temperature of the GC oven was maintained at 40 ºC with an initial 5 min hold and then increased to 220 ºC at 10 ºC min^{-1} and maintained at 220 ºC for the final 4 min hold time. The GC was equipped with a mass selective detector (MSD) 5975C inert XL EI/CI MSD and a flame ionization detector connected by capillary flow technique via a two-way splitter (Madžgalj et al., 2023a). The transfer line and the ion source of the MSD were at 230 and 280 ºC, respectively. The mass selective detector operated in positive ion electron impact (EI) mode. Electron impact spectra were collected in scan mode at 70 eV in the mass range from 35 to 500 m/z (Madžgalj et al., 2023a). The temperature of the FID detector was 300 ºC (Madžgalj et al., 2023b).

The aromatic compounds were identified by comparison with reference mass spectra (Wiley and NIST databases). For the quantitative determination of aromatic compounds, an internal standard 4-methyl-1-pentanol of known concentration was used.

The (relative) percentages of identified aromatic compounds were calculated using the peak area of the gas chromatogram. The concentration of each aromatic compound was determined from the peak area of 4-methyl-1-pentanol and expressed as the relative concentration of each compound in the analysed sample.

Statistical analysis

The statistical software R (R Core Team, 2022) was used to perform statistical analysis. The experimental data were analysed using one-way analysis of variance (ANOVA) with the Tuckey's post-hoc test. ANOVA was perfomed to compare the influence of two lactic acid bacteria strains and bentonite on the aromatic compound content.

Tuckey post-hoc test was used to compare the means and the results were considered significant if the p value was < 0.05. Principal component analysis (PCA) was used to determine the differences between the wine samples based on the concentrations of aromatic compounds.

RESULTS AND DISCUSSION

Influence of *O. oeni* and *L. plantarum* on the aroma content of Krstač and Žižak wines

The content of total aroma compounds ranged from 248.16 to 266.47 mg/L for Krstač wines and from 186.10 to 245.08 mg/L for Žižak wines (Tables 3 and 4).

By using the Tukey's *post-hoc* test, a statistically significant difference in the concentration of total aroma compounds was established between wines from Žižak variety CTRL and MFLP, MFOO. The concentration of total aroma compounds was lower in the wines from Krstač and Žižak in which malolactic fermentation was conducted.

The lactic acid bacteria *O.oeni* caused a greater reduction in total aroma compounds in the wines from Krstač and Žižak compared to *L. plantarum*. *L. plantarum* is considered to have a greater sensory influence on wine because they have more enzymatic activity, especially esterases (Capozzi et al., 2021).

Higher alcohols

In wines that underwent malolactic fermentation, the content of higher alcohols was lower than in control wines (CTRL). *L. plantarum* yielded a higher content of higher alcohol than *O. oeni*. The concentration of total higher alcohols ranged from 208.33 to 225.58 mg/L for Krstač wines and from 158.52 to 213.63 mg/L for Žižak wines (Tables 3 and 4). In addition, there was a statistically significant lower content of total higher alcohols in wines from Žižak, where MLF was carried out than in control wine (CTRL). Higher alcohols are precursors of esters (Diez-Ozaeta et al., 2020; Kong, Ma, Yin, Zhao & Tao), therefore the decrease in the concentration of total higher alcohols could be explained through the synthesis of esters and an increase in their concentrations.

The content of 2-phenylethyl alcohol was higher in ZMFLP wine than in control wine (ZCTRL), which is consistent with the results in the literature (Knoll et al., 2012). Sumby et al. (2019) reported that the concentration of 2-phenylethyl alcohol can increase or decrease during MLF. ZMFLP had a statistically significant higher content of all higher alcohols compared to ZMFOO. Some authors reported that MLF affected the content of higher alcohols (Knoll et al., 2012; Pozo-Bayón et al., 2005), while others found no significant changes (de Revel, Martin, Pripis-Nicolau, Lonvaud-Funel & Bertrand, 1999).

Fatty acids

Medium-chain fatty acids (hexanoic, octanoic, and decanoic acids) were detected in the GC/FID-MS analysis. The content of fatty acids ranged from 5.11 to 6.46 mg/L in the wines from Krstač and from 4.11 to 6.37 mg/L in wines from Žižak (Tables 3 and 4). Krstač and Žižak wines, produced with malolactic fermentation, had a statistically significant lower content of total fatty acids. Since fatty acids are precursors of esters (Diez-Ozaeta et al., 2020; Kong et al., 2021), the decrease in the total fatty acid concentration could be explained by an increase in the concentration of esters. Octanoic (3.84 mg/L, 3.61 mg/L) and hexanoic acid (2.17 mg/L, 2.10 mg/L) had the highest concentrations in the wines from

Krstač and Žižak. Using the ANOVA test, a statistically significant difference was found in the content of all acids between ZMFOO and the control wine sample (ZCTRL).

In addition, a statistically significant difference in the concentration of octanoic (K) and decanoic acid (K and Z) was found between MFLP (*L. plantarum*) and MFOO *(O. oeni)* in the wine samples.

Previous studies confirmed that the octanoic and decanoic acid content depends on the LAB strain (Pozo-Bayón et al., 2005). In contrast, de Revel et al. (1999) re-ported that the content of fatty acids did not change under the influence of LAB.

Esters

Esters are very important for fruit aroma and the quality of wine (Cappello et al., 2017; Diez-Ozaeta et al., 2020; Wang et al., 2021). In this study, the total ester content was between 30.28 and 32.70 mg/L in Krstač wines, and between 19.35 and 23.21 mg/L in Žižak wines.

During MLF, *Lactobacillus plantarum* influenced the synthesis of esters more than *O.oeni*. This can be explained by a lower ester hydrolysis activity of *L. plantarum* compared to *O.oeni* (Maicas et al., 1999). *L plantarum* has a significant source of esterases and due to their enzymatic activity can modulate wine volatile profiles more efficiently than *O. oeni* (Cappello et al., 2017). How many esters are synthesized strongly depends on LAB strains during MLF (Gammacurta et al., 2018; Knoll et al., 2012; Knoll et al., 2011; Lasik-Kurdyś et al., 2018; Maicas et al., 1999; Sumby et al., 2019; Ugliano & Moio, 2005; Virdis et al., 2021).

In this experiment, *O.oeni* and *L. plantarum* had a statistically significant effect on the concentration of most esters. MLF decreased the content of total higher alcohols and total fatty acids and increased the content of total esters.

Esters can be esterified or hydrolysed by esterases of LAB during MLF (Diez-Ozaeta et al., 2020; Virdis et al., 2021), leading to an increase or decrease in the concentrations of aromatic compounds (Cappello et al., 2017; Sumby et al., 2013; Sumby et al. 2019). Esterase is an enzyme that catalyzes the synthesis and hydrolysis of esters during winemaking (Kong et al., 2021).

In addition, ester precursors (higher alcohols and fatty acids) significantly influence fruit ester production (Diez-Ozaeta et al., 2020; Kong et al., 2021).

Esters can consist of fatty acids (ethyl hexanoate, ethyl octanoate, ethyl decanoate), organic acids (ethyl lactate, diethyl succinate) and higher alcohols (isoamyl acetate, 2-phenylethyl acetate) (Inês & Falco, 2018).

These aromatic compounds are produced during alcoholic and malolactic fermentation (Inês & Falco, 2018). The levels of ethyl butyrate (K and Z), ethyl hexanoate (Z), ethyl decanoate (K) and isoamyl acetate (K, Z) decreased, which is consistent with literature data (Herjavec, Tupajić & Majdak, 2001; Jeromel et al., 2008). Isoamyl acetate gives pleasant fruity aromas and is formed from isoamyl alcohol and acetic acid, intermediate metabolites of alcoholic and malolactic fermentation (Lasik-Kurdyś et al., 2018). *O. oeni* caused a greater reduction in the levels of ethyl butyrate and ethyl octanoate than *L. plantarum*.

This can be explained by the greater ester hydrolytic activity of *O. oeni* bacteria (Inês & Falco, 2018; Sumby et al., 2013). The concentrations of ethyl octanoate in Krstač wines and ethyl decanoate in Žižak wines were in the trace range.

Krstač wines had a significantly higher concentration of ethyl lactate (16.68 mg/L) compared to Žižak wines (3.38 mg/L). The content of ethyl lactate was higher in ZMFLP wines than in ZCTRL, which is consistent with the literature (Jeromel et al., 2008; Pozo-Bayón et al., 2005). Ethyl lactate is synthesized during MLF by esterification of ethanol and lactic acid.

The amount of ethyl lactate depends on the ethanol concentration (Knoll et al., 2011), pH value and the initial concentration of malic acid (Pozo-Bayón et al., 2005). *Lactobacillus plantarum* influenced the higher synthesis of ethyl lactate in Žižak wines, compared to *O. oeni*. *L. plantarum* is a heterofermentative bacteria and it is recommended for wines which have a higher pH (Gammacurta et al., 2018) such as Žižak wine (pH=3.44). *L. plantarum* has increased tolerance to higher pH, and concentration of SO_2 and ethanol. Lasik-Kurdyś et al. (2018) reported that ethyl lactate is one of the most characteristic compounds

formed during MLF. Diethyl succinate is a volatile compound which contributes to the aroma of wine. Succinic acid is a by-product of microbial α-keto-glutarate metabolism (Lasik-Kurdyś et al., 2018).

Diethyl succinate is obtained by esterification of succinic acid (Lasik-Kurdyś et al., 2018). In this study, the content of diethyl succinate ranged from 2.23 to 2.45 mg/L for Krstač wines and from 2.73 to 3.41 mg/L for Žižak wines, which is consistent with literature data (Lasik-Kurdyś et al., 2018). Malolactic fermentation increased the content of diethyl succinate in all Krstač and Žižak wines. Two lactones, γ-butyrolactone and γ-ethoxy butyrolactone, were detected in Krstač and Žižak wines. Malolactic fermentation influenced the increase of γ-butyrolactone content in the wines of Krstač and Žižak, which is consistent with the literature (Celik, Cabarouglu & Krieger-Weber, 2018). Butyralactone is a by-product of α-ketoglutarate metabolism in lactic acid bacteria and gives the wine a sweet and caramel aroma (Celik et al., 2018).

Table 3.
The content of aromatic compounds in Krstač wines after malolactic fermentation with lactic acid bacteria *O. oeni* and *L. plantarum*

	Samples (mg/L)			F	p
Compounds	**KCTRL**	**KMFLP**	**KMFOO**		
1-Hexanol	2.82 ± 0.28	2.70 ± 0.17	2.77 ± 0.30	0.17	0.8469
Isobutyl alcohol	18.19 ± 0.85^{a}	15.62 ± 0.82^{b}	14.66 ± 0.94^{b}	13.24	0.0063
Isoamyl alcohol	157.82 ± 13.50	148.29 ± 4.10	142.69 ± 7.40	2.07	0.2073
4-Methyl-1-pentanol	8.13	8.13	8.13		
3-(Methylthio)-1- propanol	0.48 ± 0.03	0.48 ± 0.05	0.49 ± 0.03	0.07	0.9287
2-Phenylethyl alcohol	38.14 ± 1.37	37.83 ± 0.63	39.59 ± 0.51	3.15	0.1161
Total higher alcohols	**225.58** ±14.28	**213.05** ± 2.85	**208.33** ± 9.12	2.423	0.169
Hexanoic acid	2.17 ± 0.02	2.07 ± 0.02	2.12 ± 0.12	1.48	0.2999
Octanoic acid	3.84 ± 0.18^{a}	2.75 ± 0.17^{b}	3.75 ± 0.22^{a}	29.62	0.0008
Decanoic acid	0.45 ± 0.01^{a}	0.29 ± 0.03^{c}	0.37 ± 0.01^{b}	43.64	0.0003
Total fatty acids	**6.46** ±0.19^{a}	**5.11** ±0.22^{b}	**6.24** ±0.19^{a}	39.14	0.0003
Ethyl butyrate	2.80 ± 0.04^{a}	2.37 ± 0.23^{b}	2.28 ± 0.25^{b}	5.90	0.0383
Ethyl hexanoate	0.15 ± 0.02	0.11 ± 0.04	0.10 ± 0.02	2.12	0.2012
Ethyl (S)-(-) lactate	16.68 ± 1.22	16.87 ± 1.90	16.75 ± 3.38	0.01	0.9950
Ethyl octanoate	t	t	t		
Ethyl 3-hydroxybutyrate	0.19 ± 0.01	0.21 ± 0.01	0.20 ± 0.00	2.42	0.1696
Ethyl decanoate	0.16 ± 0.00^{a}	0.14 ± 0.00^{b}	0.13 ± 0.01^{b}	24.71	0.0013
Diethyl succinate	2.23 ± 0.06^{b}	2.45 ± 0.18^{a}	2.38 ± 0.01^{ab}	3.21	0.1129
Ethyl 4- hydroxybutanoate	0.52 ± 0.02^{a}	0.50 ± 0.02^{a}	0.38 ± 0.04^{b}	23.56	0.0014
Diethyl hydroxybutanedioate	0.75 ± 0.15	0.86 ± 0.12	0.84 ± 0.25	0.30	0.7484
Diethyl 2-hydroxy-3-methylsuccinate	0.27 ± 0.03	0.29 ± 0.03	0.30 ± 0.05	0.50	0.6284
Ethyl-2-hydroxy-3-phenyl propaonate	0.15 ± 0.02	0.16 ± 0.01	0.16 ± 0.00	0.58	0.5867
Ethyl hydrogen succinate	7.17 ± 0.06^{c}	8.71 ± 0.20^{a}	6.66 ± 0.32^{b}	69.60	0.0001
Isoamyl acetate	0.18 ± 0.01^{a}	0.03 ± 0.03^{c}	0.10 ± 0.02^{b}	33.73	0.0005
2-Phenylethyl acetate	0.12 ± 0.01	ND	ND		
Total esters	**31.37** ± 1.25	**32.70** ± 1.96	**30.28** ± 2.99	0.929	0.445
2H Pyran-2.6(5H)-dione	0.33 ± 0.03	0.39 ± 0.04	0.38 ± 0.05	2.06	0.2088
Cis 4-hydroxymethyl 2-methyl 1,3-dioxolane	t	0.33 ±0.03^{a}	0.10 ± 0.08^{b}	20.46	0.0106
γ-Butyrolactone	2.59 ± 0.12	2.69 ± 0.14	2.83 ± 0.12	2.72	0.1440
γ-Ethoxy butyrolactone	0.14	t	t		
Total other compounds	**3.06** ± 0.11^{b}	**3.41** ± 0.12^{a}	**3.31** ± 0.12^{a}	14.31	0.0052
Total aromatic compounds	**266.47** ± 13.07	**254.27** ± 3.07	**248.16** ± 6.09	3.492	0.0987

All experimental data are expressed as the means (n=3) ± standard deviation;
$^{a, b, c}$ Means followed by different letters within the same row are significantly different as $p < 0.05$;
Krstač (K) wines: CTRL-no addition of lactic acid bacteria, MFOO-with addition of lactic acid bacteria O. oeni, MFLP-with addition of lactic acid bacteria L. plantarum. t-trace (below limit of quantification = 0.01 mg/L); ND-not detected

Table 4.
The content of aromatic compounds in Žižak wines after malolactic fermentation with lactic acid bacteria *O. oeni* and *L. plantarum*

	Sample (mg/L)			F	p
Compounds	**ZCTRL**	**ZMFLP**	**ZMFOO**		
1-Hexanol	1.66 ± 0.07[a]	1.67 ± 0.09[a]	1.30 ± 0.05[b]	26.14	0.0011
Isobutyl alcohol	14.60 ± 0.82[a]	12.61 ± 0.50[b]	9.30 ± 0.35[c]	61.87	0.0001
Isoamyl alcohol	152.32 ± 5.10[a]	141.75 ± 3.10[b]	108.57 ± 5.20[c]	75.06	0.0001
4-Methyl-1-pentanol	8.13	8.13	8.13		
3-(Methylthio)-1- propanol	0.34 ± 0.03[a]	0.36 ± 0.01[a]	0.25 ± 0.03[b]	14.69	0.0049
2-Phenylethyl alcohol	36.58 ± 0.35[b]	38.98 ± 0.69[a]	30.97 ± 0.18[c]	238.42	0.0000
Total higher alcohols	**213.63** ± 5.17[a]	**203.50** ± 2.35[b]	**158.52** ± 4.86[c]	138.49	0.0000
Hexanoic acid	2.10 ± 0.01[a]	2.03 ± 0.07[a]	1.52 ± 0.02[b]	176.19	0.0000
Octanoic acid	3.61 ± 0.07[a]	2.49 ±0.02[b]	2.21 ± 0.40[b]	30.22	0.0007
Decanoic acid	0.66 ± 0.04[a]	0.28 ± 0.01[c]	0.38 ± 0.01[b]	168.29	0.0000
Total fatty acids	**6.37** ± 0.02[a]	**4.80** ± 0.06[b]	**4.11** ± 0.41[c]	70.147	0.0000
Ethyl butyrate	2.10 ± 0.19[a]	1.54 ± 0.12[b]	1.41 ± 0.04[b]	23.72	0.0014
Ethyl hexanoate	0.29 ± 0.02[a]	0.17 ± 0.01[b]	0.19 ± 0.01[b]	54.79	0.0001
Ethyl (S)-(-) lactate	3.38 ± 1.09	3.59 ± 2.21	2.65 ± 2.32	0.19	0.8311
Ethyl octanoate	0.12 ± 0.01	0.13 ± 0.01	t	1.95	0.2353
Ethyl 3-hydroxybutyrate	0.10 ± 0.00	0.10 ± 0.01	t	0.00	1.0000
Ethyl decanoate	t	t	t		
Diethyl succinate	2.73 ± 0.09[c]	3.41 ± 0.11[a]	2.95 ± 0.21[b]	17.14	0.0033
Ethyl 4- hydroxybutanoate	0.43 ± 0.09[c]	0.45 ± 0.07[b]	0.56 ± 0.15[a]	152.34	0.0000
Diethyl hydroxybutanedioate	2.16 ± 0.15[b]	3.02 ± 0.40[a]	2.14 ± 0.12[b]	11.58	0.0087
Diethyl 2-hydroxy-3-methylsuccinate	0.52 ± 0.02[b]	0.61 ± 0.03[a]	0.35 ± 0.02[c]	99.49	0.0000
Ethyl-2-hydroxy-3-phenyl propaonate	0.20 ± 0.00[ab]	0.22 ± 0.03[a]	0.17 ± 0.01[b]	6.94	0.0275
Ethyl hydrogen succinate	8.91 ± 0.43[b]	9.89 ± 0.15[a]	8.69 ± 0.27[b]	13.22	0.0063
Isoamyl acetate	0.25 ± 0.03[a]	0.08 ± 0.01[b]	0.11 ± 0.04[b]	30.99	0.0007
2-Phenylethyl acetate	t	t	0.13 ± 0.01		
Total esters	**21.19** ± 0.97	**23.21** ± 1.99	**19.35** ± 1.98	4.054	0.077
2H Pyran-2.6(5H)-dione	0.42 ± 0.04[b]	0.60 ± 0.02[a]	0.48 ± 0.03[b]	30.00	0.0008
Cis 4-hydroxymethyl 2-methyl 1,3-dioxolane	0.18 ± 0.05[a]	t	0.80 ± 0.06[b]	174.12	0.0002
γ-Butyrolactone	3.29 ± 0.21[b]	3.65 ± 0.07[a]	2.84 ± 0.08[c]	27.39	0.0010
γ-Ethoxy butyrolactone	t	t	t		
Total other compounds	**3.89** ± 0.14[b]	**4.25** ± 0.09[a]	**4.12** ± 0.07[a]	8.98	0.0157
Total aromatic compounds	**245.08** ± 4.12[a]	**235.76** ± 2.05[b]	**186.10** ± 4.97[c]	197.90	0.0000

All experimental data are expressed as the means (n=3) ± standard deviation;
[a, b, c] Means followed by different letters within the same row are significantly different as $p < 0.05$;
Žižak (Z) wines: CTRL-no addition of lactic acid bacteria, MFOO-with addition of lactic acid bacteria O. oeni, MFLP-with addition of lactic acid bacteria L. plantarum. t-trace (below limit of quantification = 0.01 mg/L); ND-not detected

Lactobacillus plantarum synthesized a higher content of γ-butyrolactone (ZMFLP) compared to *O. oeni* and the control wine (ZCTRL). The concentration of γ-butyrolactone depends on the grape variety (the precursor concentration in grapes) and the enzymatic activity of the LAB strain.

The contents of diethyl succinate (KMFLP, Z), ethyl 4-hydroxybutanoate (Z), diethyl hydroxybutanedioate (ZMFLP), ethyl hydrogen succinate (KMFLP, ZMFLP) were higher than in control wine (CTRL). These compounds are mainly synthesized by yeasts, while lactic acid bacteria synthesize them in a smaller amount. Malic acid is the precursor of diethyl hydroxybutanedioate. In addition, 4-hydroxy-butanoate is produced from glutamic acid via 4-hydroxybutanoic acid (Madžgalj et al., 2023a). Compared to *O. oeni*, *L. plantarum* had statistically significantly higher contents of diethyl succinate (ZMFLP), diethyl hydroxybutanedioate (ZMFLP), diethyl 2-hydroxy-3-methylsuccinate (ZMFLP), and ethyl hydrogen succinate (KMFLP, ZMFLP).

The increase in the content of these compounds can be explained by the greater enzymatic activity of *L. plantarum* depending on the physico-chemical properties of Žižak wines (higher pH, higher ethanol content) (Sumby et al., 2019). Ethyl lactate and diethyl succinate give the wines buttery and creamy aromas (Sereni et al., 2020).

Isoamyl acetate contributes to a pleasant fruity aroma (banana, pear), 2-phenylethyl acetate (rose), ethyl butyrate (floral, fruity), ethyl hexanoate (apple, banana), ethyl decanoate (floral), and ethyl octanoate (pine-apple, pear) (Lambrechts & Pretorius, 2000; Selli, Canbas, Cabaroglu, Erten & Günata, 2006).

Influence of bentonite treatment on aroma compounds in Krstač and Žižak wines

Tables 5 and 6 show the content of aroma compounds in the wines from Krstač and Žižak using different bentonite concentrations. Higher alcohols, fatty acids, esters and other volatile compounds were detected in the GC/FID-MS analysis.

Table 5.
The content of aromatic compounds in Krstač wines, with increasing concentrations of bentonite treated (0, 100, 200 g/hL)

	Samples (mg/L)			F	p
Compounds	**KCTRLB**	**KB100**	**K B200**		
1-Hexanol	2.32 ± 0.12	2.65 ± 0.51	2.44 ± 0.31	0.68	0.5435
Isobutyl alcohol	13.52 ± 0.38^{b}	14.58 ± 0.58^{ab}	15.12 ± 0.73^{a}	5.88	0.0386
Isoamyl alcohol	125.11± 6.00^{b}	142.23 ± 5.90^{a}	142.23 ± 3.50^{a}	10.71	0.0105
4-Methyl-1-pentanol	8.13	8.13	8.13		
3-(Methylthio)-1- propanol	0.41 ± 0.03	0.42 ± 0.06	0.44 ± 0.05	0.30	0.7499
2-Phenylethyl alcohol	32.31 ± 0.52^{b}	36.67 ± 1.54^{a}	35.94 ± 0.66^{a}	15.91	0.0040
Total higher alcohols	**181.80** ± 5.84^{b}	**204.68** ± 4.29^{a}	**204.30** ±3.47^{a}	23.94	0.0014
Hexanoic acid	1.86 ± 0.02^{b}	2.01 ± 0.04^{a}	1.90 ± 0.03^{b}	20.11	0.0022
Octanoic acid	3.81 ± 0.15	3.56 ± 0.39	3.39 ± 0.21	1.85	0.2370
Decanoic acid	0.69 ± 0.08	0.59 ± 0.10	0.72 ± 0.07	2.01	0.2150
Total fatty acids	**6.36** ± 0.20	**6.16** ± 0.50	**6.01** ± 0.26	0.763	0.5069
Ethyl butyrate	2.30 ± 0.40	2.21 ± 0.29	2.02 ± 0.39	0.47	0.6486
Ethyl hexanoate	0.32 ± 0.05	0.33 ± 0.05	0.28 ± 0.01	1.16	0.3756
Ethyl (S)-(-) lactate	13.20 ± 0.96	14.81 ± 1.62	14.63 ± 1.92	0.97	0.4325
Ethyl octanoate	t	0.16 ± 0.05	0.18 ± 0.02	0.42	0.5521
Ethyl 3-hydroxybutyrate	0.16 ± 0.06	0.16 ± 0.04	0.15 ± 0.06	0.03	0.9693
Ethyl decanoate	0.13 ± 0.07	0.15 ± 0.05	0.14 ± 0.03	0.11	0.8986
Diethyl succinate	1.87 ± 0.15^{a}	1.68 ± 0.14^{a}	1.32 ± 0.18^{b}	9.13	0.0151
Ethyl 4- hydroxybutanoate	0.40 ± 0.11	0.42 ± 0.04	0.44 ± 0.07	0.19	0.8328
Diethyl hydroxybutanedioate	0.82 ± 0.21	0.61 ± 0.32	0.59 ± 0.14	0.88	0.4616
Diethyl 2-hydroxy-3-methylsuccinate	0.23 ± 0.16	0.20 ± 0.11	0.18 ± 0.05	0.15	0.8658
Ethyl-2-hydroxy-3-phenyl propaonate	0.15 ± 0.01	0.12 ± 0.01	0.15 ± 0.02	3.39	0.1036
Ethyl hydrogen succinate	5.90 ± 0.59	6.30 ± 0.24	5.90 ± 0.36	0.91	0.4532
Isoamyl acetate	0.50 ± 0.06^{a}	0.44 ± 0.02^{a}	0.34 ± 0.03^{b}	10.65	0.0106
2-Phenylethyl acetate	0.12 ± 0.01	0.11 ± 0.00	0.11 ± 0.01	3.53	0.0970
Total esters	**26.10** ± 1.421	**27.70** ± 1.97	**26.43** ± 1.56	0.772	0.5029
2H Pyran-2.6(5H)-dione	0.30 ± 0.03^{a}	0.19 ± 0.07^{b}	0.15 ± 0.06^{b}	6.15	0.0352
Cis 4-hydroxymethyl 2-methyl 1,3-dioxolane	ND	ND	t		
γ-Butyrolactone	2.27 ± 0.24	2.53 ± 0.04	2.43 ± 0.48	0.53	0.6135
γ-Ethoxy butyrolactone	t	t	t		
Total other compounds	**2.57** ± 0.23	**2.72** ± 0.03	**2.58** ± 0.43	0.262	0.778
Total aromatic compounds	**216.83** ±7.14^{b}	**241.26** ± 1.81^{a}	**239.32** ± 4.49^{a}	22.30	0.0017

All experimental data are expressed as the means (n=3) ± standard deviation;
$^{a, b, c}$ Means followed by different letters within the same row are significantly different as $p < 0.05$;
Krstač (K) wines: CTRLB-no addition of bentonite, B100-with addition of 100 g/hL bentonite, B200-with addition of 200 g/hL bentonite. t-trace (below limit of quantification = 0.01 mg/L); ND-not detected

Table 6.
The content of aromatic compounds in Žižak wines, with increasing concentrations of bentonite treated (0, 100, 200 g/hL

	Samples (mg/L)			F	p
Compounds	**ZCTRLB**	**ZB100**	**ZB200**		
1-Hexanol	1.70 ± 0.15	1.78 ± 0.18	1.58 ± 0.28	0.69	0.5361
Isobutyl alcohol	13.92 ± 0.41	13.95 ± 0.23	14.29 ± 0.42	0.96	0.4357
Isoamyl alcohol	148.46 ± 10.30	148.43± 8.70	155.15 ± 9.70	0.49	0.6358
4-Methyl-1-pentanol	8.13	8.13	8.13		
3-(Methylthio)-1- propanol	0.33 ± 0.11	0.31 ± 0.05	0.30 ± 0.06	0.12	0.8922
2-Phenylethyl alcohol	36.78 ± 0.26	35.86 ± 0.18	36.36 ± 0.61	4.10	0.0756
Total higher alcohols	**209.32** ± 9.70	**208.46** ± 8.75	**215.81**± 10.01	0.536	0.6105
Hexanoic acid	2.00 ± 0.01	1.98 ± 0.05	2.03 ± 0.06	0.95	0.4381
Octanoic acid	4.31 ± 0.25	4.24 ± 0.26	3.94 ± 0.34	1.41	0.3138
Decanoic acid	0.99 ± 0.07	0.88 ± 0.04	0.84 ± 0.09	3.64	0.0920
Total fatty acids	**7.30** ± 0.27	**7.10** ± 0.24	**6.81** ± 0.26	2.751	0.1419
Ethyl butyrate	1.72 ± 0.26[a]	1.66 ± 0.34[a]	1.09 ± 0.11[b]	5.67	0.0414
Ethyl hexanoate	0.45 ± 0.03[a]	0.39 ± 0.04[a]	0.30 ± 0.02[b]	17.65	0.0031
Ethyl (S)-(-) lactate	3.11 ± 0.81	2.98 ± 2.13	3.07 ± 1.57	0.01	0.9948
Ethyl octanoate	0.34 ± 0.08	0.26 ± 0.07	0.22 ± 0.04	2.64	0.1502
Ethyl 3-hydroxybutyrate	t	t	t		
Ethyl decanoate	t	t	t		
Diethyl succinate	2.63 ± 0.16	2.25 ± 0.44	1.89 ± 0.29	4.04	0.0774
Ethyl 4- hydroxybutanoate	0.36 ± 0.03	0.35 ± 0.08	0.35 ± 0.04	0.03	0.9671
Diethyl hydroxybutanedioate	2.12 ± 0.57	2.03 ± 0.36	1.86 ± 0.13	0.33	0.7298
Diethyl 2-hydroxy-3-methylsuccinate	0.47 ± 0.16	0.44 ± 0.07	0.38 ± 0.11	0.44	0.6629
Ethyl-2-hydroxy-3-phenyl propaonate	0.17 ± 0.00	0.18 ± 0.00	0.16 ± 0.03	1.22	0.3602
Ethyl hydrogen succinate	7.38 ± 0.84	7.50 ± 0.54	7.97 ± 0.61	0.64	0.5605
Isoamyl acetate	0.29 ± 0.03	0.30 ± 0.12	0.21 ± 0.05	1.28	0.3432
2-Phenylethyl acetate	t	t	t		
Total esters	**19.04** ± 1.01	**18.34** ± 3.31	**17.50** ± 2.16	0.3219	0.7365
2H Pyran-2.6(5H)-dione	0.29 ± 0.03	0.40 ± 0.04	0.23 ± 0.12	4.16	0.0734
Cis 4-hydroxymethyl 2-methyl 1,3-dioxolane	ND	ND	ND		
γ-Butyrolactone	3.38 ± 0.45	3.19 ± 0.06	3.12 ± 0.18	0.68	0.5433
γ-Ethoxy butyrolactone	t	t	t		
Total other compounds	**3.67** ± 0.47	**3.59** ± 0.10	**3.35** ± 0.21	0.891	0.4584
Total aromatic compounds	**239.33** ± 10.25	**237.49** ± 6.40	**243.47** ±12.03	0.290	0.7580

All experimental data are expressed as the means (n=3) ± standard deviation;
[a, b, c] Means followed by different letters within the same row are significantly different as $p < 0.05$;
Žižak (Z) wines: CTRLB-no addition of bentonite, B100-with addition of 100 g/hL bentonite, B200-with addition of 200 g/hL bentonite. t-trace (below limit of quantification = 0.01 mg/L); ND-not detected

The concentration of total aromatic compounds ranged from 216.83 to 241.26 mg/L for Krstač wines and from 237.49 to 243.47 mg/L for Žižak wines. The addition of increasing concentrations of bentonite did not affect the content of higher alcohols in Žižak wines from 181.80 to 204.68 mg/L for Krstač wines and from 208.46 to 215.81 mg/L for Žižak wines.

Medium-chain fatty acids (hexanoic, octanoic and decanoic acids) were detected in the wines from Krstač and Žižak. Bentonite addition lowered the content of total fatty acids in all Krstač and Žižak wines. The lowest content of total fatty acids was found in B200 wines. In addition, most treatments with bentonite lowered the content of octanoic and decanoic acids.

In the production of white wines, bentonite is used as a technique for removing the proteins, which are the source of haziness in wine (Lambri et al., 2013). Bentonite is negatively charged and the mechanism of action is based on the principles of electrostatic interaction with positively charged proteins and their precipitation. Bentonite reacts not only with proteins but also with other compounds such as

esters, fatty acids, due to mutual flocculation with positively charged colloids and adsorption (Lambri et al., 2010). As a result, bentonite influences the removal of certain compounds (Lambri et al., 2010). Vincenzi et al. (2015) reported a significant effect on the removal of decanoic acid.

The total ester content was between 26.10 and 27.70 mg/l in Krstač wines and between 17.50 and 19.04 mg/L in Žižak wines. As a result of the ANOVA test, a statistically significant lower content of diethyl succinate and isoamyl acetate was found in Krstač wine (K B200) compared to the control wine (KCTRL).

In Žižak wines, the addition of 200 g/hL bentonite caused a statistically significant decrease in the content of ethyl butyrate and ethyl hexanoate. These compounds are hydrophobic molecules and bentonite has a high capacity for their adsorption (Lambri et al., 2013). The presence of protein in bentonite-treated wine tends to increase the loss of esters with long carbon chains (Vincenzi, Panighel, Gazzola, Flamini & Curioni, 2015).

The decrease in the content of ethyl esters of fatty acids could be explained by a higher protein content in Žižak wines compared to Krstač wines. These compounds have hydrophobic properties and are removed by deproteinization in the presence of proteins (Lambri et al., 2010), as only a few aromatic compounds are directly adsorbed by bentonite (Lambri et al., 2010; Vincenzi et al., 2015).

PCA analysis

Principal component analysis (PCA) was conducted for the differentiation of Krstač and Žižak wines produced with the addition of different lactic acid bacteria (Fig. 1a). The first two PCs could explain 78.3% of the variability. PC1 accounted for 56% and PC2 for 22.3% of the total variance. All wines obtained in this experiment are clearly divided into 5 groups. Figure 1a shows that Žižak wines are on the right side of the PCA plot and are clearly separated from Krstač wines.

This difference between Krstač and Žižak wines is probably the result of the different contents of amino acids in grapes.

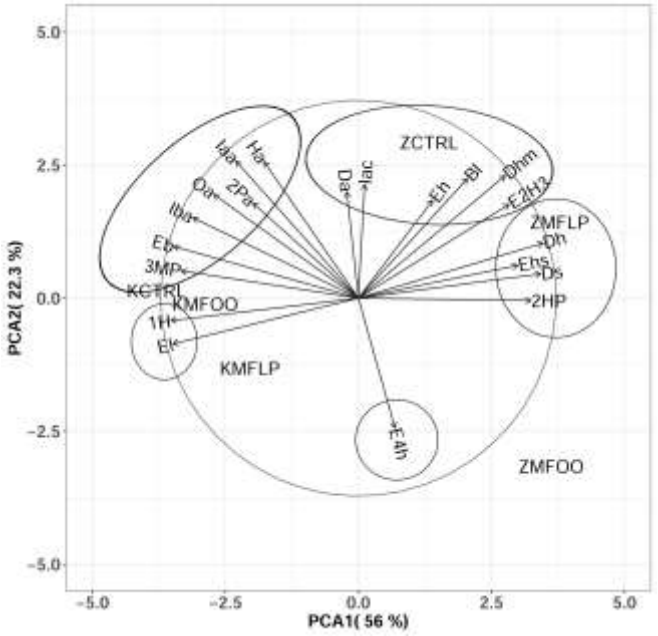
The content of amino acids is a criterion for classifying grapes and wines. Amino acid content depends on the grape variety, climatic conditions, grape cultivation and the vinification process (Scutaraşu, Luchian, Cioroiu, Trincă & Cotea, 2022). Amino acids are the main precursors of esters, fatty acids and higher alcohols, which are produced by the amino acid metabolic pathway (Scutaraşu et al., 2022).

Based on PCA analysis, medium-chain fatty acid (decanoic acid), esters (ethyl hexanoate and isoamyl acetate) and γ-butyrolactone were characterized for ZCTRL wine. Medium-chain fatty acids have an inhibitory effect on the growth of lactic acid bacteria, while malolactic fermentation reduces the concentrations of ethyl hexanoate and isoamyl acetate. ZMFLP was rich in diethyl succinate, butyrolactone, ethyl hydrogen succinate, diethyl hydroxylbutanedioate, etc. KCTRL was rich in higher alcohols. The precursors of isobutyl and isoamyl alcohols are valin, leucin and isoleucin (Scutaraşu et al., 2022). The main compounds characterizing malolactic fermentation were ethyl lactate (KMFLP) and diethyl succinate (ZMFLP).

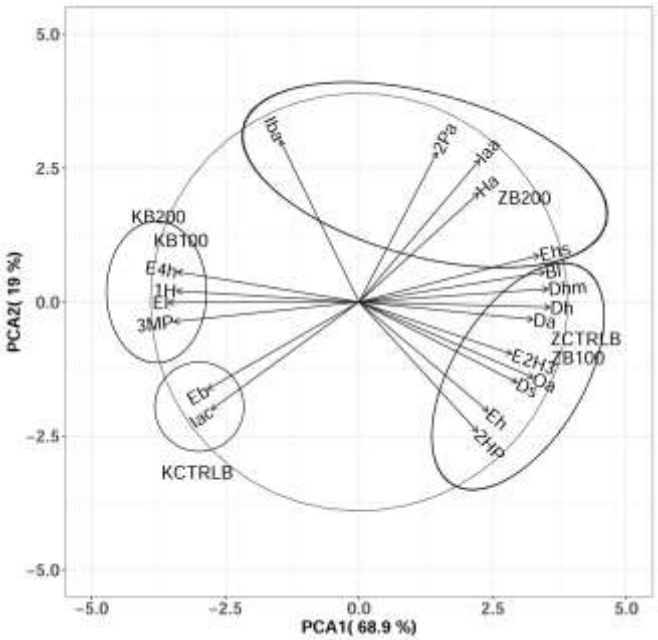
The PCA (Fig. 1a) shows that there is a greater difference between Žižak wines inoculated with *L. plantarum* (ZMFLP) and *O. oeni* (ZMFOO) than Krstač wine. This variability can be explained by different enzymatic activity of LAB and their different influence depen-ding on the physico-chemical conditions of wine such as pH, concentration of SO_2 and ethanol (Capozzi et al., 2021). *L. plantarum* has a higher enzymatic activity (Cappello et al., 2017) and higher tolerance to high pH (3.44 of Žižak wine), content of SO_2 and ethanol compared to *O.oeni* (Capozzi et al., 2021). *O. oeni* has a greater tolerance to lower pH and temperature ranges (Pannella et al., 2020).

In the wine samples from Krstač and Žižak with bentonite (Fig. 1b), the first principal component (PC1) accounted for 68.9% of the total variance, and the second principal component (PC2) for 19%. Figure 1b shows that all Krstač and Žižak wines were divided into 4 groups. Žižak wines were clearly separated on the right side of the PCA graph, while Krstač wines were on the left side. The difference between wines of the Krstač and Žižak varieties can be explained by the different chemical composition of the grapes, different concentrations of alcohols, fatty acids and esters precursors in the grape, the composition of wine, etc.

Žižak ZB200 was rich in higher alcohols (isoamyl, isobutyl, 2-phenylethyl). ZCTRLB and ZB100 were characterized by medium-chain fatty acids (octanoic and decanoic), diethyl succinate, diethyl hydroxybutanedioate, ethyl hexanoate and γ-butyrolactone. KCTRLB was rich in ethyl butyrate and isoamyl acetate, while KB100 and KB200 were characterized by ethyl lactate, 1-hexanol and 3-(methylthio)-1-propanol.



(a)



(b)

Figure 1. Results of the PCA analysis: a) Krstač and Žižak wines (malolactic fermentation), b) Krstač and Žižak wines (bentonite treatment); 1H: 1-hexanol; Iba: isobutyl alcohol; Iaa: isoamyl alcohol; 3MP: 3-(methylthio)-1-propanol; 2Pa: 2-phenylethyl alcohol; Ha: hexanoic acid; Oa: octanoic acid; Da: decanoic acid; Eb: ethyl butyrate; Eh: ethyl hexanoate; El: ethyl lactate; Ds: diethyl succinate; E4h: ethyl 4-hydroxybutanoate; Dh: diethyl hydroxybutanedioate; Dhm: diethyl 2-hydroxy-3-methylsuccinate; E2H3: ethyl-2-hydroxy-3-phenyl propaonate; Ehs: ethyl hydrogen succinate; Iac: isoamyl acetate; 2HP: 2H Pyran-2.6(5H)-dione; Bl: γ-butyrolactone

Figure 1b revealed that ZB200 wine was different in the content of aroma compounds from ZCTRLB and ZB100 wines Žižak wines were characterized by more aromatic compounds than Krstač wines.

The reason for this can be the higher concentration of precursor aroma compounds in the grapes. Bentonite in a concentration of 200 g/hL reduced the content of octanoic acid, decanoic acid, ethyl hexanoate, diethyl succinate, butyrolactone in Žižak wines. The removal of compounds depends on the characterristics and concentration of bentonite as well as the characteristics of aromatic compounds (polarity, solubility and volatility).

CONCLUSIONS

Wines of Krstač and Žižak which were inoculated with *L. plantarum* and *O.oeni* had lower content of total fatty acids and total higher alcohols, while wines inoculated with *L. plantarum* had a higher esters content compared to the control wine.

The content of diethyl succinate, γ-butyrolactone, ethyl 4-hydroxybuta-noate, diethyl hydroxylbutanedioate and ethyl lactate mostly increased, while the content of ethyl butyrate, ethyl hexanoate, isoamyl acetate decreased with the addition of LAB. The concentrations of higher alcohols, medium-chain fatty acids and esters in Krstač and Žižak wines depended on the grape variety and the strain of lactic acid bacteria.

L. plantarum yielded a higher concentration of 2-phenylethyl alcohol in the wines from Žižak compared to control wines. ZMFLP had a statistically significant higher content of all higher alcohols compared to ZMFOO. The highest content of higher alcohols was detected in control wines. In addition, a statistically significant difference in the concentration of octanoic and decanoic acid was found between MFLP and MFOO for Krstač and Žižak wines. In the wines of Krstač and Žižak, *L. plantarum* during MLF synthesized a higher content of esters compared to *O. oeni*.

The reason for a greater synthesis of esters was due to *L. plantarum* has a significant source of esterases and better tolerance to the physicochemical conditions of wine (higher pH and alcohol concentration). Different strains of bacteria have different adaptations to the changing wine conditions.

The addition of increasing concentrations of bentonite did not affect the content of higher alcohols in Žižak wines. Z B200 wine was rich in higher alcohols and had the lowest concentration of fatty acids, ethyl butyrate and ethyl hexanoate. Volatile compounds in wine consist of compounds with different chemical characterristics (polarity, solubility and volatility), so their removal depends on these characteristics. Aroma compounds can be removed indirectly (by deproteinization) or directly (by adsorption of bentonite).

Global warming affects the accumulation of proteins in the Krstač and Žižak varieties, which is why higher concentrations of bentonite are needed to remove them. In addition, climate change and higher temperatures affect the rapid degradation of malic acid in the grape berries and thus the aromatic profile of the wine.

The results of these studies would help oenologists find the optimal amount of bentonite needed to remove proteins without damaging the wine's aroma complex to a greater extent. For the first time, the effects of malolactic fermentation on these grape varieties and its influence on the aroma complex of wines were also investigated. As these are autochthonous varieties, their main advantage for consumers is the aromatic character of wine, which distinguishes them from other commercial varieties. Further studies can be extended to a larger number of samples of the same varieties and a comparison of several varieties (autochthonous and international).

AUTHOR CONTRIBUTIONS

Conceptualization, A.V.P., V.B.M. and N.M.Ž.; Methodology, V.B.M., N.M.Ž., I.V.S., V.V.T. and A.V.P.; Formal analysis, A.V.P., V.B.M. and N.M.Ž.; Investigation, V.B.M., N.M.Ž, I.V.S., V.V.T., and A.V.P.; Resources, I.V.S. and V.V.T.; Writing - creation of the original draft, V.B.M.; Writing review and editing, A.V.P.; Visualization, V.B.M. and N.M.Ž.; Supervision, A.V.P.; Project administration and fundraising, A.V.P.

DATA AVAILABILITY STATEMENT

Data contained within the article.

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CONFLICT OF INTEREST

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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UTICAJI MALOLAKTIČKE FERMENTACIJE I TRETMANA BENTONITOM NA AROMU VINA AUTOHTONIH SORTI KRSTAČ I ŽIŽAK

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Sažetak: Cilj ovog rada bio je da se ispita uticaj dva soja bakterija mlečne kiseline (*Lactobacillus plantarum*, *Oenococcus oeni*) i tretmana bentonitom na sadržaj aromatičnih jedinjenja u vinima autohtonih sorti grožđa Krstač i Žižak. GC/FID-MS analizom su detektovani viši alkoholi, srednjelančane masne kiseline (C6, C8, C10), estri, i druga isparljiva jedinjenja. Koncentracija viših alkohola bila je niža u vinima Krstača i Žižka u kojima je sprovedena malolaktička fermentacija. Rezultati ovog istraživanja su pokazali da sadržaj aromatičnih jedinjenja zavisi od sojeva bakterija mlečne kiseline. *L. plantarum* je obezbedio viši sadržaj ukupnih viših alkohola i estara u poređenju sa *O.oeni*. Sadržaj ukupnih estara se kretao od 30.28 do 32.70 mg/L za Krstač vina i od 19.35 do 23.21 mg/L za Žižak vina. *O. oeni* i *L. plantarum* su imali statistički značajan uticaj na koncentraciju većine estara. Bakterije mlečne kiseline značajno su smanjile sadržaj etil butirata, etil heksanoata, etil dekanoata i izoamil acetata. Takođe, sadržaj etil laktata, dietil hidroksibutandioata, dietil sukcinata i etil hidrogen sukcinata bio je viši u vinima proizvedenim sa *L. plantarum*. Dodavanje bentonita u rastućim koncentracijama nije imalo uticaja na koncentraciju viših alkohola u vinima Žižak. Najniži sadržaj masnih kiselina je detektovan u vinima proizvedenim sa dodatkom 200 g/hL bentonita.

Ključne reči: *jedinjenja arome, L. plantarum, O. oeni, bentonit, GC/MS-FID, bela vina*

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