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Original scientific paper

TIMING OF INOCULATION WITH SELECTED WINE BACTERIA ON THE KINETICS OF MALOLACTIC FERMENTATION AND SENSORY PROPERTIES OF SYRAH WINES FROM THE REPUBLIC OF NORTH MACEDONIA

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To improve the quality of the wine the use of selected strains of lactic acid bacteria become regular and important tool in modern wine making practice. As a result of metabolic activity of the lactic acid bacteria in the fermentation process the wines acidity is reduced and the wines flavour is more shaped. For the study four commercial LAB strains form the producer Lallemand were used in the fermentation of Syrah grapes: Lalvin VP41, O-MEGA, ML-Prime, PN4. The objective of this study was to determine the sensorial impact between co-inoculation and sequential application of four different lactic acid bacteria strains. From the obtained results co-inoculation samples resulted in higher level of esters and higher fruit intensity. Some strains contributed to more freshness and varietal characters of the wines and other increased the wine mouthfeel and red berry flavours.

Key words: Syrah grapes co-inoculation; lactic acid bacteria; kinetics; sensorial impact

INTRODUCTION

Malolactic fermentation (MLF) occurs in wine as a result of the metabolic activity of wine lactic acid bacteria (LAB). MLF reduces wine acidity and shapes wine flavour, both of which are considered to be beneficial to wine quality. Additionally, the use of selected strains of wine bacteria allows better control of the timeframe of L-malic acid degradation.

Since the quality of wine is the main objective of winemakers, the use of selected wine bacteria is more and more recognized as an important tool for winemakers leading the MLF process. Sensory studies show that flavour compounds produced by wine LAB bring recognizable changes to the flavour characteristics of wine [1–4]. Several studies show that different strains of wine LAB will have different sensory impacts in wines [1, 5–9]. The timing of the bacterial addition and the number of cells in the wine after inoculation will also influence the sensory profile [10]

Although associated with some risk, MLF can be conducted by indigenous wine LAB present in the winery infrastructure, which may grow during alcoholic fermentation (AF) or immediately after its completion. Inoculation with selected wine LAB cultures allows for a better control over one of the last steps of vinification and traditionally inoculation was performed at the completion of AF. Beelman and Kunkee explored the possibility of inoculating wine LAB into juice along with the yeast used to conduct AF [11].

Current thinking identifies the following timing during wine production when selected wine LAB can be added (Figure 1).

• Co-inoculation: Selected wine LAB added 24 to 48 hours after yeast addition (or 48 to 72 hours if 80 to 100 ppm of SO_2 is added at crushing)

• Early inoculation: Selected wine LAB added during active AF or at an approximate density of 1030/1040 kg/m^3 (8°/10°Brix)

• Post-alcoholic fermentation inoculation: At the end of, or just after, completion of AF

• Delayed inoculation: 2 to 6 months after completion of AF

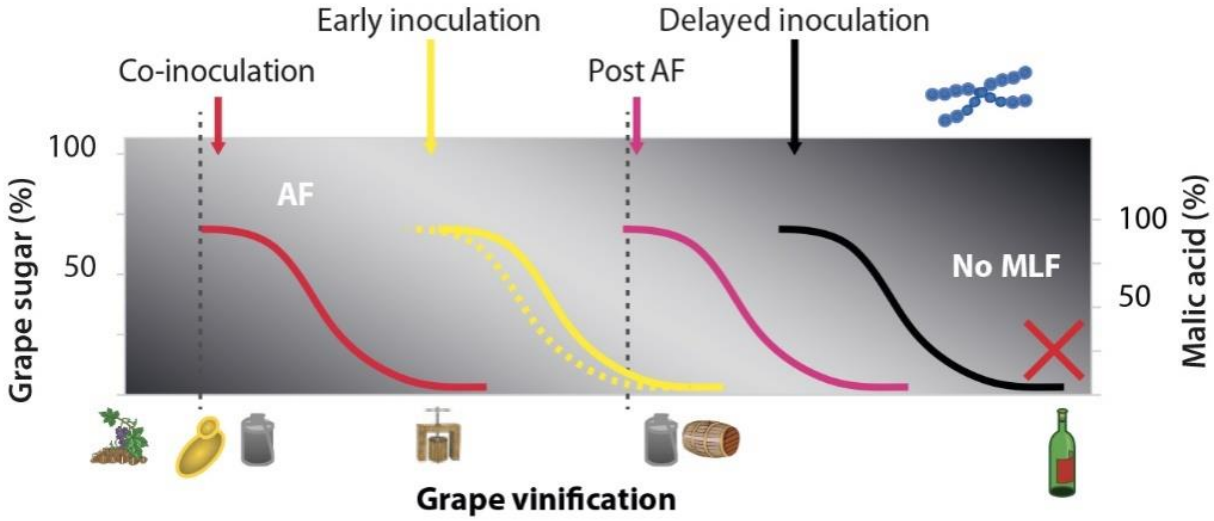

Figure 1. Inoculation regimes for selected wine lactic acid bacteria (Adapted from Bartowsky, AWRI, 2010)

The tendency to harvest high maturity grapes, resulting in higher pH and alcohol wines, seems to be more favourable to the development of indigenous bacteria flora. To limit the development of unknown indigenous bacteria, co-inoculation is an interesting winemaking option. It is a good practice to suppress at an early stage the growth of undesirable wild bacteria that can produce negative metabolites which can affect the quality of the wines, either directly by the production of negative aroma active aromas (mousy off-flavour, volatile acidity), or precursors boosting the volatile phenol productions by *Brettanomyces*, or masking the fruity varietal characters of the wines due to the production of compounds such as biogenic amines and diacetyl or acetaldehyde.

Grapes contain various aroma precursor compounds, glycosides, particularly linalool, nerol, and geraniol, which play an important role in red wine aroma. Other compounds such as phenolic compounds (astringency, bitterness) and nor-isoprenoids are aroma enhancers and are also be influenced by the activity of glycosidases. Higher aldehydes can contribute to green, herbaceous and vegetative aromas. Recent studies of Ramón Mira de Orduña have shown that certain wine bacterial strains are able to degrade some of these aldehydes and may contribute to the reduction of green and vegetative aromas. Finally, diacetyl play a role in red wines as providing an element of complexity. Concentration of diacetyl is dependent on numerous parameters including wine bacteria strain used, the timing of inoculation and the citric acid content of the wines [12].

MATERIALS AND METHODS

Production of Syrah wines from Macedonia with different wine bacteria

The climate in Macedonia is very suited for cultivation of red grape varieties. Due to good climatic conditions (a good ratio between the number of sunny days and rainfall), the grapes are with very good quality and are used exclusively for the production of premium wines for example from Syrah grape variety. Macedonian Syrah grape juice contains L-malic acid in the ranges of 0.5 to 2 g/l.

In order to obtain more balanced and microbiological stable wine with a refined aroma, this study was undertaken to investigate the influence of different wine L-lactic acid bacteria species and strains on the sensory quality of Macedonian Syrah wines. For the study, one yeast strain and four different strains of wine LAB were used and co-inoculation strategy was compared to inoculation post alcoholic fermentation.

Methodology

Mature and healthy Syrah grapes from the South East part of the Macedonia (Strumica vine-

growing district, Vardar River valley) were harvested by hand. The sugar content of the harvested grapes was 235 g/l; total acidity (tartaric acid) 5.3 g/l; L-malic acid 1.35 g/l and pH = 3.7.

The grapes were immediately destemmed and crush on a small electric crusher and 30 mg/l SO_2 was added. The grape must was divided into 5 stainless steel tanks of 30 kg each. After addition of 1 g/hl EX-V enzyme each modality was inoculated with selected active dry yeast Lalvin ICV D-254™ at 25g/hl. 24 hours after yeast addition four lots were inoculated with different wine bacteria strains as outlined below. The dosages that we used were 1g/hl for Lalvin VP41; O-MEGA and PN4 and 10g/hl for ML Prime as suggested by the manufacturer. The control sample was without bacteria inoculation.

- Control (Lalvin ICV D254™)
- Variant 1 co-inoculation (Lalvin ICV D254™ + Lalvin VP 41™)
- Vaiant 2 co-inoculation (Lalvin ICV D254™ + O-MEGA™)
- Varant 3 co-inoculation (Lalvin ICV D254™ + ML Prime™)
- Varint 4 co-inoculation (Lalvin ICV D254™ + PN4™)

During the alcoholic fermentation (AF) the cap was plunged daily 3 times. Fermaid E™ nutrient was added 15 g/hl at the temperature during fermentation did not exceed 25°C. L-malic acid was analysed every 3 days. The wine was pressed after 14 days of fermentation and left to settle for 2 days. After racking of the wine a complete chemical analysis was conducted. At the end of the alcoholic fermentation the control wine was divided in 5 equal parts and sequentially inoculated with the same bacteria strains previously used in the co-inoculation trial. Along with the different strains of LAB, a bacteria nutrition addition was also made with Opti'Malo™ 20 g/hl

- Control
- Variant 1 sequent. Lalvin VP 41™ + Opti'Malo™
- Variant 2 sequent. O-MEGA™ + Opti'Malo™
- Variant 3 sequent. ML Prime™ + Opti'Malo™
- Variant 4 sequent. PN4™ + Opti'Malo™

Enzymatic L-malic acid analyses

L-malic and L-lactic acid concentrations were determined using Oenolab enzymatic kit on an Agilent 8453 UV-VIS spectrophotometer.

Analysis of wine volatile components

The analysis of the volatile components was carried out using Varian Inc GC-MS (Varian 3900 GC, Saturn 2100T MS and Autosempler CP 8400). The working parameter of the instrument and the liquid-liquid extraction was used for isolation of the volatile components from the wine samples. The analysis was performed according to the described method of Ivanova [13].

Quantitative descriptive analysis

The sensory descriptive analysis was performed according to the method of Ubigli. Seven wine experts were involved for the descriptive evaluation of the investigated wines. The panel proposed 11 descriptors for the final evaluation. All wine samples were evaluated during one tasting session. All results of the tasting were presented in Radar chart type [14].

Statistical Analysis

Wine aroma results obtained from the GC-MS analysis were statistically processed by statistical package SPSS 13.0.

RESULTS AND DISCUSSION

Fermentation performance and wine chemistry

The AF was completed after 8-10 days and the wine was racked after 14 days (for the extraction of phenolic components). Kinetics of alcoholic fermentation was regular and didn't differ between the modalities. Analytical data of the wines after alcoholic and malolactic fermentation are shown in Table 1.

TA are total acidity (expressed as tartaric acid), VA are volatile acidity (expressed as acetic acid).

Samples marked with "CO" were produced with co-inoculations; samples marked with "Seq" were produced with the post-alcoholic fermentation inoculations.

The total acidity (TA) of the Control sample was higher than the other treatments because it was made only with a partial MLF process. This sample contained 1.35 g/L residual malic acid. All other treatments had undergone complete malic acid degradation.

Table 1. Physicochemical analysis of the wines after alcoholic and malolactic fermentation

	Sp. Gravity 20/20	Alchool vol%	Total extract g/l	TA g/l	VA g/l	ph	Free SO_2 mg/l	Total SO_2 mg/l
VP 41™ - CO	0.9934	13.30	27.1	4.5	0.38	3.55	16.64	37.64
ML Prime™ - CO	0.9930	13.48	27.1	4.5	0.43	3.65	21.76	45.25
Omega™ - CO	*0.9929*	*13,65*	*27.4*	*4.5*	*0.52*	*3.53*	*21.76*	*48.96*
PN4™ – CO	0.9934	13.48	27.9	4.4	0.51	3.62	28.00	55.12
VP 41™ - Seq	*0.9930*	*13.74*	*27.9*	*4.4*	*0.43*	*3.64*	*32.00*	*55.12*
ML Prime™- Seq	0.9932	13.56	27.9	4.5	0.48	3.55	25.60	44.00
Omega™ - Seq	0.9929	13,65	27.4	4.7	0.45	3.50	25.60	52.32
PN4™ - Seq	0.9928	13.74	27.4	4.7	0.52	3.55	21.76	48.52
Control	0.9930	13.65	27.4	5.2	0.58	3.64	25.60	51.25

The results in Figures 2 & 3 shown that in all the treatments with co-inoculation, the L-malic acid was metabolized into L-lactic acid except in the control wine where L-malic acid was unchanged. The MLF kinetics shown that *L. plantarum* ML Prime™ was very effective and able to degrade the L-malic acid within 6 days, followed by *O. oeni* PN4™ strain, which started L-malic acid degradation after a short lag phase, and completed MLF within 10 days. The other two LAB strains were slightly slower but still very efficient for the L-malic acid degradation. They finished the MLF within 17 days.

Using the traditional inoculation technique (sequential), inoculation (sequential) with selected wine LAB after alcoholic fermentation, kinetics of malic acid degradation had been almost identical between the for selected wine LAB strains. Although not recommended for sequential inoculation in red wines, the *L. plantarum* strain started the malic acid degradation faster than the *O. oeni* strains, but all LAB strains degraded malic acid within 3 weeks (Figures 2).

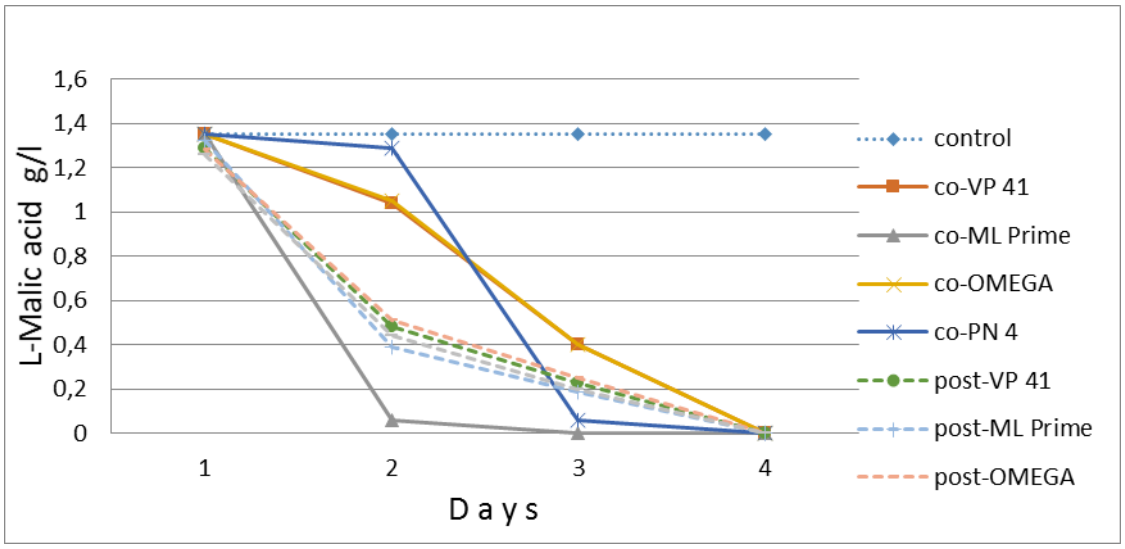

Figure 2. Kinetics of degradation of L-malic acid by four different selected wine lactic acid bacteria strains by co-inoculation and postalcoholic fermentation (sequential) inoculation

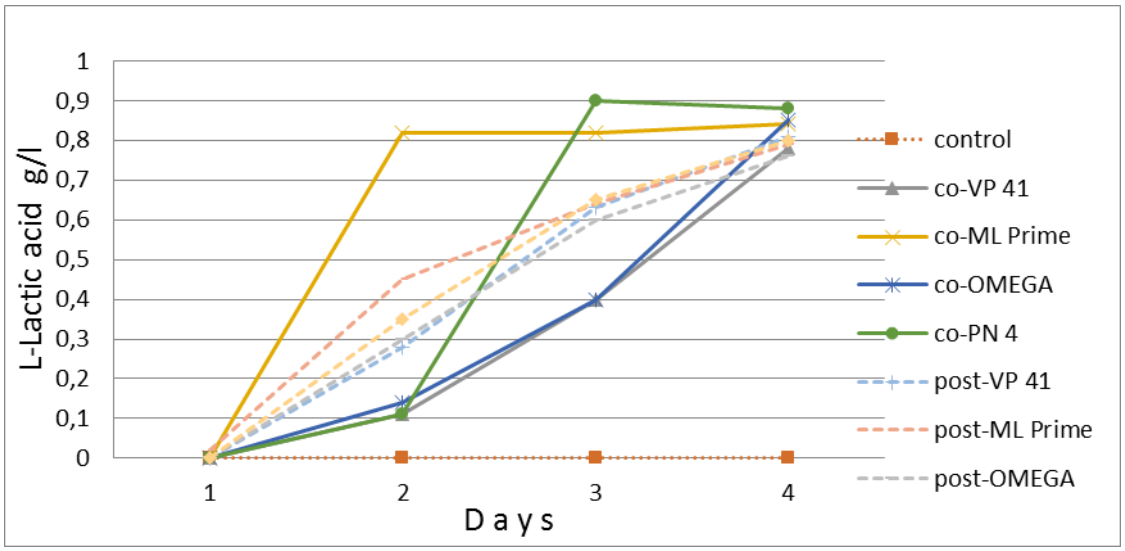

Figure 3. Kinetics of L-lactic acid formation by four different selected wine lactic acid bacteria strains by co-inoculation and post-alcoholic fermentation (sequential) inoculation

Wine aroma and sensory analyses

Volatile aroma compounds were analysed by gas chromatography-mass spectrometry (GC-MS). The results are presented in Table 2. From the obtained result we can see that statistically proven differences were found between the variants for different wine aromas. For the content of 2-phenylethanol the lowest level was analysed in the Co-inoculated variant with VP-41 (37921µg/l) and the highest level was analysed in Co and Seq-inoculated variants with PN4 strain (49652 and 49184 µg/l, respectively). For isoamyl acetate and 2-phenylethyl acetate the Co-inoculated samples had statistically proven higher values than the Sequentional and Control variants with the exception of the Co-inoculation variant with VP-41. For ethyl hexanoate two groups were formed. First group was with highest statistically proven values. It included the variants with Co-inoculation VP-41 and PN4 (366,9 and 357,1 µg/l, respectively). The second group was with lowest value. It included the Sequentional inoculation with ML Prime (292,2 µg/l).

Bacteria co-inoculation overall resulted in a higher increase of ethyl esters with the exception of phenyl ethanol in the wine co-inoculated with Lalvin VP 41™ and ML Prime™.

For the C13 nor-isoprenoids statistically proven differences were analysed for α-ionone for the variant Seq. inoculation with ML Prime (0.03 µg/l), for β-damascenon the highest level was analysed for Co-inoculation with VP-41. There were some changes in the terpene alcohols following MLF (increase in geraniol and linalool, decrease in citronellol) however, little impact was observed between the strains or timing of inoculation.

Overall the co-inoculated wines had higher levels of fruity esters compared to the wines with sequential MLF and the control wine. 2-phenyl ethanol, a major volatile compound formed during the AF decreased in concentration only for co–inoculated wines with Lalvin VP 41™ and ML Prime™. The concentrations of isoamyl acetate and 2-phenyl acetate increased with ML, overall higher with co-inoculation, especially with strains PN4™, O-MEGA™ and ML Prime™

The 2016 Syrah wines from Strumica vine-growing district (Vardar River valley) were accessed by a sensory panel (Figures 4 and 5) which highlights how these wines have been shaped during MLF with using of different selected wine bacteria strains. Figure 4 shows the wines, which have been co-inoculated (24 hours after the yeast inoculation). There is a clear sensory impact of the specific wine LAB strain. For example, the wine inoculated with LAB strain O-MEGA™, was described as more acidic, with more body balance and red fruit aromas. This corresponded with the potential of O-MEGA™ to protect the varietal aromas, to increase the aromatic intensity and bring freshness to Syrah wine resulting from grapes with high maturity. This result was in contrast to the control wine, which was dominated by bitterness and astringency. Overall co-inoculated wines were fruitier which correlated with the overall higher fruity ester concentrations obtained in these wines. This was in agreement with other studies too [15, 16].

Table 2. Volatile aroma compounds (μg/L) in x Syrah wines, vintage 2016 from Macedonia after malolactic fermentation by different selected wine LAB strain and two timing of inoculation

	Higher alcohol	Esters										
Analyzed aroma component	2-phenylethanol	isoamyl acetate	2-phenylethyl acetate	ethyl decanoate	ethyl hexanoate	ethyl octanoate	ethyl butanoate	ethyl 2-hydroxypropanoate	ethyl 3-hydroxybutanoate	ethyl 2-methylbutanoate	ethyl 2-methylpropanoate	ethyl 2-hydroxyisocaproate
Variants	(μg/l)	(μg/l)	(μg/l)	(μg/l)	(μg/l)	(μg/l)	(μg/l)	(μg/l)	(μg/l)	(μg/l)	(μg/l)	(μg/l)
Control	4383 c	671 d	31 c	29,6 bc	326,7 ab	154,1 c	538,5 ef	13684,3 i	585,3 a	13,6 a	106,4 ab	49,1 b
Co-inc. VP-41	37921 f	784 c	29,7 c	30,4 bc	366,9 a	174,9 b	654,8 c	70057,1 a	520,7 ab	10,6 a	87,2 b	56,8 ab
Co-inc. ML Prime	39832 e	1329 a	65,6 a	50,4 a	346,2 ab	194,7 a	607,2 d	43078,9 g	381,2 b	13,1 a	94,1 ab	51,9 ab
Co-inc. Omega	41052 d	1194 b	55,6 b	32,9 bc	313,3 ab	172,8 b	717,4 b	61432,9 d	442,5 ab	13,6 a	105,8 ab	46,9 b
Co-inc. PN-4	49652 a	1118 b	49,9 b	36,1 bc	357,1 a	182,6 ab	804,7 a	68033,4 b	520,9 ab	11,5 a	102,9 ab	46,8 b
sec. VP-41	43935 c	707 cd	33,4 c	26,9c	307,9 ab	136,1 d	547,4 e	55182,6 e	583,3 a	12,5 a	100,6 ab	60,1 ab
Sec.ML Prime	45480 b	704 cd	32,7 c	33,7bc	292,2 b	154,9 c	508,5 f	52497,6 f	585,5 a	13,1 a	102,3 ab	56,2 ab
Sec. Omega	43407 c	715 cd	32 c	38,1 b	317,7 ab	142,8 cd	471,3 g	65190,6 c	549,9 b	12,6 a	100,6 ab	65,3 a
Sec. PN-4	49184 a	723 cd	32,1 c	32,2 bc	313,2 ab	142,7 cd	553,6 e	31222,1 h	531,ab	13,1 a	110,9 a	54,3 ab

	C13-norisoprenoids				Terpene alcohols					
Analyzed aroma component	1,1,6-trimethyl-1,2-dihydronaphtalene	α-ionone	β-damascenone	β-ionone	linallol	nerol	geraniol	citronellol	alpha terpineol	rose oxyde
Variants	(μg/l)	(μg/l)	(μg/l)	(μg/l)	(μg/l)	(μg/l)	(μg/l)	(μg/l)	(μg/l)	(μg/l)
Control	0,048 b	0,017 b	5,1 b	0,22 a	6,8 cd	3,4 d	7,7 f	17,9 a	3,1 cd	0,12 a
Co-inc. VP-41	0,041 c	0,021 b	5,3 a	0,14 a	19,1 a	4,4 ab	13,8 d	16,4 b	6,2 a	0,13 a
Co-inc. ML Prime	0,03 f	0,029 a	3,4 d	0,11 a	5,7 d	3,7 cd	12,7 e	15,2 c	2,2 d	0,11 a
Co-inc. Omega	0,027 g	0,025 b	3,9 c	0,11 a	6,1 cd	3,6 cd	12,4 ge	13,4 d	2,5 d	0,09 a
Co-inc. PN-4	0,037 d	0,021 b	3,3 d	0,11 a	6,6 cd	4,3 ab	14,7 c	15,7 bc	2,6 d	0,13 a
sec. VP-41	0,046 b	0,028 b	4,8 b	0,24 a	8,7 b	3,5 d	14,5 cd	15,5 bc	3,2 bc	0,08 a
Sec.ML Prime	0,034 e	0,03 ab	4,9 b	0,23 a	8,1 bc	4,7 a	16,9 b	18,1 a	3,6 b	0,09 a
Sec. Omega	0,042 c	0,027 b	4,9 b	0,20 a	8,9 bc	4,0 bc	19,8 a	16,4 b	3,6 b	0,09 a
Sec. PN-4	0,054 a	0,018 b	5,1 b	0,19 a	8,9 b	4,3 ab	13,9 cd	18,6 a	3,6 b	0,11 a

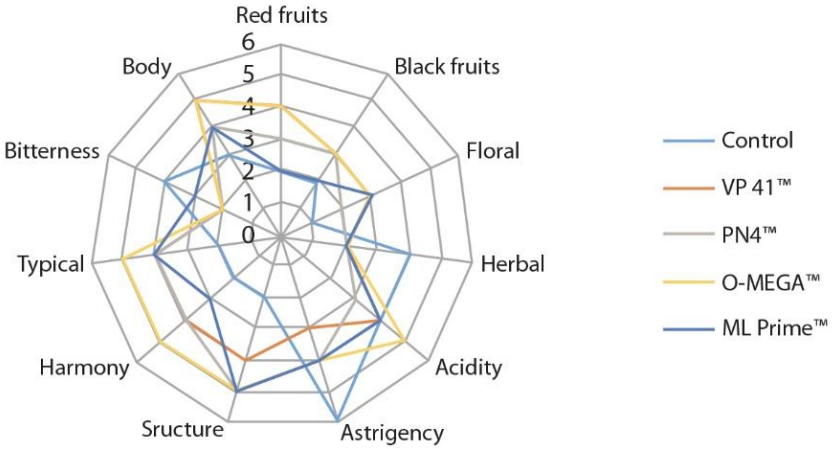

Figure 4. Sensory description of 2016 Syrah wine co-inoculated with 4 selected wine lactic acid bacteria strains compared to a control wine without MLF

Wine LAB strain PN4™ is recommended to bring more structure, creaminess and more red berry fruit sensations to wine, which was observed in this study with the sequentially inoculated Syrah wine (Figure 5). Again the control wine showed more astringency and bitterness than the wine sequentially inoculated wine LAB. The LAB strains had positive sensory impact on the body, structure and harmony of the wine and decrease the impact of herbal notes that can have a negative influence on the overall aroma of wines.

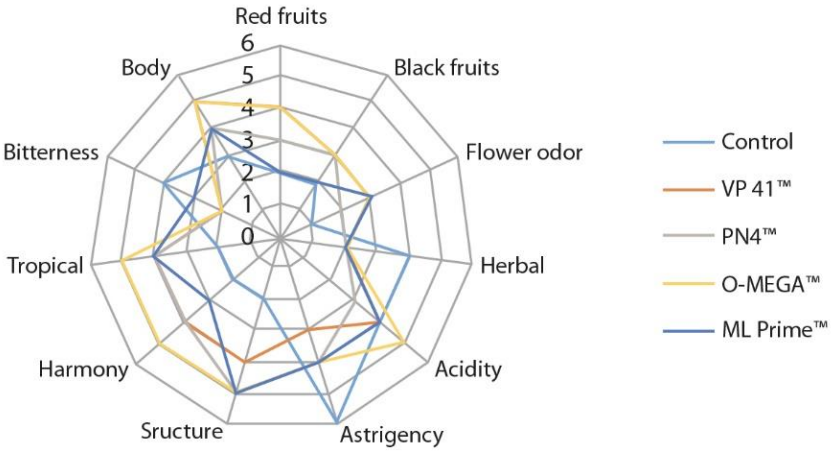

Figure 5. Sensory description of 2016 Syrah wine inoculated after alcoholic fermentation with 4 selected wine lactic acid bacteria strains compared to a control wine without MLF)

CONCLUSION

Today we have a range of reliable selected wine LAB strains available to the wine industry which do not only degrade malic acid to lactic acid in an acceptable time frame, but consistently produce favourable products with no defects. The choice of LAB strains provide an essential winemaking decision tool to fine-tune the sensory style of red wines through varied metabolism which is dependent on wine bacteria strain selected for the MLF through their esterase and glycosidase enzymes activities, as well as the citric acid metabolism.

The study shown that the use of selected wine LAB strain can help to assure faster malolactic fermentation, regardless of MLF inoculation strategy, co- or sequentially inoculation.

In this study we showed that the ester profile of the wines was modified during the course of MLF in wine. The highest volatile ester concentrations were observed in the wines where bacteria had been inoculated 24 h after the yeast (co-inoculation). The success and convenience of selected MLB strains is due to their ability to produce desirable metabolites with minimal or no production of undesirable compounds.

Co-inoculation resulted in higher ester levels and higher fruit intensity. Some strains contributed to more freshness and varietal characters, other strains increased mouthfeel and red berry flavours and all wines with malolactic fermentation were described as having lower acidity and astringency.

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ВРЕМЕ НА ИНОКУЛАЦИЈА СО СЕЛЕКЦИОНИРАНА ВИНСКА БАКТЕРИЈА ВРЗ КИНЕТИКАТА НА МАЛОЛАКТИЧКАТА ФЕРМЕНТАЦИЈА И СЕНЗОРНИТЕ КАРАКТЕРИСТИКИ НА ВИНА ОД СИРА ОД РЕПУБЛИКА СЕВЕРНА МАКЕДОНИЈА

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За да се подобри квалитетот на виното, употребата на селекционирани видови млечно-кисели бактерии станува редовна и важна алатка во современата винарска практика. Како резултат на метаболичката активност на млечно-киселите бактерии во процесот на ферментација, киселоста на вината се намалува, а вкусот на вината е позаокружен. За студијата, во процесот на ферментација на грозјето од сортата *сира* беа користени четири комерцијални соеви ЈМБ од производителот Lallemand: Lalvin VP41, O-MEGA, ML-Prime, PN4. Целта на оваа студија беше да се утврди сензорното влијание помеѓу варијантите со коинокулација и секвенцијална апликација на четири различни соеви на млечно-кисели бактерии. Од добиените резултати, примероците со коинокулација резултираа со зголемено ниво на естри и поголем интензитет на овошни ароми. Некои од соевите придонесоа за поголема свежина и сортен карактер на вината, а други за зголемено времетраење на ефект на вкусот и аромат на црвени зрнести плодови.

Клучни зборови: коинокулација на грозје од сира; млечно-кисели бактерии; кинетика; сензорно влијание