SHORT COMMUNICATION

Effects of co-fermentation with *Lachancea thermotolerans* or *Metschnikowia pulcherrima* on concentration of aroma compounds in Pinot Blanc wine

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Summary
Slovakian strains of *Lachancea thermotolerans* and *Metschnikowia pulcherrima* were used in sequential co-fermentation with *Saccharomyces cerevisiae* in small-scale production of Pinot Blanc wine from the Small Carpathian wine region in Slovakia. Aroma compounds of the produced wines were analysed using solid-phase microextraction coupled to gas chromatography-mass spectrometry. Thirty-six aroma compounds were quantified, demonstrating no significant differences in concentrations of almost half of them, including acetic acid, ethyl acetate, 2,3-butanediol and butanoic acid. Wines produced with non-*Saccharomyces* yeasts did not contain increased concentrations of aroma-active esters, but contained increased concentrations of methionol and decreased concentrations of furfural. Wine produced with *L. thermotolerans* contained increased concentrations of 2-phenylethanol, diethyl succinate and phenylethyl acetate, together with an increased concentration of 3-methylbutanoic acid. Wine produced with *M. pulcherrima* contained increased concentrations of 2-phenylethanol and diethyl succinate, together with a decreased concentration of acetaldehyde. Results of the study demonstrate that *L. thermotolerans* and *M. pulcherrima*, when used in a co-culture with *S. cerevisiae*, can modulate the composition of Pinot Blanc wine regarding aroma compounds, thereby positively contributing to its quality.

Keywords
wine; *Lachancea*; *Metschnikowia*; aroma

Non-*Saccharomyces* yeasts are becoming widely considered for production of wines with alternative or more complex aroma profiles. Various non-*Saccharomyces* yeast species and strains have been shown to provide specific metabolic products during fermentation of grape must, such as terpenoids, esters, higher alcohols, glycerol, acetaldehyde, acetic acid or succinic acid. In order to guarantee successful fermentation, non-*Saccharomyces* yeasts are used in mixed cultures with *Saccharomyces* strains, while sequential inoculation facilitates stronger contribution of the former to organoleptic properties of wine [1–3].

A widely studied non-*Saccharomyces* yeast, which is already commercially available and practically used, is *Lachancea thermotolerans.* This yeast is promoted as aroma and flavour enhancer, producing wines with increased concentrations of lactic acid, glycerol and 2-phenylethanol by mixed fermentation of grape must. Fortunately, the increase in glycerol is not accompanied by an increase in acetic acid concentration in wines produced by co-fermentation with *L. thermotolerans* [4–6].

Another widely studied non-*Saccharomyces* yeast, which is also commercially available and

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practically used, is *Metschnikowia pulcherrima*. It is promoted as a high producer of esters, in particular the pear-associated ethyl octanoate [7, 8]. Various white wines obtained by sequential fermentation with *M. pulcherrima* and *Saccharomyces cerevisiae* showed higher quality scores than control wines obtained by fermentation solely with *S. cerevisiae* [2, 9].

In this study, *L. thermotolerans* and *M. pulcherrima* were applied in small-scale production of Pinot Blanc wine from the Small Carpathian wine region in Slovakia. Two non-*Saccharomyces* yeast strains of Slovakian origin were used for sequential inoculation together with a *S. cerevisiae* strain of the same origin. Aroma compounds of the produced wines were analysed using solid-phase microextraction coupled to gas chromatography-mass spectrometry.

MATERIALS AND METHODS

Yeast strains and inoculum preparation

Lachancea thermotolerans 5-1-1, *Metschnikowia pulcherrima* 11-1-7 and *Saccharomyces cerevisiae* PDA W10 were from the Culture Collection of Wine Yeasts (Food Research Institute, National Agricultural and Food Centre, Bratislava, Slovakia). These strains were previously isolated in Slovakia and were selected based on their enzymatic potential, enological properties and results of preliminary microvinification experiments (Ženišová and Sidari, unpublished results). A loopful of each strain was inoculated to 10 ml of sterile must with potassium metabisulfite (to form 20 $mg \cdot l^{-1}$ of free SO_2) and incubated statically for 24 h at 25 °C. Then, 1 ml of the prepared culture was transferred to 100 ml of sterile must with potassium metabisulfite (to form 20 $mg \cdot l^{-1}$ of free SO_2) and incubated statically for 24 h at 25 °C. Finally, 10 ml of this culture was transferred to 1 000 ml of sterile must with potassium metabisulfite (to form 20 $mg \cdot l^{-1}$ of free SO_2) and incubated with shaking of 2 Hz for 24 h at 25 °C. The must used for inoculum propagation was Pinot Blanc (20 °Brix, pH 3.0, acidity of 9.4 $g \cdot l^{-1}$).

Wine samples

Mature grapes of Pinot Blanc were collected from a small vineyard in Modra, Slovakia, Small Carpathian wine region (vintage 2018; collection date 18 September 2018) and processed in a traditional way [10]. The must (24 °Brix, acidity of 9.4 $g \cdot l^{-1}$) was decanted, divided to batches of 90 l and individual batches were inoculated with *L. thermotolerans, M. pulcherrima* or *S. cerevisiae* at 1% (v/v). With a delay of 24 h, the first two batches were additionally inoculated with *S. cerevisiae* at 1% (v/v). All inocula contained 10^6–10^7 $CFU \cdot ml^{-1}$ yeasts. Upon the onset of fermentation, cooling devices were applied and fermentation took place at 16 °C. After the end of fermentation, the wine was separated and filtered through a sheet filter Hobrafilt N S15N (cellulose, diatomaceous earth and perlite, pore size 2 μm; Hobra – Školník, Broumov, Czech Republic).

Analytical methods

Solid phase microextraction (SPME) was carried out using a polydimethylsiloxan-divinylbenzene fibre, coating thickness 65 μm (Supelco, Bellefonte, Pennsylvania, USA) immersed in 10 ml of wine sample mixed at 6 Hz on a magnetic stirrer during 30 min at 20 °C. The extracted compounds were analysed by gas chromatography-mass spectrometry (GC-MS) using a 6890N gas chromatograph (Agilent Technologies, Santa Clara, California, USA) coupled to a 5973 mass spectrometric detector (Agilent Technologies). The SPME fibre was placed in the inlet of the chromatograph for 2 min at 250 °C so as to desorb the extracted compounds. The gas chromatographic separation took place in a high polarity polyethyleneglycol column DB-WAXetr (length 30 m, inner diameter 0.25 mm, stationary phase thickness 0.5 μm; Agilent Technologies) using a temperature programme of 35 °C for 1 min, 5 $°C \cdot min^{-1}$ and 250 °C for 1 min. The split ratio was 10:1. An average velocity of He carrier gas was 34 $cm \cdot s^{-1}$ at constant flow. Ionization voltage of 70 eV was used. Identification of compounds was done by comparison of mass spectra with NIST 14 MS library (National Institute Standards and Technology, Gaithersburg, Maryland, USA). For quantification, total ion current was detected, 4-methyl-2-pentanol was used as an internal standard and individual peaks were calibrated using authentic standards (all from Sigma, St. Louis, Missouri, USA).

Other analyses were performed in accordance with the official methodology of International Organisation of Vine and Wine [11].

RESULTS AND DISCUSSION

Pinot Blanc wines produced using co-fermentation with *L. thermotolerans* or *M. pulcherrima* were characterized and compared to that produced solely with *S. cerevisiae.* The wine samples were prepared in conditions that mimicked small-scale production by traditional producers. Chemical parameters of the produced wines are presented

Tab. 1. Chemical parameters of Pinot Blanc wine fermented using *S. cerevisiae* with or without pre-inoculation with *L. thermotolerans* or *M. pulcherrima.*

	L. thermotolerans + *S. cerevisiae*	*M. pulcherrima* + *S. cerevisiae*	*S. cerevisiae*
Ethanol (v/v) [%]	13.8	14.0	13.8
pH	3.4	3.4	3.6
Total acidity [g·l^{-1}]	4.8^{a}	4.9^{a}	4.4^{b}
Sugar [°Brix]	5.5^{a}	6.0^{b}	6.0^{b}

Mean values of three measurements are presented. Values in rows marked by different superscript letters are significantly different at $p < 0.05$ as tested by one-way ANOVA with Tukey's test.

Tab. 2. Concentrations of aroma compounds in Pinot Blanc wine fermented using *S. cerevisiae* with or without pre-inoculation with *L. thermotolerans* or *M. pulcherrima.*

Compound [mg·l^{-1}]	*L. thermotolerans* + *S. cerevisiae*	*M. pulcherrima* + *S. cerevisiae*	*S. cerevisiae*
Ethyl formate	0.00 ± 0.00^{a}	0.31 ± 0.12^{b}	0.45 ± 0.14^{b}
Methyl acetate	0.51 ± 0.09^{a}	0.27 ± 0.04^{b}	0.33 ± 0.08
Ethyl acetate	24.53 ± 0.55	22.89 ± 1.52	21.47 ± 2.22
2-Methylpropyl acetate	0.05 ± 0.01	0.04 ± 0.02	0.06 ± 0.01
Ethyl butanoate	0.14 ± 0.01^{a}	0.13 ± 0.01^{a}	0.16 ± 0.01^{b}
1-Propanol	11.25 ± 2.73	9.84 ± 1.86	7.57 ± 1.81
Ethyl 3-methylbutanoate	0.01 ± 0.00^{a}	0.02 ± 0.00^{b}	0.02 ± 0.00^{b}
Butyl acetate	0.72 ± 0.22	1.05 ± 0.56	0.61 ± 0.26
3-Methylbutyl acetate	0.79 ± 0.01^{a}	0.58 ± 0.04^{b}	1.37 ± 0.08^{c}
1-Butanol	0.35 ± 0.02	0.38 ± 0.09	0.32 ± 0.03
Pentyl acetate	0.00 ± 0.00	0.03 ± 0.03	0.01 ± 0.01
2-Methyl-1-butanol	46.66 ± 0.59	42.60 ± 1.22	47.01 ± 4.00
3-Methyl-1-butanol	133.58 ± 2.44	122.46 ± 3.85	138.10 ± 12.24
Ethyl hexanoate	0.39 ± 0.03	0.32 ± 0.04	0.34 ± 0.03
Hexyl acetate	0.01 ± 0.00^{a}	0.01 ± 0.00^{b}	0.02 ± 0.00^{c}
3-Methyl-1-pentanol	0.17 ± 0.01	0.16 ± 0.00	0.18 ± 0.02
1-Hexanol	0.87 ± 0.03^{a}	0.84 ± 0.01	0.76 ± 0.05^{b}
Ethyl octanoate	0.34 ± 0.07	0.26 ± 0.06	0.26 ± 0.05
1-Heptanol	0.22 ± 0.01^{a}	0.17 ± 0.01^{b}	0.28 ± 0.01^{c}
Furfural	0.32 ± 0.06^{a}	0.21 ± 0.07^{a}	1.78 ± 0.49^{b}
Acetic acid	135.83 ± 10.40	113.66 ± 13.87	123.36 ± 22.94
2,3-Butanediol	786.40 ± 134.02	623.79 ± 54.75	615.53 ± 122.23
Linalool	0.01 ± 0.00^{a}	0.01 ± 0.00^{a}	0.01 ± 0.00^{b}
Ethyl decanoate	0.10 ± 0.02	0.10 ± 0.02	0.08 ± 0.02
Butanoic acid	1.62 ± 0.14	1.43 ± 0.08	1.47 ± 0.17
Diethyl succinate	0.85 ± 0.05^{a}	0.77 ± 0.02^{a}	0.40 ± 0.05^{b}
3-Methylbutanoic acid	1.61 ± 0.26^{a}	0.82 ± 0.03^{b}	1.03 ± 0.02^{b}
Methionol	0.63 ± 0.07^{a}	0.49 ± 0.14^{a}	0.24 ± 0.03^{b}
Phenylethyl acetate	0.63 ± 0.07^{a}	0.46 ± 0.03^{b}	0.59 ± 0.02^{a}
Geraniol	0.02 ± 0.01^{a}	0.01 ± 0.00	0.00 ± 0.00^{b}
Hexanoic acid	2.34 ± 0.08^{a}	2.00 ± 0.01^{b}	2.27 ± 0.08^{a}
2-Phenylethanol	63.11 ± 2.13^{a}	57.58 ± 1.26^{b}	45.30 ± 2.50^{c}
Octanoic acid	2.81 ± 0.12	2.57 ± 0.19^{a}	3.10 ± 0.10^{b}
2-Methoxy-4-vinylphenol	0.15 ± 0.01^{a}	0.09 ± 0.00^{b}	0.11 ± 0.01^{c}
Decanoic acid	0.32 ± 0.04	0.30 ± 0.05	0.28 ± 0.02

Wine samples were analysed in triplicate. Values represent mean ± standard deviation. Concentrations of 2,3-butanediol isomers were summarized. Values in rows marked by different superscript letters are significantly different at $p < 0.05$ as tested by one-way ANOVA with Tukey's test.

in Tab. 1, they all fell within the usual ranges of this wine variety [10]. The values for individual samples were similar but the wines produced using non-*Saccharomyces* yeasts had slightly higher total acidity with corresponding slightly lower pH. White wines are generally more acidic than red wines, while acidity gives the wine crispness on the palate. Acidity of a wine is one of its attractive properties, as it improves its refreshing, crisp qualities and it makes possible to combine wines successfully with certain food [10, 12].

Results of GC-MS analysis of aroma compounds are presented in Tab. 2. Thirty-six compounds that are known to contribute to wine aroma [12–14] were identified and quantified. Concentrations of almost half of them did not differ significantly between the analysed samples, including acetic acid or ethyl acetate, as well as those of 2,3-butanediol and butanoic acid, which are taken as off-flavours. No desirable increase was observed in wines produced with non-*Saccharomyces* yeasts regarding aroma-active esters, such as ethyl hexanoate, ethyl octanoate or ethyl decanoate. However, interesting was the increase in concentration of methionol, which is a common component of wine and is characterized by soupy, onion or cooked vegetable flavour. Concentration of furfural was decreased in both wines produced with non-*Saccharomyces* yeasts.

Positive effects were observed in wine produced by co-fermentation with *L. thermotolerans* regarding increased concentrations of 2-phenylethanol (floral flavour), diethyl succinate (fruity flavour) and phenylethyl acetate (honey flavour), while the increased concentration of 3-methylbutanoic acid can be taken as detrimental as the compound is an off-flavour. Positive effects were observed also in wine produced by co-fermentation with *M. pulcherrima*. These included an increase in concentration of 2-phenylethanol and diethyl succinate, which are floral and fruity flavours, accompanied with a decrease in the concentration of acetaldehyde, which is taken as an off-flavour. The concentrations of aroma compounds determined in this study fall within the ranges previously reported for Central European Pinot Blanc wines and correspond to wines of good quality [10, 14–16].

CONCLUSIONS

Although several properties of non-*Saccharomyces* yeasts in winemaking are apparently strain-dependent, our results overall confirm that *L. thermotolerans* and *M. pulcherrima*, when used in a co-culture with *S. cerevisiae*, can modulate the composition of wine regarding aroma compounds, thereby positively contributing to its quality.

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