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Effects of co-fermentation with Lachancea thermotolerans or Metschnikowia pulcherrima on concentration of aroma compounds in Pinot Blanc wine. Katarína Ženišová - Tereza Cabicarová - Rossana Sidari - Emil Kolek - Domenico Pangallo - Tomáš Szemes – Tomáš Kuchta Katarína Ženišová, Tereza Cabicarová, Emil Kolek, Tomáš Kuchta, Food Research Institute, National Agricultural and Food Centre, Priemyselná 4, 82475 Bratislava, Slovakia. Rossana Sidari, Department of AGRARIA, Mediterranean University of Reggio Calabria, loc. Feo di Vito, 89122 Reggio Calabria, Italy. Domenico Pangallo, Institute of Molecular Biology, Slovak Academy of Sciences, Dúbravská cesta 21, 84551 Bratislava, Slovakia. Tomáš Szemes, Department of Molecular Biology, Faculty of Natural Sciences, Comenius University, Ilkovičova 6, 84215 Bratislava, Slovakia; Science Park, Comenius University, Ilkovičova 8, 84104 Bratislava, Slovakia; Geneton Ltd., Ilkovičova 8, 84104 Bratislava, Slovakia. Correspondence author Katarína Ženišová, e-mail: katarina.zenisova@nppc.sk 

### 68 Summary

The Slovakian strains of Lachancea thermotolerans and Metschnikowia pulcherrima were used in 69 sequential co-fermentation with Saccharomyces cerevisiae in small-scale production of Pinot Blanc 70 wine from the Small Carpathian wine region in Slovakia. Aroma compounds of the produced wines 71 were analysed using solid-phase microextraction coupled to gas chromatography-mass 72 spectrometry. Thirty-six aroma compounds were quantified, demonstrating no significant 73 differences in concentrations of almost half of them, including acetic acid, ethyl acetate, 2,3-74 butanediol and butanoic acid. Wines produced with non-Saccharomyces yeasts did not contain 75 76 increased concentrations of aroma-active esters, but contained increased concentrations of methionol and decreased concentrations of furfural. Wine produced with L. thermotolerans 77 contained increased concentrations of 2-phenylethanol, diethyl succinate and phenylethyl acetate, 78 79 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.

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#### 87 Keywords

- 88 wine; *Lachancea*; *Metschnikowia*; aroma
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Non-Saccharomyces yeasts are becoming widely considered for production of wines with 101 alternative or more complex aroma profiles. Various non-Saccharomyces yeast species and strains 102 have been shown to provide specific metabolic products during fermentation of grape must, such as 103 terpenoids, esters, higher alcohols, glycerol, acetaldehyde, acetic acid or succinic acid. In order to 104 guarantee successful fermentation, non-Saccharomyces yeasts are used in mixed cultures with 105 Saccharomyces strains, while sequential inoculation facilitates stronger contribution of the former 106 to organoleptic properties of wine [1-3]. 107

A widely studied non-Saccharomyces yeast, which is already commercially available and 108 practically used, is Lachancea thermotolerans. This yeast is promoted as aroma and flavour 109 enhancer, producing wines with increased concentrations of lactic acid, glycerol and 2-110 phenylethanol by mixed fermentation of grape must. Fortunately, the increase in glycerol is not 111 accompanied by an increase in acetic acid concentration in wines produced by co-fermentation with 112 *L. thermotolerans* [4–6]. 113

Another widely studied non-Saccharomyces yeast, which is also commercially available and 114 115 practically used, is Metschnikowia pulcherrima. It is promoted as a high producer of esters, in particolar the pear-associated ethyl octanoate [7, 8]. Various white wines obtained by sequential 116 117 fermentation with *M. pulcherrima* and *Saccharomyces cerevisiae* showed higher quality scores than control wines obtained by fermentation solely with S. cerevisiae [2, 9]. 118

In this study, L. thermotolerans and M. pulcherrima were applied in small-scale production of Pinot 119 Blanc wine from the Small Carpathian wine region in Slovakia. Two non-Saccharomyces yeast 120 strains of Slovakian origin were used for sequential inoculation together with a S. cerevisiae strain 121 of the same origin. Aroma compounds of the produced wines were analysed using solid-phase 122 microextraction coupled to gas chromatographymass spectrometry. 123

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#### **MATERIALS AND METHODS** 125

#### 126 Yeast strains and inoculum preparation

Lachancea thermotolerans 5-1-1, Metschnikowia pulcherrima 11-1-7 and Saccharomyces cerevisiae 127 PDA W10 were from the Culture Collection of Wine Yeasts (Food Research Institute, National 128 Agricultural and Food Centre, Bratislava, Slovakia). These strains were previously isolated in 129 130 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 131 each strain was inoculated to 10 ml of sterile must with potassium metabisulfite (to form 20 mg $\cdot$ l<sup>-1</sup> 132 of free SO<sub>2</sub>) and incubated statically for 24 h at 25 °C. Then, 1 ml of the prepared culture was 133 transferred to 100 ml of sterile must with potassium metabisulfite (to form 20 mg $\cdot$ l<sup>-1</sup> of free SO<sub>2</sub>)

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·l<sup>-1</sup> of free SO<sub>2</sub>) 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·l<sup>-1</sup>).

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## 140 Wine samples

Mature grapes of Pinot Blanc were collected from a small vineyard in Modra, Slovakia, Small 141 Carpathian wine region (vintage 2018; collection date 18 September 2018) and processed in a 142 traditional way [10]. The must (24 °Brix, acidity of 9.4  $g \cdot l^{-1}$ ) was decanted, divided to batches of 90 143 1 and individual batches were inoculated with L. thermotolerans, M. pulcherrima or S. cerevisiae at 144 1 % (v/v). With a delay of 24 h, the first two batches were additionally inoculated with S. cerevisiae 145 at 1 % (v/v). All inocula contained  $10^6 - 10^7$  CFU·ml<sup>-1</sup> yeasts. Upon the onset of fermentation, 146 cooling devices were applied and fermentation took place at 16 °C. After the end of fermentation, 147 the wine was separated and filtered through a sheet filter Hobrafilt N S15N (cellulose, 148 diatomaceous earth and perlite, pore size 2 µm; Hobra – Školník, Broumov, Czech Republic). 149

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### 151 Analytical methods

Solid phase microextraction (SPME) was carried out using a polydimethylsiloxan-divinylbenzene 152 fibre, coating thickness 65 µm (Supelco, Bellefonte, Pennsylvania, USA) immersed in 10 ml of 153 wine sample mixed at 6 Hz on a magnetic stirrer during 30 min at 20 °C. The extracted compounds 154 were analysed by gas chromatography-mass spectrometry (GC-MS) using a 6890N gas 155 chromatograph (Agilent Technologies, Santa Clara, California, USA) coupled to a 5973 mass 156 157 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 entracte compounds. The gas 158 chromatographic separation took place in a high polarity polyethyleneglycol column DB-WAXetr 159 160 (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 min<sup>-1</sup> and 250 °C for 1 min. The split ratio 161 was 10:1. An average velocity of He carrier gas was 34 cm  $s^{-1}$  at constant flow. Ionization voltage 162 of 70 eV was used. Identification of compounds was done by comparison of mass spectra with 163 NIST 14 MS library (National Institute Standards and Technology, Gaithersburg, Maryland, USA). 164 For quantification, total ion current was detected, 4-methyl-2-pentanol was used as an internal 165 standard and individual peaks were calibrated using authentic standards (all from Sigma, St. Louis, 166 Missouri, USA). Other analyses were performed in accordante with the official methodology of 167 168 International Organisation of Vine and Wine [11].

#### 169 **RESULTS AND DISCUSSION**

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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 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

176 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 180 181 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 182 183 or ethyl acetate, as well as those of 2,3-butanediol and butanoic acid, which are taken as offflavours. No desirable increase was observed in wines produced with non-Saccharomyces yeasts 184 185 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 186 component of wine and is characterized by soupy, onion or cooked vegetable flavour. 187 Concentration of furfural was decreased in both wines produced with non-Saccharomyces yeasts. 188 Positive effects were observed in wine produced by co-fermentation with L. thermotolerans 189 regarding increased concentrations of 2-phenylethanol (floral flavour), diethyl succinate (fruity 190 flavour) and phenylethyl acetate (honey flavour), while the increased concentration of 3-191 methylbutanoic acid can be taken as detrimental as the compound is an off-flavour. Positive effects 192 were observed also in wine produced by co-fermentation with M. pulcherrima. These included an 193 194 increase in concentration of 2-phenylethanol and diethyl succinate, which are floral and fruity

195 flavours, accompanied with a decrease in the concentration of acetaldehyde, which is taken as an 196 off-flavour. The concentrations of aroma compounds determined in this study fall within the ranges 197 previously reported for Central European Pinot Blanc wines and correspond to wines of good 198 quality [10, 14–16].

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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
рН	3.4	3.4	3.6
Total acidity [g·l-1]	4.8ª	4.9ª	4.4 <sup>b</sup>
Sugar [°Brix]	5.5ª	6.0 <sup>b</sup>	6.0 <sup>b</sup>

206 Mean values of three measurements are presented. Values in rows marked by different superscript letters are 207 significantly different at p < 0.05 as tested by one-way ANOVA with Tukey's test.

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- 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*.

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Compound [mg·l-1]	L. thermotolerans + S. cerevisiae	M. pulcherrima + S. cerevisiae	S. cerevisiae
Ethyl formate	$0.00 \pm 0.00^{a}$	0.31 ± 0.12 <sup>b</sup>	0.45 ± 0.14 <sup>b</sup>
Methyl acetate	0.51 ± 0.09ª	0.27 ± 0.04 b	$0.33 \pm 0.08$
Ethyl acetate	24.53 ± 0.55	22.89 ± 1.52	21.47 ± 2.22
2-Methylpropyl acetate	$0.05 \pm 0.01$	$0.04 \pm 0.02$	$0.06 \pm 0.01$
Ethyl butanoate	0.14 ± 0.01 ª	0.13±0.01ª	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	$0.72 \pm 0.22$	$1.05 \pm 0.56$	0.61 ± 0.26
3-Methylbutyl acetate	0.79 ± 0.01 ª	0.58 ± 0.04 b	1.37 ± 0.08°
1-Butanol	$0.35 \pm 0.02$	$0.38 \pm 0.09$	$0.32 \pm 0.03$
Pentyl acetate	$0.00 \pm 0.00$	$0.03 \pm 0.03$	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	$0.39 \pm 0.03$	$0.32 \pm 0.04$	$0.34 \pm 0.03$
Hexyl acetate	0.01 ± 0.00 ª	0.01 ± 0.00 b	0.02 ± 0.00°
3-Methyl-1-pentanol	$0.17 \pm 0.01$	0.16±0.00	$0.18 \pm 0.02$
-Hexanol	0.87 ± 0.03 ª	$0.84 \pm 0.01$	0.76 ± 0.05 b
Ethyl octanoate	$0.34 \pm 0.07$	$0.26 \pm 0.06$	$0.26 \pm 0.05$
-Heptanol	0.22 ± 0.01 ª	0.17 ± 0.01 b	0.28 ± 0.01 °
Furfural	$0.32 \pm 0.06$ <sup>a</sup>	0.21 ± 0.07 ª	1.78 ± 0.49 <sup>b</sup>
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 <sup>b</sup>
Ethyl decanoate	$0.10 \pm 0.02$	$0.10 \pm 0.02$	$0.08 \pm 0.02$
Butanoic acid	$1.62 \pm 0.14$	$1.43 \pm 0.08$	$1.47 \pm 0.17$
Diethyl succinate	0.85 ± 0.05 ª	0.77 ± 0.02 ª	0.40 ± 0.05 b
3-Methylbutanoic acid	$1.61 \pm 0.26^{2}$	0.82 ± 0.03 b	$1.03 \pm 0.02^{b}$
Methionol	$0.63 \pm 0.07$ <sup>a</sup>	0.49 ± 0.14 ª	0.24 ± 0.03 b
Phenylethyl acetate	$0.63 \pm 0.07^{a}$	0.46 ± 0.03 b	$0.59 \pm 0.02^{a}$
Geraniol	0.02 ± 0.01 ª	$0.01 \pm 0.00$	0.00 ± 0.00 b
Hexanoic acid	2.34 ± 0.08 ª	2.00 ± 0.01 b	$2.27 \pm 0.08^{2}$
2-Phenylethanol	63.11 ± 2.13 <sup>a</sup>	57.58±1.26 <sup>b</sup>	45.30 ± 2.50°
Octanoic acid	2.81 ± 0.12	2.57 ± 0.19ª	3.10±0.10b
2-Methoxy-4-vinylphenol	$0.15 \pm 0.01$ <sup>a</sup>	0.09 ± 0.00 b	0.11 ± 0.01 °
Decanoic acid	$0.32 \pm 0.04$	$0.30 \pm 0.05$	$0.28 \pm 0.02$

 $238 \qquad \text{Wine samples were analysed in triplicate. Values represent mean} \pm \text{standard deviation. Concentrations of 2,3-butanediol}$ 

isomers were summarized. Values in rows marked by different superscript letters are significantly different at p < 0.05as tested by one-way ANOVA with Tukey's test.

#### 254 CONCLUSIONS

- Although several properties of non-*Saccharomyces* yeasts in winemaking are apparently straindependent, 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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