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

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

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Summary

The 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

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

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

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

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

124

125 **MATERIALS AND METHODS**

126 **Yeast strains and inoculum preparation**

127 *Lachancea thermotolerans* 5-1-1, *Metschnikowia pulcherrima* 11-1-7 and *Saccharomyces cerevisiae*
128 PDA W10 were from the Culture Collection of Wine Yeasts (Food Research Institute, National
129 Agricultural and Food Centre, Bratislava, Slovakia). These strains were previously isolated in
130 Slovakia and were selected based on their enzymatic potential, enological properties and results of
131 preliminary microvinification experiments (Ženíšová and Sidari, unpublished results). A loopful of
132 each strain was inoculated to 10 ml of sterile must with potassium metabisulfite (to form 20 mg·l⁻¹
133 of free SO₂) and incubated statically for 24 h at 25 °C. Then, 1 ml of the prepared culture was
134 transferred to 100 ml of sterile must with potassium metabisulfite (to form 20 mg·l⁻¹ of free SO₂)

135 and incubated statically for 24 h at 25 °C. Finally, 10 ml of this culture was transferred to 1 000 ml
136 of sterile must with potassium metabisulfite (to form 20 mg·l⁻¹ of free SO₂) and incubated with
137 shaking of 2 Hz for 24 h at 25 °C. The must used for inoculum propagation was Pinot Blanc (20
138 °Brix, pH 3.0, acidity of 9.4 g·l⁻¹).

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140 **Wine samples**

141 Mature grapes of Pinot Blanc were collected from a small vineyard in Modra, Slovakia, Small
142 Carpathian wine region (vintage 2018; collection date 18 September 2018) and processed in a
143 traditional way [10]. The must (24 °Brix, acidity of 9.4 g·l⁻¹) was decanted, divided to batches of 90
144 l and individual batches were inoculated with *L. thermotolerans*, *M. pulcherrima* or *S. cerevisiae* at
145 1 % (v/v). With a delay of 24 h, the first two batches were additionally inoculated with *S. cerevisiae*
146 at 1 % (v/v). All inocula contained 10⁶–10⁷ CFU·ml⁻¹ yeasts. Upon the onset of fermentation,
147 cooling devices were applied and fermentation took place at 16 °C. After the end of fermentation,
148 the wine was separated and filtered through a sheet filter Hobrafilt N S15N (cellulose,
149 diatomaceous earth and perlite, pore size 2 µm; Hobra – Školník, Broumov, Czech Republic).

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151 **Analytical methods**

152 Solid phase microextraction (SPME) was carried out using a polydimethylsiloxan-divinylbenzene
153 fibre, coating thickness 65 µm (Supelco, Bellefonte, Pennsylvania, USA) immersed in 10 ml of
154 wine sample mixed at 6 Hz on a magnetic stirrer during 30 min at 20 °C. The extracted compounds
155 were analysed by gas chromatography-mass spectrometry (GC-MS) using a 6890N gas
156 chromatograph (Agilent Technologies, Santa Clara, California, USA) coupled to a 5973 mass
157 spectrometric detector (Agilent Technologies). The SPME fibre was placed in the inlet of the
158 chromatograph for 2 min at 250 °C so as to desorb the entracte compounds. The gas
159 chromatographic separation took place in a high polarity polyethyleneglycol column DB-WAXetr
160 (length 30 m, inner diameter 0.25 mm, stationary phase thickness 0.5 µm; Agilent Technologies)
161 using a temperature programme of 35 °C for 1 min, 5 °C·min⁻¹ and 250 °C for 1 min. The split ratio
162 was 10:1. An average velocity of He carrier gas was 34 cm·s⁻¹ at constant flow. Ionization voltage
163 of 70 eV was used. Identification of compounds was done by comparison of mass spectra with
164 NIST 14 MS library (National Institute Standards and Technology, Gaithersburg, Maryland, USA).
165 For quantification, total ion current was detected, 4-methyl-2-pentanol was used as an internal
166 standard and individual peaks were calibrated using authentic standards (all from Sigma, St. Louis,
167 Missouri, USA). Other analyses were performed in accordance with the official methodology of
168 International Organisation of Vine and Wine [11].

169 RESULTS AND DISCUSSION

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171 Pinot Blanc wines produced using co-fermentation with *L. thermotolerans* or *M. pulcherrima* were
172 characterized and compared to that produced solely with *S. cerevisiae*. The wine samples were
173 prepared in conditions that mimicked small-scale production by traditional producers. Chemical
174 parameters of the produced wines are presented in Tab. 1, they all fell within the usual ranges of
175 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.

177 White wines are generally more acidic than red wines, while acidity gives the wine crispness on the
178 palate. Acidity of a wine is one of its attractive properties, as it improves its refreshing, crisp
179 qualities and it makes possible to combine wines successfully with certain food [10, 12].

180 Results of GC-MS analysis of aroma compounds are presented in Tab. 2. Thirty-six compounds that
181 are known to contribute to wine aroma [12–14] were identified and quantified. Concentrations of
182 almost half of them did not differ significantly between the analysed samples, including acetic acid
183 or ethyl acetate, as well as those of 2,3-butanediol and butanoic acid, which are taken as off-
184 flavours. No desirable increase was observed in wines produced with non-*Saccharomyces* yeasts
185 regarding aroma-active esters, such as ethyl hexanoate, ethyl octanoate or ethyl decanoate.
186 However, interesting was the increase in concentration of methionol, which is a common
187 component of wine and is characterized by soupy, onion or cooked vegetable flavour.
188 Concentration of furfural was decreased in both wines produced with non-*Saccharomyces* yeasts.
189 Positive effects were observed in wine produced by co-fermentation with *L. thermotolerans*
190 regarding increased concentrations of 2-phenylethanol (floral flavour), diethyl succinate (fruity
191 flavour) and phenylethyl acetate (honey flavour), while the increased concentration of 3-
192 methylbutanoic acid can be taken as detrimental as the compound is an off-flavour. Positive effects
193 were observed also in wine produced by co-fermentation with *M. pulcherrima*. These included an
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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203 **Tab. 1.** Chemical parameters of Pinot Blanc wine fermented using *S. cerevisiae* with or without
204 pre-inoculation with *L. thermotolerans* or *M. pulcherrima*.

	<i>L. thermotolerans</i> + <i>S. cerevisiae</i>	<i>M. pulcherrima</i> + <i>S. cerevisiae</i>	<i>S. cerevisiae</i>
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

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

Compound [mg·l ⁻¹]	<i>L. thermotolerans</i> + <i>S. cerevisiae</i>	<i>M. pulcherrima</i> + <i>S. cerevisiae</i>	<i>S. cerevisiae</i>
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

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

254 **CONCLUSIONS**

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

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