Effect of non-*Saccharomyces* yeast strains on 3-isobutyl-2-methoxypyrazine concentration and aroma properties in Sauvignon Blanc wines during fermentation

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Abstract

Background and Aims: 3-Isobutyl-2-methoxypyrazine (IBMP) is a compound whose aroma is reminiscent of green capsicum and is found in many winegrape cultivars, such as Cabernet Sauvignon and Sauvignon Blanc. A high concentration in grapes can lead to excessive greenness in the resulting wine products, thus reducing quality. This study sought to determine the impact of using non-*Saccharomyces* yeast during fermentation on the concentration and perception of IBMP in wines.

Methods and Results: As a potential postharvest remediation strategy, 11 strains of non-*Saccharomyces* were evaluated through fermentation of juices containing IBMP. Wines fermented with *Kazachstania servazzii, Metschnikowia pulcherrima, K. aerobia, Hanseniaspora uvarum, Meyerozyma guillermondii* and *Candida krusei* were rated with a higher level of fruitiness and less greenness in sensory analysis, even though no significant difference was observed amongst yeast treatments for IBMP concentration.

Conclusions: In mixed fermentation, in which *Saccharomyces cerevisiae* yeast strain EC1118 was sequentially inoculated, several non-*Saccharomyces* yeast strains differentially masked the perception of IBMP.

Significance of the Study: The selective use of non-*Saccharomyces* yeast may be a strategy for modulating the excessive perception of greenness in wines derived from grapes containing a high concentration of IBMP.

Keywords: *aroma masking effect, IBMP, non*-Saccharomyces, *sequential inoculation*

Introduction

Winemaking, specifically grape juice fermentation, is a complicated biochemical process involving the conversion of sugar to alcohol and the production of various secondary metabolites, in which wine yeast play significant roles and are one of the key factors determining wine quality and style. As the primary wine yeast, *Saccharomyces cerevisiae* has been thoroughly studied in terms of its ecology, physiology, biochemistry and molecular biology and how these are involved in wine production to influence wine chemistry and sensory properties (Pretorius et al. 1999, Fleet 2003, Ribéreau-Gayon et al. 2006). Specifically, it is responsible for generating yeast-derived volatiles recognised as secondary aromas, which either enhance aroma complexity and add distinctiveness to certain types of wine or else impart undesired aromas and flavours. The first property is well researched and extensively applied in winemaking in order to bring out the full potential of various grape cultivars.

It is clear that fermenting grape must contains a mixture of yeast species other than *S. cerevisiae*, whether indigenous or inoculated, and that 'wild' non-*Saccharomyces* yeasts and their impacts are typically numerous. Originally seen as spoilage yeasts responsible for producing a high concentration of negative metabolites such as volatile acids (van der Walt and van Kerken 1959, Rankine 1972), the merits of some non-*Saccharomyces* yeasts are now well recognised.

Spontaneous fermentations, which are fermentations conducted by mixed cultures of yeasts undergoing sequential dominance, have been recognised for the crucial part indigenous yeasts play in bringing out unique characteristics compared to *S. cerevisiae* (Comitini et al. 2017). Though exposed to a higher risk of spoilage, wines made in such manner are generally reported to have improved quality through better flavour integration and more complexity (Heard and Fleet 1985, Gil et al. 1996, Lema et al. 1996, Soden et al. 2000, Varela et al. 2009, Izquierdo Canas et al. 2011). Many of the enzymatic mechanisms (both desirable and undesirable) have been elucidated, and this knowledge has been used in the search for potentially useful yeasts [e.g. *Pichia anomala* possessing β-glucosidase (Charoenchai et al. 1997)]. This has led to a re-evaluation of the role of non-*Saccharomyces* during fermentation, with selected strains being used to impart a positive effect on wine quality. Beyond the contribution from non-*Saccharomyces* yeasts to improved aroma and flavour complexity, some yeast species have shown the ability to alleviate some modern winemaking issues such as excessive ethanol yield (Ciani et al. 2016). Non-*Saccharomyces* yeasts in winemaking have therefore been widely applied in deliberate inoculation for production of wine with specific aims, such as the reduction of alcohol concentration.

Deliberate inoculation of non-*Saccharomyces* strains during fermentation has been reported for species including

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Torulaspora, *Metschnikowia*, *Hanseniaspora*, *Lachancea*, *Pichia* and *Candida* (Jolly et al. 2014). *Saccharomyces cerevisiae* is normally used to complete the alcoholic fermentation, as few of the non-*Saccharomyces* ferment well, and most typically cannot finish fermentation. Therefore, the common practice of sequential inoculation is used, whereby one or more non-*Saccharomyces* strains are inoculated into the musts to initiate the fermentation, with the duration of different fermentation periods (ranging from 1 h to 15 days) allowed before inoculation of *S. cerevisiae* to complete the fermentation (Ciani and Ferraro 1998, Ferraro et al. 2000, Jolly et al. 2003).

3-Isobutyl-2-methoxypyrazine (IBMP), a potent aroma compound present naturally in winegrapes, reminiscent of capsicum, can influence wine aroma profiles at different concentration and is especially undesirable at high concentration. Remediation of this compound in grapes and wine is thereby required for improvement of wine quality. Such amelioration has been mostly achieved for grapes containing a high concentration of IBMP through viticultural practices (Marais 1994, Noble et al. 1995, Sala et al. 2004). In comparison, the available oenological methods are limited and non-specific (Pickering et al. 2006, 2014, Ryona et al. 2012, Botezatu et al. 2016), and little is known about the effect of yeast selection on wine versus juice IBMP concentration. In a preliminary study, Treloar and Howell (2006) reported the capacity of several commercial *S. cerevisiae* yeast strains to affect IBMP concentration during fermentation. These authors determined the IBMP concentration in finished Cabernet Sauvignon wines fermented by different strains, and reported a statistically significant difference amongst certain yeast treatments, namely, 4.45 ± 1.33 ng/L for Lalvin ICV D-21 and 2.92 ± 0.36 ng/L for Lalvin BM-45. The limited description of the analytical methods, however, makes it difficult to assess the robustness of the findings. Even so, the research was the first to investigate the association of wine-related yeasts and IBMP concentration in the resultant wine, and encourages further exploration to validate the findings and define the mechanisms therein. Wine yeasts, as a dominant factor determining wine quality, as well as harbouring diverse enzymes with versatile metabolic activities, could potentially contribute to IBMP remediation. Despite the little research to date on the potential impacts of various species of wine yeasts on IBMP concentration being somewhat disappointing, the topic remains worthy of investigation. Given the great metabolic versatility of non-*Saccharomyces* yeasts, this project aims to investigate the possible effects of different non-*Saccharomyces* yeast strains in sequential inoculation on IBMP concentration and the perception of IBMP.

Materials and methods

Vinification methods

Sauvignon Blanc grapes (100 kg) were harvested from a vineyard located in the Adelaide Hills wine region (34°59′S, 138°47′ E, 378 masl), when the TSS reached 23°Brix, and were transferred directly to The University of Adelaide, Waite Campus. The mean January temperature, an indication of the hotness of a region during the growing season, was 21.5°C at this vineyard site with the highest temperature reaching 41°C, and 8 days of a maximum daily temperature exceeding 30°C. The average January rainfall is 33.5 mm (2010–2020) with the 2020 January rainfall being 42.8 mm. Grapes were crushed and pressed, with the addition of 30 mg/L of SO_2 in the form of potassium metabisulfite (PMS). The juice (25 L) was then settled for 3 days at 4°C before racking off of gross lees and spiking with 52 ng/L of IBMP which was confirmed by GC analysis. Before inoculation the juice had the following basic composition: 8.2 g/L TA, pH 3.51, 23°Brix, 245.9 mg/L yeast assimilable nitrogen, 3.54 g/L malic acid, and no volatile acidity. The juice (500 mL per replicate) was distributed into 1 L fermentation vessels and each strain treatment was in triplicate (36 vessels for 11 strain treatments and 1 Control). Eleven non-*Saccharomyces* yeast strains (Table 1) were pre-cultured overnight in yeast extract-peptone-dextrose (YEPD) media at 28°C and then sub-cultured at the inoculation rate of 1×10^6 cells/mL into the starter medium [1:1 YEPD and Sauvignon Blanc juice (v/v)] and incubated overnight at 23°C, before inoculation into the juice fermentations at an inoculation rate of 5×10^6 cells/mL. Ferments were initially kept at 20°C for 5 days and then transferred to 16°C after sequential inoculation with *Saccharomyces cerevisiae* (EC1118, Lallemand, Montréal, QC, Canada), and were sampled daily to monitor sugar consumption kinetics. Fermentations were deemed finished once the sugar concentration was below 2.5 g/L, upon which 90 mg/L of SO_2 in the form of PMS was added, and wines from replicates were combined into one container with minimal headspace and cold stabilised at 4°C for 14 days. Wines were subsequently bottled in 750 mL glass bottles sealed with screw caps, with the headspace filled with nitrogen to minimise oxidation and stored at 4°C for sensory analysis. Samples from each replicate were collected at the following timepoints: (i) after non-*Saccharomyces* inoculation; and (ii) on the completion of fermentation, and were analysed for IBMP concentration by GC-MS/MS.

Follow-up trials—100 mL fermentation in sterile Sauvignon Blanc juice

Grape juice pressed from the same batch of Sauvignon Blanc juice was used for 100 mL fermentations. The IBMP was spiked into the grape juice at a concentration of 30 ng/L (confirmed by GC analysis). A lower concentration of IBMP was obtained in sterile juice compared to those used in the 500 mL scale trial (50 ng/L), mainly due to the possible binding effects and volatilisation during juice sterilisation. Differences between the trials are not important, however, since the impact on IBMP concentration was evaluated between treatments within each trial. Juice was sterilised (0.2 μm) before distribution into individual 250 mL flasks equipped with a fermentation lock and ports for aseptic sampling. Pre-cultivation of yeast strains was the same as with the 500 mL scale trial. Two inoculation rates

Table 1. List of yeast species and isolates used in this study.

Isolate	Species	Source
MF_9_W14	*Kazachstania aerobia*	Shiraz must[†]
PF_9_W21	*Aureobasidium pullulans*	Shiraz must
PF_9_W13	*Meyerozyma guillermondii*	Shiraz must
EF_7_L1	*Wickerhamomyces anomalus*	Shiraz must
PF_9_W20	*Kazachstania servazzii*	Shiraz must
EF_8_L1	*Torulaspora delbrueckii*	Shiraz must
H11_G1_2	*Hanseniaspora uvarum*	Malvasia juice[‡]
M. pulcherrima 1	*Metschnikowia pulcherrima*	NRLL/ARS
C. krusei 1	*Candida krusei*	NRLL/ARS
L. thermotolerans	*Lachancea thermotolerans*	AWMCC
S. ludwigii	*Saccharomycodes ludwigii*	AWMCC
Lalvin EC1118™	*Saccharomyces cerevisiae*	Lallemand, France

[†]Spontaneous fermentations of Shiraz grape must (Hardy's vineyard, McLaren Vale, SA, Australia; 2007). [‡]Malvasia juice (Heathcote winery, Heathcote, Vic., Australia; 2018); NRLL/ARS, NRRL Agriculture Research Service culture collection, USA; AWMCC, The Australian Wine Research Institute Wine Microorganism Culture Collection, Australia.

were applied for every strain tested, one of which was identical to the previous fermentation, and the other was six-times the original dosage (i.e. 3×10^7 cells/mL), with each treatment in triplicate. Strain EC1118 was inoculated at 5×10^6 cells/mL into all treatment groups after 5 days of fermentation by the pure culture. All fermentations were incubated with shaking (120 rpm) at 16°C. Fermentation kinetics were monitored by weighing each flask daily for CO_2 loss, and completion of alcoholic fermentation was confirmed by sugar analysis. Samples for IBMP analysis were collected at two timepoints, immediately after inoculation and at the end of fermentation.

Measurement of basic composition of the juice and wine

Titratable acidity and pH were measured by an InMotion Flex Autosampler connected with a Mettler Toledo T50 titrator (Mettler-Toledo, Port Melbourne, Vic., Australia). Alcohol concentration was determined by an Anton Paar DMA 4500 M density meter (Anton Paar Australia, North Ryde, NSW, Australia). Sulfur dioxide concentration of wine in both free and bound forms was analysed by the aspiration/titration method (Rankine and Pocock 1970). Sugar concentration was quantified by reacting with Megazyme hexokinase/glucose-6-phosphate dehydrogenase (Deltagen Australia, Kilsyth, Vic., Australia) and Megazyme phosphoglucose isomerase (Deltagen Australia) in reaction buffers and measuring the absorbance (340 nm) of the NADPH by-product from the reactions (Henniger and Mascaro Jr. 1985). Malic acid, acetic acid and glycerol were measured by HPLC analysis with undiluted final wine samples (Gardner et al. 2005). For HPLC analysis, samples were clarified by centrifugation (10 000 × *g*, 3 min) and analysed with an Aminex HPX-87H column (300 mm × 7.8 mm; Bio-Rad Laboratories, Hercules, CA, USA), and a working temperature of 60°C with 2.5 mmol/L of H_2SO_4 at a flow rate of 0.5 mL/min. A RID-10A refractive index detector (Shimadzu, Kyoto, Japan) was used for peak detection; compound determination and quantification was achieved by comparing with standards prepared in chemically defined grape juice Medium (CDGJM) (Henschke and Jiranek 1993, McBryde et al. 2006) using Delta integration software (DeltaWare Dataworks, Brisbane, Qld, Australia).

GC-MS/MS analysis of IBMP in juice and wine

3-Isobutyl-2-methoxypyrazine was determined by the method of Sanders et al. (2022).

Sensory analysis of finished wines

The aroma amongst the first batch of wines (500 mL scale) including the intensity of 'greenness' were different; therefore, a sensory study was applied to profile the aroma characters of each wine using the Rate-All-That-Apply (RATA) method (Ares et al. 2014). Since IBMP, a non-food grade chemical, was used for spiking during the winemaking process, the sensory study was constrained only to an evaluation of aroma attributes. A pilot panel ($n = 4$) was hosted prior to the formal sensory trial to characterise each sample and generate aroma descriptors for use in the RATA survey (aroma attributes used in RATA survey described in Table S1). Participants ($n = 50$; 19 males and 31 females) convened from The University of Adelaide and The Australian Wine Research Institute (AWRI) were required to rate the perceivable aroma characters with a scale of seven levels ranging from extremely low to extremely high, and scores from 0 to 7 were obtained accordingly to quantify the results for data analysis. Specifically, different types of greenness related to IBMP were summarised from the panel discussion and such categories were designated as an imperative section for the evaluation instead of an optional selection as for other attributes based on the perception of each participant. This design enabled a full profile of green character evaluation to be depicted for each wine sample to comprehensively assess the outcomes of individual yeast treatments.

Wines were kept at 4°C and were presented as 20 mL samples in four-digit coded, plastic lid-covered ISO standard wine glasses. Sensory evaluation was performed in isolated booths under sodium lights at 22–23°C. Twelve wines were presented to each participant in random order generated by RedJade software (RedJade, Martinez, CA, USA), which was also used as the survey tool during the RATA tests. Participants were required to rest for 30 s between samples and for 2 min after every four samples to avoid sensory fatigue as well as to obtain greater evaluation accuracy. Data were collected by RedJade software and analysed by XLSTAT statistical software (AddinSoft, Paris, France).

HS-SPME-GC-MS analysis of major fermentation-derived aroma profiles

Wine samples were prepared in duplicate and spiked with 10 µL of internal standard mix solutions (Table S2) in 10 mL volumetric flasks, after which tenfold dilution of wine samples was achieved by adding 0.5 mL of the mixture to 4.5 mL of Milli-Q water in a 20 mL SPME vial (Supelco). Vials were then sealed for GC-MS analysis after adding 2 g of NaCl.

For GC-MS (Wang et al. 2016), sample analysis was achieved by a Gerstel selectable 1D/2D-GC-MS system (Lasersan Australasia, Robina, Qld, Australia) using an Agilent 7890 GC equipped with a Gerstel MPS autosampler and low thermal mass (LTM) series II external column modules coupled to an Agilent 5897 mass selective detector (Agilent Technologies Australia, Mulgrave, Vic., Australia). For 1D separations, a deactivated 0.75 mm i.d. Supelco SPME inlet liner (Sigma-Aldrich, North Ryde, NSW, Australia) and J&W DB-Wax LTM column module (30 m, 0.25 mm i.d., 0.25 µm film thickness; Agilent Technologies Australia) were used. Carrier gas was provided at a constant flow of 1 mL/min with ultrapure helium (Coregas, Cavan, SA, Australia). The temperature program for the LTM module began at 40°C for 1 min, increasing to 135°C at 2°C/min, then to 212°C at 5°C/min, and finally to 250°C at 15°C/min, after which the temperature remained at 250°C for 10 min, allowing a total run time of 76 min. The transfer line was programmed at 200°C, and positive ion electron impact spectra were set at 70 eV for recording the scan runs with m/z ranging from 35 to 350.

For qualitative and quantitative analysis of major fermentation volatiles, samples were incubated at 50°C with agitation (500 rpm) for 10 min, and then extracted with a DVB/CAR/PDMS SPME fibre (50/30 µm, 1 cm, 23 gauge) at the same temperature and agitation conditions. The injection mode was splitless with desorption at 240°C for 10 min. New fibres were conditioned in the injection port for 1 h at 270°C and pre-baked for 10 min before every sample to avoid carryover. Blank runs were run routinely after every five samples. The IBMP was identified by the determination of retention indices for the DB-Wax column using a series of alkanes (C7-C40, Sigma-Aldrich), and with

the help of mass spectral library matches (NBS 75K). Compound information (CAS number, retention time, quantifier/qualifier ions) is listed in Table S3.

Quantitative analysis of 27 volatiles was achieved with available reference standards. Calibration and validation were performed with a series of duplicate addition of authentic standards into model wine solution spiked with internal standard mixture. The internal standards were selected based on chemical similarity, retention time and coefficient of determination (R^2). There were 12 points (six concentration values in duplicate) for each calibration function evenly spaced to cover 0–150% of the estimated analyte concentration in wine samples. Linearity of calibration curves was assessed from an inspection of residual plots and R^2 values.

Statistical analysis

Chemical and sensory data were processed with Microsoft Excel 2012. All data are presented as means with SD from replicates. Sensory data and volatile data from GC analysis were processed by one-way ANOVA using the statistical add-in package XLSTAT version 2020.5 (AddinSoft). Significantly different means were analysed for Pearson's type principal component analysis (PCA), and for partial least squares regression (PLS-R) analysis, using XLSTAT. The heatmap for sensory data was generated by GraphPad Prism version 9.0.0 (GraphPad, San Diego, CA, USA).

Results

Composition of grape juice and finished wines

Fermentation kinetics of 11 mixed fermentations (non-*Saccharomyces* yeast strains sequentially inoculated with *S. cerevisiae* EC1118) and a Control group (EC1118 monoculture) were monitored via sugar consumption (Figure 1). It is interesting to note that most fermentations inoculated with non-*Saccharomyces* strains, such as *Saccharomycodes ludwigii*, *Kazachstania aerobia* and *Lachancea thermotolerans*, progressed faster than EC1118 prior to sequential inoculation. Given that fermentations took place in non-sterile grape musts, it is therefore conjectured that indigenous *S. cerevisiae* from the must contributed before sequential inoculation.

Some distinctive physicochemical parameters were recorded for each treatment group (Table 2). While alcohol yield was fairly consistent ranging from 14.13 to 14.47% (v/v), different yeasts yielded a different wine acidity concentration, with the decrease in TA ranging from 1.63 to 2.33 g/L. The pH of the wines was diverse, with both increases and decreases noted, and specifically, *H. uvarum* producing the lowest pH of 3.41, while *S. ludwigii* and *M. pulcherrima* produced wines with an increased pH value of 3.67. The concentration of malic acid was 3.4 g/L in the grape musts, with EC1118 consuming only 0.19 g/L, while *M. guillermondii* consumed 0.87 g/L.

Concentration of IBMP during fermentation

The concentration of IBMP was measured by GC-MS/MS in samples collected from two time points during fermentations. Despite measurement of the initial concentration of IBMP in grape musts after spiking, the concentration differed in samples after inoculation with the non-*Saccharomyces*. Changes in concentration within each treatment were therefore calculated and used for data analysis. No significant difference was observed between any treatment and the Control (Table 3). An increased IBMP concentration was observed in the some of the finished wines, which was unexpected since some IBMP loss was anticipated through adsorption of biomass.

Delta values ($\text{Conc.}_{\text{finish}} - \text{Conc.}_{\text{day 0}}$) were used for pairwise comparisons, which were used for statistical analysis between treatments and the Control (Table 3), and between every two treatments (Table 4) to determine differences amongst yeast strains. No significant difference was observed between treatments and the Control, potentially suggesting that the 11 non-*Saccharomyces* strains in mixed

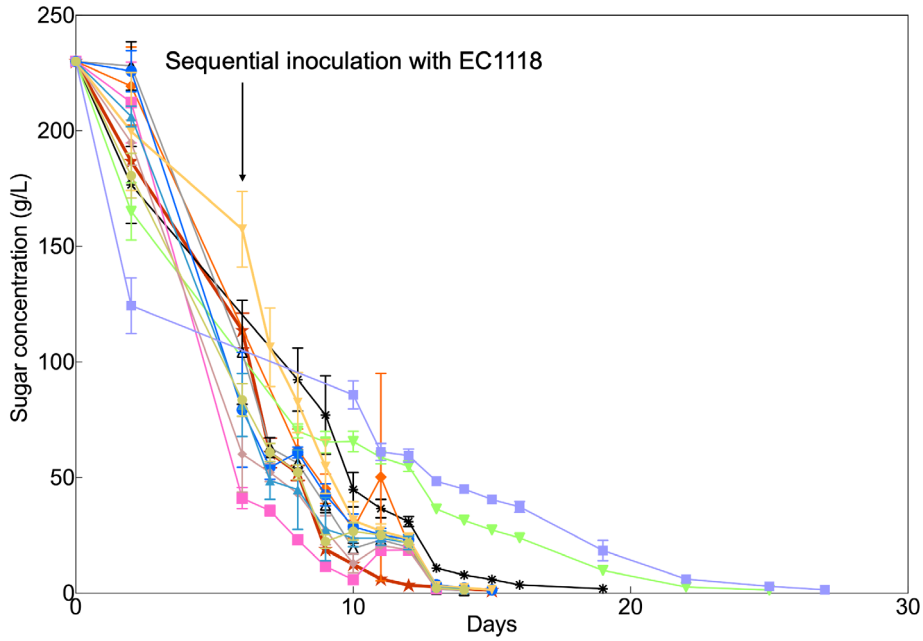

Figure 1. Fermentation kinetics of 12 yeast strains (11 mixed fermentation by non-*Saccharomyces* yeast strains sequentially inoculated with *Saccharomyces cerevisiae* EC1118, one Control group of single fermentation by EC1118 (★). Sequential inoculation of treatments occurred 5 days after initial inoculation. *Kazachstania aerobia* (●), *Aureobasidium pullulans* (■), *Hanseniaspora uvarum* (▲), *Candida krusei* (▼), *Wickerhamomyces anomalus* (◆), *K. servazzii* (●), *Saccharomycodes ludwigii* (■), *Metschnikowia pulcherrima* (△), *Lachancea thermotolerans* (▼), *Meyerozyma guillermondii* (◆), *Torulaspora delbrueckii* (✳).

Table 2. Composition of finished wines produced by co-fermentation of several non-*Saccharomyces* yeast strains and *Saccharomyces cerevisiae* (EC1118), with single-yeast fermentation by EC1118 as the Control.

Treatment	TA (g/L)	pH	Sulfur dioxide (mg/L)		Residual sugar (g/L)	Ethanol [% (v/v)]	Malic acid (g/L)	Glycerol (g/L)	Acetic acid (g/L)
			Free	Total					
Kazachstania aerobia	6.35d	3.52f	n.d.	57.6d	1.42de	14.20 g	3.17d	8.76b	0.09ef
Aureobasidium pullulans	5.87i	3.64b	n.d.	59.2c	1.45d	14.47ab	2.70 g	7.34j	0.15d
Hanseniaspora uvarum	6.21e	3.41 h	8.0a	57.6d	1.66c	14.32e	2.85f	8.31c	0.07f
Candida krusei	6.08 g	3.48 g	n.d.	57.6d	1.37e	14.46bc	3.33c	7.44 h	0.18d
Wickerhamomyces anomalus	6.44c	3.66a	0.8d	63.2b	1.28f	14.13i	3.34c	7.39i	0.43a
K. servazzii	6.41c	3.51f	0.8d	58.4d	1.42de	14.28f	3.18d	9.16a	0.08f
Saccharomycodes ludwigii	6.20ef	3.67a	4.0b	40.8 g	1.99a	14.43c	3.50a	7.01 k	0.13de
Metschnikowia pulcherrima	6.57a	3.67a	0.8d	57.6d	1.08 g	14.25f	3.46b	7.90f	0.07f
Lachancea thermotolerans	6.16f	3.61d	0.8d	52.0e	1.31f	14.40d	2.57 h	7.61 g	0.18d
Meyerozyma guillermondii	5.99 h	3.55e	0.8d	63.2b	1.91b	14.40d	2.10i	8.14d	0.08f
Torulaspora delbrueckii	6.50b	3.62 cd	1.6c	42.4f	1.86b	14.17 h	3.08e	7.32j	0.40b
Saccharomyces cerevisiae EC1118	6.48b	3.63bc	1.6c	84.0a	1.12 g	14.49a	3.34c	7.93e	0.29c

Data for each parameter are presented as mean value ($n = 3$); lower case letters indicate a significant difference within the column ($P < 0.05$) based on one-way ANOVA with least significant difference (LSD) pairwise comparison; n.d., not detected.

Table 3. Concentration of 3-isobutyl-2-methoxypyrazine in wine samples collected both at the beginning and end of alcoholic fermentation by non-*Saccharomyces* yeast over inoculated with *Saccharomyces cerevisiae* after 5 days.

Treatment	3-Isobutyl-2-methoxypyrazine concentration (ng/L)			*P*-value
	Day 0	Finish	Delta	
Kazachstania aerobia	47.80 ± 2.66	48.74 ± 0.40	0.95 ± 2.95	0.9714
Aureobasidium pullulans	47.20 ± 1.68	48.93 ± 0.41	1.73 ± 1.28	0.6590
Hanseniaspora uvarum	48.42 ± 1.39	49.40 ± 1.10	0.98 ± 1.35	0.9621
Candida krusei	50.55 ± 0.78	51.61 ± 1.48	1.06 ± 1.67	0.9982
Wickerhamomyces anomalus	47.57 ± 0.49	51.14 ± 0.66	3.57 ± 1.11	0.3294
K. servazzii	50.57 ± 2.00	51.01 ± 2.02	0.45 ± 3.97	0.1531
Saccharomycodes ludwigii	47.88 ± 0.47	50.79 ± 0.69	3.17 ± 1.21	0.7732
Metschnikowia pulcherrima	47.25 ± 0.33	50.25 ± 0.24	3.00 ± 0.39	0.4658
Lachancea thermotolerans	47.06 ± 3.83	50.19 ± 2.22	3.14 ± 5.32	0.2456
Meyerozyma guillermondii	45.44 ± 1.53	51.36 ± 1.00	5.92 ± 1.91	0.0621
Torulaspora delbrueckii	43.82 ± 3.54	51.18 ± 0.31	7.36 ± 3.84	0.2521
Saccharomyces cerevisiae EC1118	50.08 ± 2.20	51.15 ± 1.30	1.07 ± 3.49	

Data are presented as the mean of these replicates ($n = 3$) ± SD. Delta concentration ($Conc._{finish} - Conc._{day\ 0}$) is used for a paired *t*-test comparison with EC1118, with no statistical difference was identified between yeast treatment and the Control. IBMP, 3-isobutyl-2-methoxypyrazine.

fermentation with EC1118 performed similarly to EC1118 with regards to influencing the IBMP concentration during fermentation. Nevertheless, it should be acknowledged that indigenous yeasts may also have been present in the musts. Such yeasts may have influenced overall fermentation performance. Further work would be required to investigate this possibility. On the other hand, two groups of pairwise comparisons (*T. delbrueckii* and *K. aerobia*, *M. guillermondii* and *A. pullulans*) gave significantly different delta values (Table 4), indicating their putatively different impact on IBMP concentration.

Fermentations (100 mL)

An increased concentration of IBMP was observed in all groups. Student's *t*-test was performed within each yeast treatment on IBMP concentration changes ($IBMP_{initial\ conc.}$ vs $IBMP_{finish\ conc.}$) during fermentation. Based on the *P*-values (Table 5) and the delta values for each treatment (Table 3), *W. anomalus* and *M. guillermondii* were selected for a targeted investigation on the unexpected concentration increase during fermentation. Grape juice was sterilised, and the fermentation was performed at a 100 mL scale, with shaking and at the same fermentation temperature.

Fermentation kinetics were monitored via CO_2 loss (Figure 2). Although each yeast fermented the juice to dryness (residual sugar <1.1 g/L), the time required varied between strains. It took much longer for the non-*Saccharomyces* yeast strains (33 days for *W. anomalus*, 21 days for *M. guillermondii*) to finish fermentation compared to EC1118 (6 days). The concentration of IBMP (Table 6) was obtained using GC-MS/MS. Delta values (Table 7) were used for statistical analysis due to the variation in the initial concentration values. Even so, no significant difference was obtained in pairwise comparisons between either treatment or treatment versus the Control (Table 7).

Table 4. *P*-values obtained from Student's *t*-test for pairwise comparison on delta values ($Conc._{day\ 0} - Conc._{finish}$) between any two treatments.

	K. a.	*A. p.*	*H. u.*	*C. k.*	*W. a.*	*K. s.*	*S. l.*	*M. p.*	*L. t.*	*M. g.*
K. a										
A. p	0.7434									
H. u	0.9904	0.3870								
C. k	0.9667	0.6909	0.9355							
W. a	0.1674	0.1618	0.1806	0.2576						
K. s	0.8938	0.4966	0.8074	0.8478	0.2962					
S. l	0.6151	0.8578	0.5578	0.3689	0.4546	0.6753				
M. p	0.3796	0.2887	0.1129	0.1213	0.5751	0.4035	0.4668			
L. t	0.6616	0.6127	0.4793	0.6009	0.9041	0.1250	0.8263	0.9694		
M. g	0.1085	**0.0232**	0.0652	0.1357	0.0880	0.0701	0.2307	0.1574	0.3851	
T. d	**0.0260**	0.1827	0.1660	0.1417	0.1987	0.2428	0.1067	0.1925	0.4990	0.6461

P-Values in bold indicate significant differences for the 3-isobutyl-2-methoxypyrazine delta concentration between two treatments; *K. a., Kazachstania aerobia; A. p., Aureobasidium pullulans; H. u., Hanseniaspora uvarum; C. k., Candida krusei; W. a., Wickerhamomyces anomalus; K. s., Kazachstania servazzii; S. l., Saccharomycodes ludwigii; M. p., Metschnikowia pulcherrima; L. t., Lachancea thermotolerans; M. g., Meyerozyma guillermondii; T. d., Torulaspora delbrueckii*.

Table 5. *P*-values obtained from Student's *t*-test on the variation in 3-isobutyl-2-methoxypyrazine concentration within each treatment during first batch of fermentations.

Treatment	*P*-value
Kazachstania aerobia	0.5751
Aureobasidium pullulans	0.1588
Hanseniaspora uvarum	0.3915
Candida krusei	0.3322
Wickerhamomyces anomalus	**0.0017**
K. servazzii	0.7993
Saccharomycodes ludwigii	**0.0146**
Metschnikowia pulcherrima	**0.0002**
Lachancea thermotolerans	0.2871
Meyerozyma guillermondii	**0.0050**
Torulaspora delbrueckii	**0.0230**
Saccharomyces cerevisiae EC1118	0.5079

P-values in bold indicate a significant difference for variation in 3-isobutyl-2-methoxypyrazine concentration within each treatment during fermentation.

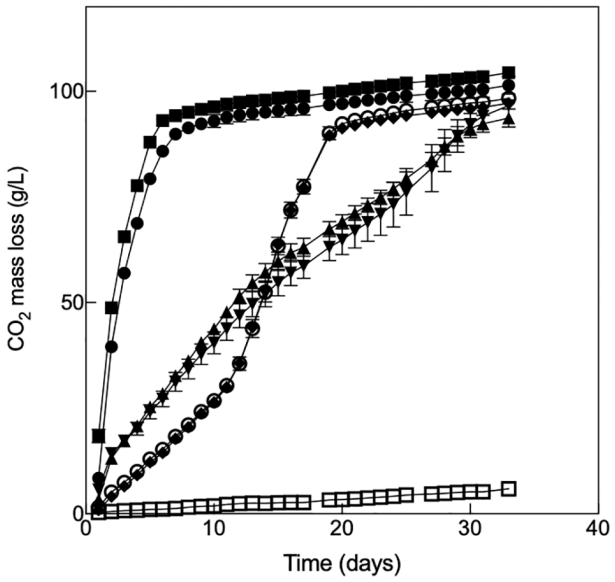

Figure 2. Fermentation kinetics of three yeast strains, determined by CO_2 mass loss. Sequential inoculation occurred 5 days after initial inoculation. 6×, the inoculation rates were six times the original dosage. EC1118 (●), EC1118 (6×) (■), *Wickerhamomyces anomalus* (▲), *W. anomalus* (6×) (▼), *Meyerozyma guillermondii* (◆), *M. guillermondii* (6×) (○), and blank (□).

Table 6. Concentration of 3-isobutyl-2-methoxypyrazine in wine samples collected both at the beginning and end of alcoholic fermentation.

	3-Isobutyl-2-methoxypyrazine concentration (ng/L)		
Treatment	**Day 0**	**Finish**	**Delta**[†]
Saccharomyces cerevisiae EC1118	29.47 ± 0.67	19.51 ± 2.00	−9.85 ± 2.92
S. cerevisiae EC1118 (6×)	29.14 ± 0.39	19.14 ± 1.09	−10.00 ± 1.02
Wickerhamomyces anomalus	30.34 ± 0.77	19.83 ± 0.80	−10.79 ± 0.05
W. anomalus (6×)	30.17 ± 0.82	19.74 ± 0.38	−10.42 ± 1.04
Meyerozyma guillermondii	29.46 ± 0.81	18.70 ± 0.97	−10.76 ± 0.23
M. guillermondii (6×)	29.33 ± 0.37	18.97 ± 0.99	−10.37 ± 0.96
Blank	29.13 ± 1.19	19.23 ± 1.62	−9.90 ± 2.64

Data are presented as the mean ± SD ($n = 3$). [†]Delta concentration: ($Conc._{finish} - Conc._{day\ 0}$).

Table 7. *P*-Value obtained from Student's *t*-test for significance check between any two groups.

	Saccharomyces cerevisiae EC1118	*S. cerevisiae* EC1118 (6×)	W. anomalus	*W. anomalus* (6×)
Wickerhamomyces anomalus	0.6960	0.3774		
W. anomalus (6×)	0.7626	0.6393		
Meyerozyma guillermondii	0.6001	0.2771	0.8869	
M. guillermondii (6×)	0.7824	0.6724	0.6015	0.9475

Sensory analysis

Differences in aroma profiles were perceived in bench-top trials amongst the first batch of wines (500 mL scale), therefore sensory analysis was undertaken to characterise wine aroma, including an evaluation of greenness for each wine using the RATA method. Even though statistical analysis of IBMP concentration in finished wines indicated there was no significant difference, informal sensory assessment suggested differences between treatments in the perceptibility of IBMP-related aromas. These wines were subjected to detailed

Table 8. Mean intensity ratings for aroma attributes evaluated in wine by rate-all-that-apply sensory analysis treatments.

	Overall aroma intensity	Vegetal†	Green capsicum†	Grassy†	Leafy†	Herbaceous†	Cooked vegetables†	Canned asparagus†	Stone fruit	Pome fruit	Banana	Floral	Confectionery	Tropical	Citrus	Boxwood	Smoky	Mineral	Solvent/ alcohol
Meyerozyma guillermondii	4.68b	2.74bcd	3.16abced	3.00ab	2.80bcde	3.02ab	2.36b	2.56bc	2.40ab	2.38abc	2.62ab	1.74abc	2.40abc	2.86ab	2.34ab	1.26c	1.02abcd	1.26ab	2.78cde
Aureobasidium pullulans	4.50bc	3.36a	3.62a	3.06a	3.16abc	3.36a	3.36a	2.94ab	1.92bc	2.18bcd	1.32d	1.16d	1.40f	2.10 cd	2.10bc	2.06a	1.28a	1.62a	2.44def
Saccharomycodes ludwigii	4.48bc	3.46a	3.34ab	3.04a	3.16abc	3.18ab	3.64a	3.10a	1.82c	2.10 cd	1.32d	1.20d	1.78def	2.06 cd	1.62c	2.14a	1.20ab	1.52ab	2.58def
Lachancea thermotolerans	4.24c	3.32a	3.52a	2.94abc	3.64a	2.82b	3.54a	3.08a	1.84c	2.00 cd	1.60 cd	1.44bcd	1.72ef	2.70ab	2.26ab	2.02a	1.14abc	1.44ab	1.94f
Kazachstania servazzii	4.54bc	2.68bcd	2.76 cde	2.64abcd	2.84bcde	2.98ab	2.00b	2.24cde	2.48a	2.76a	2.48ab	1.72abc	2.08bcde	2.72ab	2.24ab	1.38bc	0.76 cd	1.36ab	3.28bc
EC1118	4.48bc	3.20ab	3.24abcd	2.84abcd	3.34ab	3.14ab	2.46b	2.50bcd	2.30abc	2.10 cd	1.80 cd	1.52abcd	1.58ef	2.56abc	2.08bc	1.44bc	0.94abcd	1.34ab	2.58def
Metschnikowia pulcherrima	4.44bc	2.92abc	2.94bcde	2.78abcd	2.86bcde	2.94ab	1.98b	2.02de	2.54a	2.30abcd	2.68a	1.80ab	2.60ab	2.50bcd	2.64a	1.28c	0.86bcd	1.28ab	2.50def
Hanseniaspora uvarum	4.50bc	2.58 cd	2.82bcde	2.48bcd	2.76 cde	2.72b	2.38b	2.14 cde	2.40ab	2.50abc	2.66a	1.90ab	2.34abcd	2.82ab	2.02bc	1.24c	0.90abcd	1.14b	3.06bcd
Torulaspora delbrueckii	4.34bc	3.08abc	3.28abc	2.60abcd	3.04bcd	2.76b	3.34a	2.90ab	2.08abc	1.76d	1.42 cd	1.26 cd	1.88cdef	2.06 cd	2.10bc	1.88ab	0.94abcd	1.40ab	2.24ef
K. aerobia	4.34bc	2.74bcd	2.70de	2.38d	2.78cde	2.74b	2.16b	2.38cde	2.44a	2.72ab	2.88a	1.66abcd	2.70a	2.60abc	1.84bc	1.08c	0.80bcd	1.10b	3.50b
Candida krusei	4.48bc	2.70bcd	2.90bcde	2.46 cd	2.42e	2.90ab	2.34b	2.10 cde	2.32abc	2.14 cd	2.48ab	1.98a	2.34abcd	3.12a	1.88bc	1.18c	0.68d	1.10b	2.58def
Wickerhamomyces anomalus	5.44a	2.34d	2.66e	2.58abcd	2.56de	2.86ab	2.28b	1.94e	1.88c	1.94 cd	2.02bc	1.56abcd	1.92cdef	1.94d	1.68c	1.08c	0.90abcd	1.38ab	5.54a
Pr > F	<0.0001	0.000	0.002	0.094	0.001	0.306	<0.0001	<0.0001	0.014	0.014	<0.0001	0.015	<0.0001	0.000	0.012	<0.0001	0.140	0.424	<0.0001
Significant	**Yes**	**Yes**	**Yes**	No	**Yes**	No	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**	No	No	**Yes**

Data for each attribute are presented as mean values ($n = 50$); lower case letters indicate a significant difference amongst intensity ratings within the column ($P < 0.05$) based on one-way ANOVA with least significant difference (LSD) pairwise comparison. †Green characters related to 3-isobutyl-2-methoxypyrazine.

sensory analysis and promising results were obtained. Amongst the 19 aroma descriptors that were rated, a significant difference was returned for 15 of them in the 12 wines examined (Table 8). Moreover, of seven IBMP-related attributes, Vegetal, Green capsicum, Leafy, Cooked vegetables, Canned asparagus, Grassy and Herbaceous aromas, the first five were perceived as being significantly different. According to the PCA of the sensory data for the different yeast treatments, the first two factors accounted for 81.7% of the total variation, with groups with similar IBMP concentration being differentiated along F1 and green characters clustering on the right-hand side of the plot and fruity attributes on the other (Figure 3). *Aureobasidium pullulans* and *K. aerobia*, which differed in IBMP concentration by only 0.19 ng/L, were positioned on opposite sides of the PCA biplot, due to opposing aroma profiles. Wines made with *A. pullulans* were associated with a strong Cooked vegetable character, and were more closely related to other green characters compared with that of wine made with *K. aerobia*, which was more closely associated with fruity and floral attributes. *Wickerhamomyces anomalus* imparted potent solvent/alcohol aromas, which adversely affected the overall aroma intensity. The popular commercial *S. cerevisiae* strain, EC1118, gave wines with an aroma profile located more at the high end of the scale for green character.

Generally speaking, six strains, *C. krusei*, *K. aerobia*, *H. uvarum*, *K. servazzii*, *M. guillermondii* and *M. pulcherrima*, gave Sauvignon Blanc wines with more desirable fruity and floral characters, which were consequently rated with less intense green attributes.

GC-MS analysis of other fermentation volatiles

To further support the grouping patterns observed in the PCA biplot of sensory data, a range of other volatile compounds in the wines was quantified by GC/MS analysis (Table 9). A sensorially-detectable concentration was observed for 21 of the 29 volatile compounds analysed, and statistically significant differences were observed for 20 of these compounds (the exception being β-ionone).

Based on PCA analysis of the volatile compounds for yeast treatments, 55.67% of the variation was explained by the first two components, with similar clustering patterns to sensory analysis observed (Figure 4). Specifically, yeasts producing wines evaluated with higher scores of fruity and floral notes in the sensory study were clustering, and more closely linked to volatiles with desirable characters, such as 2-phenylethanol recognised as floral and rosy (Flavornet).

Discussion

Strain effects on wine composition

The concentration of ethanol and TA differed between strains. Although statistically significant, differences in ethanol production were small [i.e. at most 0.36% (v/v)]. Where wines possessed a similar ethanol concentration, it could be assumed that these represented a similar matrix for sensory analysis, and that solvent properties should not significantly influence the headspace concentration of the aroma compounds evaluated (Robinson et al. 2009, King et al. 2013, Villamor et al. 2013, Longo et al. 2017). Accordingly, any sensory differences perceived (discussed below) could be considered genuine.

In the case of TA, wine produced by *L. thermotolerans* was surprisingly low (i.e. 6.16 g/L compared to 6.57 g/L for *M. pulcherrima*). *Lachancea thermotolerans* often displays an

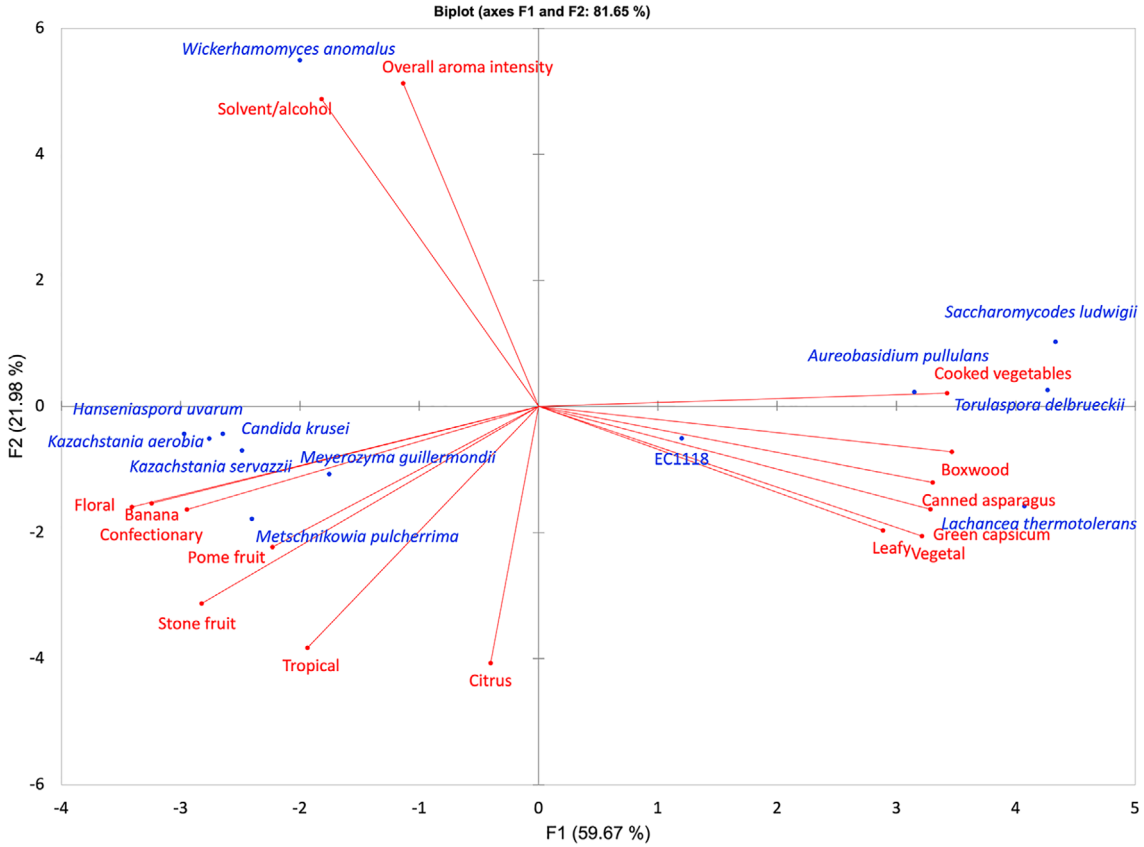

Figure 3. Principal component analysis biplot presenting scores and loadings of the standardised mean values for significant ($P < 0.05$) sensory attributes (red) and different treatments (blue).

ability to acidify wine through lactic acid production (Gobbi et al. 2013, Benito et al. 2016, Benito 2018, Hranilovic et al. 2021), but the *L. thermotolerans* used in this study failed to acidify the wine, actually consuming malic acid at a relatively high rate. Explanations may include inherent features of the strain used or involvement of other indigenous yeasts from the grape musts. Moreover, fermentation kinetics of the 12 groups (Figure 1) implied the presence of indigenous yeasts in the musts, and potentially an efficient fermenter such a *S. cerevisiae*, given that most non-*Saccharomyces* groups consumed sugar at a faster rate than the Control prior to sequential inoculation.

Although the grape musts used likely contained indigenous strains, this population would have initially been consistent across all treatments since the juices were well homogenised before use. Differences seen between treatments in physicochemical parameters, volatile compounds and sensory profiles, can therefore still be attributed to the significant inoculum of the selected non-*Saccharomyces* strain (5×10^6 cells/mL) used in each case. Our findings, though preliminary, shed light on the potential application of non-*Saccharomyces* yeasts in modulating the perception of IBMP. Further work along these lines is, however, warranted, specifically to define the contribution from both the indigenous yeasts on grapes as well as the inoculated strains. A detailed monitoring of yeast populations throughout the fermentation will help reveal the roles played by the different yeasts in the complex winemaking environment.

Interpretation of IBMP concentration

The IBMP concentration obtained at two sampling points (i.e. at the start and finish of fermentation) was unexpected. Specifically, considerable variation was observed between the IBMP concentration measured at the first timepoint; the concentration ranged from 42.78 to 52.31 ng/L across treatments, despite each fermentation being spiked at 52 ng/L. Possible explanations for these differences include analytical errors during sample preparation and processing, or rapid and differential adsorption of IBMP by the yeast inoculum. Based on the large SDs calculated for each treatment (~7.4 ng/L), it appears that analytical errors might be a significant contributor to the variation in concentration within and amongst treatments. In contrast, adsorption might also explain the decreased IBMP concentration, particularly for treatments showing small differences (e.g. *M. pulcherrima*, 46.96–47.61 ng/L for replicates at the first sampling timepoint). Hence, both factors are proposed to be involved in the variation in concentration observed at the first sampling timepoint.

An increased IBMP concentration (statistically significant difference for *W. anomalus*, *S. ludwigii*, *M. pulcherrima*, *M. guillermondii* and *T. delbrueckii*) was observed in all finished wine samples (Table 3), with potential explanations investigated. First, volume decrease due to evaporation during alcoholic fermentation could have led to such results, assuming that the absolute concentration of IBMP remained the same. Another hypothesis for an increase in IBMP concentration is production by yeasts. The precursor of IBMP, IBHP, was found in substantial quantities in harvested Cabernet Franc grapes (Ryona et al. 2010), suggesting the presence of IBHP in grape must. Additionally, putative methyltransferases, though not proven to be related to IBHP methylation, have been identified in *S. cerevisiae* (Niewmierzycka and Clarke 1999), which

Table 9. Concentration of volatile compounds measured by GC-MS in Sauvignon Blanc wines made with different treatments.

	Concentration (μg/L)											
	Candida krusei	*Wickerhamomyces anomalus*	*Metschnikowia pulcherrima*	*Meyerozyma guillermondii*	*Kazachstania aerobia*	*Lachancea thermotolerans*	*Hanseniaspora uvarum*	*Torulaspora delbrueckii*	*K. servazzii*	*Saccharomyces cerevisiae* **EC1118**	*Aureobasidium pullulans*	*Saccharomycodes ludwigii*
Ethyl esters												
Ethyl butanoate	455.8 cd	492.8bc	604.3a	463.5bcd	452.9 cd	244.9f	503.0b	320.8e	440.5d	443.8d	262.2f	275.3f
Ethyl decanoate	688.2a	649.4b	667.1ab	635.9b	514.0d	371.9 fg	569.9c	308.3 h	489.4d	457.0e	397.7f	342.4 g
Ethyl hexanoate	1368.2a	1204.6c	1235.9b	1102.8d	861.7 g	722.3 h	967.9e	442.9j	856.3 g	941.9f	302.7 k	472.1i
Ethyl lactate†	6594.5 L	9539.9i	9753.0 h	28 262.9a	11 314.9e	25 858.2b	14 672.2d	8333.8j	10 863.8f	10 055.5 g	21 242.2c	7041.4 k
Ethyl 2-phenylacetate†	1.9a	1.3b	0.9e	1.0d	0.9de	1.0c	0.8f	0.6 g	1.0 cd	0.8f	0.5 h	0.5 gh
Ethyl propanoate†	668.6d	704.3c	637.7e	670.7d	828.5b	479.8 g	722.9c	1117.5a	852.7b	555.2f	416.4 h	1113.9a
Ethyl 2-methylbutanoate	10.8a	9.7bc	9.1 cd	8.5de	8.5de	10.9a	8.2e	9.5c	8.3de	9.8bc	9.1 cd	10.4ab
Ethyl isobutyrate	376.0 cd	350.6de	354.0de	337.1e	331.8e	470.9a	352.8de	420.3b	352.4de	353.3de	423.3b	398.9bc
Ethyl isovalerate	14.6a	7.7b	7.0 cd	6.1 fg	6.4ef	6.7de	5.9 gh	7.3c	6.5e	8.0b	5.9 gh	5.6 h
Ethyl acetate	185 218.1f	644 915.8a	167 432.4 g	189 763.9e	260 989.8b	54 206.8 L	248 147.7c	106 366.4 h	231 145.0d	74 993.8j	55 765.8 k	81 258.8i
Acetate esters												
Isoamyl acetate	7453.2f	11 058.8a	10 132.2b	7631.8f	9797.4c	1121.0i	7922.1e	2889.5 h	9523.5d	4694.6 g	996.2i	454.7j
Hexyl acetate†	140.1f	277.5 cd	229.8e	338.2a	308.6b	19.3 h	359.9a	52.5 g	293.1bc	256.4d	23.7 h	18.4 h
Alcohols												
1-Butanol	1166.3 cd	1229.2bc	1088.6e	1231.2bc	1376.1a	1301.8b	1104.3de	1230.9bc	1270.1b	1290.3b	1384.6a	884.0f
1-Hexanol	841.5 h	1501.5f	1080.4 g	1460.5f	1846.7c	2183.3b	1692.2d	1706.9d	1706.8d	1642.6e	2283.6a	1612.6e
1-Propanol	56 959.9a	47 812.5f	54 104.7b	49 766.8e	52 996.2c	45 607.5 g	49 131.8e	48 167.9f	49 116.4e	50 812.2d	49 318.1e	26 875.6 h
2-Ethyl-1-hexanol†	15.6a	14.7bc	14.6bc	13.0e	15.2ab	13.4de	15.2ab	12.9e	11.6f	13.5de	14.0 cd	12.8e
2-Phenylethanol	32 201.7f	37 681.8d	52 428.6a	34 866.7e	41 423.8c	32 904.1f	29 774.7 g	42 741.8b	37 251.4d	31 712.9f	32 921.7f	13 408.6 h
3-Methyl-1-butanol	276 106.9a	234 186.8d	251 301.5b	224 669.4e	251 428.8b	253 766.0b	209 523.8f	273 692.9a	235 082.9d	218 217.3ef	247 630.6bc	239 248.6 cd
3-Octanol†	4.7c	5.7ab	1.3f	6.0a	2.4e	3.4d	5.1bc	3.8d	5.8ab	1.6f	1.1f	3.4d
4-Methyl-2-pentanol	135.0abc	125.7bcd	146.2ab	130.2bc	137.9abc	50.5e	111.6 cd	541e	51.0e	49.9e	163.2a	99.7d
Benzyl alcohol†	81 265.1a	186.2b	145.0b	137.7b	140.4b	124.9b	130.3b	130.7b	144.1b	126.0b	140.7b	121.8b
Isobutanol	33 564.5c	29 631.1d	54 171.3a	22 617.3e	11 127.1f	45 359.6b	10 670.2f	30 535.8d	10 830.4f	23 521.0e	44 127.9b	44 940.4b
Isoprenoids												
β-Damascenone	15.4ab	15.2abc	15.8a	15.8a	13.1e	14.6 cd	15.3ab	15.5a	14.7bc	15.8a	14.8bc	14.0d
1,8-Cineole	13.7b	24.1a	13.6b	13.9b	13.9b	13.7b	13.7b	13.7b	13.6b	13.7b	13.6b	13.6b
Acids												
3-Methylbutanoic acid	1808.5a	801.3b	615.0c	592.0 cd	546.7ef	604.2c	516.0 fg	614.2c	557.0de	779.7b	610.3c	503.3 g
Butanoic acid	43 996.0a	41 386.9b	40 247.6b	38 666.5c	26 404.6e	10 012.4i	31 319.1d	11 405.8 h	23 470.9f	18 068.9 g	12 214.0 h	1928.2j
Isobutyric acid	4051.7c	2497.4e	1895.4fgh	2011.6f	1681.6 gh	6453.4a	1827.3fgh	4468.1b	1626.8 h	1964.2 fg	4541.1b	3390.0d
Others												
β-Ionone	92.7ab	90.7 cd	91.7abcd	91.8abcd	90.2d	92.4abc	93.5a	91.2bcd	91.9abcd	91.0bcd	90.4d	91.4bcd

Data for each attribute are presented as mean values ($n = 3$); lower case letters indicate a significant difference amongst treatments ($P < 0.05$) based on one-way ANOVA with least significant difference (LSD) pairwise comparison. †The concentration obtained for these compounds was below the detection threshold (detection thresholds listed in Table S3).

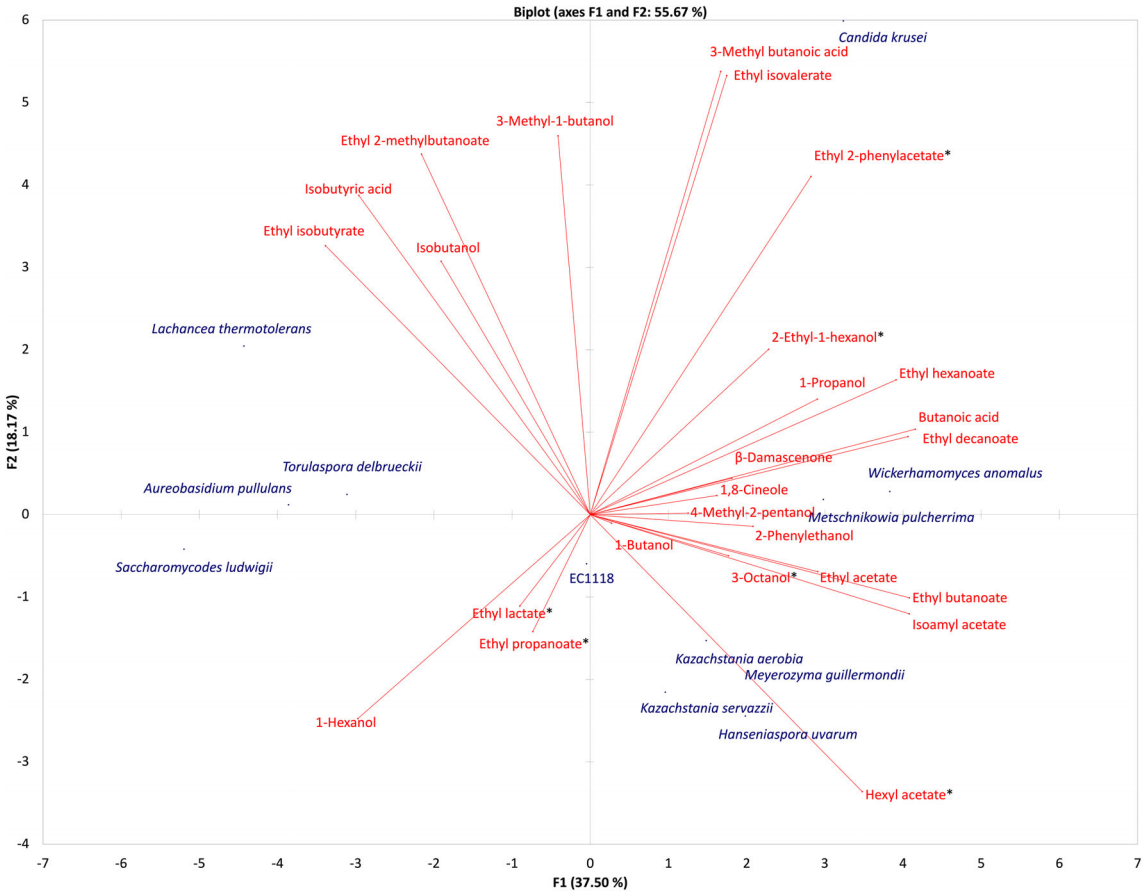

Figure 4. Principal component analysis biplot presenting scores and loadings of the standardised mean values for significant ($P < 0.05$) major volatile compounds (red) and different treatments (blue). *, concentration of these compounds was below detection thresholds.

would also be required for this hypothesis to be proven. Theoretically, however, the increase in IBMP concentration (~7.36 ng/L) observed in this experiment, accounted for only 3.13% of the IBHP pool reported for harvested Cabernet Franc grapes (Ryona et al. 2010). A similar investigation into a putative IBMP-producing ability of commercial wine yeasts during fermentation was conducted and no increase in IBMP concentration was observed (Harris 2012), which further suggests that the increase in IBMP concentration seen in the experiments reported here was not due to yeast activity.

A follow-up trial was established for further validation. Different fermentation kinetics were observed potentially due to the compositional changes of the fermentation matrices. Nevertheless, no significant difference in IBMP concentration (either increase or decrease) was observed compared to that of the Control. A decreased concentration was noticed in all groups, potentially due to evaporation by flask shaking during fermentation, which was supported by the similar extent of decline observed in the blank where no fermentation had occurred.

This follow-up experiment indicated that IBMP production by yeasts during fermentation was unlikely to occur. Therefore, the reasons for the slight increase of IBMP concentration in wines between inoculation and completion were concluded to be a result of volume loss during fermentation or analytical errors or both. Further work along these lines must therefore first secure a robust, accurate and reproducible analytical method for fermentation samples.

Sensory analysis

Despite the insignificant effects from yeast strains on IBMP concentration during fermentation, wine, as a complex matrix, comprises an array of flavour compounds that interact in a sophisticated manner to influence overall sensory quality. Therefore, an investigation of the impact of the different yeasts on wine sensory profiles was included to enable a more comprehensive evaluation of yeast performance. Diverse aroma profiles, including perception of green characters, were obtained via sensory analysis of wines from the different treatments. The PCA plot (Figure 3) and sensory data (Table 8) suggested that aroma attributes for these wines were clustered by yeast treatments, the majority of which were perceived by the RATA panel to be significantly different.

A range of green characters, typically associated with IBMP, were specifically included as descriptions that participants were asked to evaluate. Despite the non-significant differences in the IBMP concentration of these wines, a statistically significant difference was observed for five out of seven relevant attributes, amongst different treatments based on sensory analysis. The reason was proposed to be an aroma masking effect. Such an effect between fruity and vegetal attributes has been investigated in a relevant study in Cabernet Sauvignon wine (Hein et al. 2009). This work, focusing on the sensory perspective, revealed the interactive masking effect of both vegetal and fruity characters. Specifically, a decreased perception of aroma intensity for either spectrum of attributes was observed due to the addition of flavouring compounds of opposing characters. It was

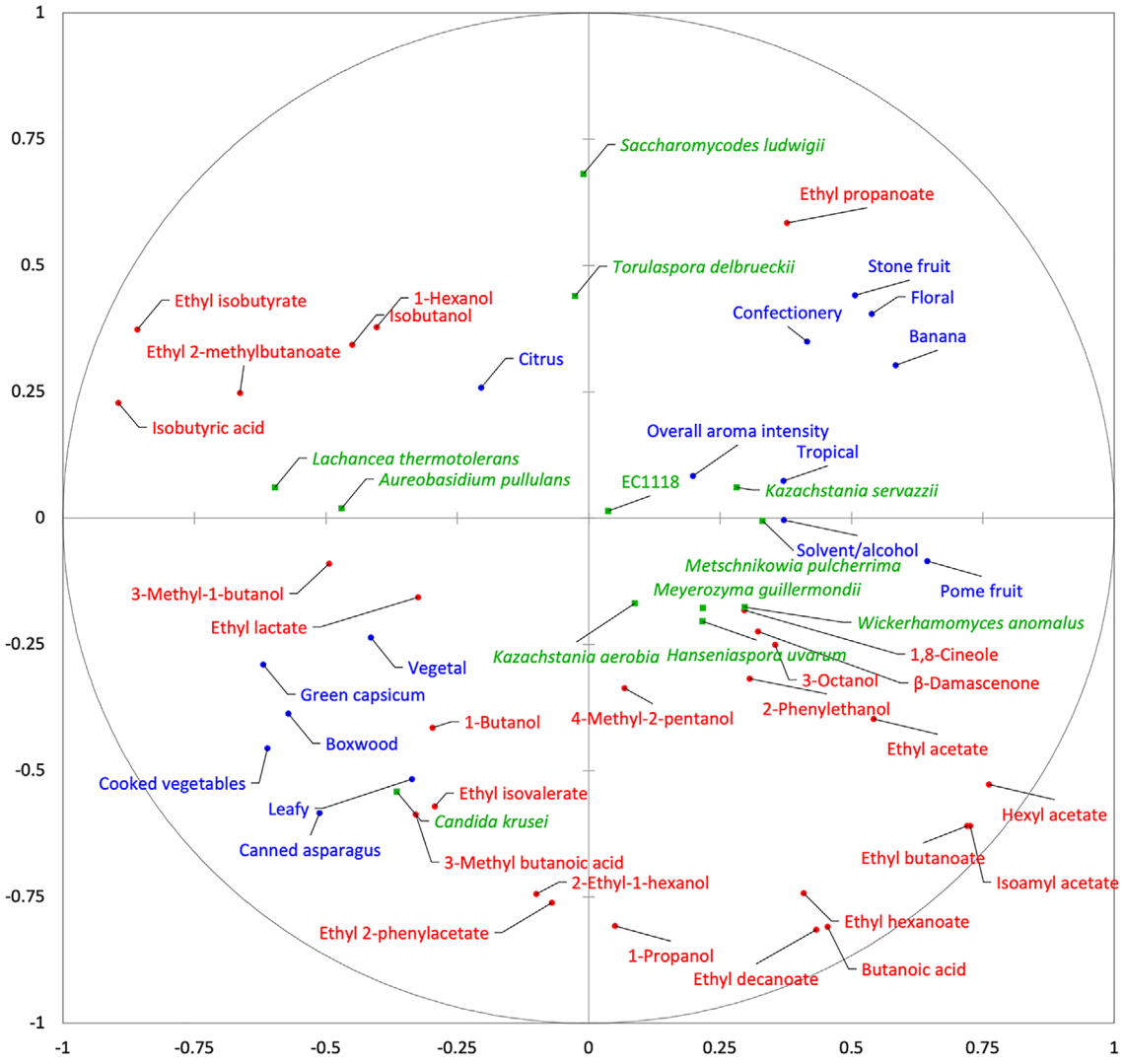

Figure 5. Partial least squares regression plot of standardised mean values for significant ($P < 0.05$) wine volatile compounds (*x*-variables, red) and standardised mean values for significant ($P < 0.05$) sensory characters (*y*-variables, blue). Treatments are shown in green. *, the concentration of these compounds was below the detection threshold.

proposed that the overall aroma profile was influenced by green characters in the presence of a high concentration of green capsicum aroma (mainly IBMP), and yet the intensity of this perception could have been diminished by elevated fruity characters. Similar sensory effects were observed in this experiment, though not in the same wine matrices. These findings form a useful starting point for future and more in-depth research.

Treatments were noticeably discriminated, with three clusters identified in the PCA plot (Figure 3), including: a group with fruit-forward aromas; one with prominent green characters; and *W. anomalus*, which was characterised by a strong solvent odour that dominated other attributes. Juice fermented using EC1118, the commercial *S. cerevisiae* strain widely used in wine industry for different grape cultivars, as the Control, resulted in wine that was classified in the vegetative cluster, with higher scores for green characters rather than fruity aromas. Juices fermented with yeasts that resulted in wines with favourable fruity aroma attributes included *C. krusei*, *K. aerobia*, *H. uvarum*, *K. servazzii*, *M. guillermondii* and *M. pulcherrima*, with the rest exhibiting more pronounced green characters. According to the PCA plot (Figure 3), greenness was perceived as fairly evident for all treatments, but at significantly different levels. Based on the non-discrimination observed for wine IBMP concentration, it was thereby reasoned that variation in the perception of green characters was largely due to different intensity of fruity aromas amongst the treatments, in agreement with the Hein et al. (2009) study.

Volatile profiles were obtained for the 12 wines to further validate the proposed aroma masking effect. Based on the partial least squares regression (PLS-R) analysis (Figure 5), wine volatile compositional data, sensory data and yeast treatments were related to investigate the underlying relationships. Such a method has been applied to evaluate multiple variables in a wine matrix (Benkwitz et al. 2012, Wang et al. 2016, Liang et al. 2018). The first two components accounted for 27.9% of volatile compounds (*x*-variables), and 13.8% of sensory data (*y*-variables). Yeasts (*K. servazzii*, *M. pulcherrima*, *K. aerobia*, *H. uvarum* and *M. guillermondii*) clustered for fruity and floral attributes in PCA analysis for sensory data (Figure 3), were observed with similar patterns in PLS-R analysis (Figure 5), more linked to the relevant aroma attributes and volatile compounds, such as 2-phenylethanol, β-damascenone and ethyl propanoate. Similar trends were

observed with the 'green-clustered' group, that is treatments evaluated with higher scores for green characters; *L. thermotolerans, T. delbrueckii, A. pullulans* and *S. ludwigii*, in both PLS-R plot (Figure 5) and PCA analysis for volatile compounds (Figure 4). Specifically, fewer fruity attributes (except citrus character) and desirable aroma compounds were linked with this cluster, indicating fewer masking effects from fruity characters. Interestingly, 1-hexanol, a C6 alcohol sensorially related to green characters in wine, was identified to be linked to the 'green-clustered' group though a concentration below the detection threshold was observed.

Analysis of other volatile compounds validated the masking effects. Specifically, the increased production of desirable aroma compounds by some yeasts explains the masking of green characters due to IBMP. For *L. thermotolerans, T. delbrueckii, A. pullulans* and *S. ludwigii*, for which pronounced green characters were perceived in the final wines, a lower production of volatiles associated with fruity/floral attributes was observed, and therefore, less effective masking. Additionally, the putative contribution of 1-hexanol to the overall perception of green character is worth further investigation. It should also be acknowledged that correlations are proposed in this experiment, and that spiking studies would be needed to further support the hypothesis.

Conclusions

As a microbiological postharvest approach to mitigating green characters in wine, this research explored the effect of 11 non-*Saccharomyces* yeasts on the concentration of IBMP and the sensory profiles of wines, following fermentation. Despite non-significant differences in IBMP concentration being observed amongst treatments, sensory analysis, coupled with instrumental analysis of the volatile compound, both indicated that yeasts producing more intense fruit notes were more closely associated with volatiles that impart pleasant aromas, rather than volatiles and sensory attributes of 'greenness'. A potential masking effect was therefore proposed whereby fruit/floral-forward characters decreased the perception of green notes.

As a pilot fermentation trial, masking of IBMP-related sensory characters was achieved and may prove to be a feasible approach for Sauvignon Blanc winemaking to mitigate the perception of greenness. As mentioned above, being non-sterile fermentations, the role of indigenous yeasts present in must needs examination, specifically in terms of the contributions of both inoculated and indigenous yeasts on aroma-masking effects. In contrast, results are likely to be highly cultivar-dependent, both in terms of the amount of IBMP present but also the diversity of aroma precursors seen across cultivars that may lead to different degrees of perception of IMBP. Further work needs to be undertaken to map the profile of yeast and cultivar interactions for the purpose of masking green characters.

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Supporting information

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Table S1. Aroma attributes used for Rate-All-That-Apply survey generated from the pilot panel.

Table S2. Qualitative information for internal standards.

Table S3. Qualitative information and method characteristics for volatile compounds determined by HS-SPME-GC-MS.