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Yeast and volatile evolution in cider co-fermentation with *Saccharomyces cerevisiae* and *Williopsis saturnus*

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Abstract This research was conducted to evaluate the impact of co-fermentation with *Saccharomyces cerevisiae* and *Williopsis saturnus* var. *mrakii* on the volatile profile of cider. Cider co-fermentation was carried out by co-inoculating *S. cerevisiae* and *W. saturnus* var. *mrakii* at a ratio of 1:100. Changes in yeast cell population, total soluble solid content (degrees Brix [°Bx]), and pH were monitored. Volatiles were analysed using headspace solid-phase microextraction/gas chromatography–mass spectrometry/flame ionization detector (HS-SPME/GC-MS-FID). A diverse group of volatiles, including fatty acids, alcohols, aldehydes, esters and ketones, were identified, among which alcohols and esters were the predominant compounds. Although most of these compounds showed similar dynamics of change, the final concentrations of some volatiles differed significantly between single-culture and co-culture fermentations. Volatiles that were indigenous to apple juice decreased during fermentation, while production of isoamyl acetate, 2-phenylethyl acetate, ethyl acetate, ethyl decanoate, ethyl dodecanoate and ethyl octanoate varied significantly among the monocultures and co-culture. Co-fermentation by *S. cerevisiae* and *W. saturnus* resulted in a more complex volatile profile, which could impact on the aromatic characteristics of cider, thus representing a novel way to modulate flavour.

Keywords Cider · Co-fermentation · Volatiles · Flavour · Yeasts

Introduction

Cider is an alcoholic beverage fermented from apple juice typically using *Saccharomyces cerevisiae* yeasts, although cider fermentation can occur spontaneously due to the presence of indigenous yeasts derived from the apple fruits or the environment. Industrially, starter cultures are used to carry out cider fermentation for better process and quality control, just like wine. The selected yeasts, commonly strains of *S. cerevisiae*, are preferred because of their reliability and their ability to induce a fermentation bouquet and to complete fermentation (Heard 1999). However, industrially produced ciders tend to lack flavor differentiation, complexity and character.

In recent years, inclusion of selected non-*Saccharomyces* yeasts as part of mixed starters together with *S. cerevisiae* in order to improve wine flavour has been suggested as a way of maximising the benefits of spontaneous fermentation without risk of spoilage (Ciani 1997; Jolly et al. 2003; Ciani and Comitini 2011; Domizio et al. 2011). The contributions of non-*Saccharomyces* yeasts include the enhancement of wine flavour due to the increased production of glycerol, esters and higher alcohols (Romano et al. 1997; Egli et al. 1998; Granchi et al. 2002; Fleet 2003).

Growth of non-*Saccharomyces* yeasts is generally limited to the first few days in spontaneous mixed-culture fermentation; they then die off gradually, and strains of *S. cerevisiae*

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become dominant until the completion of fermentation (Constanti et al. 1998). Several studies have shown that some non-*Saccharomyces* yeasts may survive longer than initially thought (Heard and Fleet 1985; Laplace et al. 1998; Zohre and Erten 2002), which would be dependent on the physiological characteristics of yeast species and strains.

The indigenous yeast profile of traditional cider fermentation is similar to that of spontaneously fermented wine, with non-*Saccharomyces* yeasts appearing initially and *Saccharomyces* yeasts taking over at a later stage (Laplace et al. 1998; Morrissey et al. 2004; Suarez et al. 2007; Pando et al. 2010). Cider fermentation with monocultures of non-*Saccharomyces* yeasts and mixed cultures has received little research attention relative to wine. Bilbao et al. (1997) studied cider fermentation with single and mixed cultures of *Kloeckera apiculata* and *S. cerevisiae* while Xu et al. (2006) investigated cider fermentation with pure and co-cultures of *Hanseniaspora valbyensis* and *S. cerevisiae*. In both reports, inhibition of non-*Saccharomyces* by *Saccharomyces* was observed, but volatile evolution during fermentation was not revealed.

Strains of *Williopsis saturnus* are high producers of fruity acetate esters and have been well characterized in mixed-culture fermentation of grape, mango, longan and papaya wines (Lee et al. 2010; Trinh et al. 2011; Li et al. 2012; Tanguler 2012). Previously, we assessed volatile changes during cider fermentation with three *Williopsis* yeasts (*W. saturnus* var. *subsufficiens* NCYC 2728, *W. saturnus* var. *saturnus* NCYC 22 and *W. saturnus* var. *mrakii* NCYC 500; Aung et al. 2014). Therefore, the present research was conducted to evaluate volatile evolution during cider co-fermentation with *S. cerevisiae* and *W. saturnus* var. *mrakii*.

Materials and methods

Yeasts and media

Williopsis saturnus var. *mrakii* NCYC 500 and *S. cerevisiae* MERIT.ferm were obtained from the National Collection of Yeast Cultures (Norwich, UK) and Chr. Hansen A/S (Hørsholm, Denmark), respectively, and were used in cider co-fermentation. The yeasts were activated in nutrient broth containing 2 % (w/v) glucose, 0.25 % (w/v) yeast extract, 0.25 % (w/v) bacteriological peptone and 0.25 % (w/v) malt extract (pH 5.0). Cultures were incubated statically at 25 °C for 48 h, then dispensed into 1-ml sterile tubes and stored at −80 °C.

Cider co-fermentation

Commercial apple juice was purchased from a local supermarket in Singapore (Marigold® 100 % apple juice; Malaysia

Dairy Industries Pte Ltd.). The juice contained 12 % total carbohydrates and 10.4 % sugar, with no added sugar, preservatives or flavouring, according to the manufacturer.

A total of 220 ml of sterile apple juice was fermented in sterile 250-ml conical flasks after inoculation with 2 % (v/v) of 10^7–10^8 CFU ml^{-1} of respective yeast culture pre-grown in the same sterile apple juice (statically at 25 °C for 2 days). Replicate fermentation for single and co-cultures was carried out for 14 days at 20 °C. Fermentation trials included a single culture of *S. cerevisiae* MERIT.ferm, a single culture of *W. saturnus* var. *mrakii* NCYC500, and a co-culture of *Saccharomyces* and non-*Saccharomyces* at a ratio of 1:100 (MERIT.ferm:NCYC500). This ratio was chosen to minimise the inhibition of the *Williopsis* yeast by the *Saccharomyces* (Lee et al. 2013).

Determination of degrees Brix (°Bx) and pH using a refractometer (ATAGO CO., LTD, Tokyo, Japan) and a pH meter (Metrohm AG, Herisau, Switzerland) was performed for each sample. Yeasts were enumerated using the spread plate method on potato dextrose agar (PDA). The two yeasts from the co-culture were differentiated by their colony appearance (the colony of strain NCYC 500 appeared dull, wrinkled and rough, while the colony of MERIT.ferm was creamy, shiny and smooth).

Analysis of volatiles

Volatiles were analysed according to a procedure that has been described elsewhere (Aung et al. 2014), by means of headspace (HS) solid-phase microextraction (SPME) using a Carboxen/polydimethylsiloxane (PDMS) fibre (85 μm; Supelco, Inc., Bellefonte, PA, USA), which was coupled with an Agilent 7890A gas chromatograph (GC)-5975 Series mass spectrometer (MS) and flame ionisation detector (FID) (Agilent Technologies Inc., Santa Clara, CA, USA). Five millilitres of samples (pre-adjusted to pH 2.5 with 1 M HCl) were tightly capped into a 20-ml glass vial, sealed with a Teflon septum, and extracted using HS-SPME at 60 °C for 30 min (stirred at 250 rpm). The fibre was then inserted into the injection port of the GC set at 250 °C for 3 min to thermally desorb analytes. A capillary column (DB-FFAP; Agilent Technologies Inc.) 60 m in length×0.25 mm internal diameter, coated with 0.25-μm-thick polyethylene glycol film (modified with nitroterephthalic acid), was used to separate volatiles. The GC oven was programmed at 50 °C for 5 min, then increased to 230 °C at 5 °C min^{-1}, and kept at this temperature for 20 min. The carrier gas was helium, with a pressure of 19.37 psi and a total flow rate of 42.7 ml min^{-1}. The mass spectra of unknown volatiles were compared with those in the Wiley database (Agilent Technologies Inc.), and identification was confirmed with respective linear retention indices. GC/MSD ChemStation software G1701EA (Agilent Technologies Inc.) was employed for data acquisition.
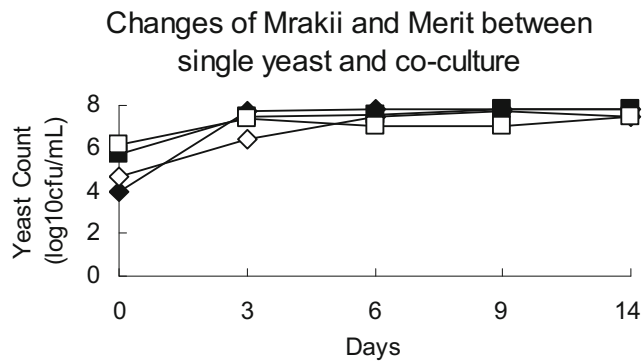
Changes of Mrakii and Merit between single yeast and co-culture

Fig. 1 Changes in yeasts during cider fermentation. ◆ and ■=single cultures of *S. cerevisiae* MERIT.ferm and *W. saturnus* var. *mrakii* NCYC 500; ◇ and □=MERIT.ferm and NCYC 500 in co-culture

Analyses were carried out in duplicate. The data shown represent the mean values from replicate fermentations.

Statistical treatment

Analysis of variance (ANOVA; Microsoft Office Excel 2003) was applied to the day-14 data to determine the differences in volatiles produced by single and mixed cultures. Results were considered significantly different if the associated *P* value was below 0.05 at a 95 % confidence level. The mean values and standard deviations were obtained from the data acquired with two independent fermentations of each treatment. Standard
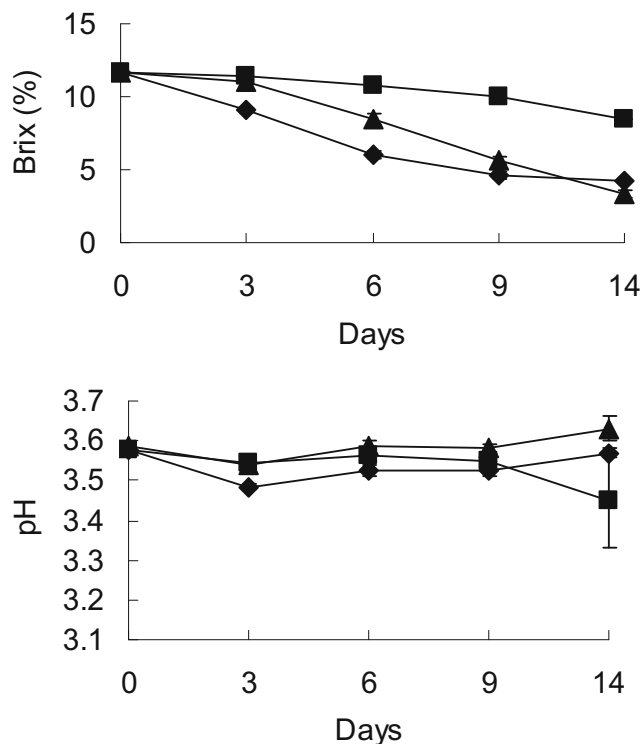
deviations expressed in this context indicate system errors (two replicate fermentations and duplicate analyses).

Results

Growth of yeasts and changes in total soluble solids and pH

Figure 1 shows the growth dynamics of *S. cerevisiae* MERIT.ferm and *W. saturnus* var. *mrakii* NCYC 500 inoculated at a ratio of 1:100 (MERIT.ferm:NCYC 500) in respective mono-culture and co-culture cider fermentation. The cell count of *W. saturnus* var. *mrakii* NCYC 500 increased by about 1 log, reaching a maximum by day 3 in both mono- and co-cultures. Its cell count in the co-culture then declined slightly from day 3, and remained consistently lower than that in the monoculture, but still remained above 10^6 CFU ml^{-1} through the end

Fig. 2 Changes in °Bx and pH during cider fermentation. ■=single *W. saturnus* var. *mrakii* NCYC 500; ▲ =co-culture (MERIT.ferm:NCYC 500=1:100); ◆=single *S. cerevisiae* MERIT.ferm
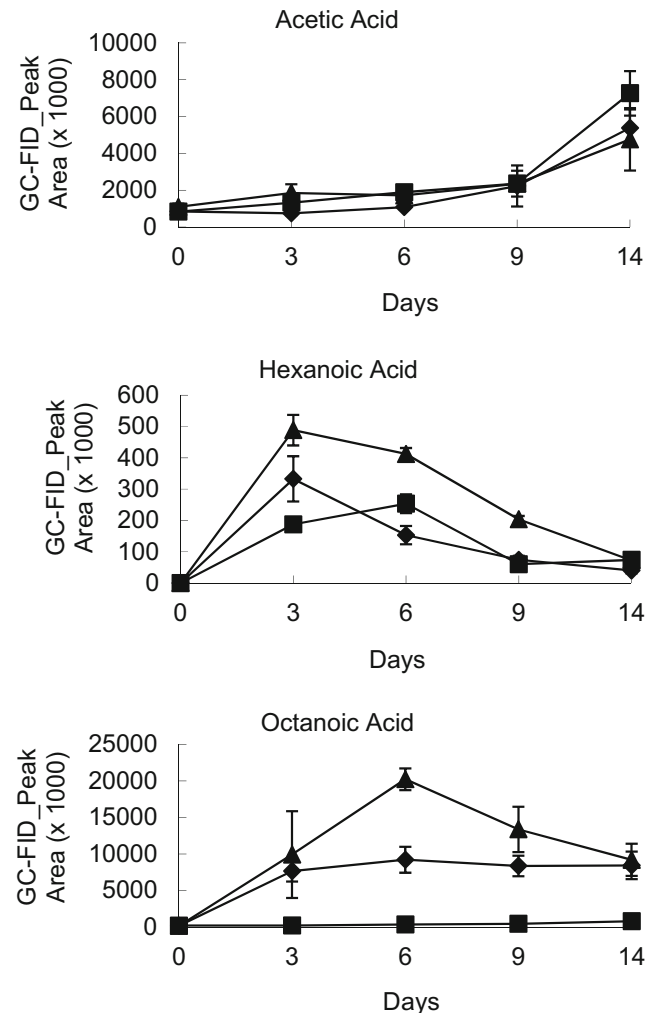

Fig. 3 Changes in acids during cider fermentation. ■=single *W. saturnus* var. *mrakii* NCYC 500; ▲ =co-culture (MERIT.ferm:NCYC 500= 1:100); ◆=single *S. cerevisiae* MERIT.ferm

of the experimental period on day 14. The cell population of
S. cerevisiae MERIT.ferm increased by approximately 3 log,
reaching a maximum by day 3 in the monoculture and by day
6 in the co-culture, and remaining above 10^7 CFU ml^{-1}
throughout the experiment in both cultures. Although the
growth of strain MERIT.ferm was delayed in the co-culture,
upon reaching a maximum count, its cell population was sim-
ilar to that in the monoculture.

Figure 2 illustrates the dynamic changes in sugars (°Bx)
and pH in the single cultures and co-culture during cider fer-
mentation. The single culture of strain MERIT.ferm had the
fastest rate of sugar consumption, with a final °Bx of 4.23. The
single culture of strain NCYC 500 had the slowest rate of
sugar consumption, with a final °Bx of 8.49, and the co-
culture had an intermediate rate, with a final °Bx of 3.28.
The profiles of pH changes for the two single cultures and
the co-culture were similar, with pH values ranging between
3.5 and 3.6, as shown in Fig. 2, with the exception of a pro-
gressive decrease in pH from day 9 onwards for the single
culture of strain NCYC 500.

Volatile evolution during fermentation of mono- and co-cultures and volatiles in final cider product

The dynamics of selected volatiles during mono- and co-
culture fermentation of apple juice are presented in Figs. 3,
4, 5, 6 and 7. The relative amounts of major volatiles in the
final cider product are shown in Table 1, and include two

acids, nine alcohols, three aldehydes, ten esters and two
ketones.

Figure 3 illustrates the evolution of acetic, hexanoic and
octanoic acids in mono- and co-cultures. Acetic acid increased
consistently in all cultures, with the co-culture containing the
lowest final level. Hexanoic acid increased initially and then
decreased in all fermentations, with similar final trace
amounts. Octanoic acid first increased and then decreased in
the co-culture, which differed from the two single cultures in
which octanoic acid increased initially, then stabilized in the
MERIT.ferm monoculture, although this acid was produced in
small amounts in the NCYC 500 monoculture throughout
fermentation.

The kinetic changes of alcohols were mostly similar in all
cultures with the final concentrations of alcohols being varied
with the alcohol and yeast culture (Figs. 4 and 5). The initially
present hexanol was produced and then was catabolised with
similar final trace amounts in all cultures. *cis*-3-Hexenol that
was indigenous to the apple juice was continuously degraded
in the MERIT.ferm single culture and co-culture, but it was
formed initially, followed by a continuous reduction in the
NCYC 500 monoculture with the highest residual content.
Ethanol, isoamyl alcohol, 2-phenylethanol and eugenol in-
creased steadily in all yeast cultures (Figs. 4 and 5). Furfuryl
alcohol initially rose sharply followed by a rapid decrease in
the MERIT.ferm monoculture and the co-culture; it was gen-
erated slowly at very low levels in the NCYC 500 single
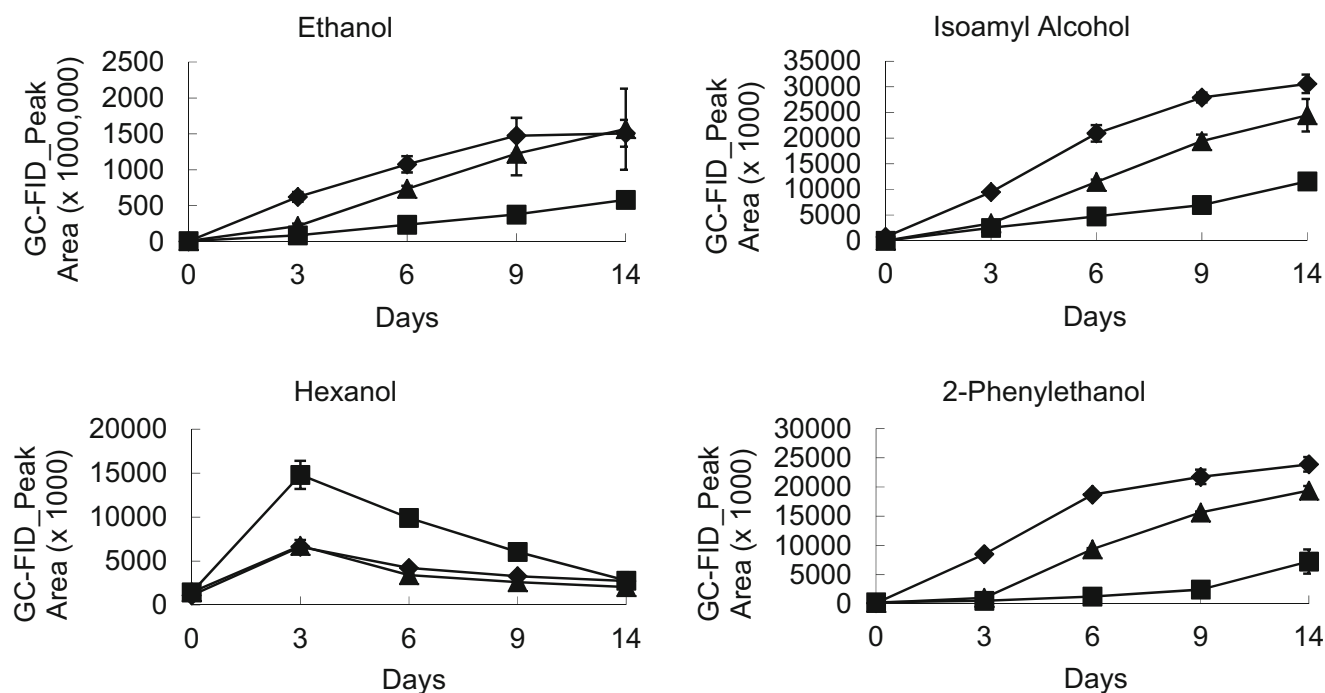culture. Ethanol, isoamyl alcohol and 2-phenethyl alcohol

Fig. 4 Changes in alcohols during cider fermentation. ■=single *W. saturnus* var. *mrakii* NCYC 500; ▲=co-culture (MERIT.ferm:NCYC 500=1:100);
◆=single *S. cerevisiae* MERIT.ferm

Fig. 5 Changes in other alcohols during cider fermentation. ■=single *W. saturnus* var. *mrakii* NCYC 500; ▲=co-culture (MERIT.ferm:NCYC 500=1:100); ◆=single *S. cerevisiae* MERIT.ferm
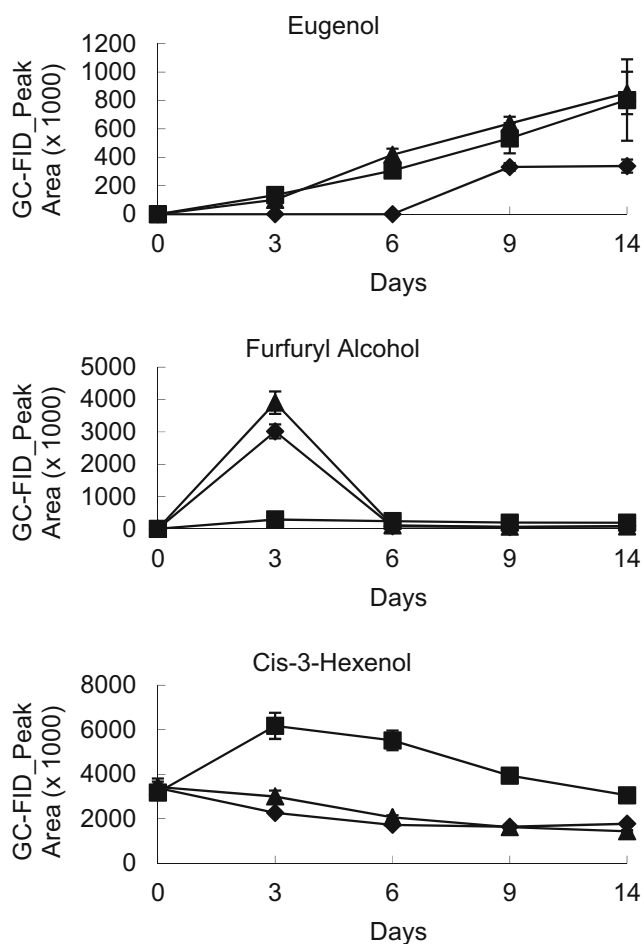
were produced mostly by the MERIT.ferm single culture, followed by the co-culture and the NCYC 500 single culture, with final levels differing significantly.

Most of the esters present in the apple juice, including hexyl acetate, ethyl butanoate, *cis*-3-hexenyl acetate, *trans*-2-hexenyl acetate and menthyl acetate, were reduced the most by the single MERIT.ferm during cider fermentation, followed by the co-culture and single NCYC 500, with the exception of *trans*-2-hexenyl acetate, which initially increased and then decreased in the co-culture and the NCYC 500 single culture (Figs. 6 and 7; Table 1). Synthesis of isoamyl acetate and 2-phenylethyl acetate was greatest with single NCYC 500, followed by the co-culture, and least by single MERIT.ferm (Fig. 6).

Ethyl acetate, ethyl octanoate and ethyl decanoate increased in all fermentations; the latter two ethyl esters declined slightly after reaching a maximum in the single MERIT.ferm and co-culture (Fig. 7). The co-culture produced an intermediate amount of ethyl acetate, between the two single cultures; it also synthesized the highest concentrations of ethyl

octanoate and ethyl decanoate. The NCYC 500 monoculture generated the highest amount of ethyl acetate but the lowest amount of ethyl octanoate and ethyl decanoate.

The final levels of aldehydes and ketones in the cider varied by culture type, with the co-culture tending to be similar to the MERIT.ferm monoculture but differing from the NCYC 500 monoculture, with exceptions such as β-damascenone (Table 1).

Discussion

This study investigated yeast and volatile evolution during cider fermentation with a pure strain of *S. cerevisiae* (MERIT.ferm), a pure strain of *W. saturnus* var. *mrakii* (NCYC 500) and a co-culture of MERIT.ferm and NCYC 500 at a ratio of 1:100. This ratio was chosen in order to avoid inhibition of the *Williopsis* yeast by the *Saccharomyces* (Lee et al. 2013). Even at this ratio, however, lower cell populations of *W. saturnus* var. *mrakii* and delayed growth of *S. cerevisiae* were observed in the co-culture (Fig. 1), indicating mutual inhibition under the current conditions. This finding is in accordance with reports that the interaction between *Saccharomyces* and non-*Saccharomyces* and their persistence during growth and fermentation are inoculum ratio-dependent (Domizio et al. 2011; Lee et al. 2013).

The ecological evolution of yeasts in spontaneous fermentation of wine and cider has been well characterized, and both share common features, with non-*Saccharomyces* yeasts initially dominant and then later overtaken over by *Saccharomyces*, presumably due to inhibition of the former by the latter as result of accumulation of high ethanol levels (Laplace et al. 1998; Morrissey et al. 2004; Suarez et al. 2007; Pando et al. 2010). In induced cider co-fermentation with non-*Saccharomyces* and *Saccharomyces*, inhibition of non-*Saccharomyces* by *Saccharomyces* has also been reported, including *Kloeckera apiculata* by *S. cerevisiae* and *Hanseniaspora valbyensis* by *S. cerevisiae* (Bilbao et al. 1997; Xu et al. 2006).

However, inhibition of *Saccharomyces* by non-*Saccharomyces* has not been noted in cider fermentation, as can be observed in Fig. 1 (retardation of growth of *S. cerevisiae* MERIT.ferm by *W. saturnus* var. *mrakii* NCYC 500), which may be attributable to mycocin (killer toxin) production by *W. saturnus* (Liu and Tsao 2009, 2010). On the other hand, multiple factors may contribute to the inhibition of non-*Saccharomyces* by *Saccharomyces*, including nutrient depletion, production of toxic substances, exhaustion of oxygen and cell-to-cell contact-mediated mechanisms (Holm Hansen et al. 2001; Nissen et al. 2003; Nissen and Arneborg 2003; Pérez-Nevado et al. 2006; Albergaria et al. 2010).

It is worth noting that the rate of sugar utilization by the co-culture was much faster than that of the monoculture of strain
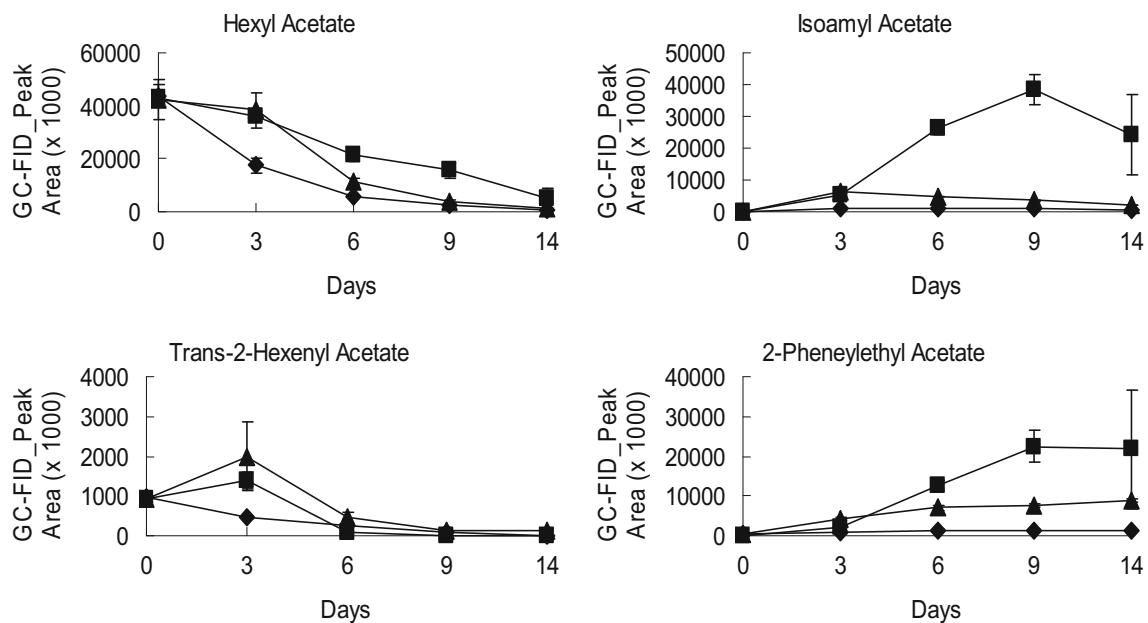
Fig. 6 Changes in acetate esters during cider fermentation. ■=single *W. saturnus* var. *mrakii* NCYC 500; ▲=co-culture (MERIT.ferm:NCYC 500= 1:100); ◆=single *S. cerevisiae* MERIT.ferm

NCYC 500, but clearly slower than that of the monoculture of strain MERIT.ferm, which corresponds to the trend of ethanol production in the respective fermentations; this pattern of sugar consumption and ethanol formation may be ascribed to the dominance of *W. saturnus* var. *mrakii* NCYC 500 during the early stage of co-fermentation (Figs. 1, 2 and 4). *Williopsis* yeasts are known to have a weaker capacity for sugar

fermentation and to produce low levels of ethanol (Erten and Campbell 2001; Lee et al. 2010; Trinh et al. 2011; Aung et al. 2014; Li et al. 2014).

Non-*Saccharomyces* wine yeasts play key roles in the synthesis of high concentrations of fermentation metabolites of oenological importance, particularly with the largest contribution of esters that enhance the characteristic fruity wine
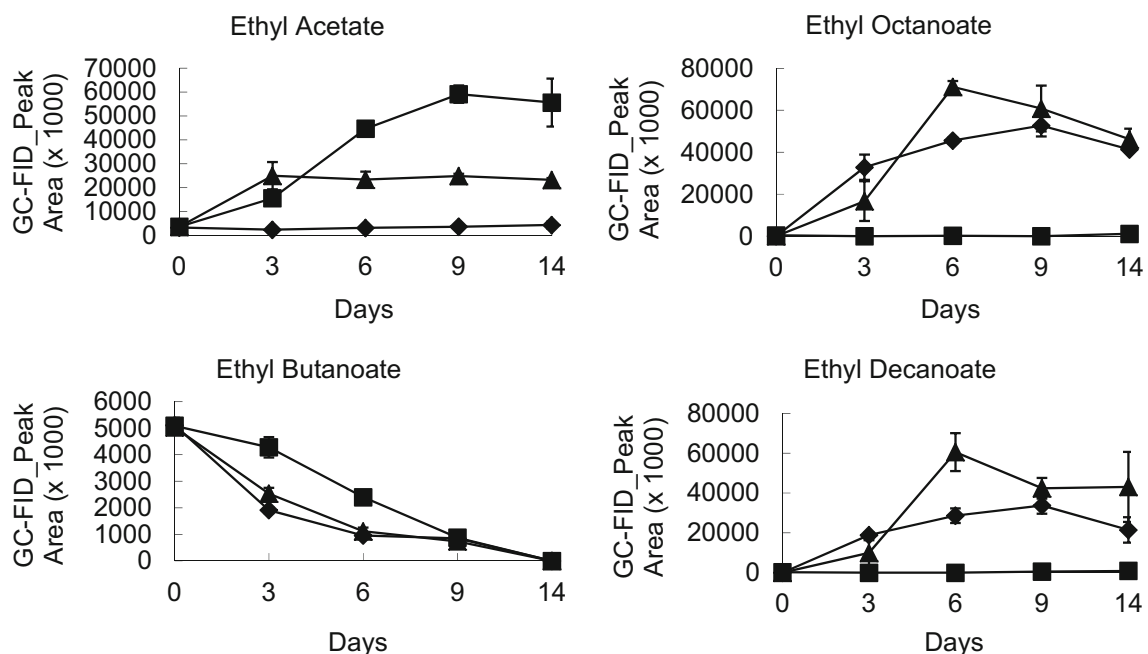

Fig. 7 Changes in ethyl esters during cider fermentation. ■=single *W. saturnus* var. *mrakii* NCYC 500; ▲=co-culture (MERIT.ferm:NCYC 500= 1:100); ◆=single *S. cerevisiae* MERIT.ferm

Table 1 Major volatiles (GC-FID peak area; mean × 10⁶±SD) identified in cider co-fermented with two single yeasts and co-culture

	Compounds	MERIT.ferm Mean±SD × 10^6	NCYC 500 Mean±SD × 10^6	Co-culture Mean±SD × 10^6	Organoleptic properties
Acid	Acetic acid	$5.38^a\pm1.00$	$7.26^a\pm1.21$	$4.76^a\pm1.70$	Vinegary
	Octanoic acid	$8.45^a\pm1.89$	$0.81^b\pm0.31$	$9.21^a\pm2.21$	Fatty, cheesy
Alcohol	Ethanol	$1507.51^a\pm186.91$	$581.66^b\pm91.03$	$1566.3^a\pm563.55$	Strongly alcoholic
	Propanol	$1.14^a\pm0.11$	$2.22^b\pm0.45$	$1.04^a\pm0.30$	Alcoholic
	Isobutyl alcohol	$3.49^a\pm0.22$	$2.55^b\pm0.19$	$3.84^a\pm1.02$	Ether-like, winey
	Isoamyl alcohol	$30.57^a\pm1.80$	$11.55^b\pm0.44$	$24.46^c\pm3.18$	Alcoholic, malty
	Hexanol	$2.74^a\pm0.82$	$2.78^a\pm0.96$	$2.04^a\pm0.16$	Green, grassy
	cis-3-Hexenol	$1.78^a\pm0.08$	$3.06^b\pm0.13$	$1.44^c\pm0.05$	Green, grassy
	Furfuryl alcohol	$0.09^a\pm0.02$	$0.19^b\pm0.004$	$0.07^a\pm0.01$	Caramel-like
	2-Phenylethanol	$23.88^a\pm1.25$	$7.26^b\pm2.07$	$19.40^c\pm0.77$	Floral, rose-like
	Eugenol	$0.34^a\pm0.05$	$0.80^b\pm0.29$	$0.85^b\pm0.15$	Clove-like, woody
Aldehyde	Hexanal	$0.12^a\pm0.02$	$0.18^b\pm0.02$	$0.09^a\pm0.01$	Fresh, green
	5-Methyl-2-furfural	$0.09^a\pm0.01$	$0.08^a\pm0.01$	$0.09^a\pm0.03$	Caramel, maple-like
	3,4-Dimethylbenzaldehyde	$2.18^a\pm0.99$	$0.97^a\pm0.26$	$6.60^b\pm4.51$	–
Ester	Ethyl acetate	$4.40^a\pm1.53$	$55.60^b\pm10.03$	$23.26^c\pm0.81$	Fruity, solvent-like
	Isoamyl acetate	$0.52^a\pm0.11$	$24.32^b\pm12.63$	$2.11^c\pm0.09$	Banana-like
	Hexyl acetate	$0.39^a\pm0.28$	$5.04^b\pm3.58$	$0.95^b\pm0.15$	Fruity, floral
	cis-3-Hexenyl acetate	$1.95^a\pm1.84$	$4.18^a\pm2.47$	$1.48^a\pm0.37$	Fruity, berry-like
	Ethyl octanoate	$41.29^a\pm1.33$	$1.15^b\pm0.49$	$46.13^a\pm5.06$	Fruity, apple-like
	Menthyl acetate	$1.17^a\pm0.28$	$2.74^b\pm0.22$	$2.13^c\pm0.34$	Fruity, minty
	Ethyl decanoate	$21.45^a\pm6.43$	$0.91^b\pm0.21$	$43.06^a\pm17.58$	Fruity, apple-like
	2-Phenylethyl acetate	$1.15^a\pm0.05$	$21.71^b\pm14.91$	$8.73^b\pm0.36$	Flowery, honey-like
	Ethyl dodecanoate	$1.33^a\pm0.36$	$0.74^a\pm0.56$	$1.46^a\pm0.21$	–
	Ethyl hexadecanoate	$2.69^a\pm1.55$	$0.17^b\pm0.01$	$1.97^a\pm1.28$	–
Ketone	Furyl methyl ketone	$0.14^a\pm0.01$	$0.19^b\pm0.03$	$0.12^a\pm0.01$	–
	β-Damascenone	$0.34^a\pm0.08$	$0.26^a\pm0.02$	$0.21^b\pm0.01$	Floral, cooked apple

[a,b,c] Statistical analysis at a 95 % confidence level, with same letter indicating no significant difference. MERIT.ferm=S. cerevisiae; NCYC 500= W. saturnus var. mrakii; Co-culture=MERIT.ferm:NCYC 500 (1:100)

fermentation bouquet (Romano et al. 2003; Ciani and Comitini 2011). However, the behaviour of non-Saccharomyces in co-fermentation would differ from that in pure cultures as a result of the interactions discussed above. In fact, volatile evolution in cider co-fermentation of S. cerevisiae and W. saturnus var. mrakii resembled or differed from that in respective single strain fermentation depending on the volatile compound (Figs. 3–7).

Acetic acid is an undesirable volatile compound in alcoholic beverages, contributing a vinegary off-flavour. In this work, the co-culture of S. cerevisiae and W. saturnus var. mrakii produced a lower amount of acetic acid compared to the respective mono-cultures, but no statistically significant differences were exhibited in the final amounts among the three fermentations. Octanoic acid was synthesized in a greater amount by the co-culture than the single NCYC 500 culture, although the co-fermentation resulted in a statistically similar amount of octanoic acid to that of the MERIT.ferm monoculture (Table 1). Strain MERIT.ferm was

likely the key contributor to octanoic acid production in the co-fermentation.

The production of higher alcohols by S. cerevisiae is generally greater than that formed by pure cultures of non-Saccharomyces and in mixed cultures (Ciani 1997; Rojas et al. 2003; Romano et al. 2003). Indeed, the major higher alcohol content (isoamyl alcohol and 2-phenylethanol) was greatest in the cider fermented by S. cerevisiae, followed by cider co-fermentation and then cider fermented with W. saturnus var. mrakii (Table 1). However, in the co-culture during cider co-fermentation, the concentration of each alcohol was affected differently, as has been reported by other researchers (Viana et al. 2009). It is noteworthy that eugenol (absent in apple juice) increased in all fermentations, especially in the co-fermentation and single fermentation of W. saturnus var. mrakii (Fig. 5). Eugenol imparts a clove and spice-like aroma, and may be released from its glucoside precursor via the action of glucosidase (Pando et al. 2012),

suggesting that *W. saturnus* var. *mrakii* might have higher glucosidase activity than *S. cerevisiae*. On the other hand, alcohols are precursors to esters, and the increased alcohol level in cider co-fermentation would be expected to raise the concentration of respective esters relative to the single fermentation of *W. saturnus* var. *mrakii*.

Low molecular weight esters, especially acetate and ethyl esters, are known to be crucial to the pleasant fruity aroma of alcoholic beverages such as cider. Non-*Saccharomyces* such as *Williopsis* and *Saccharomyces* such as *S. cerevisiae* yeasts are regarded as the principal producers of acetate and ethyl esters, respectively (Romano et al. 1997; Egli et al. 1998; Fleet 2003; Lee et al. 2010; Li et al. 2012; Tanguler 2012). Indeed, acetate ester production was significantly enhanced in the co-fermented cider compared to the *S. cerevisiae*-fermented cider; conversely, ethyl ester formation in the co-fermented cider was on par with that in the *S. cerevisiae*-fermented cider but was much higher than that in the *W. saturnus* var. *mrakii*-fermented cider (Table 1, Fig. 6 and 7).

In this study, sensory evaluation was not conducted, and this should be considered in any follow-up research. Furthermore, only volatiles that could be identified with confidence by the GC-MS system are presented in Table 1. There may well be aroma-active volatiles that were present at levels below the detection limits, in addition to volatiles that could not be identified with confidence, both of which could exert a sensory impact and should be further characterized with other appropriate methods such as solvent-assisted flavour extraction.

HS-SPME is commonly used today in volatile analysis, despite its inherent limitation in producing results prone to variation. Nonetheless, peak area differences between the two single-strain fermentations with *W. saturnus* var. *mrakii* NCYC 500 (Aung et al. 2014 and the current study) were relatively small for most volatiles, considering larger standard deviations in some cases. The main exception was acetic acid, which may be the result of batch-to-batch biological variation. For example, acetic acid can sometimes be activated to generate acetyl-CoA, which may enter the tricarboxylic acid (TCA) cycle or may be converted into various acetate esters (Ugliano and Henschke 2009; Cherry et al. 2012). This could well happen, given the comparatively aerobic nature of *Williopsis* yeasts (Erten and Campbell 2001). Indeed, the lower amount of acetic acid correlated with the higher level of acetate esters detected in our previous single fermentation with strain NCYC 500 (Aung et al. 2014) as compared with the current study.

In conclusion, incorporation of a strain of *Williopsis saturnus* into the conventional cider starter culture *S. cerevisiae* at the correct ratio may be a novel approach for modulation of cider flavour by modifying the profile of volatile compounds such as acetate esters, ethyl esters and higher alcohols.

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