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Bioflavoring by non-conventional yeasts in sequential beer fermentations

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ABSTRACT

Non-conventional yeast species have great capacity for producing diverse flavor profiles in production of alcoholic beverages, but their potential for beer brewing, in particular in consecutive fermentations with *Saccharomyces cerevisiae*, has only poorly been explored. We have screened 17 non-conventional yeast species for production of an appealing profile of flavor esters and phenolics in the first phase of alcoholic fermentation, followed by inoculation with *S. cerevisiae* to complete the fermentation. For measurement of phenolic compounds and their precursors we developed an improved and highly sensitive methodology. The results show that non-conventional yeast species possess promising potential for enhancement of desirable flavors in beer production. Notable examples are increasing isoamyl acetate (fruity, banana flavor) by application of *P. kluyverii*, augmenting ethyl phenolic compounds (spicy notes) with *Brettanomyces* species and enhancing 4-vinyl guaiacol (clove-like aroma) with *T. delbrueckii*. All *Pichia* strains also produced high levels of ethyl acetate (solvent-like flavor). This might be selectively counteracted by selection of an appropriate *S. cerevisiae* strain for the second fermentation phase, which lowers total ester profile. Hence, optimization of the process conditions and/or proper strain selection in sequentially inoculated fermentations are required to unlock the full potential for aroma improvement by the non-conventional yeast species.

1. Introduction

In spite of their great potential for flavor production, it is surprising that only few non-conventional yeast species have been evaluated in beer fermentations, which is reflected in recent reviews (Basso et al., 2016; Michel et al., 2016b). Most examples are from beers with an outspoken phenolic profile, influenced by 4-vinyl guaiacol (4VG) with “clove-like” descriptor, 4-vinyl phenol (4VP) with “phenolic” descriptor, 4-ethyl guaiacol (4EG) with “spicy”, “clove-like” and “vanilla” descriptors, and 4-ethyl phenol (4EP) with “spicy”, “smokey” and “horsy” descriptors (Callemien and Collin, 2009; Mukai et al., 2010). The 4VG derived clove-like aroma is essential for the flavor of some wheat and blond beer styles, which enjoy considerable commercial success. *Saccharomyces cerevisiae* brewing yeasts that are classified as POF+ (phenolic off-flavor positive), contain active hydroxycinnamate decarboxylase enzymes, encoded by *FDC1* and *PAD1*, that can transform *trans*-ferulic acid into 4VG and p-coumaric acid into 4VP (Mukai et al., 2010). Conventional POF + *S. cerevisiae* brewing yeasts are used in the fermentation of beers in which a clove-like aroma is desired, except for some Hefeweizen beers which are presumably fermented with *Torulaspora delbrueckii* (Tataridis et al., 2013).

The most commonly used non-conventional brewing yeast is *Brettanomyces*, which is inoculated in the fermentation of some Trappist and sour beers. It is also found in spontaneous fermentations of Gueuze and Lambic beers, in which it contributes to the typical sourness and unique complex flavor profile (Bokulich and

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Bamforth, 2013; Schifferdecker et al., 2014; Steensels et al., 2015). *Brettanomyces* yeasts contain vinyl phenol reductases that catalyze the transformation of vinyl phenols and vinyl guaiacols into the ethyl forms 4EP and 4EG (Granato et al., 2014). In contrast to wine, where high levels of 4EP are mostly associated with "medicinal" and "band aid" off-flavors, the spicy "Brett" character in beer is usually dominated by higher levels of 4EG and is considered positive for some beer styles, especially spontaneously fermented Gueuze and Lambic beers. The concentrations of 4EG and 4EP in the beer are determined by the hydroxycinnamic acid precursor levels in the fermentation media and *Brettanomyces* yeast's ability to transform the compound (Steensels et al., 2015). Some *Brettanomyces* yeasts also have high esterase activity, which is unwanted because it destroys the acetate esters which are essential for the fruity flavors of beer (Steensels et al., 2015; Verstrepen et al., 2003a). For application of candidate *Brettanomyces* strains in beer brewing, it is therefore important to evaluate the levels produced of both phenolics and acetate esters.

Non-conventional yeasts have already shown a great potential for enhancement of flavor-active esters in wine making, both in mixed or sequentially inoculated with *S. cerevisiae* (Table 1; for reviews see Ciani and Comitini, 2010; Ciani et al., 2010; Jolly et al., 2014). However, depending on the composition of the grape must, the application of non-conventional yeast in the wine fermentation can modulate the flavor profile in an unpredictable manner. For example, application of *Candida stellata*, *Torulaspora delbrueckii or Pichia anomala* has been shown to either increase or decrease the acetate ester and higher alcohol levels (Table 1). Here, we have assessed their potential for bioflavoring of beer by performing sequential inoculations with non-*Saccharomyces* and the *Saccharomyces* MauriBrew Ale 514 yeast. This approach was shown to be promising also in beer production with sequential inoculation of *Pichia kluyverii* and *S. cerevisiae* using commercially available *P. kluyverii* yeast, which is frequently used for enhancing tropical fruit aromas in Sauvignon blanc wines (Saerens and Swiegers, 2013). Previously, beer fermentations with a single and sequential inoculum of *T. delbrueckii* and *S. cerevisiae* have been evaluated under standard gravity (12°P) conditions, but the level of the important ester isoamyl acetate was negligible (≤0.01 mg/L) (Canonico et al., 2016; Michel et al., 2016a). *Saccharomycodes ludwigii* and *Zygosaccharomyces rouxii* have also been evaluated for production of low alcohol beer, but they did not produce a commercially-acceptable flavor profile in single strain fermentations (De Francesco et al., 2015).

In this study, we evaluated 17 non-conventional yeast species for production of flavor esters and phenolics, in order to possibly serve as starter cultures in sequential beer fermentations with *S. cerevisiae*. Initial observations showed that *P. kluyverii* was able to produce very high amounts of isoamyl acetate and ethyl acetate, while *P. anomala* and *P. kudriavzevii* produced extremely high levels of ethyl acetate, but low levels of isoamyl acetate. This shows that *P. kluyverii* may have unique potential for enhancing fruitiness in beer. In sequential fermentations with *P. kluyverii* (2 days) followed by *S. cerevisiae* to complete the high-gravity beer fermentation, we indeed observed a higher isoamyl acetate/isoamyl alcohol ratio although the total level of isoamyl acetate was much lower than expected. Further optimization of the process conditions and/or selection of appropriate *S. cerevisiae* strains for the second inoculation can allow to fully profit from the unusual capacity of *P. kluyverii* as starter culture to produce a highly attractive flavor profile.

Table 1
Comparison of fermentation studies performed up till now with non-*Saccharomyces* yeast co- or sequentially inoculated with *Saccharomyces* to complete the alcoholic fermentation. The studies are primarily done in wine fermentations for which the grape cultivar is indicated. CD positive, cinnamic acid decarboxylase positive; + increase in the production; − decrease in the production; = the levels were similar; n.a., information not available. A slash represents two fermentation trails in the same study. Bold indicates the genera of the yeasts.

Strain	Acetate esters	Fusel alcohols	Ethyl esters (C6-C10)	CD positive	Reference	Grape must (cultivar), media, or beer	Inoculation type
Candida *stellata*	−	=	+		Sadoudi et al. (2012)	Sauvignon Blanc	Co
stellata	+	−	+		Ciani and Ferraro (1998)	Pinot grigio	Co, Seq
stellata	+	+	+		Andorrà et al. (2010)	Macabeo	Co
Hanseniaspora *osmophilia*	+	=	−		Viana et al. (2009)	Bobal	Co
uvarum	+	+	+		Andorrà et al. (2010)	Macabeo	Co
uvarum	+	−	=		Moreira et al. (2008)	Cultivar not specified	Co
Lachancea *thermotolerans*	=	+	=		Comitini et al. (2011)	Airen	Seq
Metschnikowia *pulcherrima*	+	+	+		Rodriguez et al. (2010)	Muscat d'Alexandrie	Co, Seq
pulcherrima	+	+	+		Sadoudi et al. (2012)	Sauvignon Blanc	Co
Pichia *anomala*	+	=			Kurita (2008)	Synthetic YN medium	Co
anomala	+	+	+		Domizio et al. (2011)	Macabeo	Co
anomala	±	−	−	=/=	Izquierdo Canas et al. (2011)	Airen	Seq
fermentans	+	+	+		Clemente-Jimenez et al. (2005)	Macabeo	Co
guilliermondii	+	=			Moreira et al. (2005)	Synthetic YM medium	Co
guilliermondii	+	−	=		Moreira et al. (2008)	Cultivar not specified	Co
kluyverii	+	=	= (C6) + (C8-C10)		Saerens and Swiegers (2013)	Wheat beer	Seq
kudriavzevii		−			Kim et al. (2008)	Campbell's Early	Co
Saccharomycodes *ludwigii*	+(EA)	-Isobutanol	n.a.		Domizio et al. (2011)	Macabeo	Co
Torulaspora *delbrueckii*	−	+	−	Low	Azzolini et al. (2015)	Soave, Chardonnay	Co
delbrueckii	±	−/+	=/-	=/+	Izquierdo Canas et al. (2011)	Airen	Seq
Zygotorulaspora *florentina*	+	+	+ (C6) - (C8-C10)		Domizio et al. (2011)	Macabeo	Co

2. Materials and methods

2.1. Chemicals

All chemicals were from Sigma Aldrich. Acetonitrile and formic acid was of UV-grade, and the ethanol used for gas chromatography was GC-grade.

2.2. Preparation of malt extract medium

Malt extract medium consisting of 220 g/L of malt extract (Brewferm spraymalt 8 EBC, Brouwland, Belgium) supplemented with 0.5 mg/L $ZnSO_4$ [as commonly applied in high gravity brewing (Gibson, 2011)], was autoclaved at 110 °C for 15 min. After autoclaving, the malt extract medium was cold settled overnight and filtered through a nylon filter (GE Healthcare) to remove insoluble precipitates. The final gravity of the malt extract medium was 19.2–20.5 °P. The malt extract medium contained 98.9 g/L maltose, 17.3 g/L glucose, 6.2 mg/L ferulic acid and 2.0 mg/L coumaric acid, as well as 0–0.3 mg/L 4VG, likely formed by degradation of ferulic acid during autoclaving.

2.3. Selection of strains from non-conventional yeast species

A collection of yeasts mainly isolated from high sugar niches such as flower nectar, cacao fermentations, grape must, sugar juice, and syrup, was pre-screened for ability to grow in high sugar concentrations, in view of their possible application in high-gravity beer fermentations. The pre-screening results and methodology have been described previously (Mukherjee et al., 2017). From the 160 strains able to grow in 40% glucose and in 40% fructose, 22 strains belonging to 17 species were selected based on previous usage in diverse alcoholic fermentations (Table 1). The selected strains included seven strains from the *Pichia* genus known for high ester production in wine fermentations (Clemente-Jimenez et al., 2005; Domizio et al., 2011; Kurita, 2008; Moreira et al., 2005, 2008), including the commercialized *Pichia kluyverii* strain from Chr. Hansen A/S (Saerens and Swiegers, 2013), and four from the *Brettanomyces* genus known for its vinyl reductase activity, including the well characterized and fully sequenced wine spoilage strain *B. bruxellensis* AWRI1499 (Curtin et al., 2012). Apart from *Pichia* and *Brettanomyces* strains, several strains from other genera previously isolated from wine and spontaneous beer fermentations, or evaluated in co- and sequential wine fermentations, were included (Table 2). Strains used in this study were either obtained from the CBS-KNAW or NRRL fungal collections, Chr. Hansen A/S, The Australian Wine Research Institute (AWRI), or investigated in-house by PCR amplification and sequencing of the D1/D2 domain of large ribosomal subunit. Briefly, genomic DNA was extracted after treatment of the cells with zymolyase (purchased from Seikagaku Biobusiness, Tokyo, Japan). A single colony was suspended into 50 μL of lysis solution (3 mg zymolyase/mL ultrapure water). The solution was heated at 37 °C for 60 min, followed by 10 min at 98 °C. The variable D1/D2 domain of the large-subunit (26S) rDNA gene was amplified using primers NL-1 and NL-4 (Kurtzman and Robnett, 1998). The PCR product was purified and single read sequencing was performed by the VIB Genetic Service Facility (Antwerp, Belgium) using Applied Biosystems 3730XL DNA Analyzer (White et al., 1990). Identification was performed by a BLASTN (version 2.2.29) search against the non-redundant nucleotide collection in GenBank.

2.4. Cell cultures and inoculation by dry weight

Strains were recovered from −80 °C glycerol stocks and plated on YPD agar (10 g/L yeast extract, 20 g/L bacteriological peptone, 15 g/L agar, and 20 g/L D-glucose) and allowed to grow for 3–5 days at 25 °C. Next, strains were pre-cultured overnight in 3 mL YPD (containing 50 mg/L ampicillin and 25 mg/L kanamycin) and then 100 μL of overnight pre-culture was re-inoculated into 5 mL of YPD and cells were allowed to grow for 3 days at 25 °C. After the growth, cell cultures were maintained on ice while the dry cell weight was measured. An aliquot of 0.5 mL was filtered through a Whatman glass microfiber filter, and dried at 65 °C for a minimum of 30 min, and the dry cell weight per volume was calculated as the net weight gain of the filter. The cells (maintained on ice) were washed with malt extract media, and inoculated at a final cell density of 100 mg dry weight/L. In sequential fermentations, a second inoculation was performed after 48 h with 100 mg dry weight/L of *S. cerevisiae* brewing yeast cell counting per mg dry weight was done with the Scepter cell counter (Millipore). One milligram of dried *S. cerevisiae* MauriBrew Ale 514 yeast cells contained approximately 1.0×10^8 cells, corresponding to an inoculation rate of 1.0×10^7 cells/mL.

2.5. High-gravity beer fermentations

Small-scale high-gravity fermentations were performed at 20 °C in a total volume of 60 mL in 100 mL tall tubes [validated against 2L fermentation tubes of the European Brewery Convention (EBC)] equipped with water locks and under continuous stirring at 130 rpm. Samples (10 mL) were taken after 48 and 72 h, yeast cells and residuals were removed by centrifugation, and the samples were kept at 4 °C until analysis. Sequential fermentations, inoculated after 48 h with *S. cerevisiae* brewing yeast, were carried out in a total volume of 250 mL in 370 mL (EBC validated) tall tubes. After 72 h of stirring at 130 rpm all fermentations were left unstirred for the remaining part of the fermentation.

2.6. HPLC-UV/fluorescence detection of phenolic compounds

A reverse phase Macherey-Nagel NUCLEOSIL 100-10 C18 column with 4 mm internal diameter was used for separation on a Shimatzu LC-20AT HPLC equipped with a SIL-HT autosampler. We have first performed a two-step linear gradient of 0.1% formic acid and acetonitrile as eluent as described for separation of polyphenolic compounds (Aznar et al., 2011). This resulted in good separation of the ferulic acid and p-coumaric acid, but poor separation of 4VP and 4VG and also suffered from a long elution time for the 4EG. We therefore tested isocratic elution with higher levels of acetonitrile from 23 to 50% to enhance the separation and reduce the run time. The final method used an isocratic eluent containing 0.1% formic acid and 25% acetonitrile. This enabled good separation of *trans*-ferulic acid, p-coumaric acid, 4-vinyl phenol (4VP), 4-vinyl guaiacol (4VG), 4-ethyl phenol (4EP), and 4-ethyl guaiacol (4EG) with elution performed for 35 min. Subsequent conditioning of the column was done with 95% acetonitrile and 5% acetonitrile for 5 min each. The HPLC was coupled in parallel to an SPD-20A UV detector and an RF-10AXL fluorescence detector. The fluorescence detector gave higher signals for all phenolic compounds than the UV detector, with the strongest signal for 4VG only measured by UV detection, being out of range with fluorescence detection. On the other hand, the ferulic acid and p-coumaric acid showed poor emission between 305 and 360 nm with excitation wavelengths between 210 and 260 nm, but good UV absorbance at 280 nm. P-coumaric acid, ferulic acid and 4VG were therefore measured by the UV detector at 280 nm, while the fluorescence detector was used for 4EG and 4EP with excitation and emission wavelengths of 210 and 340nm, respectively. Standards were prepared in 100% methanol (kept at −20 °C) and spiked (1:20) into malt extract medium to

Table 2
Strains used in this study. Genera are indicated with bold. n.a., information not available.

Strain number	Species	Origin	Geo	Collection ref.
Ale 514	***Saccharomyces*** *cerevisiae*	Commercial wine strain. Ab Mauri, Australia	n.a.	
Zymaflore VL3	*cerevisiae*	Commercial wine strain. Laffort, France	France	
Bmo	***Blastobotrys*** *mokoenaii*	Soil	South Africa	x9113
Ban	***Brettanomyces*** *anomalus*	n.a.	n.a.	X9073
Bbr1	*bruxellensis*	Beer	UK	CBS 3025
Bbr2	*bruxellensis*	Wine spoilage. Isolated by AWRI, Australia	Australia	AWRI1499
Bna	*naardenensis*	Dr. Pepper soda	USA	NRRL Y-5740
Cst	***Candida*** *stellata*	Wine grapes	Germany	X9023
Cma	***Citeromyces*** *matritensis*	Beet sugar thick Juice	Belgium	ST1312/081
Dha	***Debaryomyces*** *hansenii*	Cacao fermentation	Ghana	x38
Koh	***Kodamaea*** *ohmeri*	Cacao fermentation	Ghana	x22
Lth	***Lachancea*** *thermotolerans*	Mirabelle plum conserve	Russia	x9005
Mre	***Metschnikowia*** *reukaufii*	Nectar (*Pulmonaria officinalis*)	Belgium	Y6.3K/FT11 B
Pan1	***Pichia*** *anomala*	Maple syrup	n.a.	x9015
Pan2	*anomala*	Cacao fermentation	Ivory Coast	x10
Pkl1	*kluyverii*	Cacao fermentation	Ghana	x21
Pkl2	*kluyverii*	Cacao fermentation	Ghana	x36
Pkl3	*kluyverii*	Commercial wine strain. Chr. Hansen, Denmark	New Zealand	
Pku1	*kudriavzevii*	Cacao fermentation	Ivory Coast	x12
Pku2	*kudriavzevii*	Ginger beer	West Africa	X9035
Sba	***Starmerella*** *bacillaris*	Wine	Italy	X9029
Sbo	*bombicola*	Nectar (*Helleborus foetidus*)	Belgium	V10.2Y A1
Tde	***Torulaspora*** *delbrueckii*	Beet sugar thick Juice	Belgium	ST1312/167
Zfl	***Zygotorulaspora*** *florentina*	Sulphited grape must	Italy	X9022

a final concentration of 0.3–10 mg/L. The injection volume was 100 μl. All compounds were well separated with retention times as follows: p-Coumaric acid at 8.7 min; Ferulic acid at 9.5 min; 4VP at 21.7 min; 4VG at 24.0 min; 4EP at 25.5 min and 4EG at 28.4 min. The detection of the standards spiked in malt extract medium was linear with the concentration for all compounds ($R^2 > 0.99$). For 4VP, however, the observed values in the final fermentations were unexpectedly higher than the range of the standards, which was possibly due to interference with an unknown compound eluting at the same time from the column. Therefore, we did not include the values for 4VP in the final data.

2.7. GC-FID of esters and alcohols

Fermentation samples with a volume of 2 mL were analyzed in 15 mL vials on a Thermoscience Trace GC, equipped with a ResTek Stabilowax polyethylene glycol column with 0.25 μm diameter. Split-injection (1:25), with a resting flow of 2 mL/min He, was performed after 10 min equilibration at 60 °C with a Thermoscience TriPlus RSH autosampler. The oven was kept at 40 °C for 2 min, heated to 240 °C with a ramping of 15 °C/min, and kept at 240 °C for 2 min. The detection was carried out with a flame ionization detector (FID), using 20 mL/min of N_2, 350 mL/min high grade compressed air, and 30 mL/min H_2 provided from a VWR H_2 generator. Concentrated standards were kept at −20 °C in GC grade absolute ethanol and brought to the final concentration in 5% ethanol in volumetric dilutions. Fermentation samples were kept at 4 °C prior to analysis.

2.8. HPLC-RID of maltose and ethanol

Maltose and ethanol concentrations in sequential fermentations were measured on a Waters HPLC using an Ion Exclusion Column, 7 μm, 7.8 × 300 mm. Column temperature was 75 °C, 5 mM H_2SO_4 was used as eluent with a flow rate of 1 mL/min and a Waters refractive index detector (RID) was used for detection. We have estimated the alcohol by volume content [ABV (v/v)] by the approximated formula ABV = ABW/(0.789 + 0.211*ABW). It uses the density of alcohol 0.789 g/L at 20 °C in the fraction of alcohol/water and the alcohol by weight [ABW (w/v)] determined by the HPLC measurements.

3. Results

3.1. A novel method for simultaneous detection of ferulic and coumaric acid, 4VG, 4EP and 4EG

Previous detection methods for beer phenolic compounds (Vanbeneden et al., 2006; Zhu and Cui, 2013) suffered from very long elution times (>90 min) of 4EG, incomplete separation of 4VG and 4EP, and from the inherent instability in sensitivity of electrochemical detectors. We therefore developed a novel method, which allows simultaneous detection of all four phenolic compounds with a sensitive fluorescence detector and the acid

precursors with a UV detector. The method uses acetonitrile and formic acid as eluent (Aznar et al., 2011; Shinohara et al., 2000). The best separation of the phenolic flavor compounds and their precursor acids was obtained with a 35 min isocratic flow at a concentration of 25% acetonitrile and 0.1% formic acid (Fig. 1A and B). The sensitivity was high, with the lowest concentration of 0.3 mg/L tested still being in the linear range of the standards (Fig. 1C). The fluorescence detection of 4VG was so sensitive that the signal was already saturated at 2 mg/L, but also with UV absorbance the sensitivity was high (Fig. 1B and C, and other data not shown). The linearity of the measurement as a function of the concentration was confirmed ($R^2 > 0.99$) by spiking standards into brewing malt extract medium (Fig. 1C). For 4VP, however, the observed values in the fermentations were unexpectedly high, possibly due to interference with an unknown compound eluting at the same position. Therefore, we eliminated the measurements of 4VP from the final data.

3.2. Evaluation of flavor compound production in fermentations

To assess flavor compound production, we carried out small-scale high-gravity beer fermentations (see materials and methods 2.5) with the 23 selected non-*Saccharomyces* strains, the standard brewing yeast Mauribrew Ale 514 and the Zymaflore VL3 wine yeast (used for production of fruity white wines). The fermentations were sampled after 48 and 72 h and the flavor metabolite profiles of phenolic compounds, esters and higher alcohols were determined and analyzed by phenotypic clustering (Fig. 2A and B). As expected, the *S. cerevisiae* Ale 514 brewing yeast consumed the highest amount of maltose (60 g/L) and produced 6.0% ABV after three days of fermentation. On the other hand, *Zygotorulaspora florentina* (Zfl) and *Lachancea thermotolerans* (Lth) were the most efficient non-conventional yeast strains for ethanol production in malt extract media with a production of 4.0 and 2.6% ABV, respectively. The Zfl strain even produced a slightly higher level of ethanol than the commercial *S. cerevisiae* Zymoflore VL3 wine yeast (3.6% ABV). It was the only non-conventional yeast species that consumed a significant amount of maltose (approximately 30 g/L) after three days of fermentation. The average ethanol production in the case of non-*Saccharomyces* yeasts was 1.4% ABV, with low consumption of maltose. Interestingly, the *Brettanomyces* (Bbr1, Bbr2 and Bna) and the *P. kluyverii* (Pkl2 and Pkl3) strains, which showed to be phenotypic outliers for production of flavor compounds, produced very low levels of ethanol (0.2–0.4% ABV), with residual glucose in the fermentations, even after three days.

3.2.1. Acetate esters

Production of ethyl acetate, generally considered as an off-flavor due to its "solvent-like" aroma, remained for most species and strains clearly below the sensory threshold of 21–30 mg/L in beer (Meilgaard, 1982). Exceptions were *T. delbrueckii* and *B. naardenensis* (71.5 and 24.3 mg/L after 72 h, respectively) and especially the *Pichia* strains, which produced very high levels in the range of 41–171 mg/L and 88–924 mg/L after 48 and 72 h, respectively (Fig. 2A and B). *P. anomala* and *P. kudriavzevii* strains differed from the other species in producing high levels only of ethyl acetate (110–249 mg/L after 72 h) without clear increases in the other acetate esters. The *Pichia* species were actually standing out in producing high levels of all acetate esters (Fig. 2 and Supplementary Fig. 1). *P. kluyverii* was unique among the *Pichia* species in producing also high levels of the beneficial flavor compound isoamyl acetate ("banana" flavor) (9.7–11.6 mg/L isoamyl acetate, after 48 h). The commercial Pkl3 *P. kluyverii* outperformed all other strains in this respect with 42.6 mg/L isoamyl acetate produced after 72 h (Fig. 2B). The isoamyl and ethyl acetate (924 mg/L) levels produced by the commercial Pkl3 *P. kluyverii* strain are 19 and 31 times higher than those of the *S. cerevisiae* brewing yeast (2.2 mg/L isoamyl acetate and 30.1 mg/L ethyl acetate). They are in the same range as those obtained with the commercial CMBS33 *S. pastorianus* lager strain with constitutive overexpression of *ATF1* (49 mg/L isoamyl acetate and 273 mg/L ethyl acetate) (Verstrepen et al., 2003b).

3.2.2. Fusel alcohols

The only yeasts that were able to form isoamyl alcohol above its sensory threshold of 70 mg/L (Meilgaard, 1975) were *Z. florentina* (Zfl) and *P. kudriavzevii* Pku1. All the other strains displayed a range between 1.5 and 59.6 mg/L after three days (Supplementary Data 1 and Fig. 2). The three *P. kluyverii* yeasts showed low to intermediate levels (11.6–33.1 mg/L), indicating that the high production of isoamyl acetate in *P. kluyverii* is not directly linked to the level of its precursor alcohol, and that the naturally occurring AATase(s) in *P. kluyverii* are highly active and/or expressed at high levels compared to other yeast species.

3.2.3. Ethyl esters

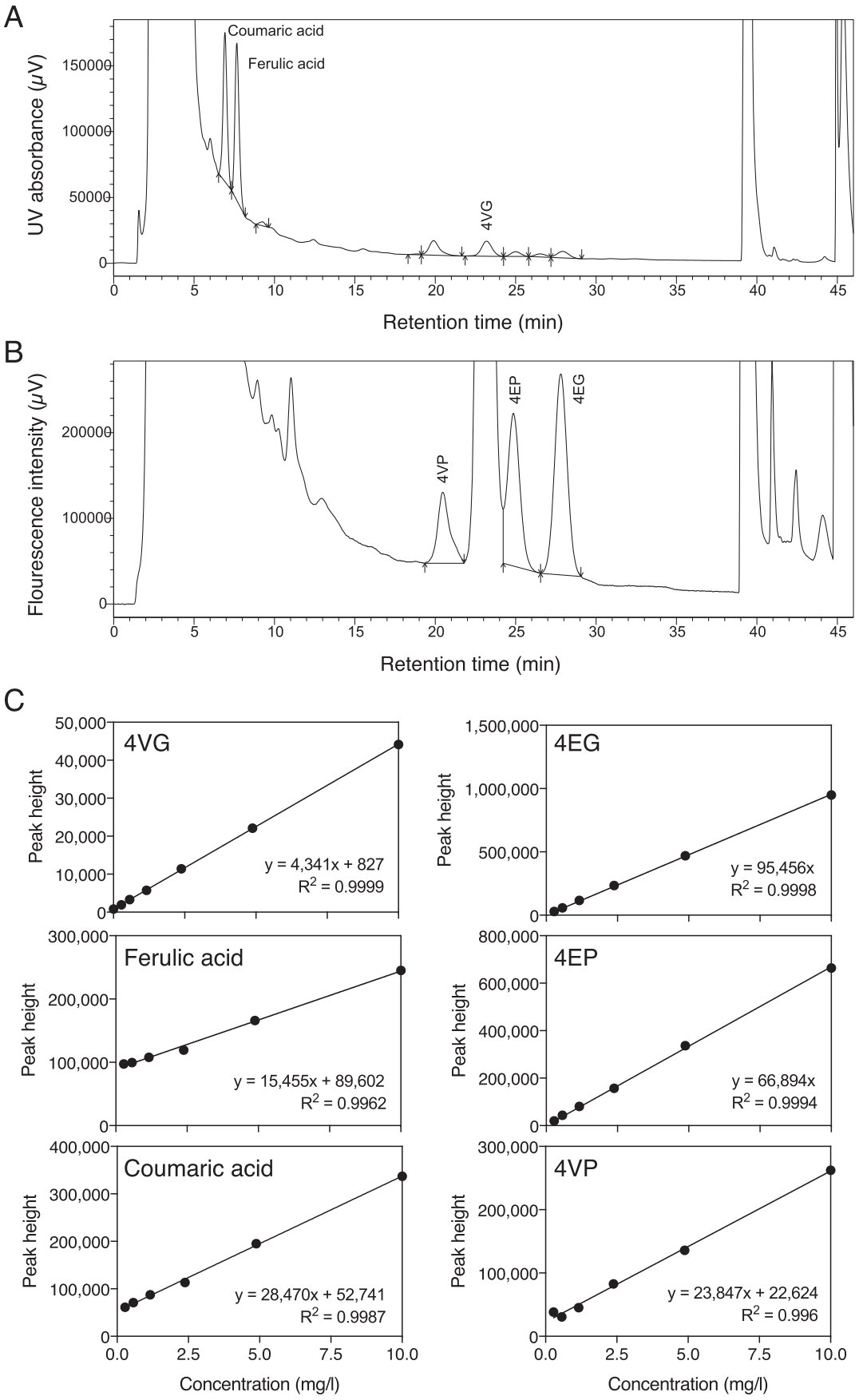
None of the non-conventional yeast strains were able to produce ethyl esters above the minimum threshold of sensory perception (Fig. 2 and Supplementary Data 1). Only the *S. cerevisiae* ale yeast was able to produce ethyl hexanoate (fruity "apple" flavor) at a level (0.60 mg/L), significantly exceeding the sensory threshold of 0.21–0.23 mg/L (Meilgaard, 1975). The production of ethyl esters was correlated to ethanol production (Supplementary Fig. 1), suggesting that the ability to form ethyl esters is somehow linked to alcoholic fermentation capacity.

3.2.4. 4VG

For all non-conventional yeast strains, production of the phenolic compounds was correlated to the capacity to transform hydroxycinnamic acids present in the malt extract medium (Fig. 2A and B). The Mauribrew Ale 514 strain was POF-, while *T. delbrueckii* and the wine yeast VL3 were POF+, transforming almost all ferulic acid into 4VG in 72 h. *C. stellata*, *Z. florentina*, and the two *P. anomala* strains were also POF+, but with intermediate levels of 4VG (Cst, 1.77 mg/L; Zfl, 1.78 mg/L; Pan1, 1.89 mg/L; Pan2, 2.89 mg/L). The *P. kluyverii* and *P. kudriavzevii* strains were POF- and showed no production of 4VG even after 72 h of fermentation.

3.2.5. 4EP and 4EG

Significant transformation of coumaric and ferulic acid into 4EP and 4EG were observed in fermentations with *Brettanomyces* strains (0.12–0.22 mg/L 4EP and 0.79–1.22 mg/L 4EG after 48 h, and 0.38–0.54 mg/L 4EP and 1.50–2.75 mg/L 4EG after 72 h), except for the *B. naardenensis* Bna strain which seemed to be POF-. The fermentations with *Brettanomyces* strains (Bbr1, Bbr2 and Ban) did not contain any detectable levels of 4VG after 48 h of fermentation, indicating that all 4VG was transformed into 4EG. The levels of ferulic and coumaric acids decreased further after 72 h, with an increase in 4VG for Ban (0.39 mg/L) and Bbr1 (0.00 and 1.65 mg/L in the fermentation replicates, respectively), while the level of 4EG strongly increased up to 2.75 mg/L. The concentrations of 4EP and 4EG in fermentations with Bbr1, Bbr2 and Ban, are all above the sensory thresholds of 0.10 mg/L for 4EP and 0.13 mg/L for 4EG, respectively (Supplementary Data 1 (Callemien and Collin, 2009)). The appearance of 4VG after 72 h suggests a two-step process, in which the bottlenecks are the decarboxylation of ferulic acid into 4VG and the subsequent reduction of 4VG into 4EG. The Ban and Bbr1 strains showed efficient conversion of ferulic acid into 4EG compared to the *B. bruxellensis* Bbr2 wine spoilage yeast, making these good candidate strains for beers in which spicy phenolic


A
Coumaric acid
Ferulic acid
4VG
UV absorbance (μV)
Retention time (min)
B
4VP
4EP
4EG
Flourescence intensity (μV)
Retention time (min)
C
4VG
y = 4,341x + 827
R2 = 0.9999
4EG
y = 95,456x
R2 = 0.9998
Ferulic acid
y = 15,455x + 89,602
R2 = 0.9962
4EP
y = 66,894x
R2 = 0.9994
Coumaric acid
y = 28,470x + 52,741
R2 = 0.9987
4VP
y = 23,847x + 22,624
R2 = 0.996
Peak height
Concentration (mg/l)

notes are desirable, while the Bna strain appears as a candidate *Brettanomyces* strain for beers in which phenolic notes are not desirable.

3.3. Sequentially inoculated beer fermentations

Next, we performed trial beer fermentations first inoculated with a non-conventional yeast species, and after 48 h of flavor development, sequentially inoculated with the Ale 514 brewing yeast. The strains evaluated in sequential fermentations were selected for high production of specific aromatic compounds and were phenotypic outliers in comparison with *S. cerevisiae* in single strain fermentations (Fig. 2 and Supplementary Data 1). This included primarily strains from the *Pichia* and *Brettanomyces* genera, as well as *Z. florentina* and *T. delbrueckii*. The *P. kluyverii* strains differed in their ability to produce acetate ester, *P. anomala* strains showed high ethyl acetate production, *T. delbrueckii* produced the highest level of 4VG (POF+), and *Z. florentina* showed the highest production of fusel alcohols. Only *Brettanomyces* strains managed to produce 4EG and 4EP, which is probably because of the unique vinyl reductase activity of these species. Fermentations were semi-static with stirring in the first 72 h to allow proliferation of both yeast species in the small tube fermentations. The formation of an aerobic cap on top of the fermentations with film-forming *Pichia* yeasts was taken as an indirect confirmation of biomass production and flavor development (Fugelsang and Edwards, 2010). The sequential combinations of non-conventional yeast species and *S. cerevisiae* mostly showed similar fermentation progress using weight loss as an indirect measure of ethanol production, except for the *Z. florentina* strain which showed intermediate fermentation capacity [Fig. 3; weight loss of 1.22% (w/v) (≈1.6% ABV) on day 2 and 1.91% (w/v) (≈2.6% ABV) on day 3] and the Bbr2 *B. bruxellensis* strain which caused stuck fermentation by *S. cerevisiae* resulting in residual maltose levels and lower ethanol yields (Fig. 3 and Supplementary Fig. 2 and Supplementary Data 1 for all quantified metabolites). Alcoholic fermentation with *Z. florentina* were also slower in the sequential fermentation compared to the corresponding single strain fermentation after three days (see section 3.2), which is likely due to inhibition on this species by *S. cerevisiae* after inoculation at 48 h of fermentation. The Bbr2 (AWRI 1499) yeast has not been investigated before in sequential fermentations such as performed in this study. *Brettanomyces* yeasts are not known to produce killer toxins and the *B. bruxellensis* Bbr2 yeast is, on the contrary, sensitive to killer toxins produced by *Klyveromyces wickerhamii* (Mehlomakulu et al., 2014). In wine sequential fermentations with *T. delbrueckii* and *S. cerevisiae* (inoculation after 48 h), sluggish fermentations were observed to be linked to low levels of assimilable nitrogen (Taillandier et al., 2014). The sluggish Bbr2-Ale 514 mixed fermentation could therefore either be due to exhaustion of essential nutrients, such as vitamins, trace metals ions (e.g. Zn^{2+}), nitrogen and lipids by Bbr2 in the malt extract medium, or due to inhibition of *S. cerevisiae* metabolism (Bisson, 1999; Ciani et al., 2016).

The flavor metabolite profiles of the sequential fermentations showed significant differences compared to single fermentation with *S. cerevisiae*, seemingly disclosing a species-dependent relationship. All *P. kluyverii* yeasts were clearly distinguishable from the other yeasts, with more resemblance to *S. cerevisiae* (Fig. 4).

The sequential fermentation with Bbr2 revealed that it was also a phenotypic outlier for aroma production (Fig. 4), leading to high levels of 4EG, 4EP, ethyl octanoate [1.01 mg/L, well above the sensory threshold of 0.9 mg/L (Meilgaard, 1975)], ethyl decanoate, acetic acid, ethyl acetate and glycerol (Supplementary Fig. 2). On the other hand, we observed lower levels of ethyl hexanoate, isobutanol, isoamyl alcohol, and an absence of isoamyl and isobutyl acetate (Supplementary Fig. 2A). The latter is consistent with previous reports that *Brettanomyces* yeast may display acetate esterase activity (Steensels et al., 2015). However, the sequential fermentations with the Bbr1 *B. bruxellensis* yeast did not blot out the isoamyl and isobutyl acetate production, while there was still production of 4EG and 4EP. These results reveal a clear difference between spoilage and beneficial *Brettanomyces* strains. In addition, the Bbr1 strain also produced the highest levels of acetaldehyde in sequential fermentations (Supplementary Figs. 2A and 4). Interestingly, sequential fermentations with *P. kluyverii* yielded lower levels of ethyl acetate than the corresponding single strain fermentations (39.4, 38.5, and 53.4 mg/L vs. 41.8, 61.1, and 83.6 mg/L after 48 h for Pkl1, 2 and 3, respectively). This is similar to the ethyl acetate yield of *S. cerevisiae* in the control fermentation (47.0 mg/L). It was also observed that P. *kluyverii* Pkl3 retained isoamyl acetate yield similar to that of the *S. cerevisiae* control fermentation (4.4 vs. 4.7 mg/L, Supplementary Fig. 2A) despite the fact that production of isoamyl acetate was inhibited in all sequential fermentations with non-conventional yeasts. On the other hand, the level of the precursor isoamyl alcohol was much lower in the P. *kluyverii* sequential fermentation than that produced in the single strain *S. cerevisiae* control fermentation (Supplementary Fig. 2A). This resulted in a higher isoamyl acetate/isoamyl alcohol conversion ratio (5.0 vs 6.7% for *S. cerevisiae* single strain control vs. *P. kluyverii*/*S. cerevisiae* sequential fermentations, respectively). Except for these differences mentioned above, the overall flavor metabolite profiles of the *P. kluyverii* sequential fermentations were similar to the complete fermentations inoculated only with *S. cerevisiae* (Fig. 4).

The production of ethyl acetate in sequential fermentations with *P. anomala* Pan1 was similar to the control single strain fermentation with *S. cerevisiae* Ale 514 (49.8 mg/L compared to 47.0 mg/L, respectively), whereas *P. anomala* strain Pan2 produced the highest ethyl acetate yield in all of the sequential fermentations (129.1 mg/L) (Supplementary Figs. 2A and 4). These results suggest lower performance for ethyl acetate production or higher esterase activity occurring in mixed fermentations with the Pan1 *P. anomala* strain compared to Pan2, and appears to be a strain-dependent trait.

The sequential fermentations using *Brettanomyces* confirmed the unique ability of *Brettanomyces* strains to produce ethyl phenols, as 4EP and 4EG were absent with any of the other non-conventional yeasts (Supplementary Fig. 2B and Supplementary Data 1).

The sequential fermentations also showed that the *P. anomala* Pan1 and Pan2 strains produced slightly lower levels of 4VG than in their corresponding single strain fermentations (1.12 and 2.46 mg/L vs. 1.89 and 2.89 mg/L after 72 h, respectively), whereas *T. delbrueckii* and *Z. florentina* produced the highest levels of 4VG in all the sequential fermentations with 3.04 and 3.40 mg/L, respectively. The similarity between *Z. florentina* and *T. delbrueckii* was striking, given that *T. delbrueckii* performed much better for 4VG

Fig. 1. UV and fluorescence HPLC chromatograms of phenolic compounds and hydroxycinnamic acid precursor standards spiked in malt extract medium. The standard shown contains 2.38 mg/L of each phenolic compound and precursor acids spiked into malt extract medium. A) UV chromatogram recorded at 280 nm. B) Fluorescence chromatogram with excitation/emission at 210/340 nm. C) Standard curves showing linearity of phenolic compounds measurement with UV and fluorescence in spiked malt extract medium. The standards included 0.28, 0.57, 1.16, 2.38, 4.87 and 10 mg/L. The x-scale indicates the phenolic compound in mg/L and the y-scale indicates the peak height of the fluorescence intensity or UV absorbance in μV. The malt extract medium contains ferulic and p-coumaric acid, a low amount of 4VG and the calibration curve therefore does not pass through zero.

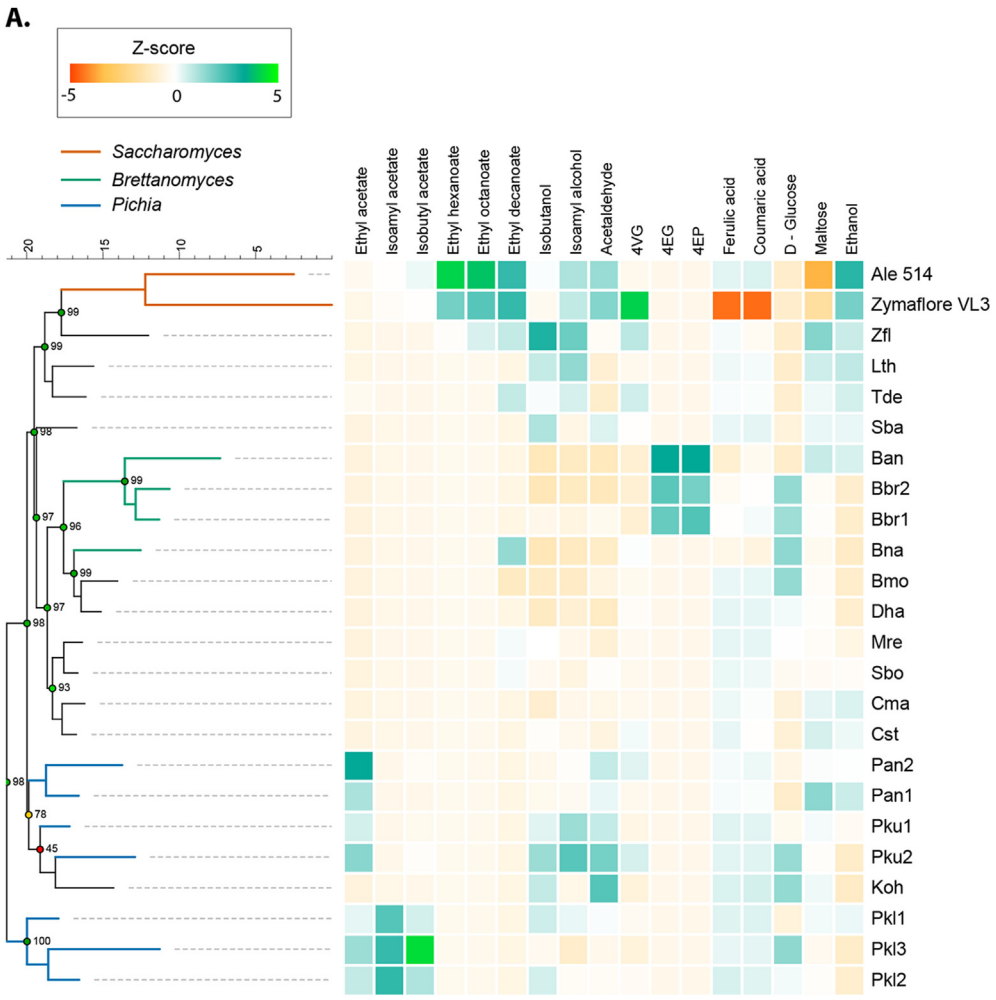


Fig. 2. Heatmap indicating secondary quantified metabolites from fermentations of single non-conventional yeast species. The fermentations were sampled after 48 h (A) and 72 h (B). The fermentations were performed in EBC-validated tubes with 60 mL 20°P malt extract media. Values of each of the metabolites (X) were normalized with the average (μ) and the standard deviation (σ), to calculate Z-scores = (X- μ)/σ, which is a measure of the deviation from the mean. Scores for all flavor compounds ranged from −5 (red), through 0 (white) indicating average measurements, to 5 (green) standard deviations. Euclidean distances calculated from the Z-scores were used to make a neighbor joining phenotypic tree with BioNumerics 7.6. Numbers on the nodes indicate the goodness of the clustering in % (cophenetic correlation coefficients). Branch lengths are the relative intercluster NJ-distances.

production in single strain fermentations (5.77 vs. 1.78 mg/L after 72 h, respectively). This revealed that the *Z. florentina* strain may be a candidate for production of beers with a phenolic profile, although it was unable to produce fruity acetate and ethyl esters, except for ethyl decanoate (Supplementary Fig. 2A and 2B).

4. Discussion and conclusions

In this work we have first developed a novel, more powerful and convenient methodology for the determination of phenolic compounds simultaneously with their hydroxycinnamic acid precursors in alcoholic fermentations. Previously, methanol has generally been used as a solvent together with electrochemical detection. Most attention has been on 4VG while the ethyl phenolics were not properly measurable. The new methodology presented in this work, which uses an acetonitrile and formic acid mixture as eluent and fluorescence detection, has a better resolution and is more sensitive, allowing accurate measurement of 4VG, 4EP and 4EG, as well as ferulic acid and p-coumaric acid. This facilitates determination of the relevant phenolics compounds more precisely in brewing and winemaking processes with non-conventional yeasts and allowed us to evaluate their use in mixed brewing fermentations.

Non-conventional yeast species generally have a lower performance for ethanol production compared to *S. cerevisiae*. As a result, they have only been rarely used as pure starter cultures for production of alcoholic beverages. However, they are generally encountered in spontaneous alcoholic fermentations, in which they impart and contribute to unique flavor profiles. However, spontaneous alcoholic fermentations generally suffer from great variability and unpredictability. These observations have raised interest in evaluating non-conventional yeast species for use in co- or sequential fermentations with *S. cerevisiae*. This has been explored previously for wine fermentations (Ciani and Comitini, 2010; Ciani et al., 2010; Jolly et al., 2014) and it has now been extended in the present work to beer fermentations.

Among the different non-conventional yeast species evaluated for flavor production in the present work, *P. kluyverii* stood out in producing exceptional levels of isoamyl acetate and ethyl acetate. Isoamyl acetate is a highly desirable flavor compound in beer fermentations, imparting a fruity and banana-like flavor, whereas ethyl acetate gives a solvent-like off-flavor. At the extreme level of

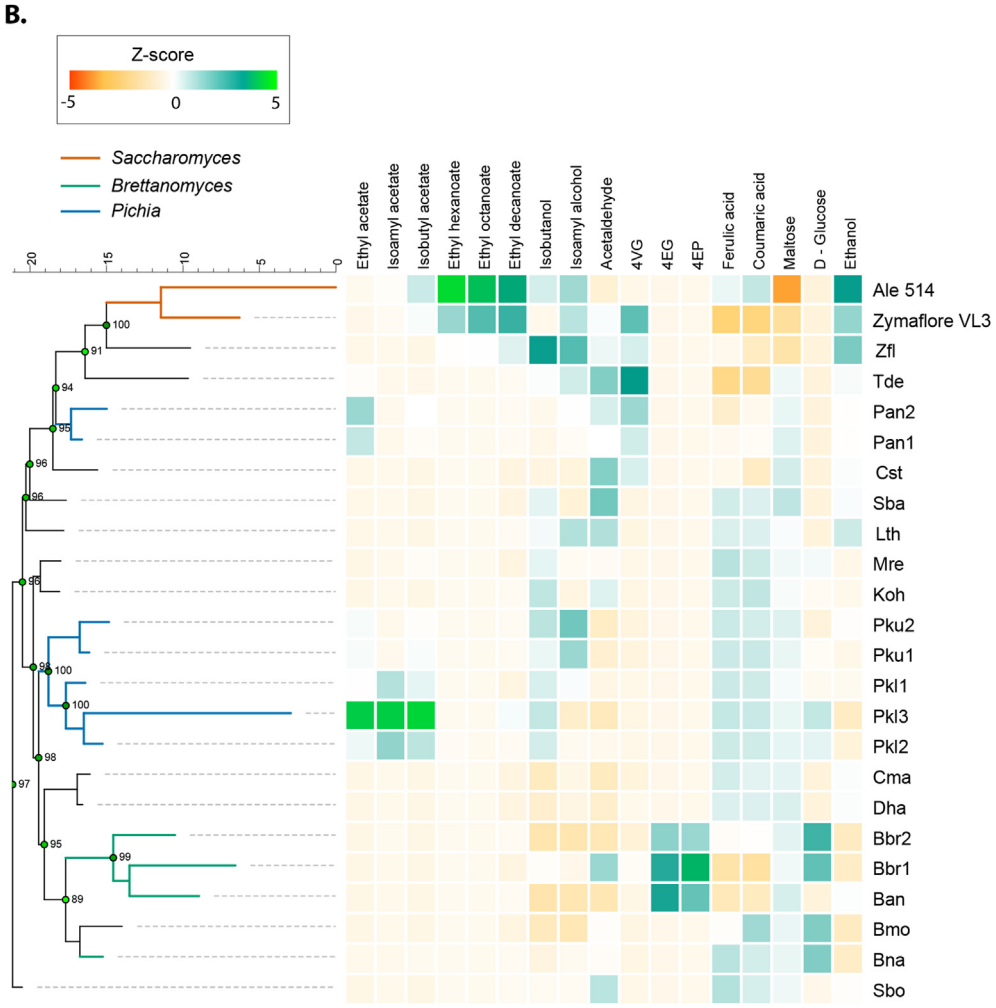


Fig. 2. (*continued*).

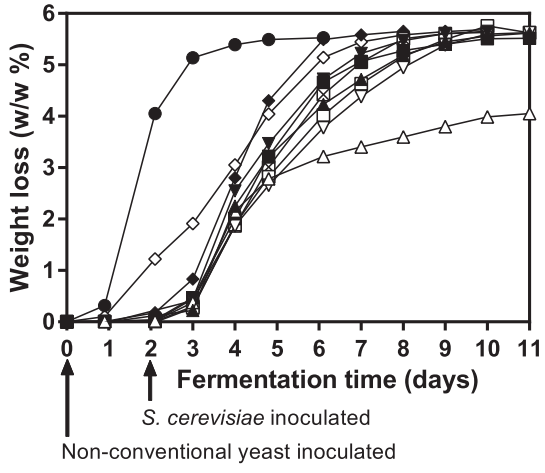


Fig. 3. Profile of fermentations sequentially inoculated with non-conventional yeast species and *S. cerevisiae*. The weight loss is shown as percentage of the starting weight of the malt extract medium. After day 2 (48 h) the fermentations were inoculated with *S. cerevisiae* Ale 514 brewing yeast to finish the alcoholic fermentation. A single inoculation of the *S. cerevisiae* Ale 514 strain was included as a reference (closed circles). Sequential inoculation with Bbr1 (closed downward triangles), Bbr2 (open downward triangles), Pan1 (closed upward triangles), Pan2 (open upward triangles), Pkl1 (closed squares), Pkl2 (open squares), Pkl3 (open squares with cross), Tde (closed diamonds), Zfl (open diamonds). After completion of the alcoholic fermentation samples were kept at 4 °C until analysis.

more than 900 mg/L ethyl acetate produced in the single fermentation at day 3, there will be a solvent-like off-flavor. However, if the fermentation with *P. kluyverii* is arrested before this excessive production (after 2 days: 83.6 mg/L ethyl acetate and 11.3 mg/L isoamyl acetate), the production of isoamyl acetate is so high that it might override the negative flavor effect of ethyl acetate.

Although the production of the two esters in single culture fermentations with *P. kluyverii* was highly pronounced, in sequential fermentations with *S. cerevisiae*, it was strongly reduced to a similar level as in *S. cerevisiae* single strain fermentations. There are multiple possible explanations that can be evaluated. First of all, it should be determined whether the removal of *P. kluyverii*, e.g. by centrifugation and subsequent separation from the medium, after the first fermentation prevents the disappearance of the huge levels of the two acetate esters in the next fermentation with *S. cerevisiae*. If this would be the case, it would point to a specific synergistic interaction between the two yeast species, triggering an increase in esterase activity much higher than the sum of that of the two yeast species separately. This could be accomplished for instance by enhanced secretion of esterases (Kurita, 2008). On the other hand, if the removal of *P. kluyverii* after the first fermentation does not prevent the disappearance of the large amounts of acetate esters accumulated, *S. cerevisiae* should in some way be responsible for it. This could be caused by intracellular esterase activity in *S. cerevisiae*, for instance encoded by *IAH1* (Fukuda et al., 2000), since acetate esters easily diffuse through cellular membranes.

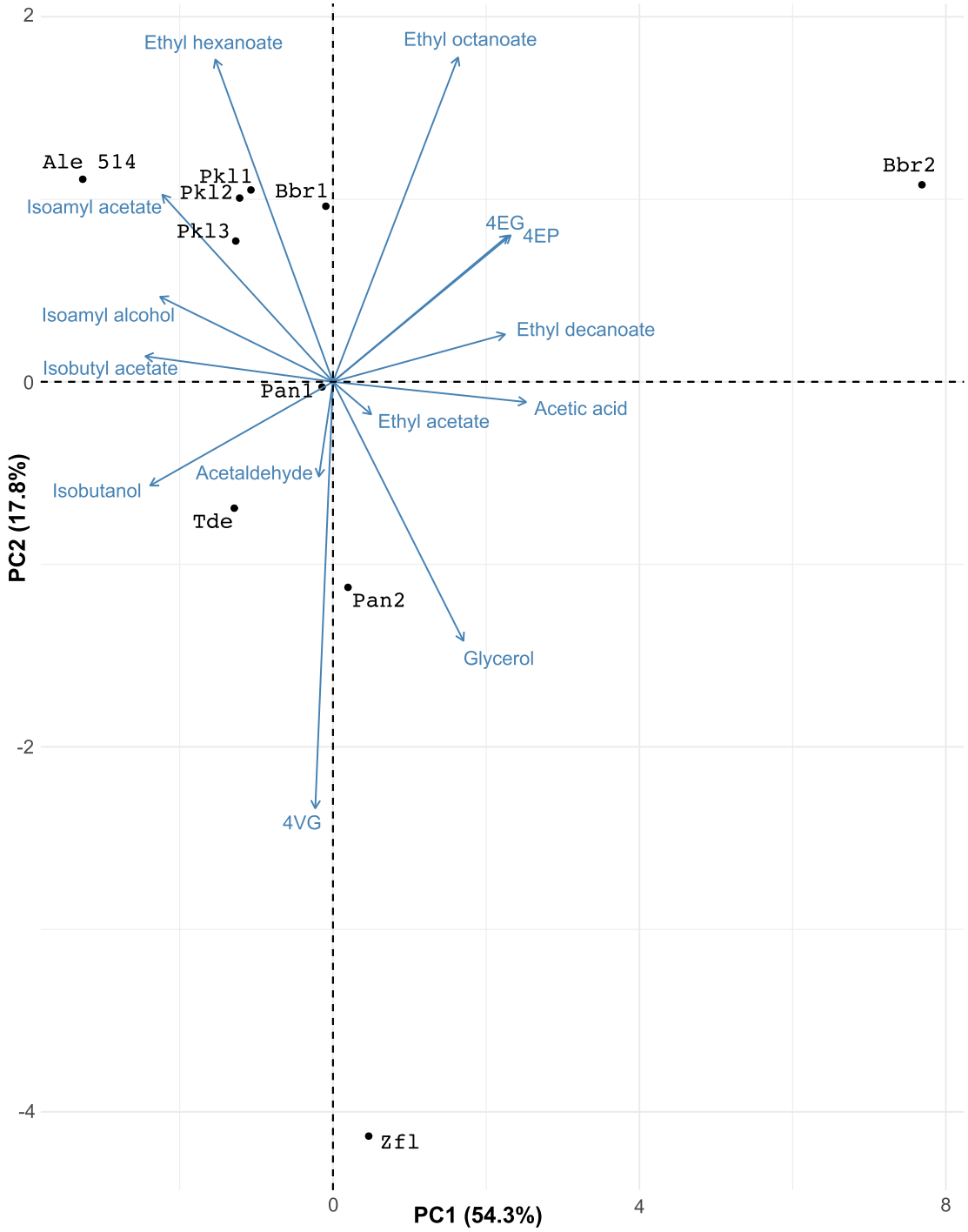


Fig. 4. Metabolites produced in sequential fermentations with *S. cerevisiae* MauriBrew Ale 514 analyzed with principle component analysis (PCA). The fermentations were first inoculated with non-*Saccharomyces* yeast, and after 48 h with the MauriBrew Ale 514 yeast to complete the alcoholic fermentation. Shown here are the first two components PC1 and PC2 that together explain 72.1% of the variance in the data. Supplementary Fig. 3 shows a scree plot of the total variances and Supplementary Fig. 4 PC3 and PC4, which together explain 19.3% of the variance. The variation in PC3 and PC4 is mainly due to differences in ethyl acetate and acetaldehyde. The data was normalized and analyzed with the R package factoextra.

Another way in which *P. kluyverii* could affect *S. cerevisiae* esterase activity or its own endogenous acetate ester production in the second fermentation, is through depletion of nutrient levels, especially nitrogen sources [for review see e.g. Verstrepen et al. (2003a)], mainly because of biomass formation in the first phase of the fermentation.

Depending on the cause of the ester disappearance, several approaches can be taken to prevent or reduce it. When the disappearance is due to *P. kluyverii* (secreted) esterase activity, many strains could be screened to identify a strain with low (secreted)

esterase activity. Alternatively, putative esterase encoding genes in *P. kluyverii* could be inactivated by targeted genetic modification, e.g. by CRISPR/Cas9 (Wagner and Alper, 2016), eliminating or reducing esterase activity. This also opens up the possibility that one or more esterases would have higher activity towards isoamyl acetate or ethyl acetate, and that the ratio between the two esters could thus be improved in this way. If *S. cerevisiae* would be responsible for the dramatic decrease in acetate ester levels, *Saccharomyces* sp. strain collections could be screened to identify strains with lower esterase activity and compatibility with *P. kluyverii* flavor production. This might also create the possibility that isoamyl acetate esterase activity would be reduced less than ethyl acetate esterase activity, resulting in an improved ratio of the two acetate esters.

A previous study in wheat beer with lower gravity (10 °P), showed a much more pronounced enhancement of isoamyl acetate in sequential fermentations with *P. kluyverii* and *S. cerevisiae* (Saerens and Swiegers, 2013). This could be due to the low-gravity conditions and/or the specific *P. kluyverii* strain or *S. cerevisiae* wheat strain used. The levels of isoamyl acetate and ethyl acetate reported were significantly lower than the levels measured in our work in the single fermentations with the same *P. kluyverii* yeast. This indicates that careful evaluation of the fermentation conditions and proper strain selection are crucial to unlock the full potential of aroma production by the non-conventional yeast species.

P. kluyverii has also been reported to convert precursors present in grapes into polyfunctional thiols for production of Sauvignon Blanc wine (Anfang et al., 2009). The polyfunctional thiols provide conspicuous tropical fruit flavors to the wine. These precursors are also present in aroma hops and a fraction is released by *Saccharomyces* yeasts during beer fermentation (Cibaka et al., 2015, 2016). Sequential or co-fermentation with *P. kluyverii* might increase the amount of polyfunctional thiols that is released from the precursors in the hop, improving beer aroma. Hence, more non-conventional yeast species could be screened for their capacity to release polyfunctional thiols from hop precursors.

Brettanomyces species also show potential for use as starter culture in sequential beer fermentations with *S. cerevisiae*. They were the only yeasts producing conspicuous levels of 4EG. It imparts spicy notes to the aroma, which is attractive in certain specialty beers. *Brettanomyces* yeasts have been identified in spontaneous beer fermentations and have also been used already as starter cultures in combination with *S. cerevisiae* (Steensels et al., 2015). *Brettanomyces* species secrete glucosidases that hydrolyze glycosylated terpenoid compounds also present in grapes and other fruits. They have been isolated from spontaneous beer fermentations for production of cherry lambic beer and likely contribute in this way to the conspicuous flavor profile of these beers. Also in this case notable differences in glycoside hydrolase activity between species and strains have been observed (Daenen et al., 2008a; Daenen et al., 2008b). Finally, King and Richard (2000) showed that *T. delbrueckii*, *Lachancea thermotolerans* as well as *S. cerevisiae* brewing yeast could transform hop monoterpene alcohols into other derivatives.

The implementation of non-conventional yeast species will introduce new challenges for breweries. For example, it will be problematic to perform serial re-pitching of the inoculum (as it will contain an unknown proportion of both *Saccharomyces* and non-*Saccharomyces* yeast), and questions also remain regarding bottling, foam stability and haze formation. The aerobic yeasts, such as *P. kluyverii*, which showed the greatest potential for production of fruity esters, require oxygen to propagate and form a layer on top of the fermentation medium. For craft brewing of ales, which is performed at 20–25 °C, the current sequential fermentation method may be implemented directly in open fermentors. On the other hand, for large-scale high-gravity beer fermentations, which is performed in closed tanks, the wort is typically oxygenated with sterile air to allow the growth of brewing yeast (Gibson et al., 2007). The sequential fermentation procedure ensures optimal flavor production by the non-conventional yeast in the first part of the fermentation, whereas inhibitory conditions such as low temperature (8–12 °C), CO_2 pressure, and an anaerobic environment would occur only later in the alcoholic fermentation when the brewing yeast is active.

In conclusion, we have developed a new HPLC-UV/fluorescence methodology that facilitates determination of phenolic compounds for alcoholic beverages, and is particularly useful for determination of ethyl phenolic compounds produced by *Brettanomyces* species. We have screened 17 non-conventional yeast species for their potential usage as natural flavor enhancers and evaluated the best candidate strains in sequential fermentations with *S. cerevisiae*. Application of *P. kluyverii* in the brewing process is a very promising method to enhance fruity acetate esters, but gave surprisingly low yields in sequential fermentations. Future studies should investigate optimization with regards to development of strains with less esterase activity and using optimal fermentation conditions, especially oxygen levels, to unlock the full potential for aroma improvement by the non-conventional yeast species.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.fm.2017.11.008.

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