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Characterisation of *Saccharomyces cerevisiae* hybrids selected for low volatile acidity formation and the production of aromatic Sauvignon blanc wine

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Wine yeasts (*Saccharomyces cerevisiae*) vary in their ability to develop the full aroma potential of Sauvignon blanc wine due to an inability to release volatile thiols. Subsequently, the use of 'thiol-releasing' wine yeasts (TRWY) has increased in popularity. However, anecdotal evidence suggests that some commercially available TRWY intermittently exhibit undesirable characteristics for example, volatile acidity (VA) formation. Therefore, a trial was undertaken to select and evaluate *S. cerevisiae* hybrids for the production of Sauvignon blanc wine with enhanced fruity and tropical aromas, but low VA. Hybrids were characterised by clamped homogeneous electrical field (CHEF) DNA karyotyping and matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS) biotyping, and subsequently trialled against top commercial TRWY that is, Zymaflore VL3 and Zymaflore X5 (Laffort Oenologie), and Fermicru 4F9 (DSM Oenology) in laboratory-scale Sauvignon blanc vinifications during 2013. Most hybrids produced wines with VA levels significantly lower than those produced with Zymaflore VL3, Zymaflore X5 and Fermicru 4F9. Low VA forming hybrids also produced wines with tropical wine aroma notes. Wines produced by Fermicru 4F9 had the lowest acetic acid (the main volatile acid) of the commercial TRWY in this study. However, some hybrid yeasts produced wines with less acetic acid on average than wines produced by Fermicru 4F9. Overall, hybrids NH 6, NH 48, NH 56, NH 88 and NH 145 produced wines with enhanced tropical fruity aroma, but lower VA compared to wines produced by commercial TRWY.

Key words: Hybrid yeasts, CHEF, MALDI-TOF/TOF MS biotyping, Sauvignon blanc, tropical fruit aroma, volatile acidity.

INTRODUCTION

Wine aroma is comprised of compounds emanating directly from the grapes, compounds produced by the yeast such as esters and higher alcohols, and yeast mediated compounds for example, volatile thiols (King, 2010; Bovo et al., 2015). Wine yeasts (*Saccharomyces cerevisiae*) vary in their ability to develop the full aroma potential of Sauvignon blanc wine due to an inability to release volatile thiols (King et al., 2011). Retention of

these bound thiols implies that the full aroma potential of the wine is not realised, as the bound thiols can only be released by wine yeasts during fermentation (Swiegers et al., 2006; Holt et al., 2011). Subsequently, the use of 'thiol-releasing' *S. cerevisiae* commercial wine yeasts (TRWY) for the production of aromatic Sauvignon blanc wine has increased in popularity (Swiegers et al., 2009). These yeast strains can release 4-mercapto-4-methylpentan-2-one (4MMP), 3-mercaptohexan-1-ol (3MH) and 3-mercaptohexyl acetate (3MHA) from the respective cysteine-bound precursors. Other yeast strains can convert the aromatic 3MH (passion fruit aroma) to 3MHA (tropical and citrus aromas). However, anecdotal evidence suggests that some commercial TRWY intermittently produce undesirable high levels of volatile acidity (VA), which imparts vinegar-like nuances to the wines (Du Toit and Pretorius, 2000; Ugliano et al., 2007; Vilela-Moura et al., 2011). Acetic acid is the main contributor to VA in wine with odour detection levels ranging between 0.7 and 1.1 g/L (Byarugaba-Bazirake, 2008; Vilela-Moura et al., 2010). Even though, excessive levels of VA are mainly caused by lactic acid bacteria, acetic acid bacteria and wild yeasts, wine yeasts also contribute to VA, by producing acetic acid during alcoholic fermentation (Cordente et al., 2013; Luo et al., 2013). Other steam distillable acids, that is lactic, formic, butyric, and propionic acids can also contribute to VA (Erasmus et al., 2004; Moss, 2015). Currently, in South Africa the legal limit of VA permissible in wine is 1.2 g/L (OIV, 2010; Sirén et al., 2015). However, the sensory threshold of VA is generally accepted to be 0.8 g/l (Du Toit, 2000).

Reduction of yeast derived VA formation can be done by using genetically modified (GM) yeasts (Swiegers et al., 2007) or improved *S. cerevisiae* hybrid yeasts bred through classical mating (Pérez-Torrado et al., 2015). Although genetic modification can address VA formation by wine yeasts, the use of genetically modified organisms (GMO) is illegal (Berrie, 2011). The Cape Winemakers Guild (CWG) and South African Wine Industry Council (SAWIC) is also largely against the use of GMO in wine production (CWG, 2015). Both CGW and SAWIC emphasises that the SA wine industry is too dependent on the highly sensitive European market for exports, which are largely against GM food products. Sauvignon blanc was chosen for this study because this cultivar was previously shown to produce grapes containing aroma-inactive, non-volatile, bound thiols (metabolites) that can only be released by the wine yeast *Saccharomyces cerevisiae* during fermentation (Von Mollendorf, 2013). Therefore, the aim of this study was to select and evaluate *S. cerevisiae* hybrids for the production of wine with enhanced fruity and tropical fruit aromas, but low VA.

MATERIALS AND METHODS

Wine yeast strains

One hundred and thirty-six hybrid strains (NH 1 to 10, 12, 13, 15 to 18, 20, 22 to 25, 27 to 78, 80 to 95, 97 to 104, 106 to 119, 121 to 145), four *S. cerevisiae* parental yeast strains (PS 1 to 4), three commercial TRWY references (Zymaflore VL3, Zymaflore X5 [Laffort Oenologie, France], and Fermicru 4F9 [DSM Oenology, Netherlands]) used in this study are conserved in the ARC Infruitec-Nietvoorbij micro-organism culture collection (ARC Inf-Nvbij CC). Hybrids were bred at the ARC Infruitec-Nietvoorbij microbiology laboratory through classical mating, as part of an ongoing hybrid breeding programme as described by Steensels et al. (2014) and Snoek et al. (2015).

Pulsed-field gel electrophoresis (PFGE)/Contour clamped homogeneous electric field (CHEF) DNA karyotyping

DNA karyotyping of yeast strains was conducted according to the embedded agarose procedure described by Carle & Olson (1985), and Van der Westhuizen et al. (1992). The procedure was adapted by conducting chromosome separation in TBE (50 mM Tris, 41.3 mM boric acid, and 0.5 mM EDTA [Sigma-Aldrich, USA]) buffer at 14°C with pulse-times of 30 and 215 sec for 34 hours using clamped homogenous electric field (CHEF) gel electrophoresis (CHEF-DR II, Bio-Rad Laboratories, Richmond, USA). Yeast strain PS1 was run parallel to CHEF DNA size marker #1703605 (Bio-Rad, Madrid, Spain) as an internal standard to determine respective chromosomal band sizes. Chromosomal banding patterns were visualised on a Bio-Rad image analyser following staining with 0.01% (v/v) ethidium bromide. Subsequently, the genetic relatedness of the various yeast strains was determined by subjecting CHEF DNA karyotypes to cluster analysis using FP Quest software FP 4.5 software (Bio-Rad, Madrid, Spain). Cluster analysis was based on the Dice coefficient and an un-weighted pair group method with arithmetic mean (UPGMA), with 1% tolerance and 0.5% optimisation.

Matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF/TOF MS) biotyping

Yeast strains were also identified by matrix-assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF/TOF MS) biotyping as an alternative to CHEF DNA karyotyping. Formic acid protein extraction for subsequent MALDI-TOF biotyping was conducted as described by Pavlovic et al. (2013). One microliter of wine yeast protein extract was spotted onto a MTP 384 polished steel target plate as described by Moothoo-Padayachie et al. (2013) and Deak et al. (2015). Thereafter, the spotted target plate was inserted into a Bruker UltrafleXtreme MALDI-TOF/TOF MS (Bruker Daltonics, Bremen, Germany) apparatus. Generation of yeast protein mass spectra using MALDI-TOF/TOF MS was conducted according to the standard National Agricultural Proteomics Research and Services Unit method (obtainable from the National Agricultural Proteomics Research and Services Unit (NAPRSU), University of the Western Cape, South Africa). Mass spectra for all strains were acquired in triplicate.

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Laboratory-scale fermentation trials

Wet culture wine yeasts were evaluated in laboratory-scale fermentation trials as described by Rossouw et al. (2010) and Maarman et al. (2014). Frozen Sauvignon blanc grape must (total sugar = 21.9°B; total acidity = 9.3 g/L; pH = 3.28) was thawed and 250 ml aliquots were transferred into fermentation vessels (340 ml glass bottles). The yeast cultures were grown at 28°C for 48 h in 10 ml YPD (1% [w/v] yeast extract, 2% [w/v] peptone, and 2% [w/v] dextrose [Biolab, Merck]), and subsequently used to inoculate the Sauvignon blanc grape must at a concentration of 2% (v/v). Commercial TRWY Zymaflore VL3, Zymaflore X5 (Laffort Oenologie, France), and Fermicru 4F9 (DSM Oenology, Netherlands) were included in fermentation trials as references. Fermentation vessels were stoppered with a fermentation lock filled with water. Fermentations were conducted on an orbital shaker in an insulated temperature-controlled room, which were electronically regulated at 14.5°C, and monitored by CO_2 weight loss for 30 days. All fermentations were conducted in triplicate in a completely randomised block design (Addelman, 1970).

Fourier transform infra-red (FTIR) spectroscopy

Wines were subjected to residual glucose/fructose, ethanol, VA, titratable acidity (TA) and pH analyses using an Oenofoss™ Fourier transform infrared (FTIR) spectrometer (FOSS Analytical A/S, Denmark) after fermentations stabilised.

Gas chromatography-mass spectrometry (GC-MS)

Wines with the most prominent fruity aromas as determined by the sensory panel were subjected to GC-MS analysis. Flavour compounds *viz*. esters, total fatty acids and higher alcohols were quantified by means of calibration mixtures of the applicable aroma compounds in conjunction with gas chromatography (GC) as described by van Jaarsveld et al. (2009), Zhang et al. (2012) and Vilanova et al. (2013).

Sensory evaluation

Wines were subjected to descriptive sensory evaluation by a panel of 14 experienced wine judges. Judges were requested to indicate aroma intensities on a unipolar six point numerical scale (absent [0], very low [1], low [2], medium [3], high [4] and very high [5]), and also to specify the most prominent aroma/s perceived that is, 'tropical fruit' for example banana, guava, peach, passion fruit and citrus; 'vegetative' for example, asparagus, herbaceous, green pepper, green beans, cut grass, green olive and gooseberry; or 'floral' for example rose, orange blossom etc. The wines were served as coded samples in international wine tasting glasses (approximately 50 ml) in a completely randomised order for each judge.

Statistical analyses

Chemical and sensory analyses data were subjected to principal component analysis (PCA) to determine the relationship between variables and treatments (yeasts) (Pearson, 1896; 1901; Zou et al., 2006). The data matrix consisted of four chemical variables that is VA, ethanol, total acidity and pH; and three sensory aroma descriptors that is, 'tropical fruit', 'vegetative' and 'floral'. Pearson's correlation was performed to study the linear relationship between the chemical and sensory variables. The Pearson's correlation matrix was used to standardise the data before performing the PCA. The PCA was performed using XLSTAT software (Addinsoft, 2013) with the principal components (PC's) as factors (that is, F1 and F2).

RESULTS AND DISCUSSION

Pulsed-field gel electrophoresis (PFGE)/Contour clamped homogeneous electric field (CHEF) DNA karyotyping

Wine chemical and sensory quality was affected by the yeast strain used to carry out the alcoholic fermentation (Sharma et al., 2012; Usbeck et al., 2014). As a result, differentiation of yeast strains is essential to ensure that the correct yeast strain is used to inoculate grape must. Previous studies showed that PFGE/CHEF DNA karyotyping allowed for the delineation of closely related yeast strains (Sheehan et al., 1991; van Breda et al., 2013). Similarly, CHEF DNA karyotyping was useful in this investigation to differentiate closely related *S. cerevisiae* hybrid strains descending from mutual parental yeast strains (Figure 1). Distinctive variations in the DNA karyotypes between hybrids can be seen especially for the smaller chromosomes (bottom bands). Four pairs of hybrids that is, NH 33 and NH 34; NH 63 and NH 64; NH 75 and NH 76; and NH 86 and NH 89 had similar DNA karyotypes, whilst the remainder of yeast strains had distinguishable DNA karyotypes. Therefore, 139 CHEF DNA karyotyping profiles of the 143 strains were generated with genetic similarity ranging from 58 to 100%. The larger chromosomes (top bands) were common to most hybrids and parental yeast strains. It is evident that chromosomal DNA of the hybrids originated from more than one parental strain. It can be envisaged that some characteristics, including flavour compound (metabolite) release during fermentation, should be similar, different or enhanced compared to parental strains.

Cluster analysis of yeast CHEF DNA karyotypes allowed for the differentiation of yeast strains with common ancestry as described by Hoff (2012), Choi and Woo (2013) and Gallego et al. (2014). A dendogram comprising of sixteen clusters (I to XVI) was observed at a genetic similarity limit of 80% for all 143 strains (Figure 1). Four hybrids that is, NH 6, NH 67, NH 73 and NH 112 exhibiting the ability to produce wines with tropical fruit aroma (hereafter abbreviated as TFPH) clustered with the commercial TRWY reference Zymaflore X5, whilst another two TFPH and low VA producing hybrids (LVPH) that is, NH 56 and NH 57 clustered with the commercial TRWY references Fermicru 4F9 and Zymaflore VL3, respectively. Both hybrids also clustered with tropical fruit wine producing PS 1 at a 74% genetic similarity cut-off. Moreover, both hybrids clustered with the lower VA producing PS 2, PS 3 and PS 4 (Figure 1). Therefore, these hybrids exhibiting the sought-after tropical fruit aroma enhancing and low VA forming qualities,

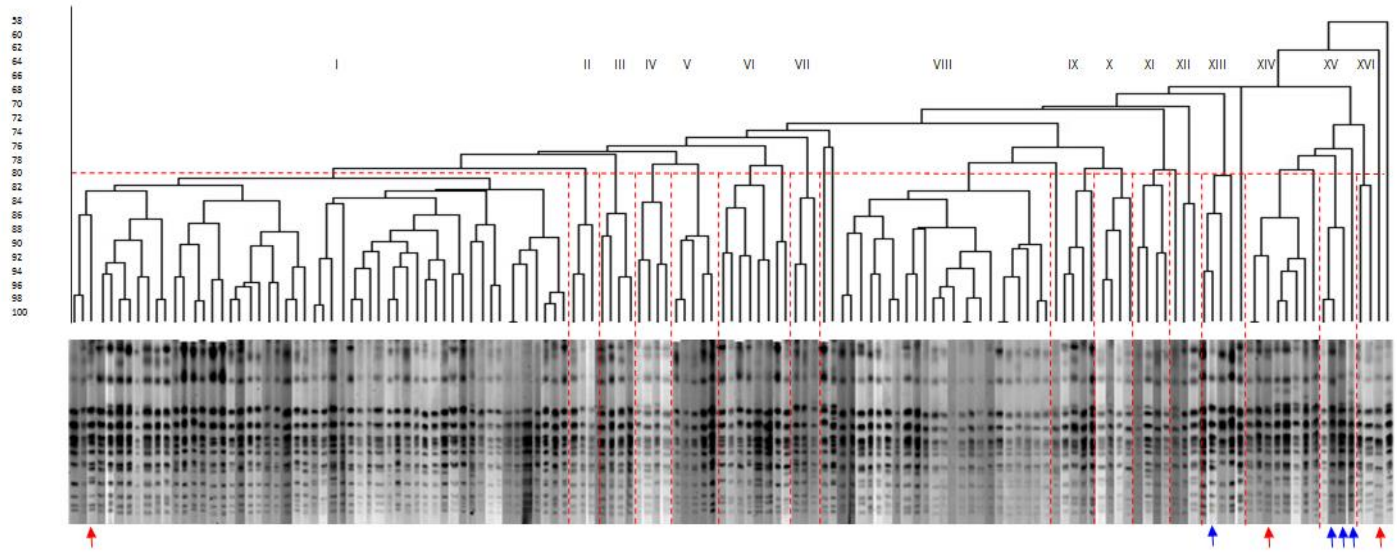

Figure 1. Dendogram showing the genetic similarity among three commercial 'thiol-releasing' wine yeasts (TRWY)(red arrows), four parental yeast (PS)(blue arrows) and 136 hybrid yeast (NH) strains. Cluster analyses was performed using a UPGMA algorithm. Yeast strains with 80% similarity (dotted line) were assigned to the same cluster indicated by Roman numerals. Dice (Opt:0.50%) (Tol 1.0%-1.0%) (H>0.0% S>0.0%) [0.0%-100.0%] **Cluster I:** NH 143, NH 132, ZYMAFLORE X5, NH12, NH 68, NH 66, NH 67, NH 125, NH 69, NH 24, NH 73, NH 10, NH 112, NH 107, NH 42, NH 113, NH 114, NH 133, NH 135, NH 134, NH 138, NH 35, NH 36, NH 117, NH 6, NH 9, NH 3, NH 47, NH 41, NH 15, NH 17, NH 54, NH 53, NH 94, NH 5, NH 7, NH 16, NH 52, NH 22, NH 25, NH 1, NH 106, NH 37, NH 13, NH 20, NH 91, NH 99, NH 33, NH 34, NH 32, NH 31, NH 4, NH 70, NH 30; **Cluster II:** NH 43, NH 55, NH 78; **Cluster III:** NH 130, NH 98, NH 48, NH 62; **Cluster IV:** NH 27, NH 81, NH 49, NH 97; **Cluster V:** NH 108, NH 127, NH 129, NH 136, NH 137; **Cluster VI:** NH 28, NH 50, NH 110, NH 29, NH 139, NH 142, NH 11, NH 18; **Cluster VII:** NH 39, NH 40, NH 38; **Cluster VIII:** NH 109, NH 2, NH 115, NH 116, NH 100, NH 45, NH 88, NH 23, NH 95, NH 44, NH 72, NH 77, NH 92, NH 93, NH 61, NH 75, NH 76, NH 74, NH 128, NH 86, NH 89, NH 83, NH 85, NH 87, NH 90; **Cluster IX:** NH 80, NH 101, NH 144, NH 71, NH 118; **Cluster X:** NH 123, NH 141, NH 103, NH 119; **Cluster XI:** NH 122, NH 8, NH 126, NH 82; **Cluster XII:** NH 121, NH 131, NH 84; **Cluster XIII:** NH 124, PS 2, NH 145, NH 140, NH 46; **Cluster XIV:** NH 63, NH 64, FERMICRU 4F9, NH 56, NH 59, NH 58, NH 60, NH 65; **Cluster XV:** NH 51, PS 1, PS 3, PS 4; **Cluster XVI:** NH 102, NH 104, ZYMAFLORE VL3, NH 57.

inherited it from the respective parental strains.

Yeast profiling with MALDI-TOF/TOF MS Biotyper

Biotyping using MALDI-TOF/TOF MS was successfully deployed to match ribosomal protein originating from commercial TRWY references, PS, and NH strains to that of a database described by Bizzini et al. (2010), Xiao et al. (2014) and Ghosh et al. (2015). All strains were identified as *Candida robusta,* the anamorph to *S. cerevisiae* (Diddens and Lodder, 1942; Kurtzman et al., 2011) following biotyping (mass spectra can be requested from the National Agricultural Proteomics Research and Services Unit (NAPRSU), University of the Western Cape, South Africa). Overall 79.72% of the strains were reliably identified as *Candida robusta* with scores of > 2 as described by Moothoo-Padayachie et al. (2013). Nonetheless no cut-off score for reliable MALDI-TOF/TOF MS biotyping was established, as all strains were shown by DNA karyotyping to be *S. cerevisiae*. Also noteworthy is that Cheng et al. (2013) showed that a lower cut-off score (1.7) sufficiently differentiate *Candida* yeast strains. Therefore, the lowest cut-off score (>1.8) for some strains used during this trial is acceptable.

A dendrogram consisting of nine clusters (I to IX) was generated following cluster analysis of the mass spectra at phylogenetic distance level of 0.80 indicated by dotted line (Figure 2). Hybrid strains were spread throughout the various mass spectral clusters. Some of the mass spectral clustering complemented DNA karyotype clustering, since TFPH, that is NH 56 and NH 57, clustered with the commercial TRWY reference Zymaflore VL3. Moreover, LVPH, that is NH 124; and NH 3, NH 88, NH 140, NH 13 and NH 81, were shown by MALDI-TOF/TOF MS biotyping (Bruker Daltonics, Bremen, Germany) to have a close phylogenetic relationship with the low VA producing PS 3 and PS 4, respectively (Figure 2). Also noteworthy is that TFPH and LVPH, that is NH 6, NH 132 and NH 134 was shown by biotyping to have a close phylogenetic relationship with parental strains that is PS 1 and PS 2, and PS 3, which was shown to produce wines with tropical fruit aroma (hereafter abbreviated to as TFPP). This provides more evidence supporting the notion that promising hybrids inherited desirable traits from the respective PS.

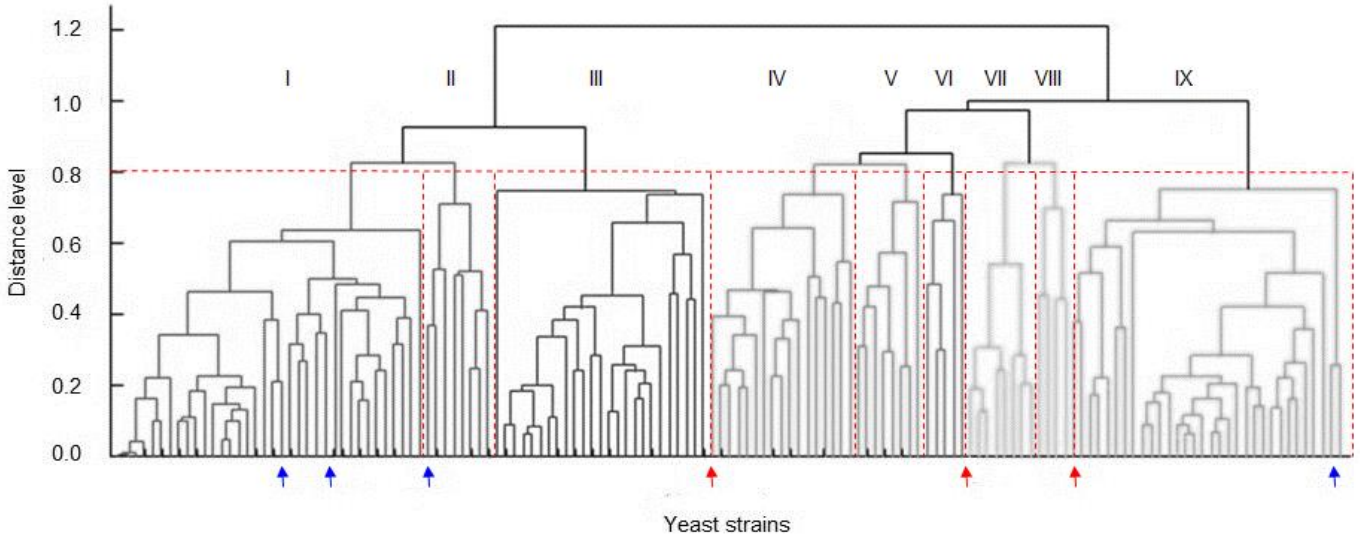

Figure 2. Principal component analysis (PCA) dendogram generated from matrix-assisted laser desorption/ionisation mass spectrometry (MALDI-TOF MS) biotyping spectra of three commercial 'thiol-releasing' wine yeasts (TRWY)(red arrows), four parental yeast (PS)(blue arrows) and 136 hybrid yeast (NH) strains, generated by cluster analysis using BIOTYPER software (Bruker Daltonics). Dendrogram based on identification score values and distance level is indicative of phylogenetic distance amongst yeast strains. Blue and red arrows indicate parental and commercial reference strains, respectively. Yeast strains were assigned to the same cluster at a 0.80 distance level (dotted line) indicated by Roman numerals. **Cluster I:** NH 104, NH 123, NH 24, NH 16, NH 47, NH 108, NH 90, NH 2, NH 9, NH 20, NH 23, NH 22, NH 44, NH 111, NH 75, NH 89, NH 145, NH 52, NH 114, NH 126, PS 1, NH 92, NH 144, NH 66, NH 107, PS 2, NH 6, NH 43, NH 82, NH 127, NH 72, NH 106, NH 29, NH 122, NH 115, NH 18 ; **Cluster II:** PS 3, NH 141, NH 103, NH 15, NH 124, NH 41, NH 93, NH 143; **Cluster III:** NH 37, NH 73, NH 86, NH 74, NH 31, NH 68, NH 132, NH 78, NH 125, NH 55, NH 87, NH 35, NH 38, NH 39, NH 134, NH 139, NH 137, NH 83, NH 36, NH 77, NH 131, NH 133, NH 46; **Cluster IV:** ZYMAFLORE VL3, NH 84, NH 91, NH 110, NH 33, NH 17, NH 56, NH 109, NH 57, NH 76, NH 61, NH 94, NH 135, NH 69, NH 112, NH 85; **Cluster V:** NH 7, NH 25, NH 48, NH 49, NH 102, NH 60, NH 65, NH 59; **Cluster VI:** NH 8, NH 50, NH 71, NH 42, NH 53; **Cluster VII:** FERMICRU 4F9, NH 40, NH 142, NH 54, NH 129, NH 51, NH 99, NH 100; **Cluster VIII:** NH 1, NH 10, NH 97, NH 101; **Cluster IX:** ZYMAFLORE X5, NH 30, NH 36, NH 140, NH 3, NH 13, NH 4, NH 5, NH 138, NH 80, NH 63, NH 32, NH 58, NH 67, NH 81, NH 98, NH 130, NH 116, NH 64, NH 45, NH 62, NH 88, NH 27, NH 28, NH 70, NH 95, NH 34, NH 118, NH 119, PS 4, NH 113.

Identification of microorganisms according to ribosomal protein spectra was reported by Gekenidis et al. (2014) and Oumeraci et al. (2015). In this study, distinctive ribosomal protein mass spectra of hybrid yeasts compared to parental strains were observed (Figure 3) (all data can be obtained from the National Agricultural Proteomics Research and Services Unit (NAPRSU), University of the Western Cape, South Africa). This study complemented research done by Bărbulescu et al. (2015), and shows that MALDI-TOF/TOF MS biotyping is a reliable yeast strain identification method that complemented CHEF DNA karyotyping. Biotyping proved to be a rapid identification method resulting in 143 mass spectra, whilst the laborious CHEF DNA karyotyping generated 139 karyotypes. However, CHEF DNA karyotyping still remains the cheaper option. Both techniques allowed for the delineation of genetically related hybrids.

Laboratory-scale fermentation trials

Most hybrids were able to ferment the grape must at a rate similar to commercial TRWY references and PS 1, PS 2, PS 3 and PS 4 (Figure 4). Most fermentations were shown to stabilise after 25 days following inoculation with the respective yeast strains. However, hybrids NH 36 and NH 34 fermented at rates noticeably different than the remaining strains included in this trial. Both hybrids produced wines with more vegetative aroma descriptors. Therefore, it can be tentatively surmised that fermentation rates nearby those of commercial TRWY references and TFPP are linked to production of wines with the sought-after fruity and tropical fruit aroma notes, since TFPH (for example, NH 56, NH 48, NH 88, NH 57, NH 3, NH 77, NH 124, NH 24, NH 29, NH 6) had similar rates to that of the commercial references and parental strains. This study complemented previous research which showed that faster fermentation rates improved the sensory quality of wines (Bell and Henschke, 2005). Also noteworthy is that, Shinohara et al. (1994) showed that hybrid yeast strains with similar fermentation rates as aromatic wine producing parental strains, was able to produced wines with aroma enhancing metabolites. Nonetheless, both NH 36 and NH 34 were shown to be LVPH and will be used in further breeding programs to

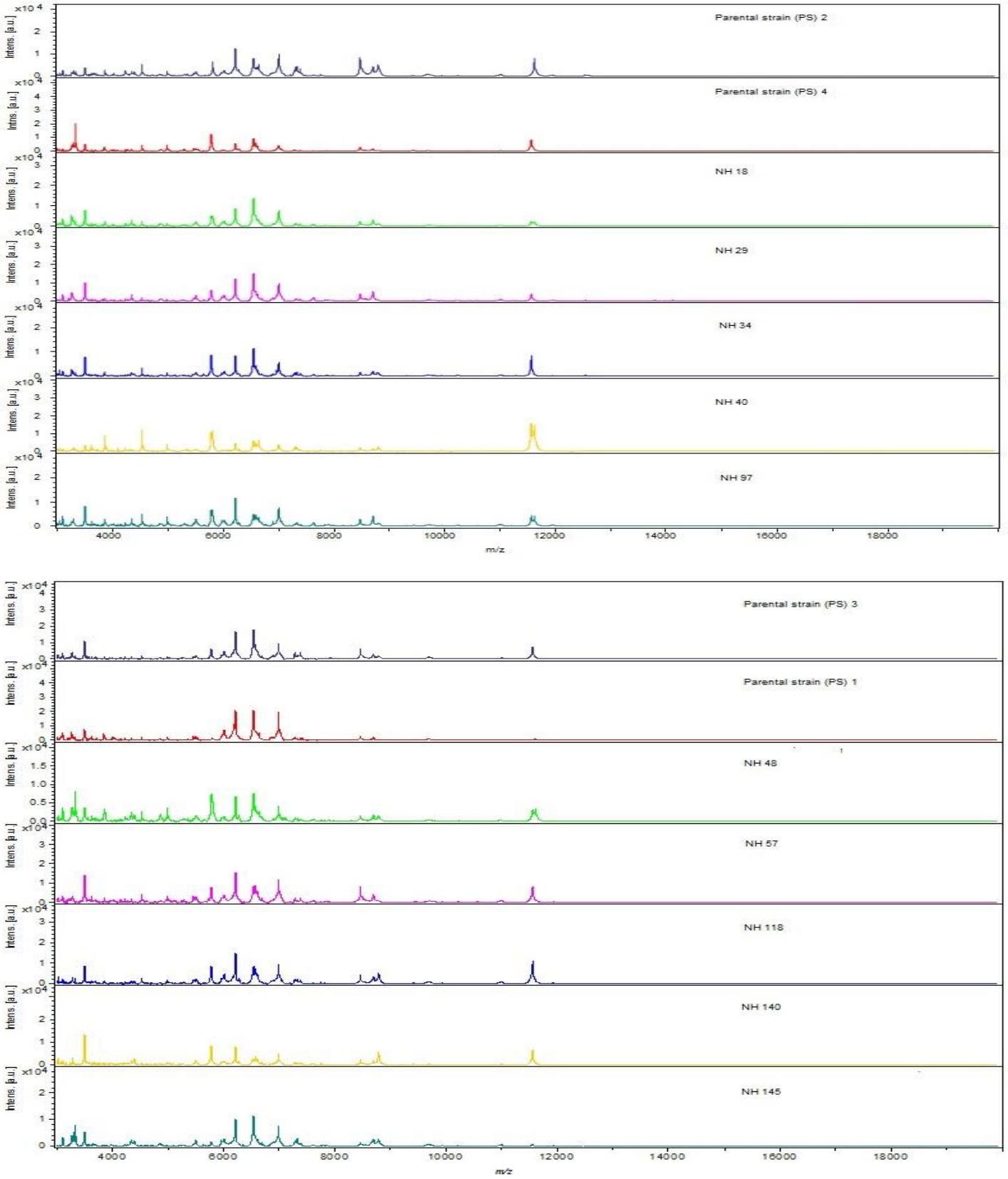

Figure 3. Matrix assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF MS) spectral fingerprints of four parental strains (PS) and ten hybrid strains (NH). The absolute intensities of the ions and mass-to-charge (m/z) ratios are represented on the y- and x-axis, respectively.

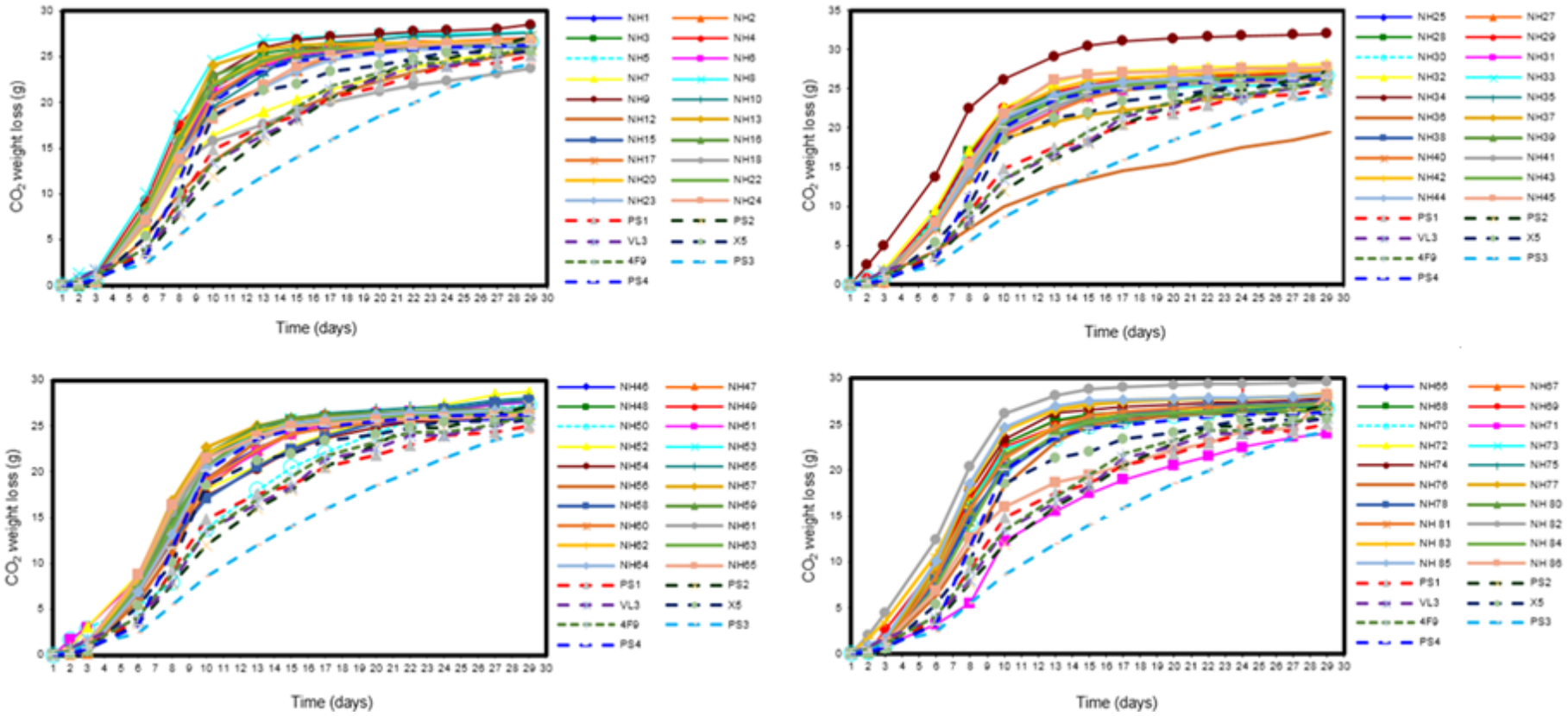

Figure 4. CO_2 weight loss of Sauvignon blanc grape must fermented at an ambient temperature of 14.5°C at the ARC Infruitec-Nietvoorbij microbiology laboratory using three commercial 'thiol-releasing' wine yeasts (TRWY), four parental yeast (PS) and 136 hybrid yeast (NH) strains.

improve progeny in this regard.

Fourier transform infra-red (FTIR) spectroscopy

Principle component analysis (PCA) biplot of FTIR spectroscopy generated data showed that promising hybrids, including NH 56, NH 48, NH 88, NH 57, NH 3, NH 77, NH 124, NH 24, NH 29, NH 6 situated in the left quadrants produced wines had a negative correlation with VA (Figure 5). The same observation was made with regard to PS 3, PS 2, PS 4 that was shown to be low VA producers (hereafter referred to as LVPP) and the commercial TRWY reference Fermicru 4F9. Overall, most hybrid strains produced wine with VA below 0.20 g/L (data not shown), whereas commercial TRWY references Zymaflore VL3 (0.31 ± 0.20 g/L) and Zymaflore X5 (0.50 ± 0.21 g/L) produced wines with significantly higher VA. These results support anecdotal evidence that some commercially available yeast strains can be implicated in VA formation. However, all commercial references produced wines with VA levels that comply with legislation. Strain PS 3 (0.02 ± 0.02 g/L) produced wines with the lowest

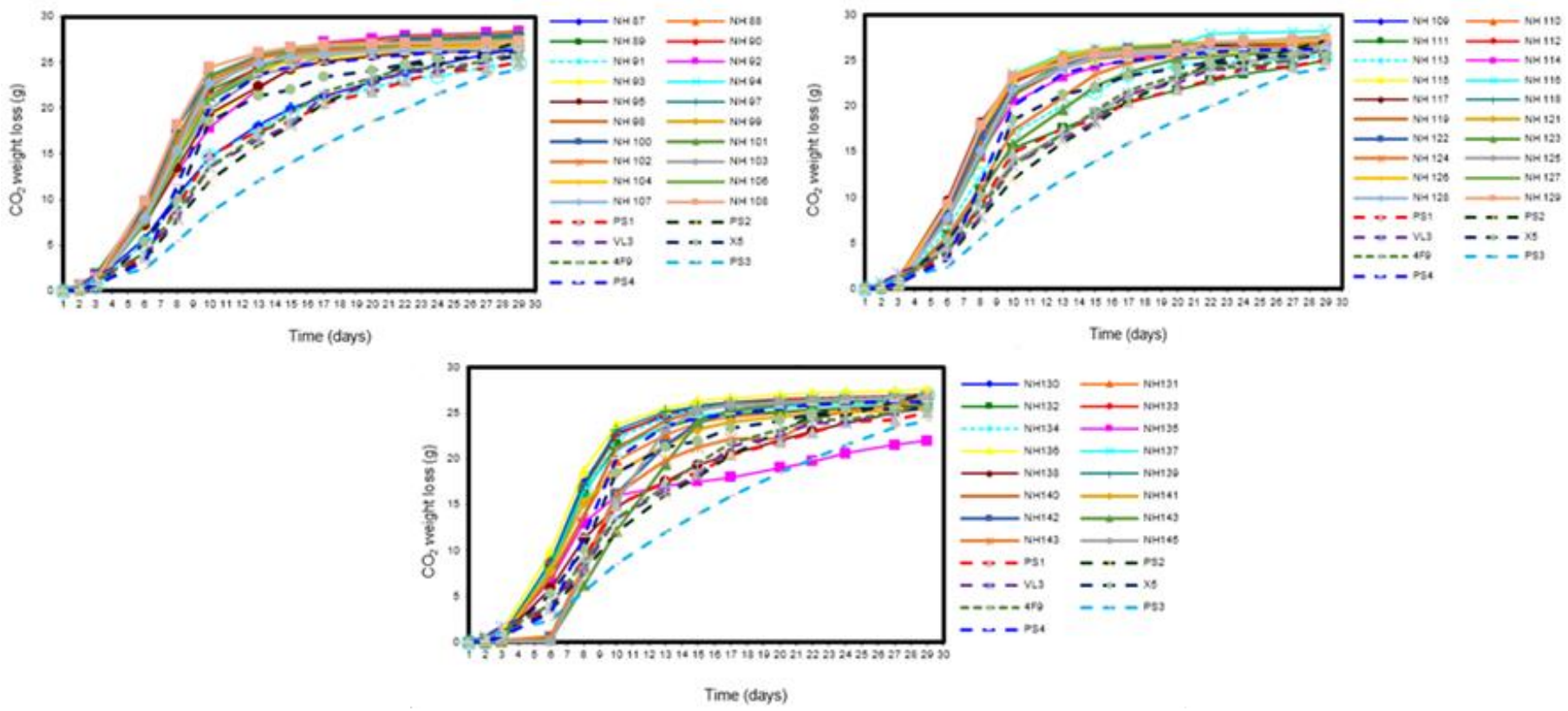

Figure 4. Contd.

VA of all the PS included in this study. Low VA forming hybrids must have inherited this trait from the respective PS that displayed this quality.

Most TFPH produced wines with a more positive association with pH compared to wines produced with commercial TRWY references that is, Fermicru 4F9, Zymaflore VL3 and Zymaflore X5 (Figure 5). However, all yeast strains included in this study on average produced wines with desired pH values (pH 3.3 ± 0.01) as described (Gauntner, 1997; Pambianchi, 2001). It was also observed that plenty of hybrids, including the TFPH already mentioned had a positive association with the titratable acidity (TA) that are closely related to pH, hence these wines were perceived to be fruitier, a wine aroma normally perceived within this pH range.

Also noteworthy is that, climate change together with a desire by wine producers to harvest grapes at optimal ripeness has led to grapes harvested with high sugar levels (Palliotti et al., 2014). Subsequently, these wines have undesirable high alcohol levels. Wine yeast strains suitable for the production of lower alcohol from grapes with higher sugar were identified as a global industry priority (Gardner et al., 2007; Contreras et al., 2014). Therefore, this study adds value to this priority, since promising LVPH (for example, NH 24, NH 73, NH 77, NH 124 and NH 145) also produced wines with lower alcohol levels

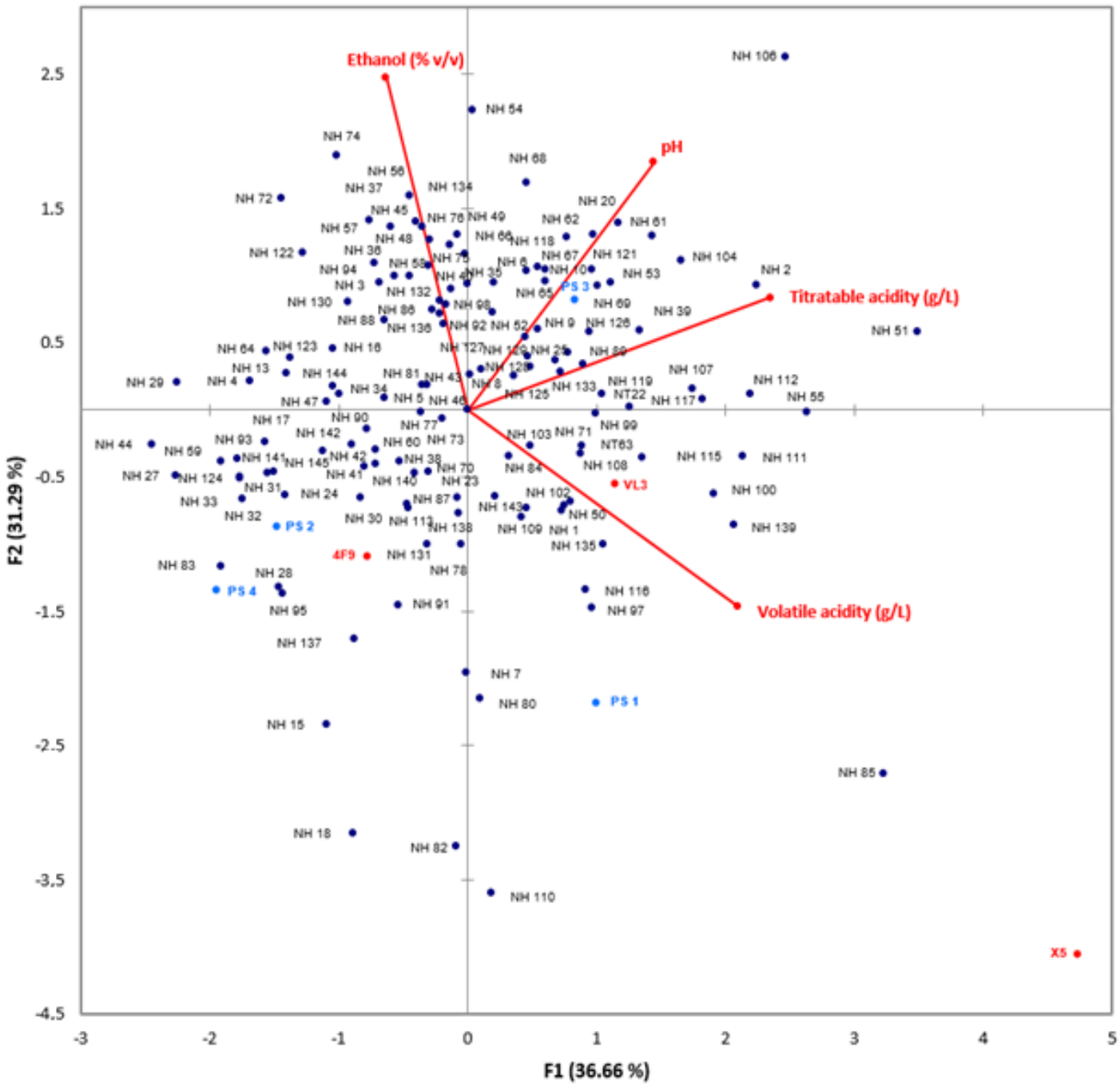

Figure 5. Biplot of basic chemical parameters of laboratory-scale Sauvignon blanc wine following fermentation by three commercial 'thiol-releasing' wine yeasts (TRWY), four parental yeast (PS) and 136 hybrid yeast (NH) strains. Average values of triplicate fermentations.

(negative association ethanol). It is envisioned that this observation will be investigated further as part of another study.

Sensory evaluation

The biplot of wine sensory data showed no distinct clusters, but rather a spread over the entire sensory space (Figure 6). Both commercial TRWY references Zymaflore VL3 and Zymaflore X5 produced wines with a positive association with tropical fruit aromas (Figure 6). Moreover, both TRWY were previously recommended for the production of aromatic white wines due to the yeast's 'thiol-releasing' abilities (Personal communication, 2010). The TRWY Fermicru 4F9 produced wines with relative

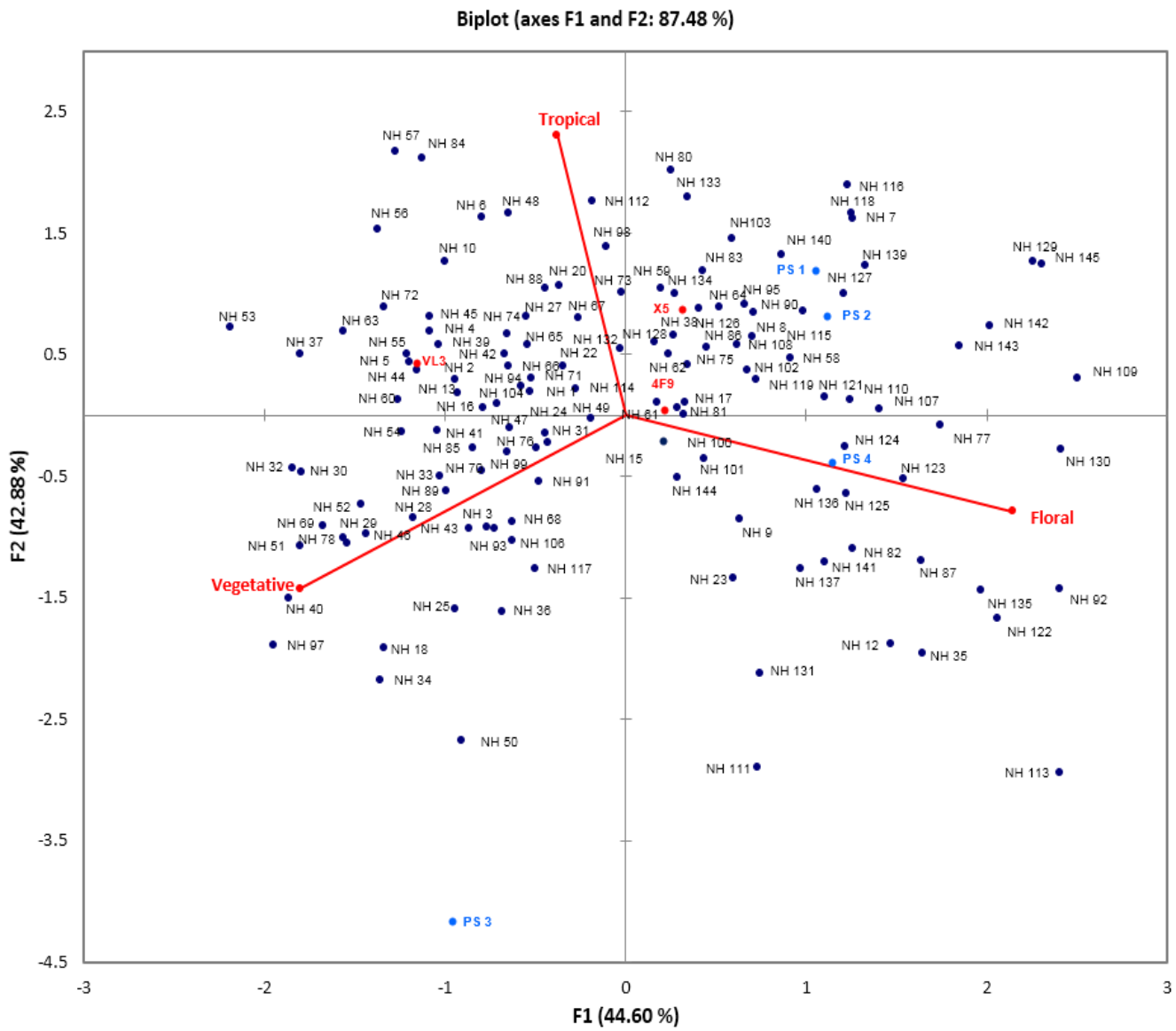

Figure 6. Biplot of descriptive sensory evaluation of laboratory-scale Sauvignon blanc wine following fermentation by three commercial 'thiol-releasing' wine yeasts (TRWY), four parental yeast (PS) and 136 hybrid yeast (NH) strains. Average values of triplicate fermentations.

less tropical fruit aroma than afore-mentioned TRWY, however the wines had a greater association with tropical aroma compared to wine produced with for example, PS 3. It is noteworthy that the Zymaflore VL3 produced wines had hints of vegetative aromas, whilst Fermicru 4F9 produced wine with a slight hint of floral aroma. It can tentatively be said that marginal vegetative aromas perceived in the Zymaflore VL3 produced wine is the result of the positive association with VA (Figure 5), whilst the hints of floral aroma perceived in the Fermicru 4F9 produced wines were due to overpowering tropical aroma. Therefore, higher VA levels observed in wines produced by Zymaflore X5 and Zymaflore VL3 were somehow masked by the overall positive aromas perceived. Nevertheless, commercial references produced wines with desired aroma notes and VA levels that complies with legislation.

Numerous hybrids, amongst others, NH 112, NH 98, NH 88, NH 84, NH 73, NH 67, NH 57, NH 56, NH 48 and NH 6 are considered TFPH, since they produced wines with enhanced tropical fruit aromas compared to commercial TRWY and TFPP. Some of these TFPH were similarly identified as LVPH (Figure 5). These hybrids, therefore, comply with both criteria put forward in the overall objective of this study. Wines with tropical fruit aroma and low VA levels are an industry priority, and the production thereof was previously achieved using co-inoculations and/or GMO (Swiegers et al., 2007).

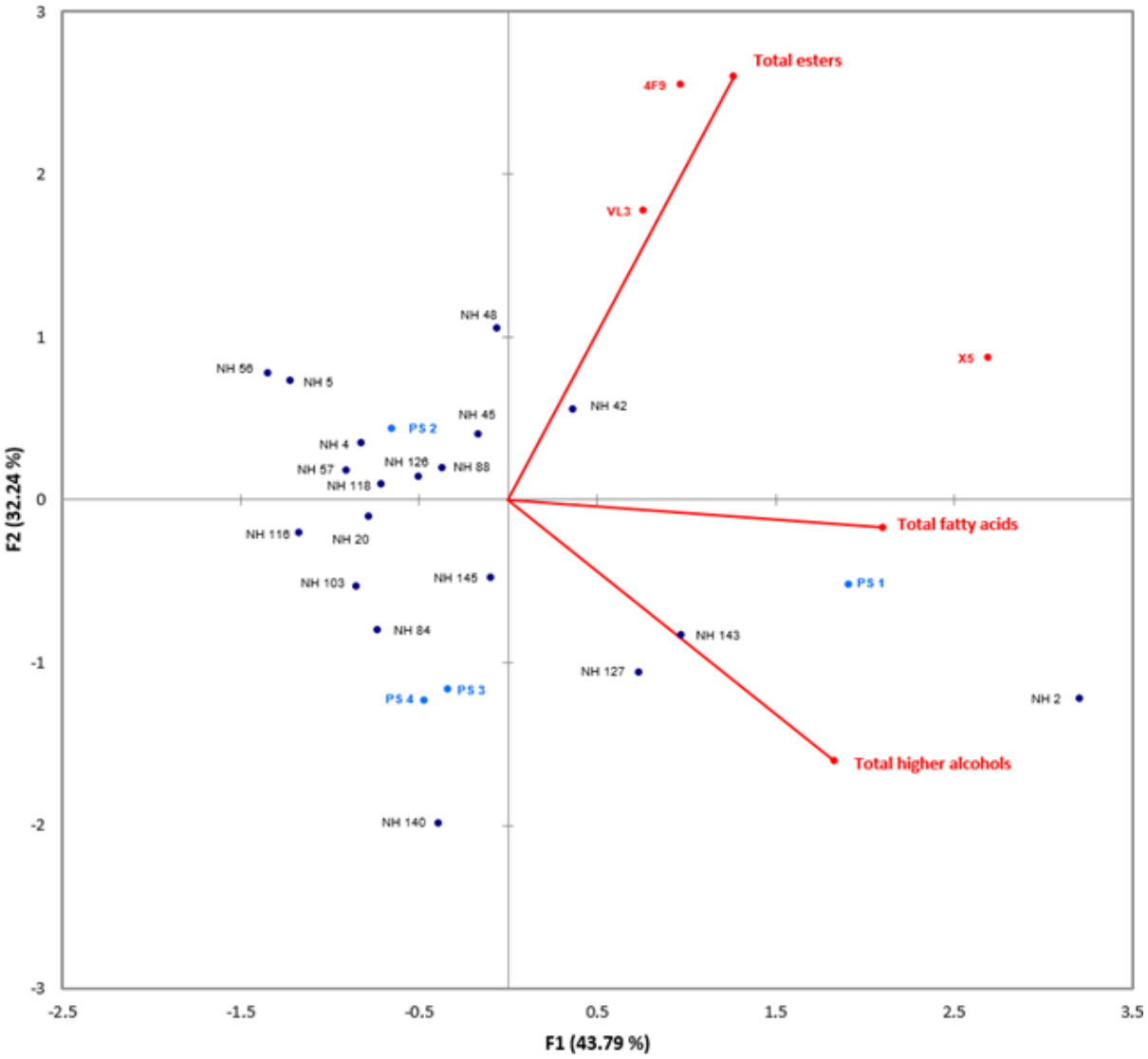

Figure 7. Biplot of aroma compounds in laboratory-scale Sauvignon blanc wine following fermentation by three commercial 'thiol-releasing' wine yeasts (TRWY), four parental yeast (PS) and selected hybrid yeast (NH) strains that produced wines with the fruitiest aroma. Average values of triplicate fermentations.

However, the use of GMO for wine production is currently illegal (Berrie, 2011). Therefore, it is envisioned that afore-mentioned TFPH and LVPH have a commercial role to play, since the fermentation potential of the parental strains were improved through natural occurring classical mating. Moreover, other hybrid strains (for example, NH 78, NH 46, NH 40, NH 34, NH 29, NH 28 and NH 18; and NH 136, NH 130, NH 124, NH 123, NH 92, NH 87, NH 82 and NH 77) that produced wines with pronounced vegetative and floral aromas, were also identified as LVPH. Two TFPP that is, PS 1 and PS 2 produced wines with tropical fruit and floral aromas, whilst the two LVPP that is, PS 3 and PS 4 produced wines with vegetative and floral aromas, respectively. In general, LVPH strains were evenly distributed on the sensory biplot, irrespective of wine sensory attributes.

Gas chromatography-mass spectrometry (GC-MS)

Gas chromatography-mass spectrometry analyses were performed on wines with the most prominent fruity aromas according to the descriptive sensory evaluation to determine aroma compounds that is, esters, total fatty acids and higher alcohols (Lambrechts and Pretorius, 2000). The PCA biplot of GC-MS data showed that the commercial TRWY reference strains Zymaflore VL3 and Fermicru 4F9 produced wines with high ester levels (Figure 7). In contrast, Zymaflore X5 and PS 1 produced wines with a positive association with total acids,

amongst others, acetic acid. Three TFPH (for example, NH 56, NH 118, and NH 145) produced wines with a negative association with total fatty acids, and therefore comply with both criteria indicated in the aims. The commercial TRWY reference Fermicru 4F9 produced wines with the highest ester levels (5.58 ± 1.42 mg/L). However, NH 48 produced wines with ester levels (4.07 ± 0.17 mg/L) that were comparable to wines produced by Zymaflore VL3 (4.80 ± 0.94 mg/L) and Zymaflore X5 (4.02 ± 0.80 mg/L), respectively. It is noteworthy that aforesaid TFPH *viz.* NH 56 (48.74 ± 0.11 mg/L); NH 118 (63.75 ± 1.03 mg/L); and NH 145 (75.26 ± 2.43 mg/L) produced wines with less acetic acid, the main volatile acid than wines produced by Fermicru 4F9 (79.01 ± 1.23 mg/L). The latter produced wines with the lowest acetic acid of the all commercial references included in this study. Therefore, GC-MS complemented FTIR spectroscopy, since LVPH also produced wines with lower acetic acid.

Conclusion

Improved hybrid strains were identified compared to commercial TRWY references and TFPP (for example, PS 1 and PS 2) and LVPP (for example, PS 3 and PS 4) included in this study. These hybrids showed lower VA formation, whilst producing aromatic and/or typical Sauvignon blanc wines. Moreover, observations during this study indicate that some commercially available yeast strains can be associated with VA formation. However, VA formation is also dependant on vintage and generalisation should be avoided. This study showed that classical mating is still practical to produce novel yeast strain with desired traits, whilst maintaining the green image of wine production.

Conflict of interest

The authors have not declared any conflict of interest.

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