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Characterisation and evaluation of thiol-releasing and lower volatile acidity forming intra-genus and inter-genus hybrid yeast strains for Sauvignon Blanc wine

R. S. Hart[1,2]*, B. K. Ndimba[1,2] and N. P. Jolly[1]

[1]Agricultural Research Council Infruitec-Nietvoorbij, Private Bag X5026, Stellenbosch 7599, South Africa.
[2]National Agricultural Proteomics Research and Services Unit (NAPRSU), Department of Biotechnology, University of the Western Cape, Private Bag X17, Robert Sobukwe Road, Bellville, 7535, South Africa.

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Wine yeast expressed proteins are influential during the production of varietal aromatic Sauvignon Blanc wines as they release or mediate aroma compounds and undesirable volatile acidity (VA). As *Torulaspora delbrueckii* in conjunction with *Saccharomyces cerevisiae* as well as a *S. cerevisiae*/*T. delbrueckii* inter-genus hybrid were previously shown to produce white wine with enhanced aroma and/or lower VA, intra- and novel inter-genus hybrids were trialled for the production of aromatic Sauvignon Blanc with lower VA. The inter-genus hybrid NH 07/1 produced wine with a more positive association with the aroma compound 3-mercaptohexylacetate (3MHA) than two commercial thiol-releasing wine yeast (TRWY) strains, Zymaflore X5 and Zymaflore VL3. The wine also had a negative association with VA, and a positive association with floral and tropical fruit aromas. Three intra-genus hybrids, NH 56, NH 57 and NH 88, produced wines with a negative association with VA, and a positive association with tropical fruit aroma. These wines also had a stronger association with the aroma compound, 3-mercaptohexanol (3MH) than wines produced with all commercial TRWY. The hybrid NH 07/1 and Zymaflore VL3 also over-expressed the lactoylglutathione lyase protein responsible for the release of the volatile thiol, 4-mercapto-4-methyl-pentan-2-one (4MMP) by cleaving its carbon-sulphur bonds. Therefore, lactoylglutathione lyase is a potential biomarker for 3MH-release, as this thiol also contains a carbon-sulphur-bond. Dehydrogenase proteins might also be useful biomarkers for VA formation by fermenting wine yeasts. Three intra- and one inter-genus hybrids with the abiliy to produce aromatic Sauvignon Blanc wines with lower VA compared to commercial TRWY references were identified.

Key words: Acetic acid, isobaric tags for relative and absolute quantitation (iTRAQ), metabolomic, Orbitrap liquid chromatography tandem mass spectrometry (LC-MS/MS), proteomic, solid phase extraction-gas chromatography coupled to tandem mass spectrometry (SPE GC-MS/MS), volatile thiols.

INTRODUCTION

Sauvignon Blanc wines are associated world-wide with either vegetative (herbaceous) or tropical fruit and/or floral aromas (Marais, 1994; Von Mollendorf, 2013; Hart et al., 2016). Key to the production of high quality

Sauvignon Blanc wines with the desired properties are wine yeasts, namely *Saccharomyces cerevisiae* that can convert relatively "neutral" grape must lacking varietal aromas into varietal-typical aromatic wines through their metabolic activity (Swiegers et al., 2006a; 2007a). Sauvignon Blanc wine aroma and flavour are the result of grape derived compounds (metabolites), *e.g.* methoxipyrazines, *de novo* synthesised metabolites or compounds released from aroma-inactive, non-volatile grape-derived precursors by wine yeast during fermentation (Bovo et al., 2015; Pinu et al., 2015). However, yeast also produces undesirable metabolites, for example, acetic acid the main contributor to volatile acidity (VA). These compounds are responsible for vinegar-like off-flavours that are detrimental to overall wine organoleptic quality (Du Toit and Pretorius, 2000; Swiegers et al., 2005). Such wines will have negative financial implications as expensive reverse osmosis techniques have to be used to remove the excessive VA. Commercial yeast strains implicated in the production of wines with higher VA values will create negative perceptions for the yeast manufacturer and result in loss of revenue due to lower yeast sales (Margaret Fundira, Personal communication, 2016).

Wine yeast expressed enzymes (proteins) during winemaking were previously reported to be key effectors of wine aroma and flavour compounds present in wines (Holt et al., 2011; Roncoroni et al., 2011). Furthermore, Holt et al. (2012) and Pretorius (2016) reported that yeast expressed proteins with carbon-sulphur *β*-lyase activity are involved in the release of the aroma enhancing volatile thiol, that is, 4-mercapto-4-methyl-pentan-2-one (4MMP). Dehydrogenase enzymes were also reported to be involved in the production of acetic acid, the main contributor to total fatty acids (Varela et al., 2012; Walkey et al., 2012). Additionally, it was reported that over-expression of dehydrogenase enzymes by wine yeast during fermentation of Sauvignon Blanc grape must resulted in wines with elevated total fatty acids (Hart et al., 2016, 2017).

The use of the yeast *Torulaspora delbrueckii* was shown to produce wines with lower VA levels, and enhancing varietal aromas when inoculated singly or sequentially with *S. cerevisiae* (Albertin et al., 2014; Renault et al., 2016). *S. cerevisiae*/*T. delbrueckii* inter-genus hybrids also produced wine with enhanced aroma and flavour upon completion of fermentation (Santos et al., 2008). Therefore, *T. delbrueckii* can be advantageous for the development of new hybrid strains with the ability to produce aromatic white wines with lower VA. For that reason, the aims of this study were to breed *S. cerevisiae*/*T. delbrueckii* inter-genus hybrids using classical mating which is naturally occuring phenomenon, characterise and evaluate these inter-genus hybrids for their fermentation potential, thiol-releasing abilities and low VA formation during the production of Sauvignon Blanc wines. Promising *S. cerevisiae* intra-genus hybrids previously identified by Hart et al. (2016) for their ability to produce wines with enhanced tropical fruit aroma (henceforth referred to as TFPH) and lower VA (henceforth referred to as LVPH) compared to commercial 'thiol-releasing' wine yeasts (TRWY) were included in this study. Additionally, wine yeast regulated proteins and aroma compounds, especially volatile thiols *viz*. 3-mercaptohexanol (3MH) and 3-mercaptohexylacetate (3MHA) as well as volatile acidity *viz*. acetic acid present at the end of fermentation and their association with final wine aroma and flavour were investigated. It is envisioned that potential protein biomarkers associated with aroma-enhancing metabolites and VA will be identified.

MATERIALS AND METHODS

Origin of yeast strains

Reference yeast strains: The following commercial *S. cerevisiae* hybrid strains, namely NT 112 and NT 116 (Anchor Yeast, South Africa) served as references for the laboratory-scale fermentations, whilst the commercial thiol-releasing wine yeast (TRWY) strains, VIN 7 and VIN 13 (Anchor Yeast, South Africa), Zymaflore VL3, Zymaflore X5 (Laffort Oenologie, France), and Fermicru 4F9 (DSM Oenology, Netherlands), were included as references for the small-scale fermentations. All TRWY were previously recommended for the production of aromatic white wines due to the yeast's 'thiol-releasing' abilities (Anonymous, Personal communication, 2005a, b, 2017a, b, c). Another commercial strain, N 96 (Anchor Yeast, South Africa) and an experimental strain, P 35 (ARC Infruitec-Nietvoorbij, South Africa) used in hybrid breeding programmes, were also included in this study as references. The latter strains have the ability to produce wine with tropical fruit aromas (henceforth abbreviated as TFPP).

Intra-genus hybrids: Ten *S. cerevisiae* intra-genus hybrids, NH 48, NH 56, NH 57, NH 84, NH 88, NH 97, NH 118, NH 140, NH 143 and NH 145, previously characterised as TFPH and LVPH were included in this study (Hart et al., 2016).

Inter-genus hybrids: Two inter-genus hybrids, NH 07/1 and NH 07/2, were generated through classical mating by fusing protoplasts originating from a *S. cerevisiae* strain MCB C6, isolated from Madeba cellar winery equipment, Robertson, South Africa and *T. delbrueckii* strain M2/1 (Van Breda et al., 2013), resulting in inter-genus hybrids. Briefly, freeze cultures containing diploid *S. cerevisiae* strain MCB C6 and the haploid *T. delbrueckii* (Sasaki and Ohshima, 1987; Kurtzman et al., 2011) strain M2/1 were

*Corresponding author. E-mail: hartr@arc.agric.za. Tel: +27-21-8093097. Fax: +27-21-8093260.

thawed and streaked onto yeast extract peptone dextrose (YPD) agar (Biolab, Merck, South Africa). Agar plates were incubated at 28°C for at least 48 h until single yeast colonies were visible. A single colony from the diploid (2n) *S. cerevisiae* yeast strain was aseptically transferred onto plates containing nitrogen-limiting growth media (0.25% [w/v] yeast extract, 0.1% [w/v] dextrose, 1% [v/v] potassium acetate, and 1% [w/v] agar) and incubated for 72 h at 28°C until asci, each containing four haploid (n) spores could be observed. Thereafter, a single colony from the sporulated *S. cerevisiae* MCB C6 culture and the *T. delbrueckii* M2/1 culture were transferred into separate tubes containing 10% (w/v) *β*-d-glucuronidase enzyme, and mixed until the suspension appeared milky. Protoplasts were generated by incubation of suspensions at 30°C for 30 min. Thereafter, sterile water was added to each micro-centrifuge tube to rinse cell residue from protoplasts. Respective supernatants were gently removed and transferred into new tubes. Thereafter, 100 µL of each protoplast-containing supernatant were streaked onto different sections of a YPDA-plate, and placed on a Singer MSM system series 200 micro-manipulator (Singer Instruments, Watched, Somerset, UK) as described (Morin et al., 2009). Protoplasts were physically disrupted using a micro-fine needle; where after haploid spores from the two parental strains were placed in close proximity on the YPDA. Thereafter, the plates were incubated at 28°C for at least 48 h to allow haploids to fuse (karyogamy) to form diploid (2n) inter-genus hybrids.

Characterisation techniques

Contour clamped homogeneous electric field (CHEF) DNA karyotyping

The CHEF DNA karyotyping was conducted according to the embedded agarose procedure used for commercial TRWY and intra-genus hybrids described by Hart et al. (2016). A Bio-Rad image analyser (Bio-Rad, Madrid, Spain) was used to visualise chromosomal banding patterns on 0.01% (v/v) ethidium bromide-stained agarose gels.

Matrix-assisted laser desorption/ionisation (MALDI) biotyping

Yeast strains were characterised by MALDI biotyping using a Bruker UltrafleXtreme MALDI-TOF/TOF MS (Bruker Daltonics, Bremen, Germany) used for commercial TRWY and intra-genus hybrids described by Hart et al. (2016).

Evaluation techniques

Laboratory-scale fermentation trials

Fermentation potential of wet culture inter-genus hybrids was evaluated in laboratory-scale vinifications of Chardonnay clarified grape must (juice) (total sugar 21.3°B; total acidity (TA) 8.1 g/L; pH 3.10), similar to vinifications with TRWY and intra-genus hybrids as described by Hart et al. (2016). Commercial yeast strains, NT 112 and NT 116 (Anchor Yeast, South Africa) were included in the trials as references. All fermentations were conducted in triplicate in a completely randomised order (Addelman, 1970) at 15°C, whilst gently shaking on an orbital shaker. Fermentations were monitored by CO_2 weight loss. Subsequently, both inter-genus hybrids were trialled in small-scale winemaking after it was established that they fermented the grape must (juice) to dryness (residual sugar <5 g/L) using a portable DMA 35 density meter (Anton Paar, Southern Africa).

Small-scale winemaking trials

Small-scale Sauvignon Blanc wines were made in triplicate using commercial TRWY, intra- and inter-genus hybrids according to a standardised cellar method as described by Hart and Jolly (2008). For each treatment replicate, nine litres Sauvignon Blanc grape must (total sugar 21.9°B; TA 9.3 g/L; pH 3.28) were dispensed into 10 L stainless steel canisters with fermentation caps, and inoculated with the respective wine yeast starter cultures. The method was adjusted by having the respective yeast inoculums cultured for 24 h in 600 mL YPD broth (Biolab, Merck, South Africa) medium. Subsequently, 180 mL of the 24 h cultures (optical density at 600 nm = 0.92 ± 0.05; cfu/mL = 10^7 ± 10^6; viability = 97.93% ± 1.67) were used to inoculate clarified Sauvignon Blanc grape must (2% inoculum). Fermenting must was sampled every 48 h to measure residual glucose/fructose (R/S), ethanol, VA, TA and pH, using an Oenofoss™ Fourier transform infrared (FTIR) spectrometer (FOSS Analytical A/S, Denmark) until fermentations went to dryness. This was repeated until the R/S concentrations were below 5 g/L, where after the free-SO_2 of the wines was adjusted to 35 mg/L, following racking. Wines were cold stabilised at 0°C for at least two weeks prior to bottling.

Gas chromatography (GC) analysis

Wine aroma metabolites, namely esters, total fatty acids and higher alcohols (fusel oils), were analysed by gas chromatography (GC) on wine samples (50 mL) taken on day 15 of fermentation as described by Hart et al. (2016, 2017).

Solid-phase extraction (SPE) and GC-MS/MS analysis

The main wine volatile thiols, 3MH and 3MHA, were pre-concentrated by deploying solid-phase extraction (SPE) as described by Hart et al. (2016, 2017). Subsequently, GC coupled to tandem mass spectrometry (GC-MS/MS) as described by Mattivi et al. (2012) was used to quantify volatile aromatic thiols. The GC-MS/MS system used in this study comprised of a GC Trace 1300/TSQ8000 mass selective detector equipped with an AI 1310 auto sampler (Thermo Scientific™ Inc, USA). Aroma compounds were separated using a 30 m × 0.25 mm × 0.25 µm Zebron WAX plus column (Phenomenex Inc., Torrance, CA, USA).

Sensory evaluation

An experienced panel consisting of 14 members conducted descriptive sensory evaluation of bottled wines. The panel was requested to indicate the intensity of aroma descriptors on a unipolar six-point numerical scale (absent [0], very low [1], low [2], medium [3], high [4] and very high [5]). Panel members also had to specify the most prominent aromas associated with Sauvignon Blanc wines *viz.* 'tropical fruit' (*e.g.* banana, guava, peach, passion fruit and citrus); 'vegetative' (*e.g.* asparagus, herbaceous, green pepper, green beans, cut grass, green olive and gooseberry); or 'floral' (*e.g.* rose, orange blossom), they perceived.

Quantitative LC-based iTRAQ proteomic analysis

Based on chemical (lower VA and total fatty acids) and sensory (tropical fruit aroma) analyses of final wines, yeast-containing ferments sampled (50 mL) in triplicate on day 15 of fermentation were selected for quantitative proteomic analysis using an iTRAQ 8-

plex reagent kit (AB Sciex, USA) in conjunction with LC-MS/MS at the mass spectroscopy unit, Proteomics laboratory, Central Analytical Facility (CAF), University of Stellenbosch (US). Briefly, proteins were extracted from the different strains, followed by alkylation in methylthiosulphonate (MMTS) and digestion at 37°C using 1 µg/µL trypsin solution (Promega, Madison, WI, USA) as described by Boutureira and Bernardes (2015). Tryptic digests originating from the eight yeasts (TRWY:VIN 7, Zymaflore VL3, Zymaflore X5, and Fermicru 4F9; the intra-genus TFPH and LVPH:NH 84; two promising natural isolates; MCB C6 and M 2/1; and one inter-genus hybrids:NH 07/1), were tagged according to manufacturer's recommendations with iTRAQ labels 113, 114, 115, 116, 117, 118, 119 and 121, respectively, as described by Kim et al. (2012). The TRWY VIN 7 served as reference as the yeast was reported to be a high 'thiol-releaser' used for the production varietal aromatic Sauvignon Blanc wines with enhanced tropical fruit aroma (Swiegers et al., 2006b; Howe, 2016). Subsequently, proteins were characterised using a mass spectrometer equipped with a nanospray flex ionisation source (Thermo Scientific™ Inc, USA) in conjunction with Mascot algorithm (Matrix DiffScience, London, UK), and SequestHT algorithm included in Proteome Discoverer v1.4. Isobaric tags for relative and absolute quantitation algorithm were used for protein quantitation. Only proteins with more than 2 peptides, but less than 20% variation, and iTRAQ ratios below 0.5 and above 2 were considered down-regulated and over-expressed, respectively. Differentially expressed proteins were also subjected to Protein ANalysis through Evolutionary Relationships (PANTHER, www.pantherdb.org/) to establish their involvement in biological processes, molecular function and protein classes (Sharma et al., 2014).

Statistical analyses

Analysis of variance (ANOVA) and principal component analysis (PCA) were conducted on data from chemical, sensory and metabolomic analyses data (Pearson, 1896, 1901; Zou et al., 2006). The linear relationship between the chemical, sensory and metabolomic variables was analysed by means of a Pearson's correlation using XLSTAT software (Addinsoft, 2013) with the principal components (PC's) as factors (that is, F1 and F2).

RESULTS AND DISCUSSION

Characterisation of yeast strains

Contour clamped homogeneous electric field DNA karyotyping

The CHEF DNA karyotyping technique was previously used to successfully differentiate between *S. cerevisiae* and *T. delbrueckii* yeast strains (van Breda et al., 2013). Additionally, CHEF could also differentiate between commercial TRWY strains, *S. cerevisiae* parental strains and *S. cerevisiae* intra-genus hybrids, NH 48, NH 56, NH 57, NH 84, NH 88, NH 97, NH 118, NH 140, NH 143 and NH 145 (Van der Westhuizen and Pretorius, 1992; Hoff, 2012; Hart et al., 2016). Subsequently, CHEF successfully differentiated *S. cerevisiae* MCB C6 and *T. delbrueckii* M2/1 parental strains from inter-genus hybrids, NH 07/1 and NH 07/2 during this investigation.

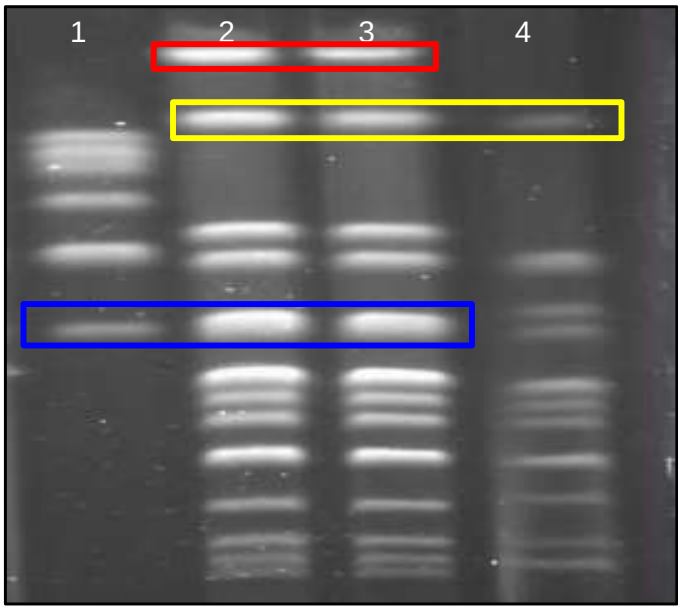

Figure 1. Contour clamped homogeneous electric field (CHEF) DNA karyotypes of parental strains, *S. cerevisiae* MCB C6 and *T. delbrueckii* M2/1, and inter-genus hybrids, NH 07/1 and NH 07/2 conserved in the ARC Infruitec-Nietvoorbij microbial culture collection (ARC Inf-Nvbij CC). Lane 1, M2/1; Lanes 2 and 3, NH 07/1 and NH 07/2; Lane 4, MCB C6.

The inter-genus hybrid strains shared similar (yellow and blue text box) and different (red text box) chromosomes in terms of size with both parental strains (Figure 1). Both inter-genus hybrids had matching DNA karyotypes, so they may be the same strain, hence MALDI biotyping was deployed as a complementary characterisation tool.

Matrix-assisted laser desorption/ionisation (MALDI) biotyping

Biotyping successfully differentiated between commercial TRWY strains, *S. cerevisiae* parental strains and *S. cerevisiae* intra-genus hybrids, NH 48, NH 56, NH 57, NH 84, NH 88, NH 97, NH 118, NH 140, NH 143 and NH 145 (Hart et al., 2016). Ribosomal proteins extracted from *S. cerevisiae* MCB C6 and *T. delbrueckii* M2/1 and inter-genus hybrids, NH 07/1 and NH 07/2 were matched to that of a database described by Hart et al. (2016, 2017). Strains MCB C6, NH 07/1 and NH 07/2 were identified as *Candida robusta,* the anamorph to *S. cerevisiae* (Diddens and Lodder, 1942; Kurtzman et al., 2011), whilst strain M2/1 was identified as *C. collucilosa,* the anamorph to *T. delburueckii* (Table 1) (Van Breda et al., 2013; Jolly et al., 2014). It can tentatively be speculated that inter-genus hybdrids were classified as *C. robusta,* as the database does not have inter-genus reference accessions.

Table 1. Matrix assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF/MS) real time classification of parental strains, *Saccharomyces cerevisiae* MCB C6 and *Torulaspora delbrueckii* M2/1 and inter-genus hybrids, NH 07/1 and NH 07/2 used for the production of varietal Sauvignon Blanc wines.

Mass spectra number	Yeast strain	MALDI-TOF MS log (score) value	Identification
1	MCB C6[3]	1.7	*Candida robusta*[1]
2	M2/1[3]	2.0	*C. colliculosa*[2]
3	NH 07/1[3]	2.1	*Candida robusta*[1]
4	NH 07/2[3]	1.9	*C. robusta*[1]

[1]***C. robusta*** *(**anamorph*** of ***S. cerevisiae***); [2]***C. colliculosa*** *(**anamorph*** of ***T. delbrueckii)***; [3]Experimental yeast (ARC Infruitec-Nietvoorbij, Stellenbosch, South Africa). *It can tentatively be speculated that inter-genus hybdrids were classified as *C. robusta,* as the database does not have inter-genus reference accessions.

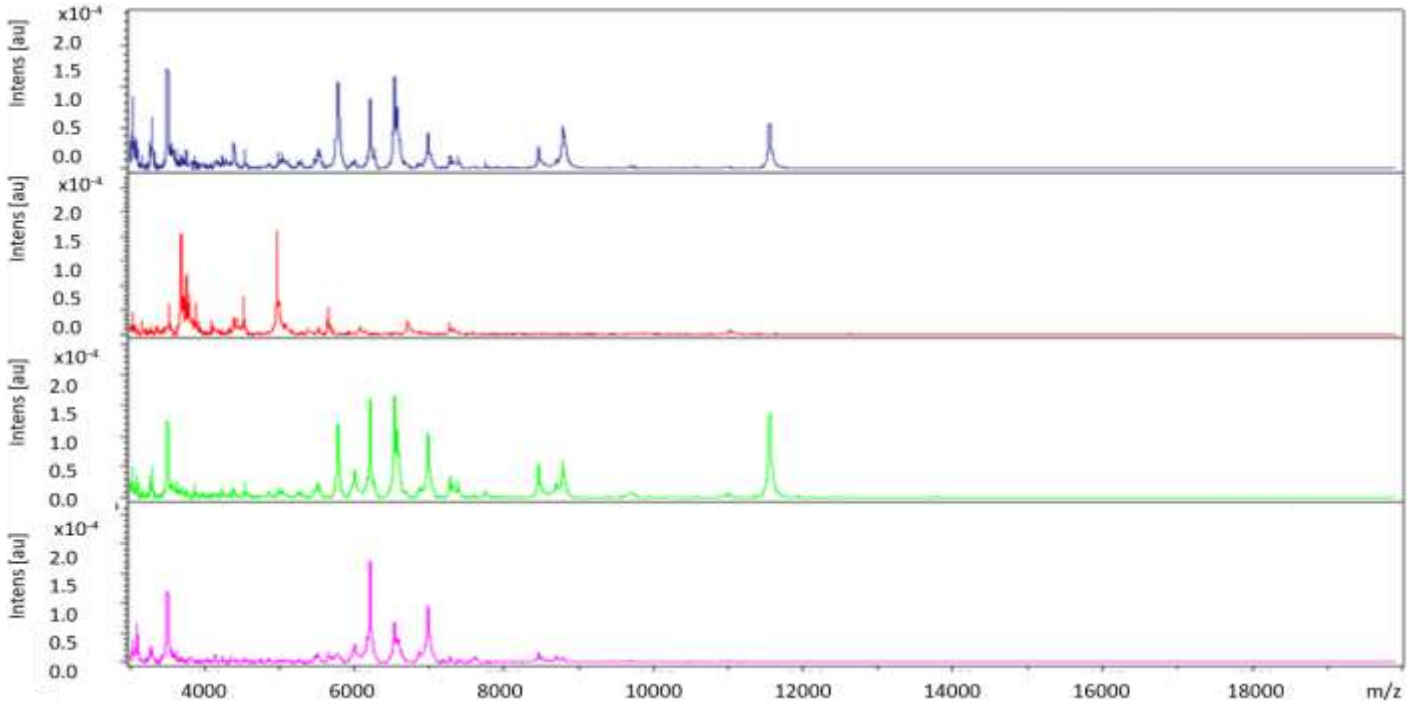

Figure 2. Matrix assisted laser desorption/ionisation-time of flight mass spectrometry (MALDI-TOF MS) spectral fingerprints of parental strains, *S. cerevisiae* MCB C6 (blue-coloured spectrum) and *T. delbrueckii* M2/1 (red-coloured spectrum) and inter-genus hybrids, NH 07/1 (green-coloured spectrum) and NH 07/2 (purple-coloured spectrum) conserved in the ARC Infruitec-Nietvoorbij microbial culture collection. Inter-genus hybrids were selected for the production of aromatic white wine, especially Sauvignon Blanc. The absolute intensities of the ions and mass-to-charge (m/z) ratios are represented on the y- and x-axis, respectively.

It is envisioned that the database will be extended by including spectral data of both novel inter-genus hybrids. Parental strains MCB C6 and M2/1 and inter-genus hybrids, that is, NH 07/1 and NH 07/2 also had distinctive mass spectra (Figure 2). Therefore, MALDI-TOF/MS biotyping proved more reliable to distinguish closely related inter-genus hybrids compared to CHEF karyotyping. Nonetheless, the two methods were complementary, as inter-genus hybrids were distinguished from parental strains.

Evaluation of yeast strains

Laboratory-scale fermentation trials

Hart et al. (2016, 2017) previously reported on the fermentation potential of intra-genus hybrids NH 48, NH 56, NH 57, NH 84, NH 88, NH 97, NH 118, NH 140, NH 143 and NH 145 compared to commercial TRWY references used in this study. Laboratory-scale white wine fermentations showed that both inter-genus hybrids,

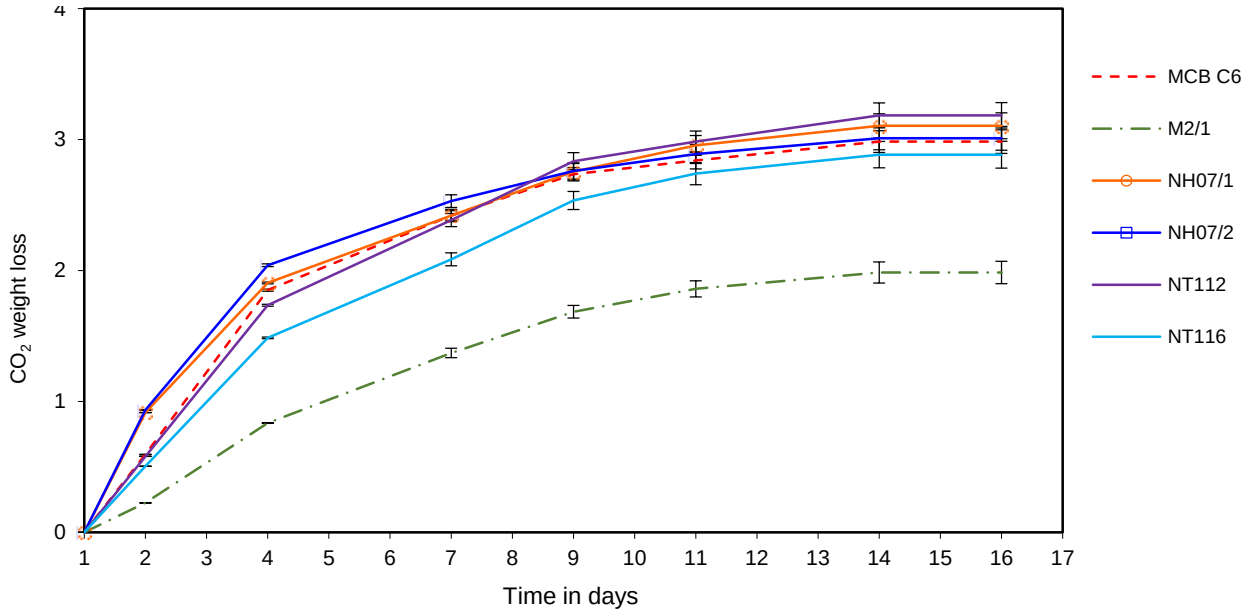

Figure 3. CO_2 weight loss of Chardonnay grape must (juice) fermented at an ambient temperature of 15°C using *S. cerevisiae* strain MCB C6, *T. delbrueckii* strain M2/1 and inter-genus hybrids, NH 07/1 and NH 07/2 in laboratory-scale vinifications.

NH 07/1 and NH 07/2 were also able to ferment grape must at a similar rate to commercial references, NT 112 and NT 116 as well as the *S. cerevisiae* strain MCB C6 parental yeast (Figure 3). The parental strain MCB C6 and inter-genus hybrids fermented at a similar rate, whilst the *T. delbrueckii* parental strain M2/1 fermented at a slower rate. Both hybrids also fermented the grape must to dryness (<5 g/L). Nonetheless, the latter was chosen as parental strain for its lower VA formation as reported by Van Breda et al. (2013). Subsequently, both inter-genus strains were compared to intra-genus hybrids and commercial TRWY references for small-scale production of varietal aromatic Sauvignon Blanc wines with lower VA.

Small-scale winemaking

Fourier transform infra-red (FTIR) spectroscopy

Principle component analysis biplot of standard wine chemical parameters showed that both parental strains *S. cerevisiae* MCB C6 and *T. delbrueckii* M2/1 and inter-genus hybrids NH 07/1 and NH 07/2 produced final Sauvignon Blanc with a negative association with VA (Figure 4). This observation with regard to *T. delbrueckii* M2/1 complements observations made by Jolly et al. (2003) and Van Breda et al. (2013). The inter-genus hybrids can also provisionally be classified as LVPH a trait inherited from the non-*Saccharomyces* parental strain. Intra-genus hybrid strains provisionally characterised as LVPH, NH 48, NH 57, NH 143, and NH 145 were positioned in the left quadrants (Figure 4), and the wines also had a negative association with VA. The yeast Zymaflore VL3, positioned in the top-right quadrant, was the only commercial TRWY reference that produced wine with a positive association with VA (Figure 4).

Sensory evaluation

Overall, none of the wines were perceived to be undesirable during descriptive sensory evaluation, but differences were evident regarding expression of tropical fruit, floral and vegetative aroma notes (Figure 5). The PCA biplot of descriptive sensory evaluation data showed that two commercial TRWY references, VIN 7 and Zymaflore X5 positioned in the bottom left quadrant, produced Sauvignon Blanc wines with a positive association with tropical fruit aromas, thereby supporting recommendations by yeast manufacturers for their use in the production of aromatic white wines, especially Sauvignon Blanc (Figure 5). The commercial TRWY, Zymaflore VL3 and Fermicru 4F9 positioned in the top quadrants, on the other hand produced wines that is positively associated with floral and vegetative aromas. The commercial TRWY VIN 13, two intra-genus TFPH and LVPH, NH 56 and NH 97 as well as the intra-genus hybrid parental strains, N 96 and P 35 positioned in the

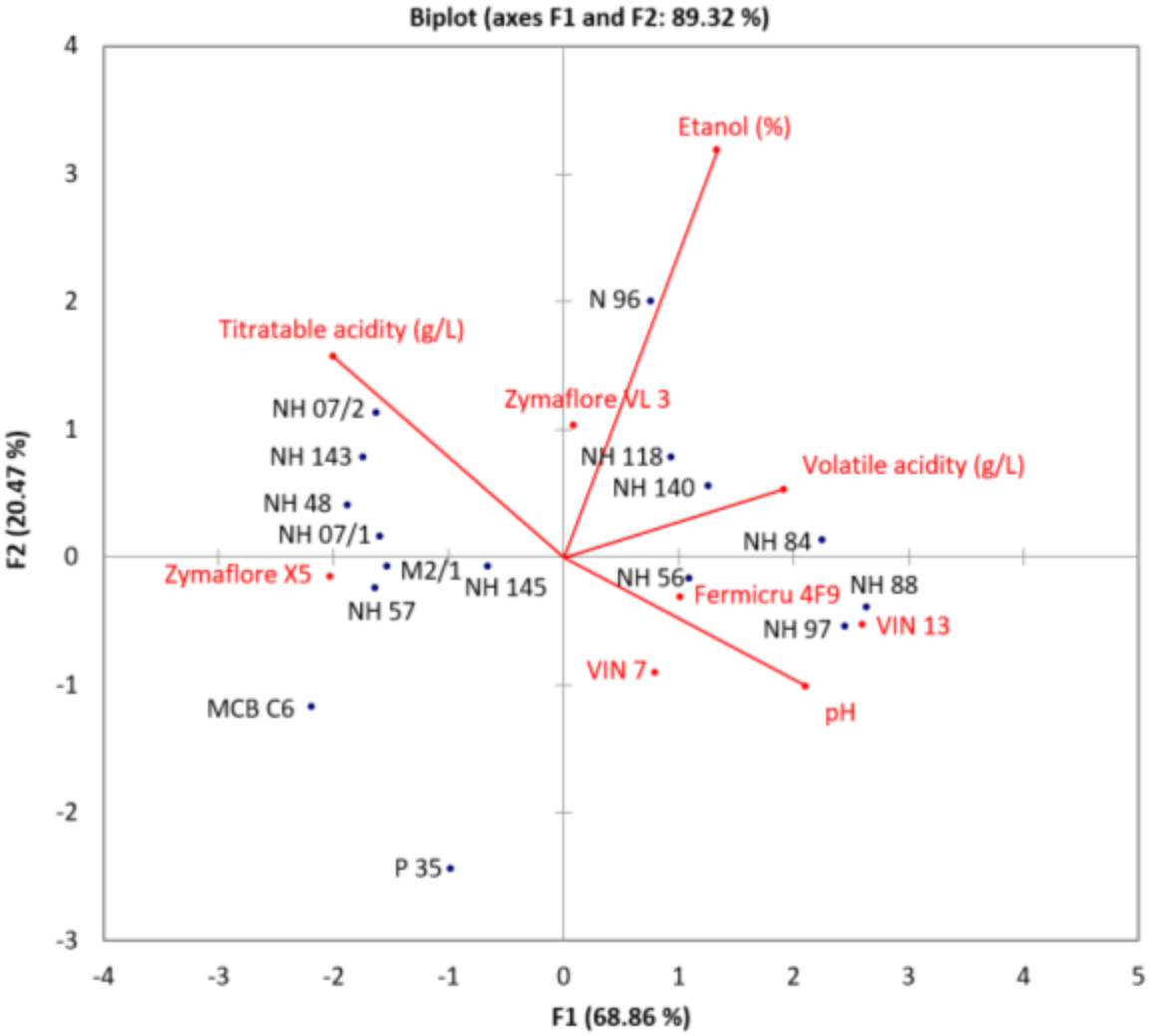

Figure 4. Biplot of basic chemical parameters of small-scale Sauvignon Blanc wine following fermentation by five 'thiol-releasing' commercial wine yeasts (TRWY), VIN 7 and VIN 13, Zymaflore VL3, Zymaflore X5, and Fermicru 4F9, two yeast strains with ability to produce wine with tropical fruit aromas, N 96 and P 35, ten intra-genus hybrids with the ability to produce wines with enhanced tropical fruit aroma and low VA, NH 48, NH 56, NH 57, NH 84, NH 88, NH 97, NH 118, NH 140, NH 143 and NH 145; and MCB C6 and M2/1 and inter-genus hybrids, NH 07/1 and NH 07/2 conserved in the ARC Infruitec-Nietvoorbij microbial culture collection. Average values of triplicate fermentations.

top left quadrant, produced wines that positively associated with both vegetative and tropical fruit aromas. Vegetative aromas associated with Sauvignon Blanc wines can be attributed to grape-derived aroma compounds for example, 2-isobutyl-3-methoxypyrazine (IBMP), especially when grapes were harvested and processed under cooler conditions (Marais, 1994; Lapalus, 2016). It can, therefore, be concluded that these compounds masked the effect of the sought-after volatile thiols (Marais, 1994) associated with tropical fruit aroma, as VIN 13 is a known TRWY strain (Swiegers et al., 2009; Von Mollendorf, 2013).

Both inter-genus hybrids, NH 07/1 and NH 07/2 as well as two intra-genus TFPH, NH 118 and NH 145 produced wines with a positive association with floral aromas (Figure 5). These hybrids can provisionally be characterised as having the ability to produce wines with floral aroma (henceforth referred to as FLPH). Both MCB C6 and M2/1, positioned in the bottom quadrants, produced wines that is associated with floral and tropical fruit aromas. This supports previous observations that a *T. delbrueckii* strain (Belda et al., 2015; Renault et al., 2016) as well as an *S. cerevisiae*/*T. delbrueckii* inter-genus hybrid (Santos et al., 2008) produced aromatic

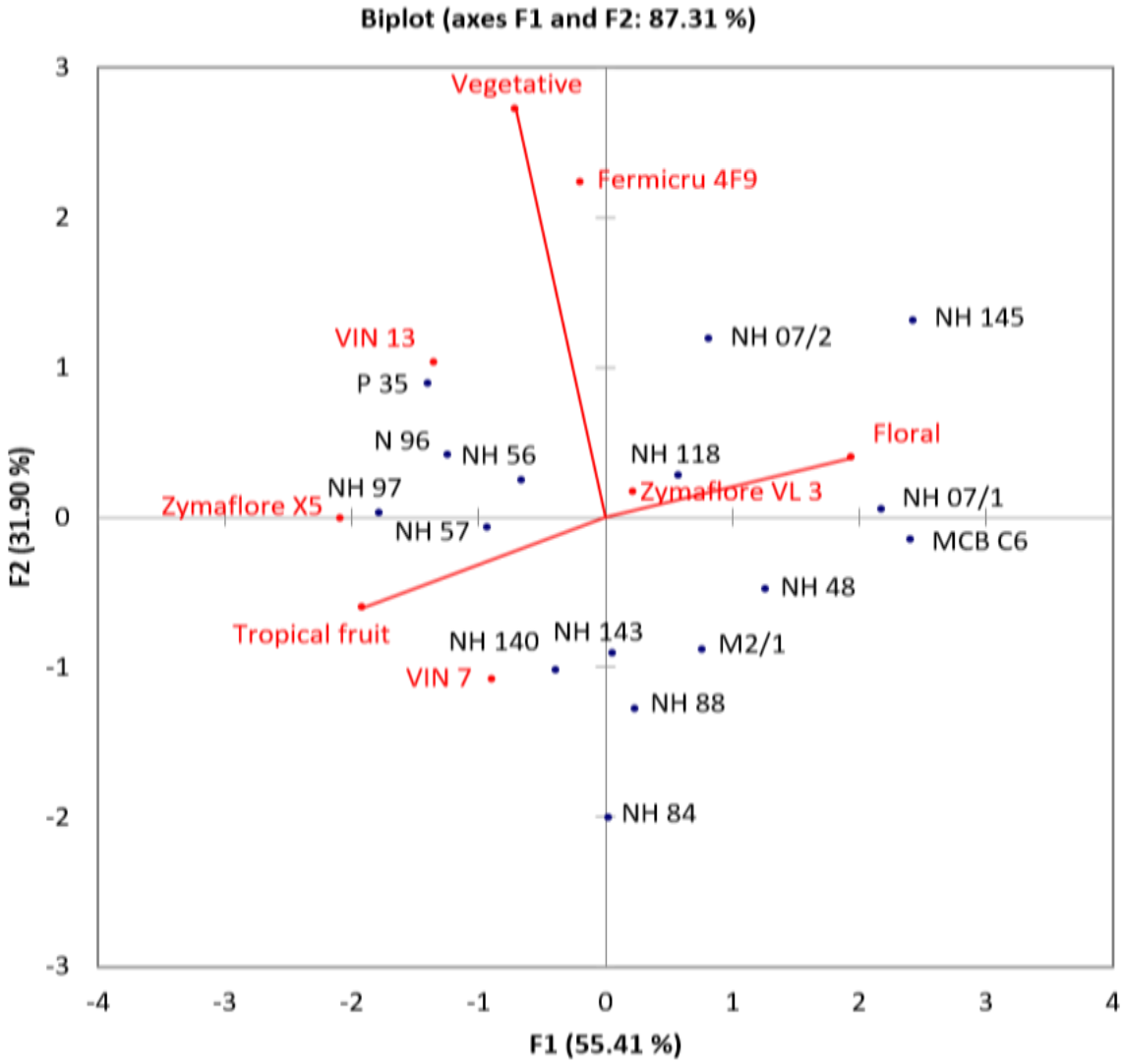

Figure 5. Biplot of descriptive sensory evaluation of small-scale Sauvignon Blanc wine following fermentation by five 'thiol-releasing' commercial wine yeasts (TRWY), VIN 7 and VIN 13, Zymaflore VL3, Zymaflore X5, and Fermicru 4F9, two yeast strains with ability to produce wine with tropical fruit aromas, N 96 and P 35, ten intra-genus hybrids with the ability to produce wines with enhanced tropical fruit aroma and low VA, NH 48, NH 56, NH 57, NH 84, NH 88, NH 97, NH 118, NH 140, NH 143 and NH 145; and MCB C6 and M2/1 and inter-genus hybrids, NH 07/1 and NH 07/2 conserved in the ARC Infruitec-Nietvoorbij microbial culture collection. Values are average of triplicate fermentations.

wines. Floral aromas, frequently associated with Sauvignon Blanc wines, are the result of yeast-mediated metabolites, namely monoterpenes produced from pre-cursors present in grape must (Von Mollendorf, 2013; Hart et al., 2017). It is apparent that the inter-genus hybrids inherited the ability to release monoterpenes from the parental strains. The intra-genus TFPH, NH 48, NH 84, NH 88 and NH 143 some of which were also shown to be LVPH (Figure 4), produced wines with a positive association with tropical fruit and floral aromas. These TFPH also produced wines with a negative association with vegetative aromas. Both inter-genus FLPH, NH 07/1 and NH 07/2, also produced Sauvignon Blanc wines with a negative association with VA (Figure 4) and can provisionally be characterised as LVPH. Two intra-genus TFPH and LVPH, NH 57 and NH 145 produced wines with a negative association with VA (Figure 4), and a positive association with tropical fruit aroma (Figure 5). Therefore, these intra- and interspecfic TFPH, FLPH and LVPH yeasts showed promise for the production of typical varietal aromatic Sauvignon Blanc wines with lower VA.

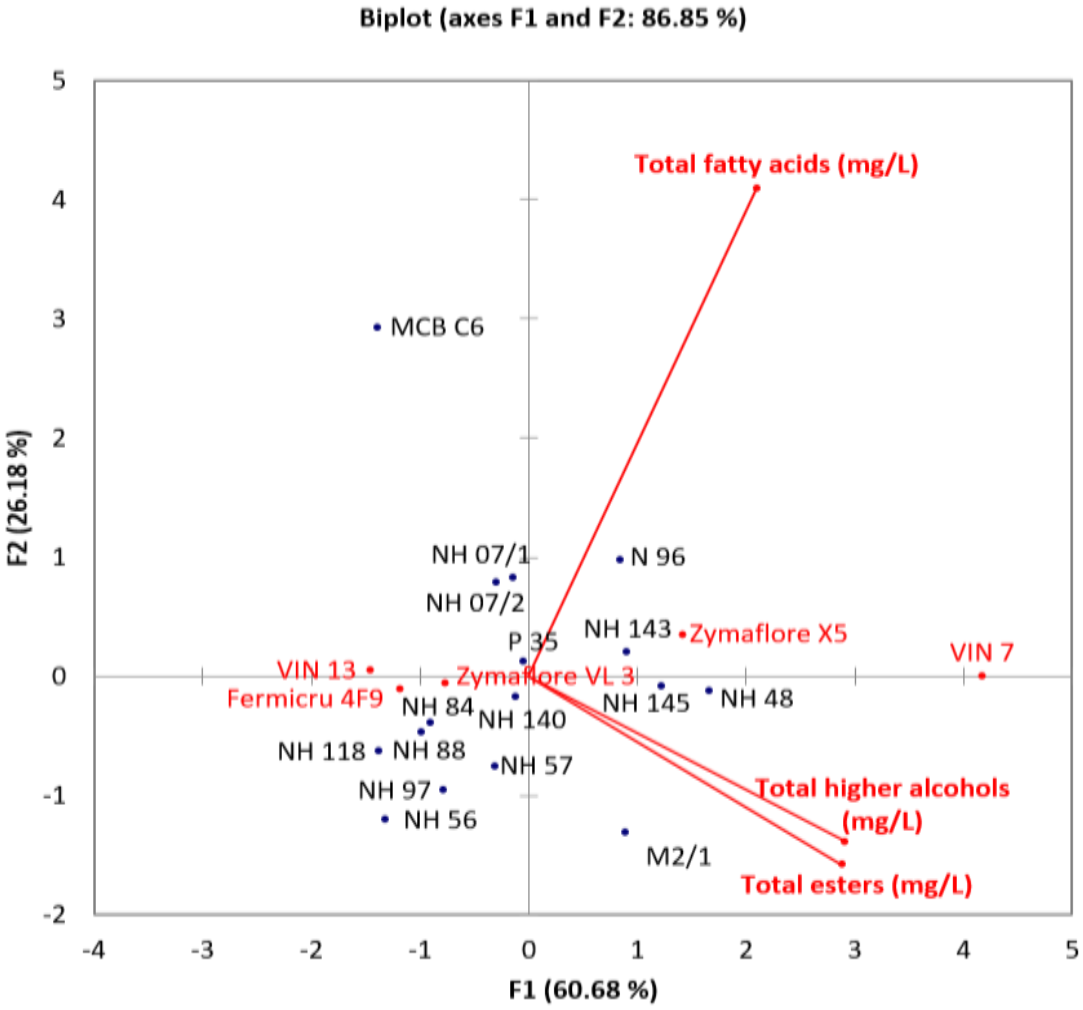

Figure 6. Biplot of aroma compounds, esters, higher alcohols and fatty acids in small-scale Sauvignon Blanc wine following fermentation by five 'thiol-releasing' commercial wine yeasts (TRWY), VIN 7 and VIN 13, Zymaflore VL3, Zymaflore X5, and Fermicru 4F9, two yeast strains with ability to produce wine with tropical fruit aromas, N 96 and P 35, ten intra-genus hybrids with the ability to produce wines with enhanced tropical fruit aroma and low VA, NH 48, NH 56, NH 57, NH 84, NH 88, NH 97, NH 118, NH 140, NH 143 and NH 145; and MCB C6 and M2/1 and inter-genus hybrids, NH 07/1 and NH 07/2 conserved in the ARC Infruitec-Nietvoorbij microbial culture collection. Values are average of triplicate fermentations.

Gas chromatography (GC) analysis

Gas chromatography was deployed to quantify wine aroma compounds, namely esters, total fatty acids and higher alcohols, most of which are associated with wine 'fermentation bouquet' and/or 'fruitiness' (Lambrechts and Pretorius, 2000; Coetzee and du Toit, 2015). The PCA biplot showed that strain *T. delbrueckii* M2/1 produced wines with a positive association with esters and higher alcohols compared to the remaining *S. cerevisiae* and hybrid yeast strains (Figure 6). Strains of *T. delbrueckii* were previously reported to produce wines with enhanced aroma (Van Breda et al., 2013; Renault et al., 2016). The TRWY reference VIN 7 and the intra-genus hybrids, NH 48 and NH 145, positioned in the right quadrant, also produced wines with a positive association with esters and higher alcohols, which imparts fruity aromas and complexity. These wines had positive associations with, amongst others, tropical fruit and floral aromas (Figure 5).

The TRWY reference, Zymaflore X5, the two TFPP, N 96 (Figure 5) as well as the intra-genus hybrid NH 143, positioned in the top right quadrant, produced wines with a positive association with total fatty acids also referred to as volatile fatty acids. Some volatile fatty acids (for example, octanoic acid, decanoic acid) were reported to be associated with faint fruity and citrus wine aromas (Lambrechts and Pretorius, 2000). However, acetic acid, responsible for vinegar-like off-flavours at higher concen-

trations still remains the main contributor to total fatty acids (Swiegers et al., 2005; Ugliano et al., 2009; Vilela-Moura et al., 2011). Nevertheless, Zymaflore X5 still produced wines with a positive association with fruity aroma, specifically tropical fruit (Figure 5). The TRWY references VIN 13, Zymaflore VL3 and Fermicru 4F9, positioned in the left quadrants, produced wines with a negative association with total fatty acids (Figure 6). These wines also had a positive association with, amongst others, tropical fruit and floral aromas (Figure 5).

Both inter-genus FLPH, NH 07/1 and NH 07/2 also produced wines with a negative association with volatile fatty acids. This can also be seen in Figures 4 and 5, there was a negative association with VA (Figure 4) and positive association with floral aroma (Figure 5). The lower production of VA by the inter-genus hybrids can be attributed to inheritance from the *T. delbrueckii* parent strain.

Seven intra-genus hybrids provisionally characterised as TFPH and LVPH, namely NH 56, NH 57, NH 84, NH 88, NH 97, NH 118 and NH 140 also produced wines with a negative association with volatile fatty acids, including acetic acid (Figure 6). Two of these intra-genus hybrids were also shown to produce Sauvignon Blanc wines with a negative association with VA (Figure 4) and positive association with floral aroma (Figure 5). These intra-genus hybrids also produced wines with a postive association with esters and higher alcohols (Figure 6). Yeast strains, namely VIN 7, M2/1, NH 48, NH 57, NH 84, NH 88, NH 140 and NH 143 produced wines with a positive association with 'fruitiness' (tropical fruit aroma) (Figure 5) and higher alcohols (Figure 6). Therefore, this observation compliments a previous study that showed higher alcohols to be the key precursors involved in ester formation (Patrianakou and Roussis, 2013). Based on this data set, intra- and inter-genus TFPH, FPH and LVPH have great potential for the production of varietal aromatic Sauvignon Blanc wines with lower VA, as they comply with yeast selection criteria set forth in the objectives.

Solid-phase extraction (SPE) and GC-MS/MS analysis

Volatile aromatic thiols; for example, 3-mercaptohexan-1-ol (3MH) and 3-mercaptohexyl acetate (3MHA), primarily responsible for passion fruit, tropical fruit and citrus aromas in Sauvignon Blanc wines, are released by fermenting wine yeasts from aroma-inactive, bound precursors present in grape juice (Swiegers et al., 2007b; Roland et al., 2011; Harsch et al., 2013). The PCA biplot of volatile thiol analyses showed that the inter-genus hybrid NH 07/1 produced wine with a more positive association with volatile thiols, 3MHA in particular, than either parental strain as well as known two commercial TRWY yeasts, Zymaflore X5 and Zymaflore VL3 (Figure 7). It is noteworthy that NH 07/1 also produced wines with a negative association with VA (Figure 4) and acetic acid (Figure 5), whilst having a positive association with floral aroma (Figure 6). These wines also had hints of tropical fruit aroma, and data suggests that inter-genus hybrids can be applied for the production of varietal aromatic Sauvignon Blanc wines with lower VA. The inter-genus hybrid NH 07/2, on the other hand, produced wines with a negative association with 3MH and 3MHA (Figure 7). This observation compliments the descriptive sensory evaluation, as NH 07/2 produced wines with a positive association with vegetative aroma (Figure 5). Nonetheless, NH 07/2 produced wines with chemically detectable 3MH and 3MHA levels, albeit lowest of all yeast strains included in this study. It can, therefore, be speculated that the tropical fruit aroma and effect of volatile thiols were masked by methoxypyrazines, another aroma compound naturally associated with this cultivar (Marais, 1994; Lapalus, 2016).

Three intra-genus hybrids, NH 56, NH 84 and NH 88 produced wines with stronger association with the volatile thiol 3MH than the commercial TRWY VIN 7 reference. The latter was also reported to be the highest producer of another volatile thiol, 4MMP, associated with 'fruity' aroma in wine during previous studies (Swiegers et al., 2009; Borneman et al., 2012). Five more intra-genus yeasts, namely NH 48, NH 118, NH 140, NH 143 and NH 145 also produced wines with a stronger association with 3MH than wines produced with the commercial TRWY Zymaflore X5 and Zymaflore VL3 (Dubourdieu, 2006; Bowyer et al., 2008). These hybrids also produced wines with a stronger association with 3MHA than wines produced with the commercial TRWY VIN 13. Three intra-genus TFPH and LVPH, NH 56, NH 57 and NH 88 also produced wines with a negative association with VA (Figure 4) and acetic acid (Figure 5), whilst having a positive association with tropical fruit aroma (Figure 6). Therefore, observations made during this study is indicative that these intra-genus hybrids can be used for the production of varietal aromatic Sauvignon Blanc wines with low VA. It is noteworthy that the intra-genus TFPH, namely NH 84 produced wines with 3MH levels that were significantly higher than its sensory detection threshold (Van Wyngaard, 2013). The 3MH levels in these wines were also discernibly higher compared to wines produced by the best commercial TRWY reference VIN 7 in this study. The TFPP N 96, considered to be a 'neutral' yeast by the manufacturer (Anchor Yeast, South Africa - N 96 product data sheet), produced wines with a more positive association with 3MH (Figure 7) than the *T. delbrueckii* M2/1 previously shown to produce aromatic wines (Jolly et al., 2003; Van Breda et al., 2013).

Indications, therefore, are that intra-genus TFPH inherited the 'thiol-releasing' abilities from both *S. cerevisiae* parental strains, that is, N 96 and P 35. The latter produced wines with a stronger association with 3MH than all commercial TRWY references included in this study (Figure 7).

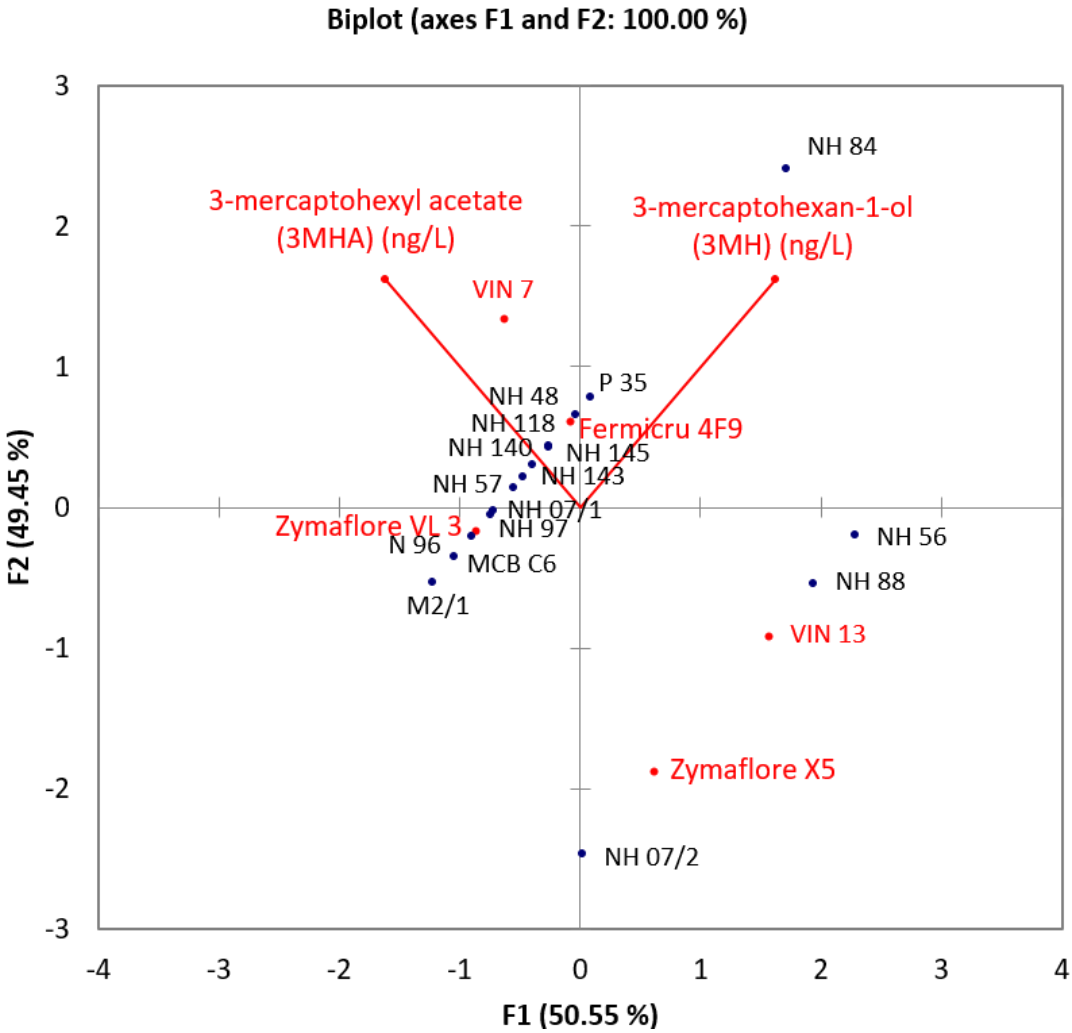

Figure 7. Biplot of volatile thiols, 3MH and 3MHA in small-scale Sauvignon Blanc wine following fermentation by five 'thiol-releasing' commercial wine yeasts (TRWY), VIN 7 and VIN 13, Zymaflore VL3, Zymaflore X5, and Fermicru 4F9, two yeast strains with ability to produce wine with tropical fruit aromas, N 96 and P 35, ten intra-genus hybrids with the ability to produce wines with enhanced tropical fruit aroma and low VA, NH 48, NH 56, NH 57, NH 84, NH 88, NH 97, NH 118, NH 140, NH 143 and NH 145; and MCB C6 and M2/1 and inter-genus hybrids, NH 07/1 and NH 07/2 conserved in the ARC Infruitec-Nietvoorbij microbial culture collection. Values are average of triplicate fermentations.

Quantitative LC-based iTRAQ proteomic analysis

Yeast-expressed enzymes (proteins) during fermentation regulate wine aroma compounds (metabolites) responsible for wine aroma and flavour (organoleptic quality) (Moreno-García et al., 2015). Analysis of the combined datasets in conjunction with Uniprot *S. cerevisiae* database identified a total of 998 yeast derived proteins (Table 2) on day 15 of fermentation when fermentations stabilised and/or were dry. Commercial TRWY (VIN 7, Zymaflore VL3, Zymaflore X5, and Fermicru 4F9), naturally isolated parental strains (MCB C6 and M2/1), as well as both intra- and inter-genus hybrids (NH 84, NH 07/1) were shown to vary in their up- and down-regulated proteins compared to the TRWY VIN 7 reference expressed proteins. Overall 25 proteins (2.51%) were down-regulated and 122 proteins (12.22%) were overexpressed. Properties and relative expression of down-regulated and overexpressed proteins of yeast strains, amongst others, the intra-genus TFPH and LVPH NH 84 and inter-genus FLPH and LVPH, NH 07/1 were established by using quantitative LC-based iTRAQ proteomic analysis (all data can be obtained from the Agricultural research council (ARC) Infruitec-Nietvoorbij, Microbiology Department, Stellenbosch, South Africa).

Proteomic analyses showed that the TRWY Zymaflore

Table 2. Number of differentially expressed proteins originating from fermenting commercial 'thiol-releasing' wine yeasts (TRWY), with ability to produce wine with tropical fruit aromas, naturally isolated parental strains, intra- and inter-genus hybrid yeast strains with the ability to produce wines with enhanced tropical fruit aroma (abbreviated as TFPH) and lower VA (abbreviated as LVPH) during the fermentation of 2013 Sauvignon Blanc grape juice.

Yeast strain	Proteomic analysis	
	Down-regulated	Over-expressed
VIN 7 TRWY Reference	998 proteins characterised	
VL3 TRWY	2	27
X5 TRWY	4	9
4F9 TRWY	1	60
NH 84 TFPH and LVPH	0	6
MCB C6	6	9
M2/1	11	3
NH 07/1 FLPH and LVPH	1	8

VL3 reference over-expressed the lactoylglutathione lyase protein, an enzyme responsible for cleaving a carbon-sulphur bond to release the volatile thiol 4MMP from its bound aroma-inactive precursor (Howell et al., 2005). Unfortunately, 4MMP was not quantified during this study. However, the TRWY produced wine with a moderate association with 3MH. It can, therefore, be speculated that aforementioned carbon-sulphur lyase enzyme is also involved in the release of 3MH from its carbon-sulphur-containing precursor (Swiegers et al., 2007a). A gene encoding for *β*-lyase involved in the release of volatile thiols, 3MH and 4MMP was previously reported (Holt et al., 2011). Therefore, lactoylglutathione lyase might be used as a protein biomarker for volatile thiol release during production of varietal Sauvignon Blanc wines. Additionally, Zymaflore VL3 produced wines with a positive association with floral aroma that is influenced by yeast-mediated released monoterpenes, which essentially are hydrocarbons and/or glycoconjugates, from its bound aroma-inactive precursors in grape juice (Von Mollendorf, 2013). Monoterpenes was shown to be released in abundance by using genetically modified (GM) *S. cerevisiae* strains expressing a S-linalool synthase (Pardo et al., 2015). Nevertheless, non-GM *S. cerevisiae* can release moderate geraniol and linalool levels (Lambrechts and Pretorius, 2000). The TRWY was also shown to produce wines with a moderate association with VA (Figure 6), which comprise acetic acid, an intermediate of long chain fatty acid production catalysed by fatty acid synthases (Tehlivets et al., 2007). However, the yeast did not regulate any synthases. Nonetheless, the association between regulated proteins, especially synthases, and their effect on VA formation should be further investigated.

The TRWY Zymaflore X5 was shown not to regulate any lyases and synthases (Table 2), which complements FTIR analyses (Figure 4), descriptive sensory evaluation (Figure 5), GC-analyses (Figure 6) and SPE-GC/MS analyses (Figure 7), as the yeast produced wines with a negative association with VA and total fatty acids (comprised mainly of acetic acid), a positive association with tropical fruit aroma, and a negative association with volatile thiols, respectively. On the other hand, the TRWY Fermicru 4F9 was shown to regulate dehydrogenases. Proteins in the same class were previously implicated in excessive acetic acid production (Varela et al., 2012; Walkey et al., 2012). However, regulated dehydrogenases by Fermicru 4F9 do not seem to have stimulated VA formation as the yeast produced wines with a negative association with VA and total fatty acids (Figures 4 and 6). The yeast also did not regulate any carbon-sulphur lyases responsible for volatile 'thiol-release' and, therefore, complements descriptive sensory evaluation as the yeast produce wines with a negative association with tropical fruit aroma (Figure 5). SPE-GC/MS analyses revealed that the TRWY produced wines with a positive association with volatile thiols 3MH and 3MHA (Figure 7). This therefore, implies that more proteins might be involved in volatile thiol-release.

Proteomic analyses further showed that the intra-genus TFPH and LVPH NH 84 was the only strain not to have down-regulated any proteins, whilst the remaining strains down-regulated from one to 11 proteins. Furthermore, NH 84 only overexpressed six proteins classed as nucleic acid 'binders', hydrolases and transporters, some of which are associated with cell proliferation and protein synthesis. As NH 84 was the only strain to have regulated these proteins, they could also be associated with higher 3MH released by this strain. These proteins will in future be further investigated as potential biomarkers, as the yeast also produced wines with a positive association with tropical fruit aroma (Figure 5) and volatile thiols, especially 3MH (Figure 7).

Both inter-genus parental strains, *S. cerevisiae* MCB

C6 and *T. delbrueckii* M2/1 were shown not to regulate the lactoylglutathione lyase protein. Nevertheless, the inter-genus hybrid, NH 07/1 provisionally characterised as a FLPH and LVPH, as observed with the TRWY Zymaflore VL3, over-expressed the lactoylglutathione lyase protein responsible for the release of the volatile thiol 4MMP (Howell et al., 2005). As previously mentioned, 4MMP was not quantified during this study. The inter-genus hybrid not only produced wine with a positive association with 3MH, it was more pronounced than wines produced with both parental strains MCB C6 and M2/1 and TRWY Zymaflore VL3 and Zymaflore X5. As 3MH release also involves enzymatic cleavage by a carbon-sulphur lyase, there is a possibility that this over-expressed protein could also be involved in the release of 3MH from its carbon-sulphur-containing precursor (Howell et al., 2005; Swiegers et al., 2007a). This observation further supports the notion that lactoylglutathione lyase might be a useful biomarker for volatile thiol release by NH 07/1 during production of varietal Sauvignon Blanc wines.

The inter-genus FLPH and LVPH, NH 07/1 also produced wines with a positive association with floral aroma that is influenced by yeast-released mono-terpenes, a metabolite that was released in abundance by a genetically modified (GM) *S. cerevisiae* strains expressing a S-linalool synthase as mentioned previously (Von Mollendorf, 2013; Pardo et al., 2015). However, the inter-genus FLPH did not regulate any synthases, which suggests that other proteins are involved in monoterpene release. Strains belonging to the same species of parental strains *S. cerevisiae* MCB C6 and *T. delbrueckii* M2/1 are known to produce monoterpenes (King and Dickinson, 2000). This warrants further investigation to identify protein biomarkers associated with floral wine aroma and monoterpene release.

Differentially expressed proteins by the intra-genus TFPH and LVPH, NH 84 and inter-genus FLPH and LVPH, NH 07/1 during the stationary phase of Sauvignon Blanc grape must fermentation were classified according to molecular function, biological process and protein class using PANTHER (Sharma et al., 2014). Classification of proteins according to molecular function showed that NH 84 regulated proteins were linked to translation regulator activity, catalytic activity and transporter activity, whilst that of NH 07/1 were linked to binding activity, structural molecule activity, catalytic activity and antioxidant activity (Figure 8a and b). Classification of proteins according to biological processes showed that NH 84 regulated proteins were linked to cellular processes, metabolic processes and localisation, whilst those of NH 07/1 were linked to response to stimuli, cellular processes, metabolic processes and cellular biogenesis (Figure 8c and d). Furthermore, NH 84 regulated proteins clustered into three protein classes *viz.* nucleic acid binding, hydrolase and transporter, whilst those of NH 07/1 also clustered into different protein classes *i.a.* hydrolases, and oxidoreductases (Figure 8e and f). It is evident that regulated proteins differed between intra-genus and inter-genus strains, explaining the production of wines with different chemical (Figure 4) and sensory (Figure 5) properties, as well as differences in aroma and off-flavour compound levels (Figures 6 and 7).

Conclusions

The inter-genus FLPH and LVPH, NH 07/1, produced wine with a more positive association with volatile thiols, 3MHA in particular, than both parental strains, *S. cerevisiae* MCB C6 and *T. delbrueckii* M2/1, as well as commercial TRWY, Zymaflore X5 and Zymaflore VL3. This hybrid also produced wines with a negative association with VA and acetic acid, but a positive association with floral aroma with hints of tropical fruit aroma. Three intra-genus TFPH and LVPH, NH 56, NH 57 and NH 88 produced wines with a negative association with VA and acetic acid, but with a positive association with tropical fruit aroma. These intra-genus hybrids also produced Sauvignon Blanc wines with a stronger association with 3MH than the commercial reference. Five more intra-genus yeasts, NH 48, NH 118, NH 140, NH 143 and NH 145 also produced wines with a stronger association with 3MH than wines produced with the commercial TRWY Zymaflore X5 and Zymaflore VL3. These hybrids also produced wines with a stronger association with 3MHA than wines produced with the commercial TRWY VIN 13. Proteomic analyses showed that NH 07/1 and Zymaflore VL3 over-expressed the lactoylglutathione lyase protein responsible for the release of the volatile thiol 4MMP by cleaving its carbon-sulphur bonds. Since 3MH release also involves enzymatic cleavage by a carbon-sulphur lyase, there is a possibility that the aforementioned over-expressed protein could also be involved in the release of 3MH from its carbon-sulphur-containing precursor. Lactoylglutathione lyase might be a useful protein biomarker for volatile thiol release by especially NH 07/1 during production of varietal Sauvignon Blanc wines. As dehydrogenases were previously implicated in VA formation, these proteins might also be useful biomarkers for VA and/or acetic acid formation by fermenting wine yeasts.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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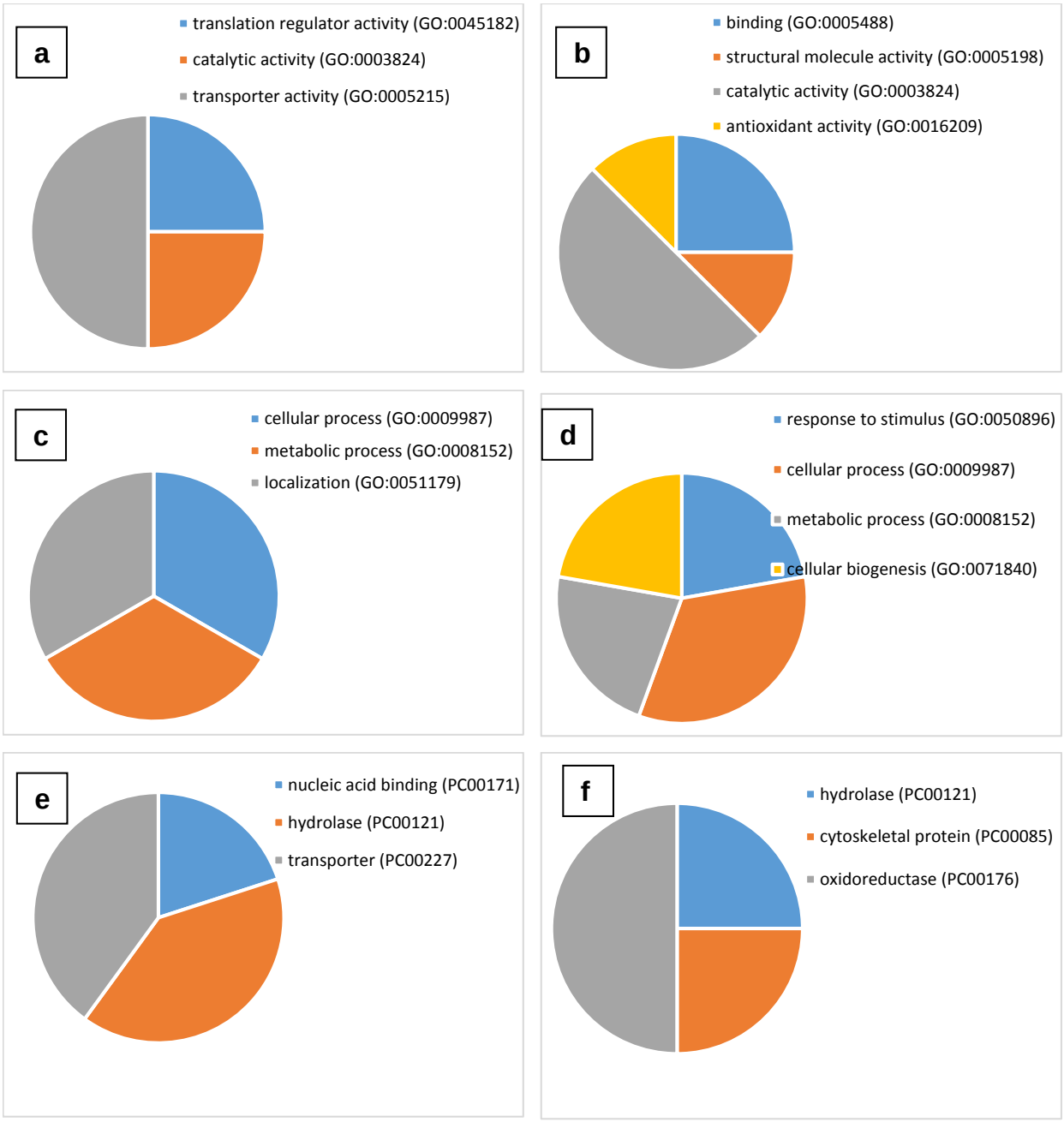

Figure 8. Classification of differentially expressed proteins by intra-genus hybrids with the ability to produce wines with enhanced tropical fruit aroma (abbreviated as TFPH) and low VA (abbreviated as LVPH), NH 84 and the inter-genus hybrid, NH 07/1 during the end of Sauvignon Blanc grape must fermentation according to (a and b) Molecular function, (c and d) Biological process, and (e and f) Protein class using Protein ANalysis THrough Evolutionary Relationships (PANTHER, www.pantherdb.org/).

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