Research Note
Screening Non-*Saccharomyces* Yeasts as Low Ethanol Producing Starter Cultures

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Non-*Saccharomyces* yeasts are known for their low fermentation rate in comparison to *Saccharomyces cerevisiae*. In this study, non-*Saccharomyces* yeasts were inoculated into Chenin blanc grape must and fermented under aerobic and anaerobic conditions. *Saccharomycodes ludwigii* displayed a strain-dependent fermentation rate, which yielded between 5.2% and 9.9% ethanol concentration under both conditions, albeit with residual sugar. Aerobic conditions favoured the production of reduced ethanol which was between 5.8% and 9.7% for non-*Saccharomyces* yeasts in comparison to *S. cerevisiae* (10%). This trend was observed for *Wickerhamomyces anomalus*, *Stamerella bacillaris* (*Candida zemplinina*), *Metshnikowia pulcherrima*, *Cyberlindnera saturnus*, *Wickerhamomyces subpelliculosus* and *Cyberlindnera jadinii*. The laboratory-scale wines prepared with the aforementioned yeasts yielded ripe fruit and floral aroma attributes while other non-*Saccharomyces* yeasts resulted in wines with spicy, acidic and solvent aroma notes.

INTRODUCTION

In winemaking, non-*Saccharomyces* yeasts are recognised as the indigenous microbial population found on the grape surface with about 10^4 to 10^6 CFU/g on ripe grapes (Fleet, 2003), 10^3 to 10^5 CFU/g on aseptically crushed ripe grapes (Ribèreau-Gayon & Peynaud, 1960) and 10^6 to 10^8 CFU/mL during fermentation (Jolly *et al.,* 2014). The ascomycetous yeast species *Hanseniaspora uvarum* (anamorph *Kloeckera apiculata*) account for 10 to 100% relative abundance of the indigenous yeast population (Jolly *et al*., 2014; Bagheri *et al*., 2015; Shekhawat *et al*., 2018) with the remainder attributed to the yeast genera *Hansenula*/*Pichia*, *Metschnikowia*, *Candida, Kluyveromyces, Schizosaccharomyces, Torulaspora* and *Zygosaccharomyces* in varying abundance (Fleet *et al*., 1984; Viana *et al*., 2008; Manzanares *et al*., 2011; Jolly *et al.*, 2014; Bagheri *et al*., 2015). Non-*Saccharomyces* yeasts are generally characterised by low fermentation vigour, low fermentation rate (Ciani & Maccarelli, 1998; Lappa *et al*., 2020) and low SO_2 resistance (Ciani *et al*., 2010).

During wine fermentation there is a sequential development of yeasts in both red and white musts, with *Saccharomyces cerevisiae* (*S. cerevisiae*) taking the lead role beyond the initial five days of fermentation (Fleet *et al*., 1984; Ciani *et al*., 2016). The dominance of the *S. cerevisiae* population is accompanied by the decline or death of the non-*Saccharomyces* population due to its sensitivity to certain metabolic compounds e.g. ethanol and killer toxins secreted by other yeasts during fermentation (Suzzi *et al*., 1995; Mehlomakulu *et al*., 2014; Vicente *et al*., 2020). Furthermore, the low levels of available oxygen and competition for nutrients during fermentation are growth limiting factors for non-*Saccharomyces* yeasts (Holm *et al*., 2001).

Since the dawn of the current millennium, non-*Saccharomyces* yeasts have gained popularity in winemaking (Jolly *et al*., 2014). These yeasts play an important role in the flavour and aroma development of wine when co- or sequentially inoculated with *S. cerevisiae* in mixed cultures (Fleet, 2003; Jolly *et al*., 2003; Ciani *et al*., 2010; Jolly *et al*., 2014; Varela *et al*., 2016). This has led to the commercial development of non-*Saccharomyces* starter cultures to enhance the aroma bouquet and flavour profile of wines (Ciani *et al*., 2010; Jolly *et al*., 2014; Vicente *et al*., 2020). These non-*Saccharomyces* yeasts include the genera *Torulaspora, Metschnikowia* and *Lachancea* (Ivit *et al.,*

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2020; Lappa *et al*., 2020).

In the last decade, grapes are often harvested at high sugar concentration as a result of climate changes affecting the physiological development of grapes (Gonzalez *et al*., 2013). Thus, in winemaking; yeasts have had to adapt to stressful environments e.g. high sugar concentration through intricate regulation of stress tolerance, growth and metabolic genes (Tondini *et al*., 2020). Thus, the utilization of the respiratory (oxidative) metabolism of non-*Saccharomyces* yeasts to lower ethanol content in wine has received attention (Erten & Campbell, 2001; Gonzalez *et al*., 2013; Quiros *et al*., 2014; Contreras *et al*., 2015; Canonico *et al*., 2016; Ciani *et al.,* 2016; Varela *et al*., 2015). In such experiments, aerobic fermentation is promoted over anaerobic fermentation; driving the carbon to other metabolites e.g. glycerol, organic acids or biomass instead of ethanol (Canonico *et al*., 2019). Apart from reducing ethanol concentration, the inoculation of non-*Saccharomyces* yeasts contributes to secondary aroma and flavour notes which give unique characteristics to the wine (Ivit *et al*., 2020). This is attributed to the Ehrlich pathway (catabolism of aromatic amino acids) and yeast secreted enzymes which aid in the catabolism of primary aroma and flavour compounds (Belda *et al*., 2017, Gamero *et al*., 2016). Known aroma compounds include alcohols, ethyl esters of fatty acids, acetate esters of higher alcohols, terpenes, sulphur compounds e.g. thiols, hydrogen sulphide, dimethyl sulphide and methanethiol. However, the production of aroma compounds is dependent on yeast species and strains (Belda *et al*., 2017).

Therefore, the aim of the study was to screen a selection of non-*Saccharomyces* yeasts for their fermentative capability under aerobic and anaerobic conditions to investigate their potential as low ethanol-producing starter cultures.

MATERIALS AND METHODS

Yeast species and culture conditions

Twenty-four yeast strains from the ARC Infruitec-Nietvoorbij gene bank and culture collection (Table 1) were aseptically streaked out on YPD (yeast peptone dextrose) agar (Biolab, Merck, South Africa) and incubated at 30°C for 3 days. A single colony from each plate was aseptically inoculated into 5 mL YPD broth (Biolab, Merck, South Africa) and incubated at 28°C with gentle agitation for 14-16 h. Each of the yeast strains was used as a pre-culture for the experiments described below.

Yeast species verification

The identities of the species were verified by a PCR method. DNA isolation was carried out by inoculating single yeast colonies in 5 mL of YM broth (10 g/L glucose, 3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone) and allowed to grow aerobically for 24-48 h at 30°C. Subsequently, these cultures were subjected to a lithium acetate (LiOAc)-SDS lysis DNA extraction method (Lõoke *et al.*, 2011). The primers NL1 (5'- GCATATCAATAAGCGGAGGAAAAG -3') and NL4 (5'- GGTCCGTGTTTCAAGACGG-3') were used to amplify the D1/D2 region of the 26S rDNA gene (O'Donnel, 1993). The reactions were performed in 0.2 mL tubes with a final volume of 50 µL. Amplification was performed in a 3Prime thermal cycler (Techne, Bibby Scientific, Lasec, South Africa) with the following reagent concentrations: 2.5 mM dNTPS, 25 mM $MgCl_2$, 0.2 mM (each) primers NL1 and NL4 (IDT, Whitehead Scientific, South Africa), 0.2 µL Super-Therm Gold Taq polymerase (5U/µL) (Separations Scientific, South Africa). The following cycling conditions were applied for the amplification: 94°C, 2 min; 30 cycles of 94°C, 1 min; 51°C, 30 s and 72°C, 4 min. A final extension step of 72°C for 5 min and a final holding temperature of 7°C, 10 min was added. Samples were electrophoresed on 1.5% agarose gels and stained with ethidium bromide (EtBr) after electrophoresis. Images were visualised with a Gel Doc XR system (BioRad, South Africa). Amplification products were sent to the Central Analytical Facility (CAF) at Stellenbosch University (South Africa) for post-amplification clean-up and sequencing. Sequences were edited using FinchTV (version 1_4_0). Edited sequences Fast Adaptive Shrinkage Threshold Algorithm (FASTA format) were used to verify yeast species identity through the National Centre for Biotechnology Information\ (NCBI) database (https://www.ncbi.nlm.nih.gov) using the Basic Local Alignment Search Tool nucleotide **(**BLASTn) search function.

Killer activity screening

All the yeast strains (Table 1) were screened for killer activity on white grape juice medium prepared with commercial 100% white table grape juice (preservative-free) and 4% bacteriological agar as described in Mehlomakulu *et al*. (2014). The 22 non-*Saccharomyces* strains were screened against *S. cerevisiae* VIN13, as was *S. cerevisiae* VIN13 against the 22 non-*Saccharomyces* strains. Briefly, 5 µL of an exponential phase growing culture of either a *S. cerevisiae* or non-*Saccharomyces* yeast strain were spotted on a plate seeded with 10^6 CFU/mL of a non-*Saccharomyces* or a *S. cerevisiae* culture. All plates were incubated at ambient temperature (20 to 22°C), and killer activity was observed as a zone of clearance around the spotted yeast colony. All experiments were done in biological triplicates.

Fermentative capability

The non-*Saccharomyces* strains were tested as axenic cultures for their fermentative capability in previously frozen Chenin blanc grape must. Chenin Blanc grapes obtained from the Nietvoorbij Research Farm (Stellenbosch, South Africa) were destemmed, crushed and pressed at 1 Bar in a small bladder press. A sedimentation enzyme (0.005 g/L Rapidase® Clear, Anchor Oenology, South Africa) and SO_2 (50 mg/L) were added to the juice. After overnight clarification at 15°C, the clear juice was racked off and frozen (-10°C) in 25 L plastic buckets. After overnight thawing and thorough mixing the juice (total sugar 211 ± 0 g/L, pH 3.37 ± 0.02, total titratable acidity 4.87 ± 0.15 for n=3) was dispensed into triplicate 50 mL Erlenmeyer flasks (30 mL must) for aerobic fermentations and 300 mL bottles (200 mL must) for anaerobic fermentations) and sterilised by autoclaving (121°C for 15 min). No ther additions were made to the grape juice.

Fermentations were carried out under aerobic and anaerobic conditions in laboratory-scale trials with *S. cerevisiae* strain 1 as the control. The inoculum for the must was prepared by inoculating 2% of the pre-culture described

TABLE 1
Yeast species used in the study.

This study reference	Yeast species	Accession number[1]	Source/Origin
1	*Saccharomyces cerevisiae*	Y0568	VIN 13, Anchor yeast (commercial hybrid wine yeast; www.anchor.co.za)
2	*Saccharomyces cerevisiae*	Y0117	Unknown
3	*Saccharomycodes ludwigii*	Y0116	Juice, Germany (CBS[2] 820)
4	*Saccharomycodes ludwigii*	Y0153	Juice, Italy (CBS 2624)
5	*Saccharomycodes ludwigii*	Y0154	Unknown (CBS 2625)
6	*Saccharomycodes ludwigii*	Y0205	Soil, South Africa (CBS 5929)
7	*Saccharomycodes ludwigii*	Y0244	Unknown (CSIR[3] Y8)
8	*Starmerella bacillaris* (*Candida zemplinina*)	Y1021	Wine, Hungary (Type strain; CBS 9494)
9	*Starmerella bacillaris* (*Candida zemplinina*)	Y1020	Grape must, South Africa
10	*Hanseniaspora uvarum*	C19V17	Unknown
11	*Wickerhamomyces anomalus*	C19V22	Grape must, South Africa
12	*Wickerhamomyces anomalus*	C40V8	Grape must, South Africa
13	*Wickerhamomyces anomalus*	C40V20	Grape must, South Africa
14	*Metschnikowia pulcherrima*	Y0839	Grape must, South Africa
15	*Candida stellata*	Y1025	Fruit, Germany (Type strain; CBS 157)
16	*Wickerhamomyces anomalus*	Y1072	CU-HUT[4] 7087
17	*Cyberlindnera saturnus*	Y1073	CU-IAM 12217
18	*Wickerhamomyces subpelliculosus*	Y1075	Unknown (CBS 1997)
19	*Lipomyces tetrasporus*	Y1077	Unknown (CBS 7939)
20	*Lipomyces starkeyi*	Y1076	Soil, South Africa (CBS 7537)
21	*Cyberlindnera jadinii*	Y1074	Italy (Type strain, CBS 567)
22	*Cyberlindnera saturnus*	Y1080	CU-NCYC 22
23	*Schizosaccharomyces pombe* (*Schizosaccharomyces malidevorans*)	C41V13	Australia (AWRI[5] 442)
24	*Candida zemplinina* (*C. stellata*)	C41V14	Australia (AWRI 1159)

[1] Gene bank or culture collection accession number, Agricultural Research Council – Infruitec-Nietvoorbij, Stellenbosch, South Africa.
[2] CBS-KNAW Collections, Westerdijk Fungal Biodiversity Institute, Utrecht, Netherlands.
[3] Council for Science and Industrial Research, Pretoria, South Africa.
[4] Cukurova University, Adana, Turkey.
[5] Australian Wine Research Institute, Glen Osmond, Australia.

above into 50 mL YPD broth and incubated for 48 h at 28°C under static conditions. Each flask and bottle of grape must was inoculated individually with 2.5% ($^{v}/_{v}$) of the respective inoculum. The aerobic fermentations were closed with cotton wool plugs to facilitate air movement into the flask and agitated at 140 rpm, while the anaerobic fermentations were fitted with fermentation air trap caps and not agitated. All the fermentations were incubated at ambient temperature (20-22°C) and weighed daily for 14 days for cumulative CO_2 loss. Sugar consumption and alcohol production were measured using a Density meter DM 35 (Anton Paar GmbH, Austria) and Alcolyzer Wine M analysis system (Anton Paar GmbH, Austria), respectively. Sugar consumption by the yeasts for the production of 1% ethanol was calculated from the initial sugar present in the must, the residual sugar in the wine and the final ethanol value using the formula below.

$$1\%\ \text{Ethanol produced} = \frac{(\text{Sugar concentration in must at day 0} - \text{Residual sugar concentration}) \times 1\%}{\text{Ethanol at end of fermentation}}$$

Yeast strains that resulted in wines with an ethanol content below 10% $^{v}/_{v}$ were selected for a second series of fermentations to evaluate the aroma of fermenting musts and wines. These selected yeast strains were prepared as axenic cultures and fermented aerobically for five days. On the fifth

day of fermentation, samples were taken for aroma evaluation. *Saccharomyces cerevisiae* strain 1 was then sequentially inoculated into the fermenting must. The inoculum of *S. cerevisiae* strain 1 was prepared and the flasks were further incubated under anaerobic conditions. The evolution of CO_2 loss for the sequential fermentations was monitored until no mass loss was recorded for three consecutive days and this was regarded as the end of fermentation.

Sensory evaluation of fermentations

Descriptive aroma evaluation of the partially fermented wine (five days) and finished wine (end of fermentation) was carried out using a panel of eight judges (male and female), with between 2 to 20 years of wine tasting experience with no collective training. The judges were asked to score the wine aroma on a scale of 1 (unacceptable) to 5 (acceptable), and to select the descriptors ("Fruity", "Overripe fruit", "Floral", "Acidic", "Cooked vegetables", "Fresh vegetables", "Spicy", "Rotten egg", "Solvent", "Oxidized", "Rotten") that matched the aroma perceived, or to provide any other descriptors perceived. The descriptors were selected based on terminology generally used in wine tasting for positive and negative traits (Nobel *et al*., 1987). The partially fermented and finished wines (*ca.* 50 mL) were served in ISO tasting glasses at an ambient temperature of 20-22°C. Wines were coded and randomised before presentation to the judges. The scores were converted to percentages for graphical representation as the total sum a descriptor was noted by a judge per wine divided by the total number of judges; then converted to a % by multiplication with 100. Descriptor noted by a judge per wine was recorded as a count of one.

Statistical analyses

Data (randomised and continuous) were subjected to analysis of variance (ANOVA) using General Linear Models Procedure (PROC GLM) of SAS software (Version 9.4; SAS Institute Inc, Cary, USA). Shapiro-Wilk test was performed on the standardized residuals from the model to verify normality after outliers were removed (Shapiro and Wilk, 1965). Fisher's least significant difference was calculated at the 5% level to compare treatment means (Ott and Longnecker, 2010). A probability level of 5% was considered significant for all significance tests.

RESULTS AND DISCUSSION

The yeast species used in this study (Table 1), by no means exhaustive, include a broader range of yeast than those normally investigated for wine production (Jolly *et al*., 2014) and contain some yeasts that have received less attention than the commercial non-*Saccharomyces* wine yeasts such as *Torulaspora delbrueckii, Metschnikowia pulcherrima* and *Lachancea thermotolerans*. Furthermore, these yeasts have been isolated from diverse areas and substrates.

The identity of these yeasts, as listed in Table 1, was verified by sequencing the D1/D2 region amplification products (data not shown) (O'Donnell, 1993). These yeast species and strains used in the study have been reported in lower alcohol production and/or wine fermentations (Erten & Campbell, 2001; Quiros *et al*., 2014; Varela, personal communication, 2014; Contreras *et al*., 2015; Englezos *et al*., 2016).

Killer activity screening

S. cerevisiae strains 1 and 2 (a laboratory reference strain) and the 22 non-*Saccharomyces* yeast strains and species were screened for killer activity against each other in white grape juice medium. None of the non-*Saccharomyces* yeast strains exhibited killer activity against the commercial wine yeast *S. cerevisiae* strain 1, except for *C. saturnus* strain 17. *S. cerevisiae* strain 1 was antagonistic towards *Cyberlindnera jadinii* strain 21, clearly exhibiting its killer positive trait as reported by the yeast producer. The genetic origin of the killer toxin secreted by strain 1 is not disclosed by the producer as either K1, K2, K28 or Klus. *S. cerevisiae* strains with the Klus genetic origin are antagonistic towards *S. cerevisiae* and non-*Saccharomyces* yeasts, as reported by Rodríguez-Cousiño *et al*. (2011). However, *S. cerevisiae* strain 1, as a commercial yeast, is routinely used in winemaking with no reported adverse effects on fermentation. Non-*Saccharomyces* yeasts are reported to secrete proteinaceous antimicrobial compounds, termed killer toxins, against other yeast species within the same environment (Philliskirk & Young, 1975; Heard & Fleet, 1987; Palpacelli *et al*., 1991; van Vuuren & Jacobs, 1992; Lowes *et al*., 2000; Mehlomakulu *et al*., 2014).

C. jadinii is referred to by its anamorph (*Candida utilis*) in literature and is used in the production of single-cell protein (Lee & Kim, 2001; Ibrahim *et al*., 2004). However, in view of the results from this study, mixed culture fermentations of *S. cerevisiae* strain 1 with *C. jadinii* strain 21 would need careful consideration.

Fermentative capability

When all the non-*Saccharomyces* yeasts were tested for their fermentative capability as axenic cultures under aerobic and anaerobic conditions, the strains were found to variably ferment grape must. Under aerobic conditions (Fig. 1), the *Lipomyces tetrasporus* strain 19 and *L. starkeyi* strain 20 had the highest residual sugar, i.e. 192.57 and 196.00 g/L, respectively, at the end of the 14-day fermentation. This was followed by *Cyberlindnera jadinii* strain 21 and *Wickerhamomyces anomalus* strain 11 with a residual sugar of 89.00 and 95.23 g/L at the same period, respectively although the latter had fermented 50% of the sugar by day four (data not shown) (Fig. 1). After 14 days of fermentation, yeasts had completed the fermentation (residual sugar <2 g/L). These were *S. cerevisiae* strain 1, *Candida zemplinina* strain 24, *Sacchromycodes ludwigii* strain 6, and *C. zemplinina* strains 8 and 9. In contrast, the *S. ludwigii* strains 3, 4, 5 and 7, *S. cerevisiae* strain 2, *Candida stellata* strain 15, *Wickerhamomyces subpelliculosus* strain 18 and *Schizosaccharomyces pombe* strain 23 had <10 g/L but >2 g/L residual sugar at day 14 (Fig. 1).

Under anaerobic conditions, all the non-*Saccharomyces* yeast strains performed poorly. All had high residual sugars, i.e. >105.5 g/L at day four, while *S. cerevisiae* strain 1 was the exception with 78.47 g/L of residual sugar (data not shown). At day 14, *S. cerevisiae* strains 1 and 2 had a residual sugar of 6.63 g/L and 33.83 g/L, respectively. The residual

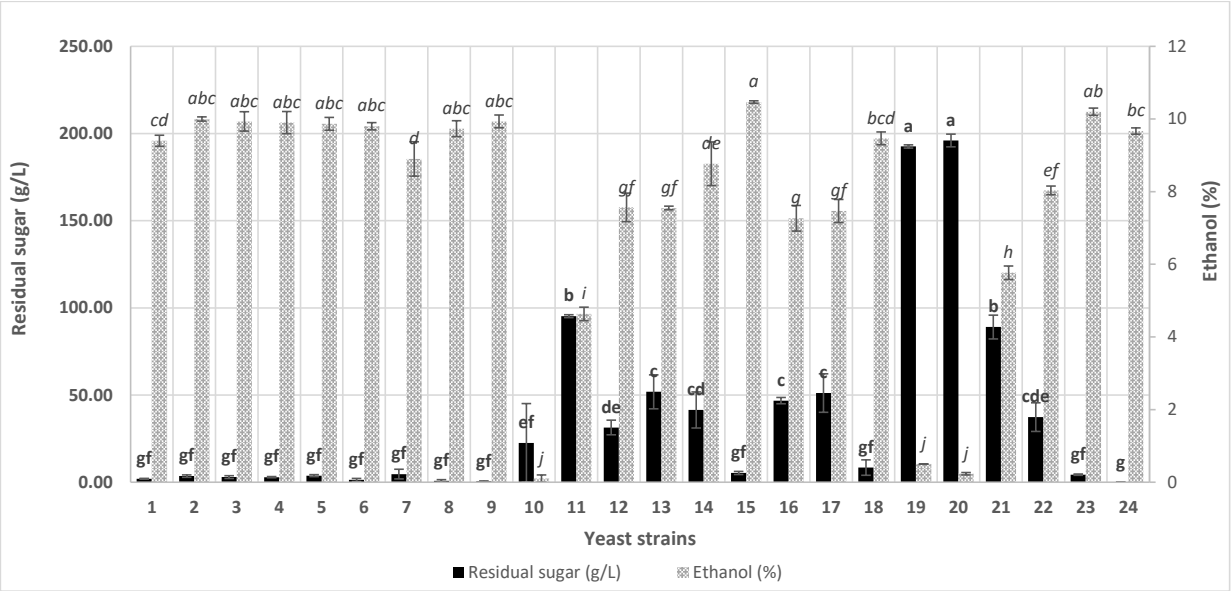

FIGURE 1

Residual sugar (g/L) and ethanol concentration (% $^{v}/_{v}$) on day 14 of aerobic fermentation for Chenin blanc wines produced with different yeast strains (see Table 1). Values are averages of three replicates and the error bars indicate the standard error. Bars within the graph with the same letters do not differ significantly ($p \leq 0.5$) for residual sugar (indicated in bold) and ethanol (indicated in italics), respectively.

sugar concentration of *S. ludwigii* strains 3, 4, 5 and 7, and *S. pombe* strain 23 fermentations was between 69.07 g/L and 107.93 g/L. The rest of the non-*Saccharomyces* fermentations showed residual sugar concentration >110 g/L (Fig. 2).

Liu *et al*. (2013) reported that the non-*Saccharomyces* yeasts – *C. stellata* (*C. zemplinina*), *K. apiculata* (*H. uvarum*) and *C. pulcherrima* (*M. pulcherrima*) were slow fermenters. The aforementioned authors found that the wines had residual sugar between 24.8 g/L and 158.9 g/L, similar to what was found in this study under aerobic and anaerobic conditions (Figs. 1 and 2, respectively). However, in this study the non-*Saccharomyces* yeasts; *C. zemplinina* and *S. ludwigii* displayed a fermentative metabolism in aerobic conditions, which was comparable to that of *S. cerevisiae*. Jolly *et al*. (2006) grouped non-*Saccharomyces* yeasts found in grape must and during fermentation into mainly oxidative yeasts; apiculate yeasts with low fermentative activity and yeasts with fermentative metabolism. In this study, *S. ludwigii*, *S. pombe*, *C. zemplinina* and *W. subpelliculosus* exhibited oxidative behaviour while *L. tetrasporus* and *L. starkeyi* can be characterised as non-fermenting yeasts.

During winemaking, sugar consumption is correlated with ethanol production. At day 14, the highest ethanol concentration between 10.0% and 10.5% for the aerobic fermentations were detected for *S. cerevisiae* strain 2, *C. stellata* strain 15 and *S. pombe* strain 23, while the lowest ethanol concentration of <5% was detected for *H. uvarum* strain 10, *W. anomalus* strains 11 and 19, and *Lipomyces starkeyi* strain 20. An ethanol concentration between 5.8% and 9.9% was detected for the rest of the yeast species studied (Fig. 1). Anaerobic fermentation resulted in 11.9% and 10.4% ethanol at day 14 for the *S. cerevisiae* strains 1 and 2, respectively (Fig. 2). Ethanol production of between 5.2% and 8.2% was detected for the *S. ludwigii* strains 3, 4, 5, 6 and 7, and *S. pombe* strain 23.

Sugar consumption from the axenic fermentations for the production of 1% ethanol was calculated from the initial sugar present in the must and the residual sugar in the wine. In the axenic fermentations, at the end of fermentation under both aerobic and anaerobic conditions, it was found that the non-*Saccharomyces* yeasts consumed sugar similarly to the *S. cerevisiae* strains, except for *L. tetrasporus* strain 19, *L. starkeyi* strain 20 and *C. jadinii* strain 21 (anaerobic only) (Figs 3.1 and 3.2). The *S. cerevisiae* strains consumed approximately 22 g/L to 17 g/L to produce 1% ethanol under aerobic and anaerobic conditions, respectively. The non-fermenting yeasts *L. tetrasporus* strain 19 and *L. starkeyi* strain 20 consumed >30 g/L under both aerobic and anaerobic conditions with *C. jadinii* strain 21 consuming 46.6 g/L of sugar under the anaerobic conditions only. *H. uvarum* strain 10, *W. anomalus* strain 13, *M. pulcherrima* strain 14 and *C. zemplinina* strain 15 consumed <19.7 g/L, compared to the *S. cerevisiae* strains 1 and 2 under aerobic conditions (Fig. 3.1), whereas *S. ludwigii* strain 5 consumed 18.6 g/L of sugar, which is similar to that of *S. cerevisiae* strains 1 and 2 under anaerobic conditions (Fig. 3.2).

S. cerevisiae is Crabtree positive, therefore can ferment sugars under respiro-fermentative conditions (García *et al*., 2016), as was found in this study. *S. ludwigii* is characterised as being a fermentative species (Ciani & Picciotti, 1995; Ciani & Maccarelli, 1998), yielding 12.63% ethanol on average and between 0.2 and 1.4 g CO_2/day over a period of three days. This metabolic activity is reported to be similar to that of *S. cerevisiae* (Ciani & Maccarelli, 1998). Similar findings were found in this study where the *S. ludwigii* strains 3, 4, 5 and 7 competed with *S. cerevisiae* strain 1 in

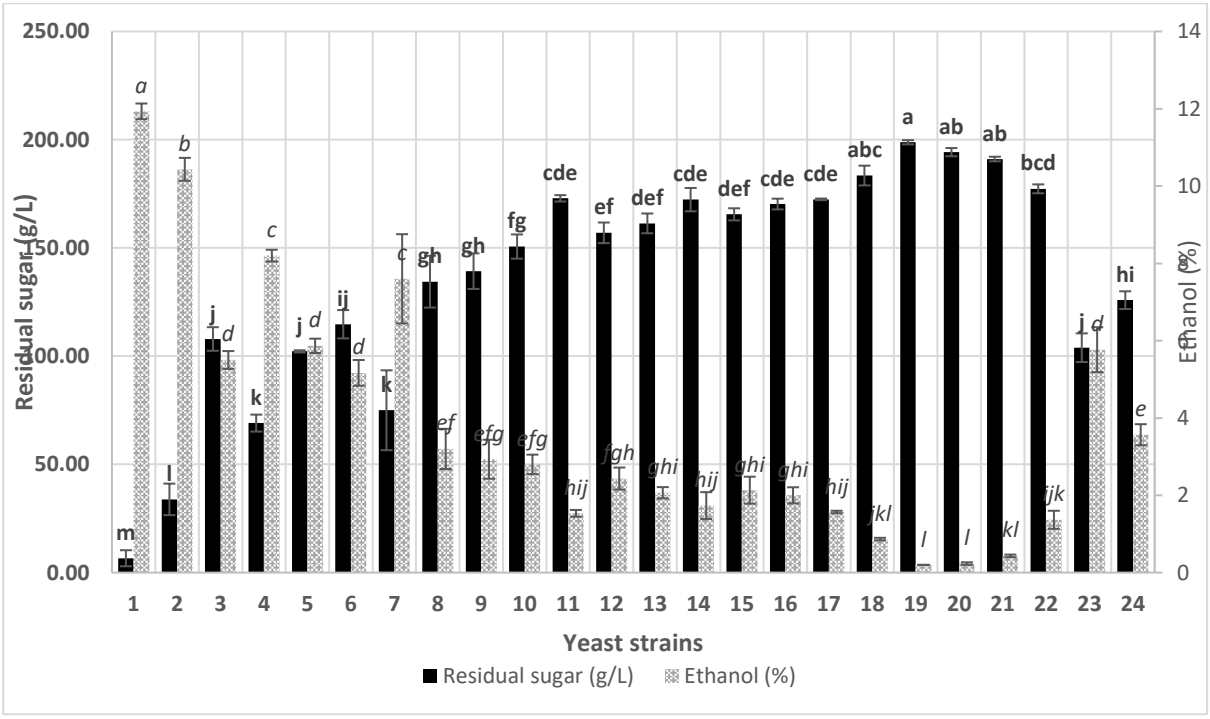

FIGURE 2

Residual sugar (g/L) and ethanol concentration (%, $^{v}/_{v}$) on day 14 of anaerobic fermentation for Chenin blanc wines produced with different yeast strains (see Table 1). Values are averages of three replicates and the error bars indicate the standard error. Bars within the graph with the same letters do not differ significantly ($p \leq 0.5$) for residual sugar (indicated in bold) and ethanol (indicated in italics), respectively.

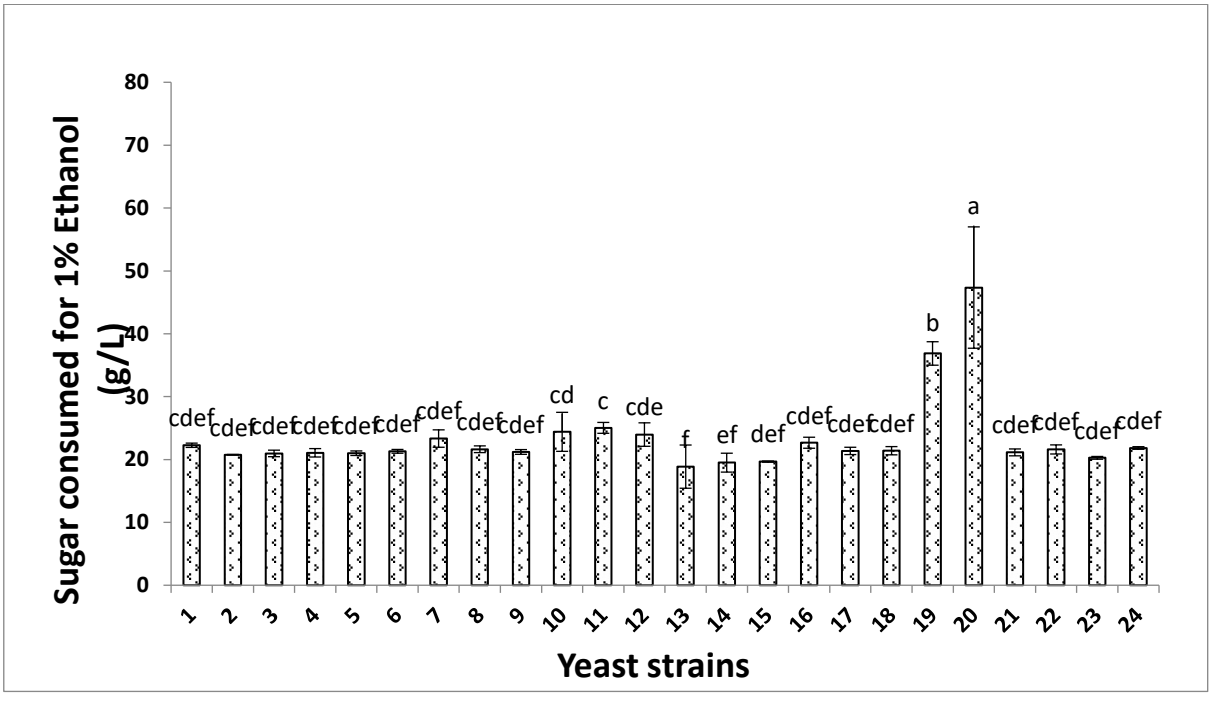

FIGURE 3.1

Sugar consumed to produce 1% ($^{v}/_{v}$) ethanol under aerobic conditions at the end of fermentation (day 14) for Chenin blanc wines produced with different yeast strains (see Table 1). Values are averages of three replicates and the error bars indicate the standard error. Bars within the graph with the same letters do not differ significantly ($p \leq 0.5$).

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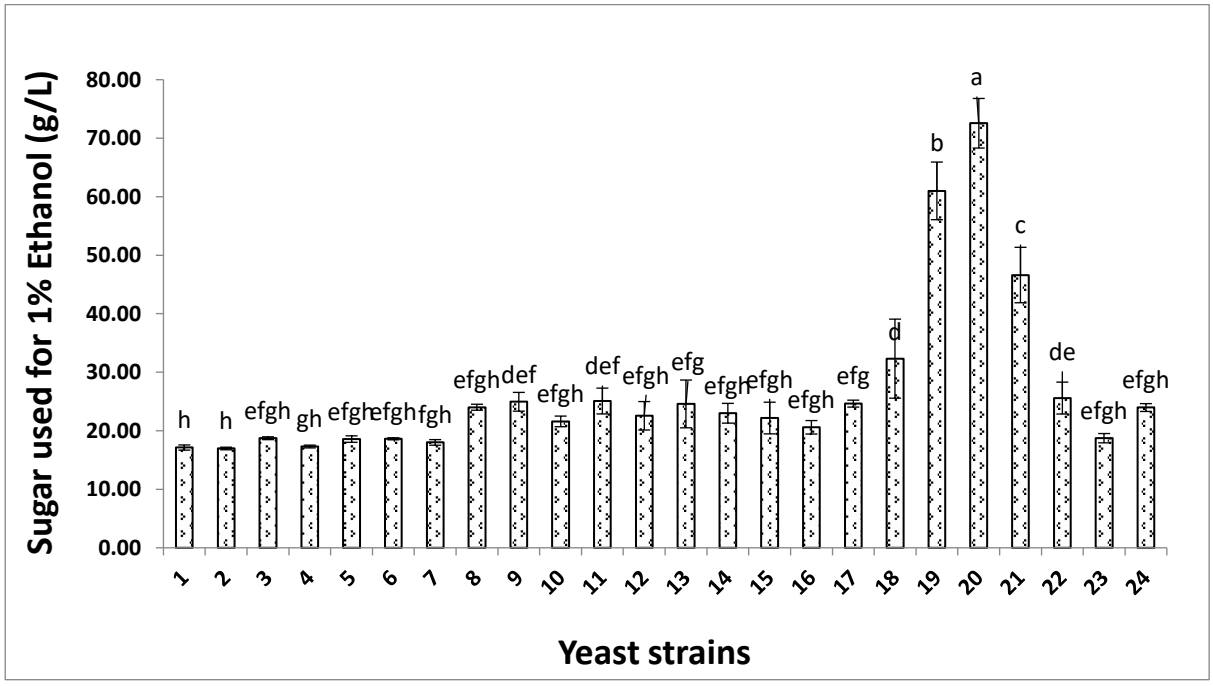

FIGURE 3.2
Sugar consumed to produce 1% ($^{v}/_{v}$) ethanol under anaerobic conditions at the end of fermentation (day 14) for Chenin blanc wines produced with different yeast strains (see Table 1). Values are averages of three replicates and the error bars indicate the standard error. Bars within the graph with the same letters do not differ significantly ($p \leq 0.5$).

sugar consumption and ethanol production.

Although grape must fermentation through the glycolysis pathway yields ethanol as the main by-product, sugar consumption by non-*Saccharomyces* yeasts is characterised by the production of other metabolites in order to maintain redox balance (Goold *et al*., 2017). In this study, sugar consumption similar to that of *S. cerevisiae* by some of the non-*Saccharomyces* yeasts was observed. This was linked to a lower ethanol yield, which can be regarded as a positive attribute in the quest to identify low ethanol yeast starter cultures. The lower ethanol yield is attributed to redox balance, as some of the carbon flux is directed to the formation of either organic acids, glycerol, ethyl acetate, acetoin, acetaldehyde (Zohre & Erten, 2002) and volatile aroma compounds (Sadoudi *et al*., 2012; Liu *et al*., 2016) by the non-*Saccharomyces* yeasts. The produced metabolites contribute to the aroma, body, protection against spoilage microorganisms and de-acidification of the fermenting must (Fleet, 2003).

The non-*Saccharomyces* yeast strains 3, 4, 12, 13, 14, 17, 22 and 24 produced approximately 1% less ethanol than the control *S. cerevisiae* yeast strain 1 under both aerobic conditions. Whereas under anaerobic conditions the reduction in ethanol was more than 2% for the same strains in comparison to the control *S. cerevisiae* yeast strain 1. The low ethanol concentration can be attributed to the carbon flux being directed to the production of other primary and secondary metabolites.

Sensory profiles

A basic aroma profiling of the fermenting must and wine using the non-*Saccharomyces* yeast strains was carried out. The purpose was to screen for yeasts producing off-odours, and thereby eliminating them as potential candidate organisms in further studies.

More than 56% of the judges found the fermenting wine at day four prepared from axenic cultures of the strains acceptable, except for the must inoculated with strain 13 (*W. anomalus*). The highest score, i.e. 88% for acceptability and tropical fruit aroma was given to for strain 17 (*C. saturnus*). The fermenting wines were also described as having a spicy aroma by 38% of the judges. Strain 13 (*W. anomalus*) was the only yeast that was found to produce a negative sulphur-like aroma as identified by 13% of the judges (Fig. 4.1).

In sequentially inoculated wines (Fig. 4.2), a fruity aroma was detected by 65% of the judges for the strains 1 (*S. cerevisiae*), 3 (*S. ludwigii*), 4 (*S. ludwigii*), 12 (*W. anomalus*) and 22 (*C. saturnus*). Strain 17 (*C. saturnus*), was found by 43%, 29% and 43% of the judges to have fruity, overripe and floral notes, respectively (Fig. 4.2). Volatile acidity was identified in strain 13 (*W. anomalus*) by 29% of the judges, with none of the judges identifying this aroma attribute in strains 3 (*S. ludwigii*), 4 (*S. ludwigii*), and 17 (*C. saturnus*). Wines prepared with strains 13 (*W. anomalus*) and 24 (*S. bacillaris*) were identified by 100% of judges to have a solvent odour, while 78% of judges identified this sensory attribute in wine prepared with strain 22 (*C. saturnus)*. All wines had some degree of oxidation as

indicated by 43% of the judges (Fig. 4.2), however, this may have been an outcome of the small fermentation volumes used.

Valente *et al*. (2018) reported that the aroma sensory attributes: acid/acidity, tropical, fruit, ripe fruit and spice were mentioned more than 50 times for Chenin blanc wines (n=39) from 20082014 by the John Platter Wine Guide to South African wines. Indeed, in this current study the same aroma attributes were mentioned by the judges, albeit this study was over a shorter period. Valente *et al*. (2018) further highlighted that residual sugar affected the sensory attributes of Chenin blanc wines. Unwooded dry wines (residual sugar <5 g/L) and semi-dry (residual sugar >5g/L but <12g/L) wines had 56% and 13%, respectively, association with juicy, guava, floral, acid, peach, pineapple and tropical aroma sensory attributes according to the judges. In this study, anaerobically fermented musts prepared only with *S. cerevisiae* strain 1 exhibited residual sugar of 6.63 g/L, i.e. semi-dry according to Valente *et al*. (2018). This semi-dry wine exhibited the same aroma attributes observed by Valente *et al*. (2018) (Fig. 4.2). Under aerobic conditions, strains 1 (*S. cerevisiae*), 12 (*W. anomalus*), 17 (*C. saturnus*) and 22 (*C. saturnus*) exhibited tropical aroma (Fig. 4.1). Only the wine prepared with strain 1 reached dryness under aerobic conditions (Fig. 1). When strain 1 (*S. cerevisiae*) was used in sequential fermentations with the non-*Saccharomyces* yeasts, the finished wines exhibited aromas such as fruity, floral and overripe fruit (Fig. 4.2). These wines were fermented to dryness over 21 days (data not shown).

Currently, non-*Saccharomyces* yeasts such as *T.delbrueckii*(BiodivaTD291™,Prelude™,Zymaflore®Alpha), *L. thermotolerans* (*Viniflora®Concerto*™), *M. pulcherrima* (Flavia™ Mp346), *Pichia kluyveri* (Frootzen®) and *S. pombe* (ProMalic®) are commercially available. These yeasts are reported to reduce acetic acid, degrade malic acid, improve wine aroma and enhance glycerol content (Ciani *et al*., 2010) through their metabolism and interactions during co- or sequential inoculation with *S. cerevisiae* or other yeasts (Ciani & Comitini, 2015). The interactions found in this study warrant further investigation into whether these yeasts can be used as low ethanol starter cultures.

CONCLUSIONS

This study investigated the production of ethanol by non-*Saccharomyces* yeasts under aerobic and anaerobic conditions. Although the wines did not ferment to dryness, fermentation under aerobic conditions yielded higher ethanol than that found under anaerobic conditions. This study revealed that the fermentative non-*Saccharomyces* yeasts produce less ethanol (1% to 2%) under aerobic and anaerobic conditions in comparison to *S. cerevisiae*. The optimal yeast under aerobic conditions was strain 13 (*W. anomalus*)

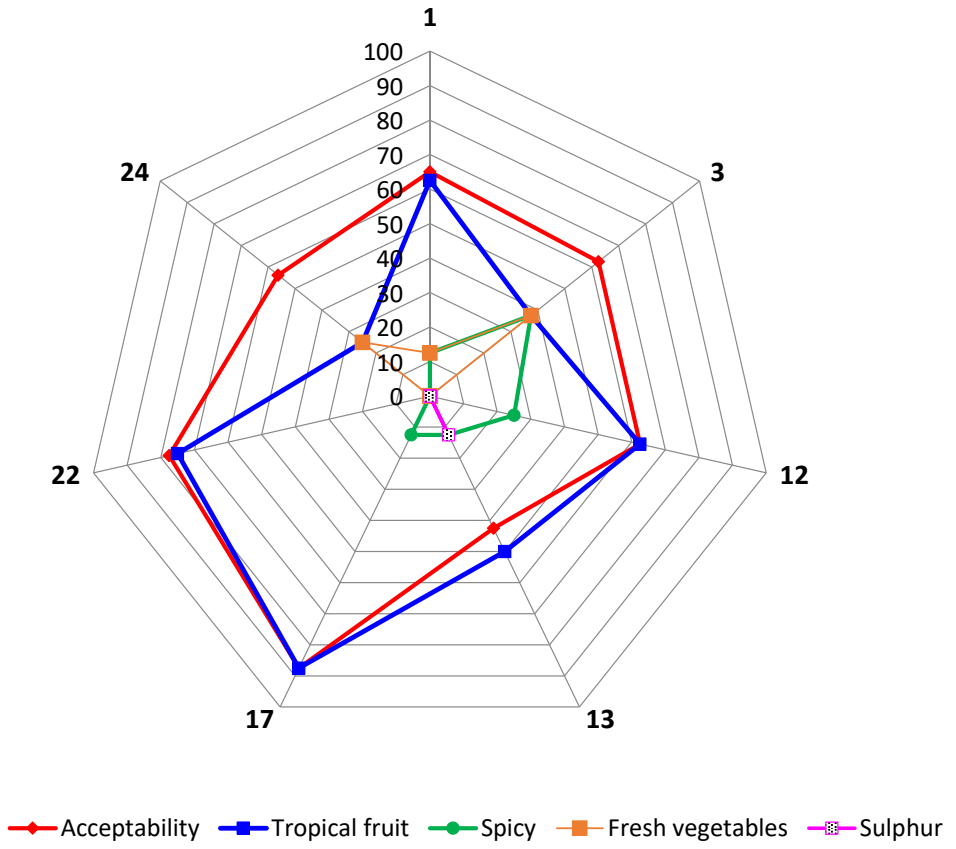

FIGURE 4.1.
Aroma profile of fermenting wine produced with axenic cultures of *S. cerevisiae* (strain 1) and non-*Saccharomyces* (strains 3, 12, 13, 17, 22 and 24) under aerobic conditions (day 4 of fermentation).

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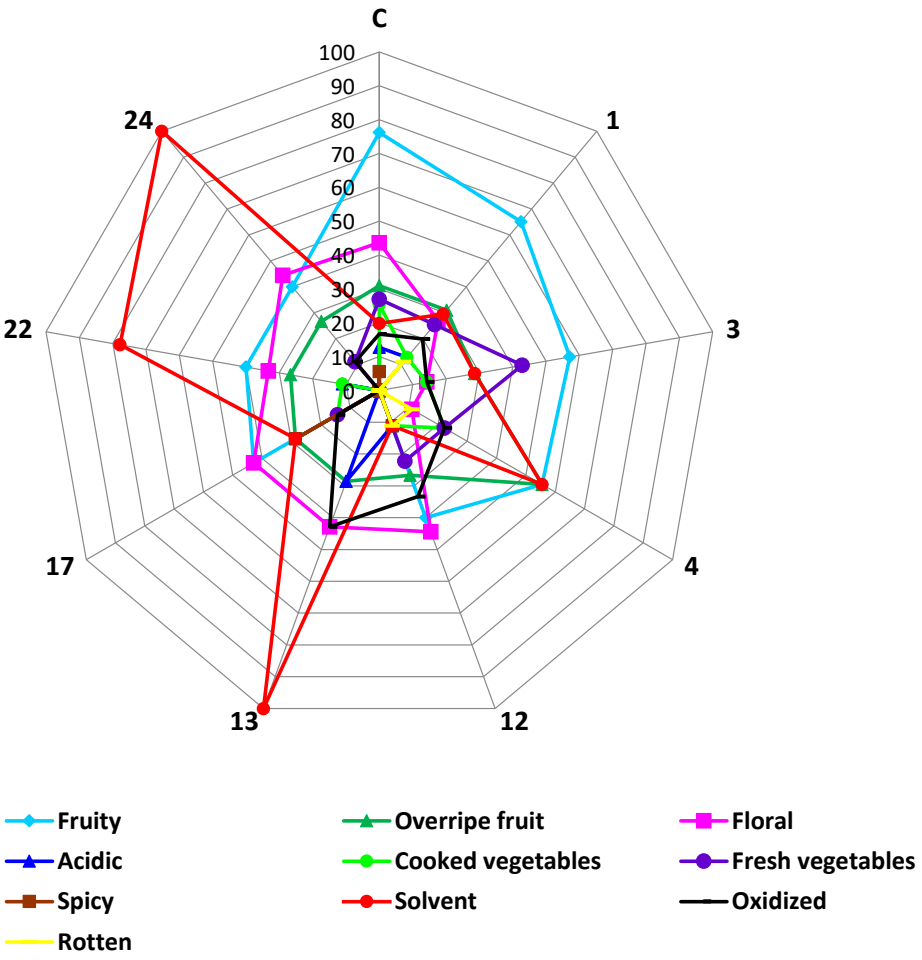

FIGURE 4.2.
Aroma profile of wines produced with non-*Saccharomyces* (strains 3, 4, 12, 13, 17, 22 and 24) sequentially inoculated with a *S. cerevisiae* strain 1 and a Control wine (C) (must where *S. cerevisiae* was inoculated at day 4).

with regards to ethanol yield. The aroma profiles of the fermenting and finished wines was similar to that reported for Chenin blanc wines produced using *S. cerevisiae* cultures. Some of the yeasts e.g. strains 3, 12 and 17 used in this study could potentially be co- or sequentially inoculated with *S. cerevisiae* to lower the ethanol content of wine and have a positive impact on the aroma and flavour of the wine. However, subsequent studies need to be conducted on the volatile and non-volatile compounds, as well as a full flavour evaluation of the musts and wines in order to elucidate the use of these non-*Saccharomyces* yeasts as starter cultures and whether the yeasts would have an effect on wine style.

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