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Aroma Profiles of *Vitis vinifera* L. cv. Gewürztraminer Must Fermented with Co-Cultures of *Saccharomyces cerevisiae* and Seven *Hanseniaspora* spp.

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Abstract: In this study, the aroma-production profiles of seven different *Hanseniaspora* strains, namely *H. guilliermondii*, *H. meyeri*, *H. nectarophila*, *H. occidentalis*, *H. opuntiae*, *H. osmophila* and *H. uvarum* were determined in a simultaneous co-inoculation with the wine yeast *Saccharomyces cerevisiae* Champagne Epernay Geisenheim (Uvaferm CEG). All co-inoculated fermentations with *Hanseniaspora* showed a dramatic increase in ethyl acetate levels except the two (*H. occidentalis* and *H. osmophila*) that belong to the so-called slow-evolving clade, which had no meaningful difference, compared to the *S. cerevisiae* control. Other striking observations were the almost complete depletion of lactic acid in mixed-culture fermentations with *H. osmophila*, the more than 3.7 mg/L production of isoamyl acetate with *H. guilliermondii*, the significantly lower levels of glycerol with *H. occidentalis* and the increase in certain terpenols, such as citronellol with *H. opuntiae*. This work allows for the direct comparison of wines made with different *Hanseniapora* spp. showcasing their oenological potential, including two (*H. meyeri* and *H. nectarophila*) previously unexplored in winemaking experiments.

Keywords: *Hanseniaspora*; mixed-starter culture fermentation; wine; aroma

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1. Introduction

Yeasts belonging to the genus *Hanseniaspora* are among the most commonly isolated in vitivinicultural settings, and their role within grape must fermentations has been the topic of investigation among wine microbiologists for many years [1–3]. They are also common isolates on other fruits and have an influential role on the outcomes of fermentations, ranging from apple cider to coffee and chocolate [4–6]. During grape must fermentations, in general, the population of *Hanseniaspora* spp. drops significantly within the first couple of days due to a number of factors, including the accumulative exposure to an anaerobic environment and, intriguingly, the killer ability of fermenting yeast, such as *S. cerevisiae* [7–9]. Nevertheless, their impact on the final wine product can be meaningful as they compete for nutrients needed for fermenting yeasts to complete the fermentation, and most importantly, are capable of producing a large array of important aroma-active compounds. *Hanseniaspora* spp. are well-known for their production of high-levels of acetate esters, particularly ethyl acetate, which would often exceed concentrations deemed to be pleasant (~150 mg/L) and imparting a solvent or nail polish remover aroma [10]. Although *Hanseniaspora* has often been cited as being a spoilage yeast within winemaking [11,12], researchers are re-evaluating the overall impact that some species of *Hanseniaspora* can have as co-partners with *S. cerevisiae* in mixed-culture fermentations, with many reporting on the beneficial effects in adding to the aroma complexity of a wine [2]. *Hanseniaspora*-initiated fermentations have also been shown to reduce final ethanol levels [13], increase overall

glycerol concentration [14], modulate acid composition [15], as well as causing a change in anthocyanin content and therefore the colour of the wine [16,17]. One species in particular, namely *H. vineae*, has shown repeatedly to provide several oenological benefits, which led to the development of a commercially available starter culture by the name of Fermivin VINEAE supplied by Oenobrands [18].

In mixed-culture fermentations, the most popular inoculation modality is a so-called sequential inoculation where the non-*Saccharomyces* yeast (NSY) starter culture is inoculated at the onset, allowing for NSY to exert an effect without the influence of *S. cerevisiae* [19]. One to three days later or once the sugar consumption has reached a certain level, the *Saccharomyces* culture is normally added. Alternatively, both starter cultures can be added simultaneously at the onset of fermentations. Studies where both modalities were conducted show the dramatic difference in the final outcome of the wine, which emphasizes the complexity and unpredictability of the interaction between the different yeasts [20–23].

The recent influx of whole-genome sequencing of representatives of each species within the *Hanseniaspora* genus, along with accompanying comparative studies, have revealed interesting data regarding the genomic make-up of this genus. Phylogenetic analyses have separated *Hanseniaspora* into two major clades, namely the fast-evolving lineage (FEL) and the slow-evolving lineage (SEL) [24,25]. Genes involved with cell cycle and genome integrity, thought to be conserved within ascomycetes, are not present within the genus. These genomic features of *Hanseniaspora* could assist in explaining the biology of the genus that becomes rapidly abundant as the sugar content in fruits increases during ripening [26]. Moreover, *H. vineae* was recently shown to undergo a rapid loss in cell viability during the stationary phase warranting more in-depth research in this genus, in particular how the two different lineages differ from each other [27].

In this study, we report on the co-fermentation of seven different *Hanseniaspora* spp. (two belonging to the SEL and five to the FEL) and how the respective species affected the aroma profile of a Gewürztraminer wine.

2. Materials and Methods

2.1. Yeast Strains Used in the Study

The *Hanseniaspora* strains *H. guilliermondii* NRRL-Y 1625, *H. meyeri* NRRL-Y 27,513 and *H. osmophila* NRRL Y-1613 were obtained from the Agriculture Resource Service Culture Collection (NRRL) (Peoria, IL, USA). The strain *H. uvarum* DSM2768 was a gift from Professor Jürgen Heinisch (University Osnabrück). The strains *H. nectarophila* GYBC-283, *H. occidentalis* GYBC-211 and *H. opuntiae* GYBC-284 were obtained from the Geisenheim yeast breeding culture collection. The *S. cerevisiae* strain Uvaferm CEG (Eaton, Nettersheim, Germany) was used as the fermenting yeast.

2.2. Microvinification

Pasteurised Gewürztraminer (GT) grape must (harvested from the vineyards belonging to the Geisenheim University in the Rheingau wine region of Germany) were used in the study. The GT must had a total sugar concentration of 247.8 g/L (123.3 g/L glucose and 124.5 g/L fructose). The main wine acids, tartaric and malic acid, were measured to be 3.7 g/L and 2.1 g/L, respectively. The must also had a citric acid content of 0.18 g/L. Its yeast available nitrogen was calculated as 65 mg/L by determining the primary free amino acid content using the spectrophotometrically-based nitrogen by the *o*-phthaldialdehyde method [28] and the free ammonium content using the rapid ammonium kit (Megazyme, Bray, Ireland). Opti-MUM White™ (Lallemand, Montreal, QC, Canada), at a concentration of 20 g/hL, was added as a supplement. Fermentations were conducted in 250 mL Schott Duran flasks filled with 150 mL of GT must. All yeasts were precultured in YPD medium (20 g/L glucose, 20 g/L peptone, 10 g/L yeast extract) and washed once with phosphate-buffered saline. The *Hanseniaspora* cultures were inoculated at a concentration of ~1×10^7 cells/mL, as determined by haemocytometer, whereas the *S. cerevisiae* strain was inoculated at a concentration of 1×10^6 cells/mL. Airlocks were added to the flasks filled

with approximately 3 mL of water. Fermentations were conducted at 22 °C and flasks were weighed daily until no further mass-loss was recorded. Samples were then centrifuged and subsequently prepared for high-performance liquid chromatography (HPLC) and gas-chromatography mass-spectrometry (GC-MS) analyses.

A one-day fermentation was also conducted where 1 µL of citronellol (Thermo Fisher Scientific, Geel, Belgium) was added to pure inoculations of *H. guilliermondii* and *H. uvarum*.

2.3. Analysis of the Must and Wines

Standard operating procedures, as established by the analysis team at the Institute of Microbiology and Biochemistry at Hochschule Geisenheim University, were followed to analyse the grape must and final wines produced in the study.

2.3.1. HPLC

For the quantification of the major organic acids, sugars, ethanol and glycerol of the wines and must, HPLC was implemented with a method previously described [29]. An HPLC Agilent Technologies Series 1100 (Agilent Technologies, Steinheim, Germany) was used built-in with an autosampler, a multi-wavelength (MWD) and refractive index (RID) detector and a binary pump. An HPLC column, 250 mm in length (Allure Organic Acids Column, Restek, Bad Homburg v. d. Höhe, Germany), with an inside diameter of 4.6 mm and a particle size of 5 µm was used for the separation of the different analytes. The MWD was set at a wavelength of 210 nm for the detection of organic acids and the RID was used to detect the sugars, organic acids, glycerol and ethanol. The isocratic eluent was comprised of deionized water with 0.5% ethanol and acidified with 0.0139% concentrated sulphuric acid (95–97%). The flow rate was 0.6 mL/min and column temperatures were 29 °C and 46 °C. Chemstation software (Agilent, Steinheim, Germany) was used to analyse, integrate and determine the concentrations of each analyte.

2.3.2. GC-MS

Aroma Bouquet Analysis

To detect and quantify the so-called aroma bouquet of the final wines comprising of expected higher alcohols; medium-chain fatty acids; and acetate and ethyl esters, a targeted headspace solid-phase micro-extraction gas-chromatography mass spectrometry analysis (HS-SPME-GC-MS) was employed using a protocol, as previously outlined [30]. Sample preparation entailed pipetting 5 mL of wine samples along with adding 1.7 g NaCl to a 20 mL headspace vial. Two internal standards, 1-octanol (600 mg/L) and cumene (52 mg/L), were also added. The GC-MS used was a GC 7890 A, equipped with an MS 5975 B (both Agilent, Santa Clara, CA, USA), an MPS robotic autosampler and a CIS 4 (both Gerstel, Mülheim an der Ruhr, Germany). A 65 µm fibre coated with polydimethylsiloxane crosslinked with divinylbenzene (Supelco, Merck, Darmstadt, Germany) was used to carry out the solid-phase microextraction. Separation of the volatiles was performed with a 60 m × 0.25 mm × 1 µm gas chromatography column (Rxi®-5Si1 MS w/5 m Integra-Guard, Restek, Bad Homburg v. d. Höhe, Germany) with helium as a carrier gas. Split mode injection was employed (1:10, initial temperature 30 °C, rate 12 °C/s to 240 °C, hold for 4 min). The initial temperature of the GC run was 40 °C for 4 min, and then increased to 210 °C at 5 °C/min and raised again to 240 °C at 20 °C/min and held for 10.5 min. Mass spectral data were acquired in a range of mass-to-charge ratio (m/z) of 35 to 250 and used to determine the concentration values. A 5-point calibration curve was used for each volatile compound within a wine model solution of 12% ethanol with 3% tartaric acid at pH 3.

Terpenes

Free terpenes and C_{13}-norisoprenoids expected in wines were also measured by means of HS-SPME-GC-MS, as detailed previously [29,31]. A GC 6890 and a 5973 N quadrupole MS (Agilent Technologies, Palo Alto, CA, USA) equipped with a Gerstel MPS2 autosampler

(Gerstel, Mülheim an der Ruhr, Germany) was used. Similar to the aroma bouquet sample preparation, 5 mL of wine samples with 1.7 g of NaCl were added to a 20 mL headspace amber vial, along with 10 µL of the internal standard, which contained 30 µg/L 3-octanol, 30 µg/L linalool-d3, 40 µg/L α-terpineol-d3, 10 µg/L β-damascenone-d4, 16 µg/L β-ionone-d3 and 12.5 µg/L of naphthalene-d8 in ethanol solution (Sigma-Aldrich, Merck KGaA, Darmstadt, Germany).

A 100 µm polydimethylsiloxane fibre (Supelco, Merck, Darmstadt, Germany) was used to carry out the headspace solid-phase microextraction. Separation of the volatiles was performed with a 30 m × 0.25 mm × 0.5 µm gas chromatography column (DB-Wax, J & W Scientific, Agilent Technologies, Palo Alto, CA, USA) with helium as a carrier gas. Splitless mode injection was employed (injector temperature: 240 °C). The initial temperature of the GC run was 40 °C for 4 min, and then increased to 190 °C at 5 °C/min and raised again to 240 °C at 10 °C/min and held for 15 min. Mass spectral data were acquired in SIM mode with characteristic ions for each analyte and used to determine the concentration values. Calibration was performed by means of the standard addition in Riesling wine. For both the aroma bouquet and terpene analysis, Masshunter workstation software version B.09.00 (Agilent Technologies, Palo Alto, CA, USA) was used to calculate the concentrations of the aroma compounds.

2.4. Statistical Analysis

All fermentations were conducted in triplicate. Results from the fermentation analyses that are shown in the Tables are the average value of the triplicates followed by the standard deviation of the mean (±). All statistical analyses were performed using Graphpad Prism version 9.4.1 (GraphPad Software, San Diego, CA, USA). The control experiment in all cases was the fermentation inoculated with only *S. cerevisiae*. Principal component analyses were performed on the fermentation data using RStudio (version 2022.07.0) along with the packages factoextra (version 1.0.7), ggbiplot (version 0.55) and ggplot2 (version 3.3.6).

3. Results and Discussion

3.1. Fermentation Curves

Co-fermentations using *Hanseniaspora* strains with *S. cerevisiae* were carried out at 22 °C using 10:1 ratios of starting cultures. The progression of these fermentations was followed by daily measurements of CO_2 mass-loss (Figure 1). All fermentations were completed by 13 days, which was also confirmed by the HPLC analysis indicating that all fermentations reached dryness (Table 1). There were some deviations in the mass-loss patterns, with some co-fermentations (*H. occidentalis*, *H. osmophila* and *H. nectarophila*) exhibiting slightly less mass-loss throughout the fermentation, yet no notable fermentation burden was observed with any of the co-inoculations. This is in concurrence with previous *Hanseniaspora*-initiated fermentations, where no meaningful influence on the fermentation rate of *S. cerevisiae* was observed [32]. CEG is known for its slow fermentation performance. This was also the reason why we decided to use this *Saccharomyces* yeast.

3.2. Organic Acids

Organic acids, such as tartaric acid, malic acid, lactic acid, acetic acid and citric acid, play a major role in the aroma profile and the mouthfeel of the wine. Table 1 shows the content of the major organic acids of the final GT wines, as determined via HPLC. All fermentations, including the pure *S. cerevisiae* inoculation, led to a reduction in malic acid, yet in some cases (with *H. occidentalis* and *H. opuntiae*) the malic acid content was indeed higher than the control. This observation, especially with *H. occidentalis*, was surprising as it was recently shown that *H. occidentalis* can consume malic acid within grape must [15]. In that study, *H. occidentalis* consumed malic acid in a sequential-type of inoculation modality without an airlock, suggestive of the importance of oxygen in the consumption of malic acid.

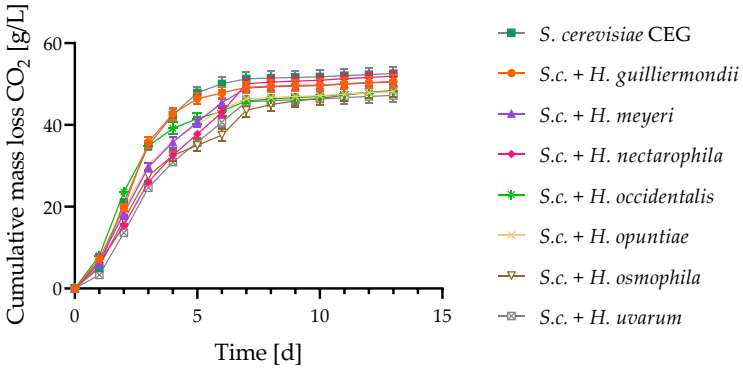

Figure 1. The mass-loss curves (g/L) of the co-fermentations of *Hanseniaspora* spp. conducted in GT must.

Table 1. The major organic acids, ethanol, glycerol and total sugar levels (all g/L) of the GT wines co-fermented with different *Hanseniaspora* spp., as determined with HPLC. Values are the means of three replicate fermentations followed by its standard deviations (±). Two-tailed unpaired *t*-tests with Welch's correction were conducted to compare values to the pure *S. cerevisiae* inoculum control. ↑ indicates significantly more than the control ($p < 0.05$), ↓ indicates significantly less than the control ($p < 0.05$). nd: not detected.

	S. cerevisiae	***H. guilliermondii***	***H. meyeri***	***H. nectarophila***	***H. occidentalis***	***H. opuntiae***	***H. osmophila***	***H. uvarum***
Total sugars	nd	nd	nd	nd	nd	nd	nd	nd
Tartaric acid	2.60 ± 0.12	2.40 ± 0.09	2.53 ± 0.03	2.59 ± 0.01	2.52 ± 0.07	2.45 ± 0.09	2.75 ± 0.02	2.56 ± 0.05
Malic acid	1.48 ± 0.06	1.35 ± 0.02	1.48 ± 0.02	1.57 ± 0.01	1.71 ± 0.05 ↑	1.65 ± 0.03 ↑	1.56 ± 0.02	1.55 ± 0.01
Lactic acid	0.25 ± 0.02	0.23 ± 0.01	0.23 ± 0.01	0.20 ± 0.01 ↓	0.22 ± 0.00	0.22 ± 0.02	0.04 ± 0.00 ↓	0.22 ± 0.01
Acetic acid	0.84 ± 0.04	0.75 ± 0.01	0.75 ± 0.03 ↓	1.10 ± 0.01 ↑	0.56 ± 0.01 ↓	0.94 ± 0.02 ↑	1.23 ± 0.01 ↑	0.68 ± 0.02 ↓
Citric acid	0.14 ± 0.01	0.12 ± 0.00 ↓	0.12 ± 0.00 ↓	0.13 ± 0.01	0.14 ± 0.00	0.11 ± 0.01 ↓	0.14 ± 0.00	0.10 ± 0.01 ↓
Ethanol	117.10 ± 3.20	114.67 ± 1.39	115.75 ± 0.91	117.35 ± 1.34	116.65 ± 2.74	116.37 ± 1.62	118.56 ± 0.59	116.31 ± 0.61
Glycerol	9.39 ± 0.35	9.28 ± 0.15	10.17 ± 0.05	10.05 ± 0.08	7.80 ± 0.12 ↓	10.10 ± 0.22 ↑	8.25 ± 0.08 ↓	9.98 ± 0.02

Regarding acetic acid, *Hanseniaspora*-inoculated fermentations resulted in both a reduction and an increase, depending on the species: for *H. nectarophila*, *H. opuntiae* and *H. osmophila,* co-fermentations led to a significant increase, whereas co-fermentations with *H. occidentalis* and *H. uvarum* led to a reduction. Acetic acid is the main contributor of volatile acidity in wine and along with its activated thioester acetyl-coenzyme (acetyl-CoA) are key participants within the central metabolism of cells. They directly take part in acetate ester formation as acetyl-CoA condenses with ethanol and other higher alcohols, which could explain the reduction in acetic acid in *H. uvarum* co-fermentations, which made the highest levels of ethyl acetate (Table 2). The divergent acetic acid levels with different *Hanseniaspora* spp. additions are consistent with the literature where no clear pattern emerges to what effect it has on the volatile acidity in wine [15,33].

Strikingly, lactic acid (the minor acid) was almost completely consumed within the co-fermentation with *H. osmophila*. It is unclear why this occurred as this has, to our knowledge, not been reported before.

3.3. Ethanol and Glycerol

A microbially-facilitated strategy to reduce ethanol levels in wine is by implementing NSY in co-culturing set-ups [34]. The strategy is based on the idea that the NSY would consume a portion of the initial sugars leaving less sugars to be fermented to ethanol by *Saccharomyces*. With the experimental set-up presented here, no significant reduction of ethanol was observed in any of the *Hanseniaspora* co-inoculums. Even though there have been reports of a reduction in ethanol in final wines with *Hanseniaspora* additions [13,20,35,36], fermentations, especially with simultaneous inoculation modalities did not observe ethanol reductions [37,38].

Table 2. Major wine aroma components of the GT wines, as determined with HS-SPME-GC-MS. Values are means of three replicate fermentations followed by the standard deviations (±). Two-tailed unpaired *t*-test with Welch's correction was conducted to compare values with the pure *S. cerevisiae* inoculum control. ↑ indicates significantly more than the control ($p < 0.05$), ↓ indicates significantly less than the control ($p < 0.05$). nq: not quantifiable.

	S. cerevisiae	***H. guilliermondii***	***H. meyeri***	***H. nectarophila***	***H. occidentalis***	***H. opuntiae***	***H. osmophila***	***H. uvarum***
Acetate esters (μg/L, except for ethyl acetate (mg/L))								
Ethyl acetate	115.80 ± 6.73	318.93 ± 28.10 ↑	332.86 ± 97.78	312.98 ± 55.29 ↑	108.19 ± 12.18	291.07 ± 21.83 ↑	151.60 ± 34.74	558.07 ± 52.66 ↑
Isoamyl acetate	1089 ± 104	3788 ± 614 ↑	2731 ± 803	2120 ± 351 ↑	646 ± 19 ↓	723 ± 51 ↓	640 ± 197 ↓	2523 ± 383 ↑
2-Phenylethyl acetate	203 ± 16	1185 ± 24 ↑	488 ± 59 ↑	269 ± 22 ↑	225 ± 2	683 ± 45 ↑	1236 ± 48 ↑	466 ± 13 ↑
2-Methylbutyl acetate	108 ± 16	416 ± 63 ↑	390 ± 134	336 ± 127	62 ± 17↓	147 ± 16 ↑	63 ± 10 ↓	339 ± 118
Hexyl acetate	3 ± 2	24 ± 5 ↑	9 ± 5	13 ± 4 ↑	nq	nq	3 ± 1	15 ± 2 ↑
Ethyl esters (μg/L)								
Ethyl propionate	217 ± 17	238 ± 24	274 ± 70	253 ± 53	482 ± 34 ↑	265 ± 19 ↑	122 ± 29 ↓	425 ± 67 ↑
Ethyl butyrate	222 ± 21	312 ± 37 ↑	248 ± 55	241 ± 39	182 ± 7	177 ± 14 ↓	227 ± 46	272 ± 30
Ethyl hexanoate	76 ± 21	92 ± 40	51 ± 59	66 ± 47	nq	nq	35 ± 32	77 ± 11
Ethyl octanoate	494 ± 99	459 ± 184	266 ± 268	541 ± 305	nq	nq	317 ± 140	473 ± 33
Ethyl decanoate	468 ± 44	525 ± 85	425 ± 88	666 ± 318	172 ± 48 ↓	106 ± 44 ↓	462 ± 138	576 ± 205
Medium-chain fatty acids (mg/L)								
Hexanoic acid	5.63 ± 0.06	5.56 ± 0.04	5.44 ± 0.05 ↓	5.53 ± 0.05	5.37 ± 0.02 ↓	5.30 ± 0.01 ↓	5.47 ± 0.02 ↓	5.57 ± 0.01
Octanoic acid	3.17 ± 0.07	3.09 ± 0.04	2.97 ± 0.03 ↓	3.02 ± 0.03 ↓	2.88 ± 0.00 ↓	2.86 ± 0.01 ↓	2.98 ± 0.03 ↓	3.02 ± 0.00
Higher alcohols (mg/L)								
Isobutanol	141.80 ± 12.75	149.54 ± 18.24	161.05 ± 41.06	150.83 ± 26.32	121.33 ± 16.12	190.04 ± 23.72	116.23 ± 13.69	140.45 ± 0.83
Isoamyl alcohol	668.82 ± 40.95	539.02 ± 54.58 ↓	581.12 ± 112.97	493.20 ± 92.87	510.68 ± 64.69 ↓	544.60 ± 66.44	429.67 ± 37.26 ↓	540.64 ± 7.27 ↓
2-Methyl butanol	125.16 ± 3.01	97.85 ± 8.83 ↓	101.78 ± 21.19	104.83 ± 19.05	53.08 ± 6.59 ↓	105.55 ± 9.04	99.63 ± 2.63 ↓	102.37 ± 1.21 ↓
1-Hexanol	1411.90 ± 71.14	1099.56 ± 46.86↓	1348.98 ± 34.27	1257.44 ± 161.69	1029.03 ± 40.98 ↓	1431.11 ± 46.72	1312.47 ± 25.20	1168.21 ± 26.82 ↓
2-Phenyl ethanol	44.34 ± 0.43	28.34 ± 0.45 ↓	41.82 ± 5.42	27.75 ± 4.54 ↓	59.40 ± 4.51 ↑	48.52 ± 2.11	36.31 ± 2.32 ↓	37.70 ± 1.17 ↓

As with acetic acid, the co-fermentations produced both significantly more (*H. opuntiae*) or less glycerol (the two members of the SEL, *H. occidentalis* and *H. osmophila*) than the control. Glycerol levels are often elevated with NSY additions [39] and often coincide with an increase in acetic acid [40]. This is often explained within the context of cofactor maintenance, as the enzymes directly responsible for glycerol and acetic acid production, glycerol-3-phosphate dehydrogenase and acetaldehyde dehydrogenase, respectively, require and produce NADH. Curiously, in this experiment, the co-fermentation with *H. occidentalis* led to an unexpected reduction in both acetic acid and glycerol, a finding which was not observed when the same yeast was co-fermented in a sequential-type inoculation [15].

3.4. Aroma Analysis

Since *Hanseniaspora* is known for acetate ester formation and produces large amounts of ethyl acetate, a solvent-like odour, too much of which can quickly spoil the wine, the volatile aroma compounds (VOC) were measured. Table 2 shows the concentrations of many of the expected esters, medium-chain fatty acids, and higher alcohols in the final wines co-cultured with *Hanseniaspora* spp. With regards to the ethyl esters and medium-chain fatty acids, little modulation was observed with the *Hanseniaspora* co-cultured fermentations, when compared to the *S. cerevisiae* control, apart from the concentration of ethyl propionate (an aroma compound imparting a pineapple-like odour). The ethyl propionate concentrations in wines fermented with *H. occidentalis* and *H. uvarum* were measured to be more than double than that of the control.

As expected, a large level of variability was measured looking at acetate esters. For all of the five acetate esters, the concentrations were significantly more in *H. guilliermondii* co-fermentations, whereas four of the five acetate esters were higher with *H. nectarophila* and *H. uvarum*. An increase in all of these esters within a wine will generally be considered as an oenological benefit as they contribute to a fruity or flowery aroma, except for ethyl acetate. Ethyl acetate measured in the five FEL members were approximately three times more than the control with *H. uvarum* co-fermentations achieving levels of more than 500 mg/L. All of these levels far exceed what is considered to be pleasant (<100 mg/L). With the ethyl acetate levels obtained from the two members of the SEL, no significant difference with the control was observed. Yet for *H. osmophila*, more than six times more 2-phenethyl acetate, a key rose-like aroma component, was recorded. Furthermore, of note are the quantitatively high levels of the beneficial acetate esters produced by *H. guilliermondii.*

The majority of the higher alcohols were either unchanged or comparatively lower than the control. This is directly connected with their conversion to their corresponding acetate esters. Only co-fermentations with *H. occidentalis*, however, showed to have higher amounts of one of the higher alcohol than the controls, namely 2-phenyl ethanol.

3.5. Terpenes

Typical of GT must is that it has a high terpene potential. Terpenes are often bound to sugar moieties in grape must and require deglycosylation for their release, in order to be perceived. This release can be facilitated enzymatically via the action of β-glucosidases or non-specific β-glucanases and it has been shown that NSY, including *Hanseniaspora* spp., have superior terpene-releasing abilities than *Saccharomyces* [38,41–43]. The conversion of terpenes can also occur, which can be catalyzed by several enzymes, including dehydrogenases, oxygenases and reductases [44]. Table 3 shows the terpene content of the GT wines co-fermented with the different *Hanseniaspora* spp. We observed in certain cases significant modulation of the terpenes within the co-fermentations. With *H. opuntiae*, in particular, many of the terpenes were significantly affected like citronellol and β-myrcene, which is suggestive of the release of terpenes from sugar moieties. Curiously, with some co-fermentations (*H. guilliermondii*, *H nectarophila* and *H. uvarum*) citronellol levels were significantly lower than those of the control. A short fermentation of must spiked with higher levels of citronellol in wines co-fermented with *H. guilliermondii* and *H. uvarum*

was conducted to see if other peaks related to citronellol could be observed. A peak corresponding to the acetate ester of citronellol (i.e., citronellyl acetate) was detected in the GC-MS chromatograms (Figure 2). This strongly suggests that these *Hanseniaspora* strains converted citronellol (which is a terpenol with a primary alcohol functional group) to its acetate ester, presumably via the same action as the esterification of other alcohols.

3.6. PCA

To analyse the overall outcome of VOC production in the different co-fermentations, a principal component analysis was conducted, including aroma compounds, organic acids, ethanol and glycerol (Figure 3). Large variations, especially in the ethyl ester content, of co-fermentations with *H. nectarophila* and *H. meyeri* replicates caused the overlaps of the data points with other groups. The PCA indicated that the two members of the SEL separated from the other groups regarding their wine profiles. It is also noteworthy that the bulk of the acetate esters production was associated with members of the FEL, such as *H. guilliermondii* and *H. uvarum.* The PCA also shows the noteworthy acid modulation displayed by the fermentation with *H. osmophila* with its high production of acetic acid coinciding with the possible consumption of lactic acid.

Table 3. Terpene and norisoprenoid content (all µg/L) of the GT wines, as measured with HS-SPME-GC-MS. Values are the means of three replicate fermentations followed by the standard deviations (±). Unpaired *t*-test with Welch's correction was conducted to compare values with the pure *S. cerevisiae* inoculum control. ↑ indicates significantly more (<0.05) than the control (*S. cerevisiae*) ↓ indicates significantly less (<0.05) than the control (*S. cerevisiae*).

	S. cerevisiae	***H. guilliermondii***	***H. meyeri***	***H. nectarophila***	***H. occidentalis***	***H. opuntiae***	***H. osmophila***	***H. uvarum***
β-myrcene	6.71 ± 0.31	9.82 ± 1.26 ↑	7.87 ± 1.05	9.37 ± 0.58 ↑	7.95 ± 1.74	9.13 ± 0.17 ↑	8.87 ± 0.67 ↑	9.06 ± 0.24 ↑
limonene	0.96 ± 0.01	0.99 ± 0.02	0.97 ± 0.02	0.99 ± 0.01 ↑	0.96 ± 0.03	1.01 ± 0.01 ↑	0.98 ± 0.01	1.02 ± 0.01 ↑
cis-rose oxide	0.42 ± 0.03	0.24 ± 0.03 ↓	0.35 ± 0.07	0.38 ± 0.04	0.19 ± 0.07 ↓	0.36 ± 0.03 ↓	0.24 ± 0.04 ↓	0.45 ± 0.02
trans-rose oxide	0.15 ± 0.01	0.09 ± 0.01 ↓	0.14 ± 0.02	0.15 ± 0.01	0.09 ± 0.02 ↓	0.13 ± 0.01 ↓	0.11 ± 0.01 ↓	0.17 ± 0.01
cis-linalool oxide	19.74 ± 1.03	19.68 ± 0.13	19.11 ± 0.19	18.37 ± 0.24	18.13 ± 0.54	18.84 ± 1.43	18.54 ± 0.65	19.12 ± 0.68
nerol oxide	1.29 ± 0.04	1.35 ± 0.07	1.27 ± 0.11	1.32 ± 0.09	1.25 ± 0.18	1.45 ± 0.05 ↑	1.29 ± 0.16	1.38 ± 0.07
trans-linalool oxide	6.20 ± 0.67	5.75 ± 0.18	5.43 ± 0.34	5.77 ± 0.21	5.45 ± 0.20	5.72 ± 0.25	5.63 ± 0.52	5.52 ± 0.03
vitispirane	0.56 ± 0.01	0.54 ± 0.00 ↓	0.55 ± 0.01	0.54 ± 0.01 ↓	0.54 ± 0.01 ↓	0.55 ± 0.01	0.52 ± 0.01 ↓	0.57 ± 0.00
linalool	72.43 ± 3.71	72.93 ± 1.80	71.43 ± 0.69	72.13 ± 3.01	71.18 ± 2.51	73.91 ± 3.70	72.04 ± 5.49	72.50 ± 0.45
hotrienol	36.61 ± 6.24	29.52 ± 1.85	31.48 ± 4.25	28.38 ± 3.47	39.98 ± 3.95	43.04 ± 3.24	35.04 ± 0.35	31.78 ± 1.97
α-terpineol	55.46 ± 9.81	47.13 ± 2.74	50.75 ± 6.32	46.91 ± 5.59	68.66 ± 7.75	72.42 ± 4.46	69.19 ± 2.49	49.01 ± 3.90
citronellol	26.97 ± 4.40	9.52 ± 0.56 ↓	18.96 ± 4.08	13.83 ± 1.22 ↓	27.37 ± 4.97	38.08 ± 1.52 ↑	20.31 ± 2.57	13.85 ± 1.88 ↓
β-damascenone	0.46 ± 0.01	0.56 ± 0.04	0.53 ± 0.05	0.61 ± 0.04 ↑	0.64 ± 0.14	0.69 ± 0.03 ↑	0.70 ± 0.10	0.55 ± 0.06

Figure 2. GC-MS chromatograms of the one-day must fermentations with *H. guilliermondii* and *H. uvarum* supplemented with higher levels of citronellol.

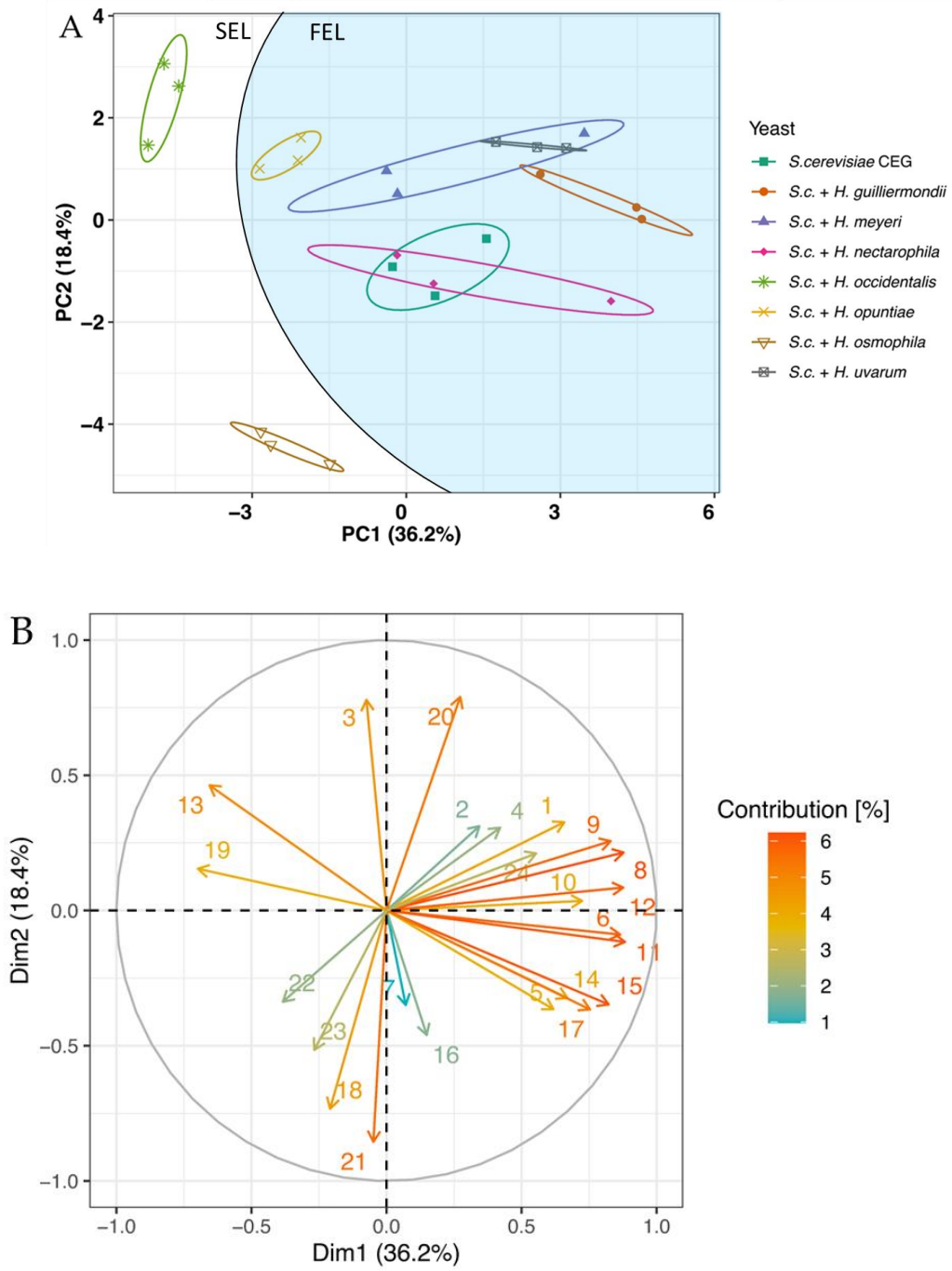

Figure 3. PCA of the final wines co-fermented with *Hanseniaspora* spp. All of the measured wine parameters, excluding the terpene data, were used to compose the PCA. Score plot (**A**) in the first two PCs: fermentation replicates are shown with the same shape and colour. The blue-and-white separation indicates the division of the two lineages (FEL and SEL). (**B**) Corresponding loading map. Each arrow relates to the tip of a vector starting from the origin. The closer the variable is to the circle, the better it is explained by the components. Contribution values (%) are shown in a gradient scale of colours with corresponding values. Numbers in the loading map correspond to: (1) ethyl acetate, (2) isobutanol, (3) ethyl propionate, (4) isoamyl alcohol, (5) 2-methyl-1-butanol, (6) ethyl butanoate, (7) hexan-1-ol, (8) isoamyl acetate, (9) 2-methyl-1-butyl acetate, (10) hexanoic acid, (11) ethyl hexanoate, (12) hexyl acetate, (13) 2-phenylethanol, (14) octanoic acid, (15) ethyl octanoate, (16) 2-phenylethyl acetate, (17) ethyl decanoate, (18) tartaric acid, (19) malic acid, (20) lactic acid, (21) acetic acid, (22) citric acid, (23) ethanol, (24) glycerol.

4. Conclusions

Even though non-*Saccharomyces* yeasts are increasingly used in wine fermentations, reproducibility is a concern. This is not only evident in our study, but also emerges from the literature on mixed-culture fermentations. The interaction of different yeast species is complex and strongly dependent on the initial conditions in the must. In conclusion, the different *Hanseniaspora* species contributed in various ways to the aroma profile of the wine. The SEL yeasts (*H. occidentalis* and *H. osmophila*) showed little change in ethyl acetate formation, which is typical for *Hanseniaspora*, in contrast to the control fermentation with *S. cerevisiae*. In addition, some of the *Hanseniaspora* spp. contributed significantly to the complex aroma profile through the depletion of lactic acid, an increase in acetate esters or terpenols, or through the conversion of citronellol to citronellyl acetate. Finally, this study shows for the first time the use of *H. nectarophila* and *H. meyerii* in winemaking and the characterization of the resulting wine. In particular, the wines fermented with *H. nectarophila* showed increased amounts of acetate esters, compared to the control wine.

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