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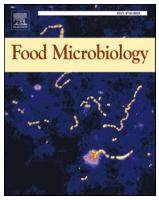

Effect of *Hanseniaspora vineae* and *Saccharomyces cerevisiae* co-fermentations on aroma compound production in beer

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ABSTRACT

In recent years, the boom of the craft beer industry refocused the biotech interest from ethanol production to diversification of beer aroma profiles. This study analyses the fermentative phenotype of a collection of non-conventional yeasts and examines their role in creating new flavours, particularly through co-fermentation with industrial *Saccharomyces cerevisiae*. High-throughput solid and liquid media fitness screening compared the ability of eight *Saccharomyces* and four non-*Saccharomyces* yeast strains to grow in wort. We determined the volatile profile of these yeast strains and found that *Hanseniaspora vineae* displayed a particularly high production of the desirable aroma compounds ethyl acetate and 2-phenethyl acetate. Given that *H. vineae* on its own can't ferment maltose and maltotriose, we carried out mixed wort co-fermentations with a *S. cerevisiae* brewing strain at different ratios. The two yeast strains were able to co-exist throughout the experiment, regardless of their initial inoculum, and the increase in the production of the esters observed in the *H. vineae* monoculture was maintained, alongside with a high ethanol production. Moreover, different inoculum ratios yielded different aroma profiles: the 50/50 *S. cerevisiae/H. vineae* ratio produced a more balanced profile, while the 10/90 ratio generated stronger floral aromas. Our findings show the potential of using different yeasts and different inoculum combinations to tailor the final aroma, thus offering new possibilities for a broader range of beer flavours and styles.

1. Introduction

The use of non-conventional yeasts (NCY) in the beverage industry has been rapidly growing in recent years (Hittinger et al., 2018). This is mainly due to the fact that these yeasts can produce beers with special characteristics, such as unique aroma profile (Bourbon-Melo et al., 2021; Dippel et al., 2022; Gamero et al., 2016; Peces-Pérez et al., 2022) low ethanol content (Bellut and Arendt, 2019; Bucher et al., 2018; Francesco et al., 2018), probiotic properties (Piraine et al., 2023; Silva et al., 2021) and better nutritional content (Canonico et al., 2021). Craft beer production has likewise flourished in the last decade: from 2012 to 2022 the number of active microbreweries in Europe has augmented a 73 % (The Brewers of Europe, 2023), and in contrast with large-scale industrial brewing, which relies on very well-established and standardized processes, craft brewing is more flexible in its approaches (Cabras and Bamforth, 2016) The utilization of NCY in craft brewing represents a good opportunity for innovation to take place, yet the biological knowledge of NCY strains is still limited compared to the conventional brewing yeasts (*i.e. S. cerevisiae* and *S. pastorianus*) (Basso et al., 2016).

High-throughput screening methods are a cost-effective approach to assess the suitability of conventional and non-conventional yeast strains for their use in brewing and have been previously employed for this purpose (Aguiar-Cervera et al., 2021; Gamero et al., 2016). These methods enable the evaluation of numerous strains, aiding in the identification of those with desirable traits and reducing the number of candidates for downstream analytics of aroma compounds, ethanol, and other secondary metabolites such as glycerol and acetic acid. These compounds are key elements for any alcoholic beverage, as their concentration greatly influences the quality of the final product (Carpena et al., 2021; Dzialo et al., 2017; Zhao et al., 2015).

Higher alcohols such as isoamyl alcohol and phenylethyl alcohol, as

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well as their ester derivatives, isoamyl acetate and 2-phenylethyl acetate, are among the key aroma compounds that positively influence beer organoleptic quality, even at very low concentrations (Dzialo et al., 2017). These molecules are largely responsible for the fruity and floral notes that are highly esteemed in many beer styles, contributing to a complex and desirable flavour profile (Pires et al., 2014). On the other hand, certain volatiles including acetic acid, butyric acid, diacetyl, and phenolic compounds, are generally considered undesirable due to their tendency to produce off-flavours like sourness, rancidity, or buttery notes (Lentz, 2018; Olšovská et al., 2019; Stewart, 2017). However, concentrations of these compounds vary greatly within different beer styles; for instance, the smoky, clove-like character of phenolic compounds such as 4-vinyl guaiacol is essential in certain traditional ales, while low levels of diacetyl are desired in certain English ales and Bohemian Pilsners for the slight buttery note it imparts (Pires et al., 2014; Verstrepen et al., 2003). This complexity indicates the need for a nuanced approach to controlling these compounds in brewing, where the balance and concentration of each can either enhance or detract from the final product's quality depending on the intended style and consumer preferences.

Besides optimizing fermentation parameters, namely growth rate, C/N ratio, oxygen, and temperature (Visinoni et al., 2022), aroma volatile compound production can be controlled through the use of hybrid yeast strains, which can produce higher levels of key aroma compounds (Giannakou et al., 2021; Mertens et al., 2015; 2019; Krogerus et al., 2023), thus making their use in the food industry an attractive approach.

Co-fermentation between *Saccharomyces* and non-*Saccharomyces* yeasts represents yet another strategy for aroma enhancement. Typically, *Saccharomyces* sp. Strains achieve robust fermentation performance, but produce low concentrations of certain desirable aroma compounds (Walker and Stewart, 2016). Conversely, non-*Saccharomyces* yeasts, while less efficient in fermentation, can generate higher levels of key flavour compounds (Basso et al., 2016). By strategically combining conventional and non-conventional yeasts, brewers can harness the strengths of each, optimizing both fermentation efficiency and aroma. This approach has been extensively explored in the wine industry (Vilela, 2020), and its use in brewing is also gaining traction in recent years.

Sequential and mixed beer co-fermentations of *S. cerevisiae* with non-*Saccharomyces* yeasts such as *Metschnikowia pulcherrima*, *Schizosaccharomyces pombe*, *Lachancea thermotolerans*, *Torulaspora delbrueckii* and several *Hanseniaspora* spp. and *Pichia* spp., have been trialled displaying very promising results (Bourbon-Melo et al., 2021; Canonico et al., 2019, 2022; Drosou et al., 2022; Han et al., 2023; Holt et al., 2018; Kayadelen et al., 2023; Larroque et al., 2021; Matraxia et al., 2021; Peces-Pérez et al., 2022; Postigo et al., 2022a, 2023; Rehorska et al., 2022; Sampaolesi et al., 2023; Toh et al., 2018; Valentoni et al., 2023; I. M.H. van Rijswijck et al., 2017; Wu et al., 2024). Here, we further expand on the potential application of eight *Saccharomyces* and four non-*Saccharomyces* yeast in the brewing industry by: ***i.*** ensuring their capacity to grow in wort through a solid media growth assessment; ***ii.*** analysing their growth kinetics and fermentation performance in micro and small-scale wort fermentations; ***iii.*** measuring their absolute aroma compound production in wort fermentation (Fig. 1A and B).

Based on our analysis, we selected two promising strains (*S. cerevisiae* WLP095 and *H. vineae* Y-17530) for subsequent mixed wort co-fermentation at different ratios and assessed their population dynamics and relative aroma compound production (Fig. 1C).

There is a vast body of evidence on the use of pure or mixed cultures of *H. vineae* for improving the quality of wine due to its enhanced production of tryptophol, tyrosol acetates, acetoin, mevalonolactone, benzyl alcohol, and especially 2-phenylethyl acetate, which is associated with a pleasant floral aroma (Carrau and Henschke, 2021; Del Fresno et al., 2021; Lleixà et al., 2016; Martin et al., 2016; Medina et al., 2013;

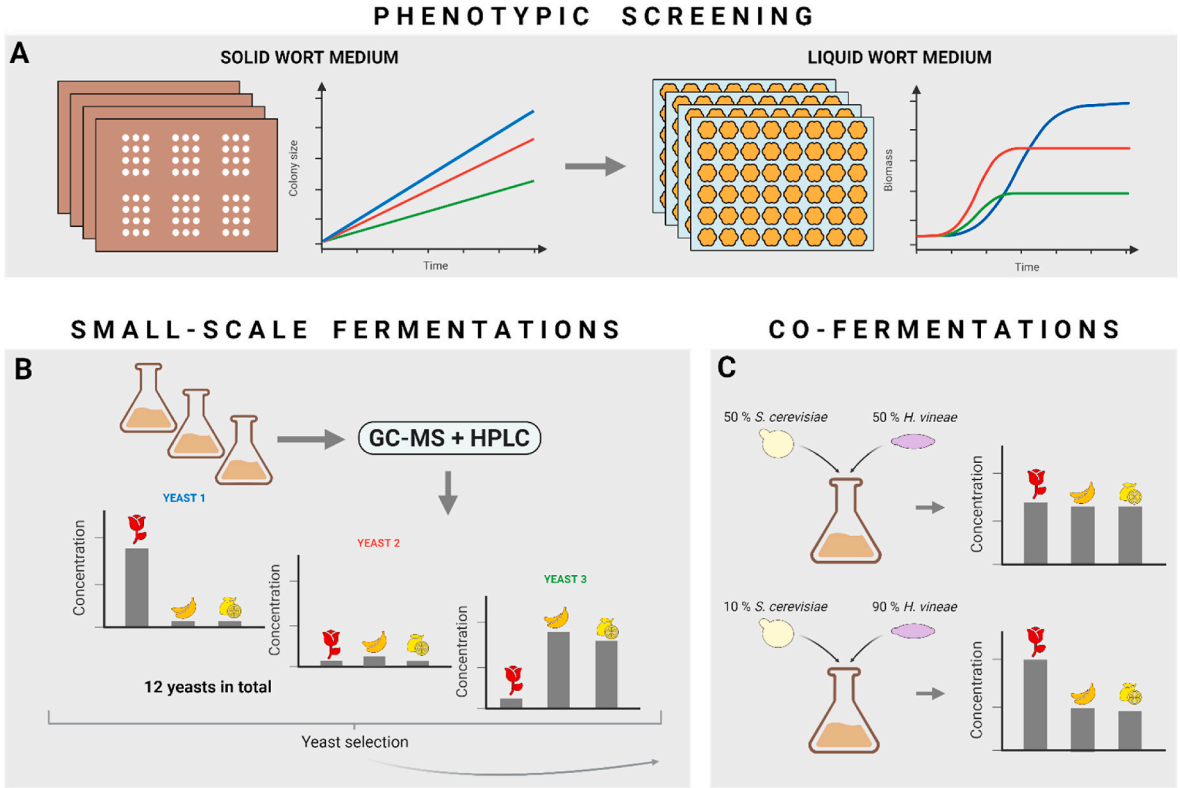

Fig. 1. Experimental strategy: Firstly, we scored the ability of 12 yeast strains to grow in wort by screening their fitness in solid and liquid wort (A). Small-scale fermentations were then carried with 12 selected yeasts, and ethanol and aroma compound production (, ,) was assessed (B). Finally, based on our results, we selected two yeast strains for mixed wort co-fermentations using two different inoculum ratios and assessed the aroma profile (C).

Valera et al., 2021; Viana et al., 2011; Zhang et al., 2020). This has allowed for the development of an *H. vineae* wine starter strain (Fermivin *VINEAE*), which has recently become commercially available. However, the use of *H. vineae* for beer production is still limited, albeit some studies have been published in recent years. These have shown that this yeast species can significantly increase the concentration of desirable aroma compounds in beer, producing fruity aroma profiles when employed in pure (Bellut et al., 2018; Osburn et al., 2018; Postigo et al., 2022b) and sequential co-fermentation with *S. cerevisiae* (Larroque et al., 2021; Peces-Pérez et al., 2022; Postigo et al., 2022c). Nonetheless, its use for simultaneous mixed wort co-fermentation has not been trialled to date. Here, we explored the effect of *H. vineae* and *S. cerevisiae* wort co-fermentation on the aroma profile and found that different *H. vineae/S. cerevisiae* inoculum ratios impact the volatile production, underlining the potential of tailoring the aroma of beer by means of different inoculation ratios in mixed co-fermentation.

2. Materials and methods

2.1. Strains and media conditions

Twelve conventional and non-conventional yeast strains were employed in the study. The microbial strains used in this work were provided by the USDA-ARS Culture Collection (NRRL), Singer Instruments Co. Ltd., or obtained from White Labs. All the strains used in this study are listed in Table 1. The non-*Saccharomyces* strains were selected based on their previously reported good aroma production (Gamero et al., 2016; Gutiérrez et al., 2018) and the lack of extensive literature exploring their use in the brewing industry. The various commercial and brewery-isolated *S. cerevisiae* strains were included with the aim of finding a strong wort fermenter with an aroma compound production that would complement that of the selected NCY in co-fermentation.

The media employed included YPD (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose; Formedium, UK) and 12 °Bx Spraymalt Amber 18 EBC Wort (Brewferm Brouland, Belgium), which had a pH of 4.9 and 0.1–0.25 g/L of free amino nitrogen (FAN; Lin et al., 2021). The wort was prepared as previously reported (James et al., 2008). The solidified media was supplemented with 20 g/L agar (Formedium, UK). A volume of 45 mL of media was poured into SBS PlusPlates (Singer Instruments, UK) and left to set on an even surface.

Table 1
Yeast strains used in the study.

Species	Strain	Abbreviation	Source	Isolated from
Saccharomyces cerevisiae	ESM356-1	Sc1	Singer Instruments	Lab strain
S. cerevisiae	Y-12632	Sc2	NRRL	Brewery
S. cerevisiae	Y-11875	Sc3	NRRL	Brewery
S. cerevisiae	Y-2432	Sc4	NRRL	Brewery
S. cerevisiae	WLP001	Sc5	White Labs	Commercial strain
S. cerevisiae	WLP008	Sc6	White Labs	Commercial strain
S. cerevisiae	WLP095	Sc7	White Labs	Commercial strain
S. cerevisiae	WLP775	Sc8	White Labs	Commercial strain
Candida milleri	Y-7248	*Cm*	NRRL	Sour dough
Hanseniaspora vineae	Y-17530	Hv	NRRL	Black-knot gall (*Dibotryum morbosum*) on *Prunus virginiana*
Kluyveromyces lactis	Y-1140	Kl	NRRL	Creamery
Pichia scutulata	Y-7663	Ps	NRRL	Slime from sandalwood (*Myoporum sandwicense*)

2.2. High-throughput fitness assessment

An adapted version of our previously developed (Aguiar-Cervera et al., 2021) colony-size-based fitness assessment was employed. This method allows for the rapid and simultaneous screening of conventional and non-conventional yeasts in solid media and was utilized to ensure that all strains were able to grow using wort as a carbon source. The yeast strains were revived from −80 °C in YPD broth and arranged in 3 × 4 squares (Fig. S1A) on SBS PlusPlates containing solidified 12 °Bx wort and YPD using the PIXL (Singer Instruments, UK) robotic platform. The plates were replicated onto agar YPD and solidified wort plates using a ROTOR HDA (Singer Instruments, UK), sealed and incubated at 20 °C for 210 h. The PhenoBooth (Singer Instruments, UK) was used to capture images every 10 h. Following background subtraction, a colony radius value measured in pixels was obtained for each colony and time point and normalised using an in-house developed javascript script (https://github.com/joseac93/Normalisation-script.git), taking into account the plate, column and row means. The screening was performed in quadruplicate, including 12 in-plate replicates for each strain.

High-throughput micro-fermentations were carried out at 20 °C using a BioLector (m2p, Germany) on YPD and wort broth, as previously reported (Giannakou et al., 2021). Briefly, overnight YPD broth cultures of the 12 strains were used to inoculate the wells of a 48-well MTP-48-B FlowerPlate (m2p, Germany) containing fresh YPD or wort in a final volume of 1.5 mL and an OD_{600} of 0.1, in triplicate. The plate was sealed with a gas-permeable foil and incubated for 72 h with 800 rpm agitation, humidity control (>85% dH_2O), and aeration (20.85 % O_2). Scattered light values at values at 620 nm were measured automatically in each sample every 7.29 min.

2.3. Small-scale fermentations

To confirm the results on a larger scale, as well as to be able to measure the aroma compound production of the strains, a scale-up experiment was performed in 500 mL flasks. Overnight YPD cultures of the 12 strains were used to inoculate 250 mL of wort at an OD_{600} of 0.1 in triplicate, then the flasks were covered with sterile cotton pads and incubated statically at 20 °C for five days, (until all metabolizable sugars were depleted). Samples for downstream analysis were collected from each flask every 24 h.

2.4. Co-fermentations

To identify the optimal inoculation ratio of Sc7 and Hv to avoid outcompetition, five wort co-fermentations and two monocultures, one for each species, were set up in triplicate in a 96-deep-well plate. The medium was inoculated to a final combined OD_{600} of 0.1 employing the following ratios: 100 % Sc7, 50/50 Sc7/Hv, 30/70 Sc7/Hv, 20/80 Sc7/Hv, 10/90 Sc7/Hv, 5/95 Sc7/Hv and 100 % Hv. The deep-well plate was covered with an aluminium seal and incubated statically at 20 °C.

The growth dynamics were studied by comparing the CFU/mL for each yeast species on each ratio. Colonies of Sc7 and Hv were easily distinguishable by eye due to their colony morphology characteristics (Fig. S2): Sc7 formed very bubbly and thick colonies, whereas Hv formed thinner colonies, which were surrounded by a halo of less dense colony material and were slightly raised at the centre. Samples for CFU assessment were taken at 0, 12, 24, 48, 72, and 96 h.

Co-fermentations were performed in 500 mL flasks, in triplicate. Cell count was carried out with a Cellometer Auto M10 (Nexcelom Bioscience, USA) to establish the initial inoculum. Two hundred and fifty mL of 12 °Bx wort were inoculated with a final concentration of cells of 1×10^5 cells/mL in the following ratios: 100 % Sc7, 50/50 Sc7/Hv, 10/90 Sc7/Hv, and 100 % Hv.

The flasks were covered with sterile cotton pads and incubated statically at 20 °C. Samples were taken at 0, 17, 24, 48, 72, 120, 192, 262 and 334 h. Population dynamics was studied with the same CFU method

as the one employed in the pre-study, based on colony morphology differences.

2.5. HPLC and GC/MS analyses

A 1260 Infinity II LC High-Performance Liquid Chromatography system equipped with a Refractive Index Detector (Agilent, USA) and a 300 × 7.8 mm Hi-Plex Exchange column (Agilent, USA) was used to quantify glucose, maltose, maltotriose, ethanol, glycerol and acetic acid at the end of the BioLector micro-fermentations and at different time-points during the course of the flasks small-scale fermentations and co-fermentations. H_2SO_4 5 mM was utilized as solvent with a flow rate of 0.8 mL/min at 55 °C. The compounds were detected by retention times and quantified using calibration curves made with analytical grade standards (Sigma-Aldrich, Germany).

A Thermo Scientific TSQ Quantum GC Triple Quadropole GC-MS was used to quantify the volatile flavour compounds in the small-scale fermentations. Twenty-five μL of internal standard (2-octanol 2.5 mg/L) and 0.5 g of NaCl were added to each sample in 20 mL vials. After 10 min of incubation at 40 °C, volatile compounds were collected on a Divinylbenzene/Carboxen/Polydimethylsiloxane 2 cm fibre (DVB-CAR-PDMS Supelco, USA) with an extraction time of 30 min. A VF-wax column (Agilent, USA) 30 m/I. D 0.25 mm/Film 0.25 μm was used for the separation. Oven temperature was set at 40 °C for 4 min and then increased 6 °C/min until 250 °C were reached. The final temperature was kept for 5 min. The injector and interface temperatures were kept at 250 °C as well. Helium was used as the carrier gas with a flow rate of 1.2 mL/min. The time for thermal desorption of analytes was 4 min. The MS detector was operated in full scan mode at 70 eV with a scan range from 40 to 300 m/z for 44 min.

Quantitative data analysis was performed using ThermoXcalibur (Version 2.2 SP1.48, Thermo scientific, USA). Identification of compounds was based on comparison with a mass spectral database (NIST version 2.0) and with pure reference standards. The calibration curves were done by adding the standards in a MQ water +5 % EtOH solution (Fig. S3).

The relative concentrations of the volatile compounds of the co-fermentations samples were assessed using a Gerstel MPS dual head system (Gerstel, Germany) and an Agilent 7890 B Gas Chromatograph (GC) in conjunction with an Agilent 5977 B Series Mass Selective Detector (MSD) (Agilent, USA). Five mL of each sample were placed in a 20 mL vial with 0.5 g of NaCl and 25 μL of the internal standard (2-octanol 2.5 mg/L). The samples were then incubated for 10 min at 30 °C to reconcentrate volatile analytes into the headspace, after which a PDMS fibre (100 μm thickness) was extracted for 5 min and injected into the front inlet. An Agilent VF-5MS column (30 m × 25 mm x 0.25 μM) with a flow rate of 1 mL min^{-1} was used to carry out the GC separation. The gradient in the oven was: hold at 40 °C for 4 min, a linear 6 °C per minute gradient to 250 °C, and a final hold at 250 °C for 5 min. The GC-MS analysis time for each sample was 44 min. The temperature conditions were as follows: inlet 280 °C, transfer line 300 °C, EI source 230 °C, quadrupole 150 °C. The MS scanned mass range was 40–400 m/z.

3. Results and discussion

3.1. Phenotyping of yeast species grown on solid and liquid wort

With the aim of finding the ideal candidates for beer co-fermentation, we first screened the ability of 12 different *Saccharomyces* and non-*Saccharomyces* yeasts (Table 1) to grow in YPD and in beer-fermentative conditions consisting of 12 °Bx wort. It is key to ensure that any potential non- *Saccharomyces* candidate for beer co-fermentations is able to reach an acceptable biomass in wort to avoid out-competition when co-fermenting with a brewing *S. cerevisiae* strain.

Phenotyping in solid media was carried out through adapting a high-throughput screening protocol that we have recently developed (Aguiar-Cervera et al., 2021). This method uses colony size in solid media as a proxy for fitness.

We found that the fitness in wort for all the strains tested was either similar or worse to the fitness in YPD, with the exception of *S. cerevisiae* WLP008 (Sc6) which grew much better in wort. The NCY *Hanseniaspora vineae* Y-17530 (Hv) and *Kluyveromyces lactis* Y-1140 (Kl) displayed the biggest disparity of fitness, having final colony sizes in YPD that doubled the ones in wort. Overall, the results confirmed that all strains could grow using wort as a carbon source (Fig. S1B).

Next, micro-fermentations were carried out to assess the ability of the strains to grow in liquid wort, to mimic their potential performance in beer-fermentative conditions. This second screening also provided information regarding the fermentation performance in terms of sugar consumption and ethanol production. In this case, maximum growth rate (max. μ), maximum biomass, and Area Under the Curve (AUC) values were calculated for the 12 strains in both liquid YPD and wort after 48 h BioLector micro-fermentations at 20 °C.

In the experiments in liquid, the final biomass and AUC values were higher in YPD for all strains, including Sc6, however, the maximum growth rate was higher in wort for 9 out of 12 strains (Tables 2 and 3). This is rather intriguing since YPD has a higher glucose concentration than wort (20 g/L vs 12 g/L in wort) hence the expectation of higher growth rates in the medium with more glucose, which is the preferable carbon source for virtually any yeast. Here, it seems like the presence of other complex sugars such as maltose in wort, bolstered growth rate. On the other hand, YPD contains a lower C/N ratio than wort (Molitor et al., 2023; Vidal, 2013), removing a growth limiting factor and allowing the accumulation of more biomass overtime.

In terms of the growth kinetics in YPD (Table 2), the *Saccharomyces* spp. *S*trains displayed max. μ values ranging from 0.16 to 0.32, whereas in the non-*Saccharomyces* spp., it ranged from 0.29 to 0.44, with Hv displaying the highest max. μ value. The major differences were observed in the max. biomass and AUC values, where the non-*Saccharomyces* spp. Strains really stood up. For example, *C. milleri* Y-7245 (*Cm*) exhibited a max. biomass of 336.11 ± 37.68, which was the smallest of the non-*Saccharomyces* species but yet similar to the max. biomass of *S. cerevisiae* Y-2432 (Sc4) (387.39 ± 65.35), which was the best performer of the *Saccharomyces* genus. Hv and Kl displayed the highest. Max. biomass and AUC values, duplicating or even triplicating the values observed in the *Saccharomyces* strains. It is interesting to observe that the lab strain *S. cerevisiae* ESM356-1 (Sc1), which should be adapted to grow in lab conditions – namely YPD, did not thrive or surpass the non-lab strains in such condition. In fact, it displayed the lowest maximum biomass and AUC values of all the studied strains in YPD. This behaviour can be explained by the strain's ploidy: Sc1 is haploid and the brewing *S. cerevisiae* strains tend to be polyploid (Gallone et al., 2016). The presence of many copies of glucose transporter genes in the polyploid brewing strains will facilitate faster glucose intake and boost fitness.

Overall, the growth kinetics results in YPD confirmed that the non-

Table 2
Growth kinetics parameters on the YPD (glucose 2 %) high-throughput micro-fermentations.

Strain	Max. μ	Max. biomass	AUC
Sc1	0.24 ± 0.02	114.72 ± 1.49	3150.56 ± 47.53
Sc2	0.32 ± 0.04	181.52 ± 0.47	6556.87 ± 11.07
Sc3	0.29 ± 0.01	182.20 ± 6.56	6175.14 ± 110.19
Sc4	0.29 ± 0.00	387.39 ± 65.35	9194.78 ± 1094.25
Sc5	0.20 ± 0.01	182.20 ± 6.56	6175.14 ± 110.19
Sc6	0.16 ± 0.02	207.37 ± 0.53	7490.57 ± 12.65
Sc7	0.20 ± 0.02	185.15 ± 0.48	6688.01 ± 11.30
Sc8	0.16 ± 0.01	185.85 ± 6.69	6298.64 ± 112.39
Cm	0.26 ± 0.01	336.11 ± 37.68	7984.90 ± 428.30
Hv	0.44 ± 0.01	677.60 ± 5.31	18702.96 ± 440.35
KI	0.27 ± 0.02	665.11 ± 5.21	19026.73 ± 653.31
Ps	0.29 ± 0.03	513.04 ± 13.55	12840.33 ± 147.95

Table 3
Growth kinetics parameters on the wort high-throughput micro-fermentations.

Strain	Max. μ	Max. biomass	AUC
Sc1	0.33 ± 0.02	27.42 ± 0.28	751.50 ± 12.68
Sc2	0.27 ± 0.00	112.88 ± 2.89	2258.51 ± 28.49
Sc3	0.23 ± 0.00	112.16 ± 1.22	2355.41 ± 20.79
Sc4	0.30 ± 0.00	109.89 ± 4.48	2195.24 ± 24.38
Sc5	0.22 ± 0.02	94.77 ± 2.56	1495.06 ± 10.68
Sc6	0.17 ± 0.02	81.60 ± 3.50	1575.16 ± 34.31
Sc7	0.22 ± 0.02	105.21 ± 5.99	1890.84 ± 44.20
Sc8	0.18 ± 0.01	95.03 ± 1.95	2284.90 ± 32.26
Cm	0.60 ± 0.10	73.15 ± 0.94	2216.76 ± 30.33
Hv	0.54 ± 0.05	79.99 ± 0.83	2193.55 ± 40.47
KI	0.29 ± 0.01	137.36 ± 4.95	3992.72 ± 73.50
Ps	0.27 ± 0.00	61.62 ± 0.39	1294.25 ± 5.20

Saccharomyces strains could generally grow as good as, or better, than the brewing *S. cerevisiae* strains.

When grown on wort, some of the non-*Saccharomyces* species displayed excellent maximum biomass and AUC values (Table 3). For example, Hv and Kl showed max. biomass values of 79.99 ± 0.83 and 137.36 ± 4.95, respectively. These values are similar and above the standard range we observed for the *S. cerevisiae* brewing strains (81.60 ± 3.50–112.88 ± 2.89). *Cm* showed a max. biomass value (73.15 ± 0.94) moderately lower than the *S. cerevisiae* brewing strains. Besides the lab strain (Sc1), *P. scutulata* Y-7663 (Ps) displayed the lowest max. biomass (61.62 ± 0.39) and AUC values in wort, so it was not deemed an ideal candidate for wort fermentation.

C. milleri is a species known for playing an important role in sourdough fermentations (Akinola and Osundahunsi, 2017), an environment rich in sugars similar to the ones encountered in wort. Likewise, *H. vineae* is known to be widely present in wine fermentations (Carrau et al., 2023), being well adapted to high concentrations of fermentation stressors such as ethanol. A good growth in beer-fermentative conditions for these two species was hence expected. *K. lactis* however, is not found in alcoholic fermentations but in dairy product fermentation. In fact, this yeast species is unable to grow in strictly anaerobic conditions and exclusively carries out respiration instead, mainly redirecting carbon into energy production and biomass anabolism at the expense of secondary metabolite production (Flores et al., 2000; Snoek and Yde Steensma, 2006). This physiological characteristic may explain the vigorous growth of Kl despite its lack of adaption for growing in wort.

In terms of fermentation performance, glucose was completely depleted in all cases, as expected, however, the consumption of maltose and maltotriose and the production of ethanol varied considerably between strains. Regarding YPD, the *S. cerevisiae* strains produced in between 5.50 ± 0.70 and 9.30 ± 0.14 g/L of ethanol, and the non-*Saccharomyces* species had a low fermentation performance, with the exception of Hv, which yielded 6.20 ± 1.13 g/L of ethanol (Table S1).

The initial sugar concentration in wort was 12.70 ± 0.0 g/L of glucose, 77.50 ± 0.76 g/L of maltose, and 33.50 ± 0.09 g/L of maltotriose (total sugars = 123.70 g/L). The strains that were able to metabolize these sugars yielded, as expected, the highest ethanol concentrations. These were the brewing strains Sc4, *S. cerevisiae* WLP001 (Sc5), Sc6 and *S. cerevisiae* WLP095 (Sc7) (Fig. 2A, Table S2). The non-*Saccharomyces* yeasts had negligible ethanol production in wort due to their inability or deficiency to ferment maltose and maltotriose, which is

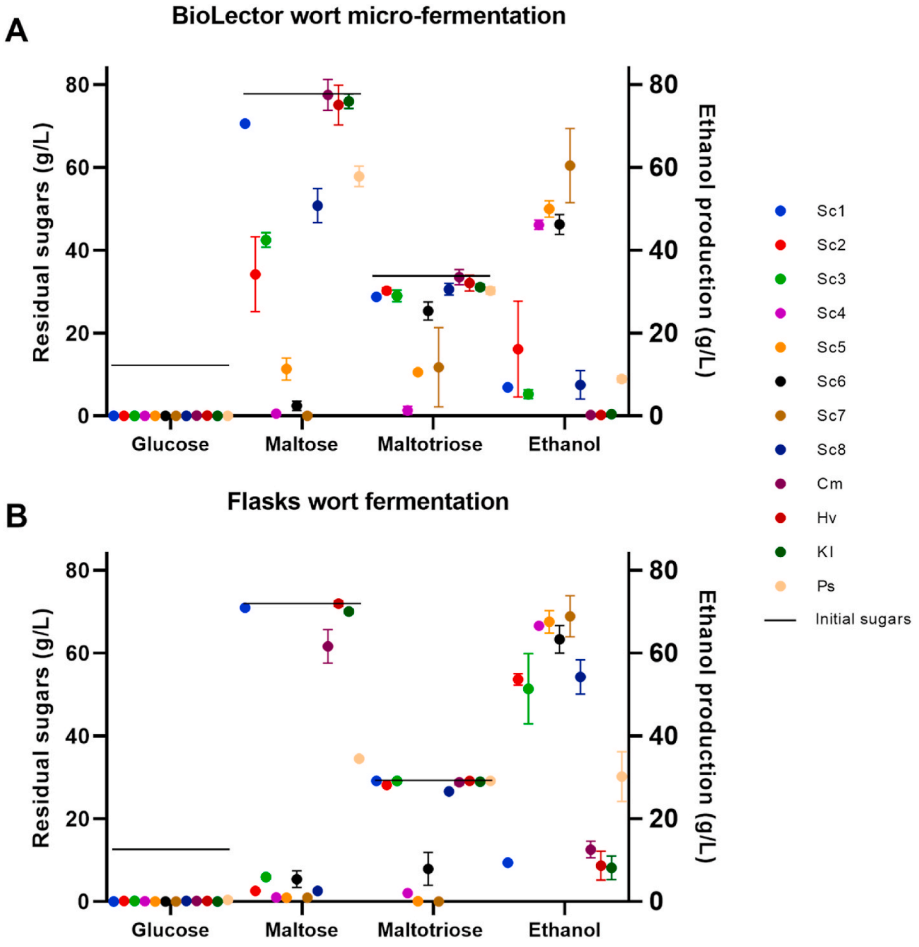

Fig. 2. Residual sugars and ethanol production in the wort micro-fermentations (A) and small-scale flasks fermentations (B). Compounds were measured through HPLC at the end of the fermentations; initial wort sugars are represented with a straight line.

common in non-*Saccharomyces* yeasts (Schinca et al., 2024). Ps was able to metabolize slowly some of the maltose and maltotriose, producing 8.91 ± 0.76 g/L of ethanol. Partial utilization of maltose and maltotriose is likely driven by genetic background, although the presence of growth-limiting factors such as nitrogen depletion, ethanol stress or low pH could also play a role. High fermentation performance is considered an important requirement for traditional brewing, however, over the last decade, the demand for low-alcoholic beverages has been growing (Bucher et al., 2018; Salanță et al., 2020); The use of low ethanol-producing non-conventional species has been previously proposed as an alternative for the production of low alcohol beer (Bellut et al., 2019; Bellut and Arendt, 2019; Francesco et al., 2018). The low-ethanol-yielding non-*Saccharomyces* yeasts of our study could be good candidates for the production of low alcoholic beer.

3.2. Small-scale fermentations and aroma compounds analysis

It is widely known that the size and the geometry of the fermentation vessel can have a profound impact on the fermentation performance mainly due to aeration and pressure (Hoogan, 1977; Shekhawat et al., 2017). So, to mimic real fermentation conditions more closely, we carried out wort small-scale fermentations in 500 mL flasks at 20 °C. Interestingly, ethanol production was significantly higher in the small-scale rather than in the micro-scale fermentations, especially in the case of *S. cerevisiae* Y-11875 (Sc2), *S. cerevisiae* Y-11875 (Sc3) and *S. cerevisiae* WLP775 (Sc8) (Fig. 2B–Table S3), likely due to the better consumption of maltose in the flasks experiment. This can be explained by the presence of oxygen in the micro-fermentations. Conversely, the small-scale fermentations were mainly anaerobic since the flasks were incubated statically and only de-caped for sampling. It is likely that the higher ethanol yield in the flasks was caused by a lower concentration of oxygen (de la Roza et al., 2003).

The production of nine key volatile compounds was measured through quantitative SPME GC-MS to help determine the ideal yeast combinations for co-fermentations (Table S4). Hv stood out as the strain with higher ethyl acetate (ethereal, fruity aroma) production, displaying an olfactory aroma value (OAV) value of 5.37 (Table 4; Fig. 3). The presence of this compound would be a novel flavour trait, although we do not know yet how it would ultimately fare in different beer styles. This strain also produced the highest amounts of 2-phenylethyl acetate (floral, rose aroma), with a concentration potentially reaching or slightly below the odour detection threshold (OAV of 0.92; Fig. 3, Table 4). This strain had a modest production of compounds such as acetaldehyde and 4-vinylguaiacol. This species is known to produce high levels of ethyl acetate and 2-phenylethyl acetate in wine (Carrau et al., 2023; del Fresno et al., 2022; Martin et al., 2018; Valera et al., 2021). However, reports for the production of this compound in beer by this species are less clear. For example, in a recent study by Postigo et al. this compound was not detected in a beer produced by this species in pure culture (Postigo et al., 2022b). In another study, by Peces-Pérez et al., the concentration of this compound in a beer sequentially fermented with *S. cerevisiae* and *H. vineae* was virtually the same as in beer fermented with *S. cerevisiae* alone (Peces-Pérez et al., 2022). In our study, we observe high production of this compound. This could be due to high strain-dependent production, as well as the influence of fermentation parameters, such as oxygen concentration, which negatively correlates with ester production (Dzialo et al., 2017). Different wort oxygen concentrations between our study, which used static fermentation, and the mentioned Postigo et al. work, which employed vigorous shaking, may account for the observed differences in 2-phenylethyl acetate production (this would need to be confirmed in a side-by-side experiment with both strains). Overall, the high production of ethyl acetate and 2-phenylethyl acetate by our Hv strain deems it as be a very good candidate for beer co-fermentations, where excessive production of those compounds should be limited.

The production of 2-phenyletylethanol (rose), isobutanol (sweet, solvent), and isoamyl alcohol (banana), was relatively similar in all the strains, and only in the case of isoamyl alcohol the production reached values beyond its sensory detection threshold (Meilgaard et al., 1982) for some of the *S. cerevisiae* strains (Table 4; Fig. 3). Acetaldehyde (apple, greasy) production was significantly higher in Sc5, Sc6 and Sc7. 3-Methyl-1-pentanol (apple, whisky) was produced in values below the sensory detection threshold in all cases, even in Kl which has been reported to be a good aroma producer (Gamero et al., 2016).

The production of citronellol (rose, citrous), which is a key terpene that provides a floral and citrous aroma at very low concentrations (Takoi et al., 2010), was produced above its detection threshold only in Sc7 (OAV of 1.31). Lastly, 4-vinylguaiacol (smoky) was produced significantly above its detection threshold in 7 out of 12 of the strains (Fig. 3, Table 4).

Overall, the aroma compound production of the commercial *Saccharomyces* strains (Sc5, Sc6, Sc7 and Sc8) was similar, and within the non-*Saccharomyces* yeasts, Hv stood up as a producer of the highest concentrations of the desirable esters ethyl acetate and 2-phenetyl acetate.

3.3. Co-fermentations in wort

After measuring the fitness, the fermentation performance and the aroma compound production for our yeast strains in wort medium, we

Table 4
OAV values in beer of the relevant aroma compounds quantified in the small-scale fermentations.

	Sensory threshold (mg/L) [b]	Sc1	Sc2	Sc3	Sc4	Sc5	Sc6	Sc7	Sc8	*Cm*	Hv	KI	Ps
Esters													
Ethyl acetate	30	Nd[a]	0.01	0.07	0.35	0.28	0.11	0.24	0.11	0.01	5.37	nd	nd
2-Phenylethyl acetate	3	nd	nd	nd	nd	nd	nd	nd	nd	0.06	0.92	0.02	nd
Higher alcohols													
2-Phenylethanol	125	0.07	0.11	0.10	0.09	0.10	0.09	0.10	0.09	0.08	0.07	0.09	0.09
3-Methyl-1-pentanol	0.83	0.00	0.04	0.04	0.04	0.01	0.02	0.01	0.05	0.02	0.00	0.13	0.03
Isoamyl alcohol	70	0.51	1.26	1.17	1.05	0.81	0.93	0.92	1.14	0.82	0.75	0.63	0.85
Isobutanol	100	0.29	0.73	0.73	0.75	0.66	0.65	0.81	0.58	0.49	0.57	0.54	0.57
Carbonyls													
Acetaldehyde	25	nd	0.24	0.49	2.26	5.77	5.46	5.27	0.81	0.42	1.94	nd	0.21
Phenolic compounds													
4-Vinylguaiacol	0.3	3.02	6.81	6.09	6.33	1.00	1.02	1.03	6.03	5.84	1.76	1.21	6.00
Terpenes													
Citronellol	0.008	0.02	0.40	0.35	0.72	0.78	0.36	1.31	0.34	0.20	0.00	0.31	0.17

[a] nd = non detected compounds.
[b] Sensory threshold values were obtained from Engan (1972); Habschied et al. (2023); Meilgaard (1975); Olaniran et al. (2017).

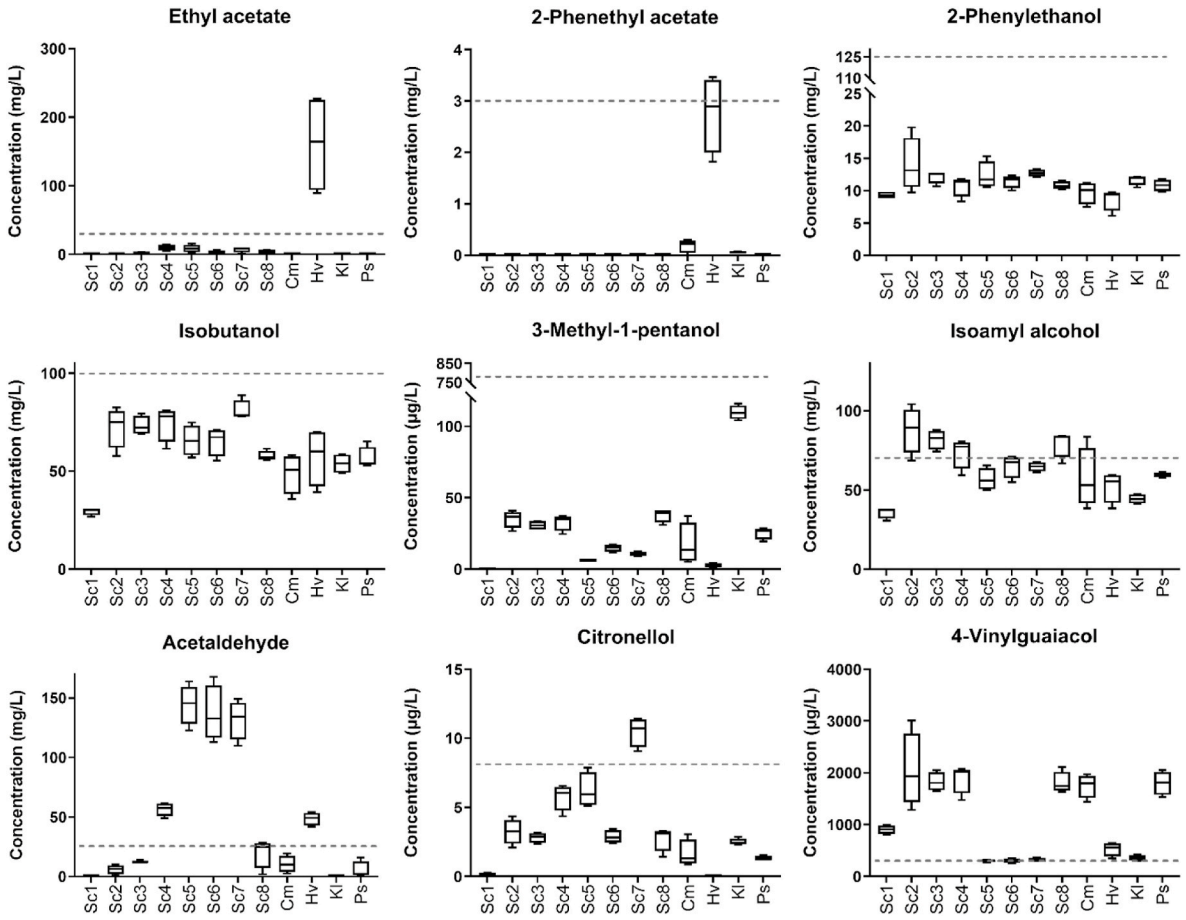

Fig. 3. Concentration of key aroma compounds at the end of the wort flasks small-scale fermentation. Errors represent the standard deviation of the mean. The dashed lines represent the sensory detection threshold of each compound in beer.

selected Sc7 and Hv for subsequent wort co-fermentations. Sc7 displayed good growth kinetics, good fermentation performance, and was the only yeast that produced citronellol above its detection threshold. Hv, despite its lack of maltose and maltotriose fermentation, displayed a high growth rate and biomass in wort and produced high amounts of ethyl acetate and 2-phenylethyl acetate. In the co-fermentations, the lack of wort sugar fermentation of Hv would be compensated by the high fermentation performance of Sc7.

Prior to the co-fermentation experiments, different ratios of competitor strains were tested to find the optimum inoculum condition that allows maintenance of both strains in the cultures over time (Fig. S4). Interestingly, we observed that Hv coped well with the presence of Sc7 in all the strain ratios, and out-competition did not take place in any case. This is remarkable since more out-competition by Sc7 was expected, as is commonly observed in *Saccharomyces* non-*Saccharomyces* co-fermentation, including Hv/Sc wine fermentations (Bordet et al., 2020; Medina et al., 2013; Zhang et al., 2018). The unexpected capacity of Hv to co-exist with Sc7 underscores its potential for wort co-fermentation.

We then carried out flask co-fermentations with the inoculation ratios of 50/50 Sc7/Hv and 10/90 Sc7/Hv. Monoculture controls were also included (*i.e.* 100% Sc7 and 100% Hv).

As observed in the ratio optimization experiment (Fig. S4), Hv and Sc7 happily co-existed throughout the co-fermentation. Sc7 displayed final CFU/mL values close to 1×10^8 when fermenting alone and in the 50/50 ratio. Hv also displayed a similar behaviour when alone or in the 50/50 ratio, with CFU/mL around 1×10^7. In the 10/90 Sc7/Hv ratio, Hv started dominating the fermentation, but after 250 h Sc7 started to prevail, yet Hv sustained high growth (Fig. 4). When nutrients became scarce at the end of the fermentation, Hv seemed to grow better in the presence of an equal amount of Sc7 cells compared to its monoculture. This could be attributed to the conversion of maltose and maltotriose to ethanol by Sc7, making the carbon on those sugars accessible for Hv. It is worth considering that *H. vineae*. can tolerate as much as 10 % v/v ethanol (Carrau et al., 2023). An increase in the ethanol levels as the one that we observed in our study should not be a major stressor for Hv. In any case, this potential symbiotic effect would need to be confirmed.

In terms of fermentation performance, all sugars were completely depleted whenever Sc7 was present (Fig. 4). The consumption of sugars was virtually identical in both Sc7 monoculture and 50/50 Sc7/Hv co-fermentations (Fig. 5). In the 10/90 Sc7/Hv co-fermentation there was a delay in the depletion of maltose, and the final concentration of ethanol was lower by a very small margin (Fig. 5; Table S5). In any case, the production of ethanol in the 10/90 Sc7/Hv co-fermentation was more than sufficient for what is expected in a normal beer product, with a value of 50.23 ± 0.47 g/L. In the Hv monoculture, only the glucose was depleted, and the ethanol production was very low.

In a relatively recent study by Larroque et al. sequential beer co-fermentation was carried out, first with *H. vineae* and then with *S. cerevisiae* (Larroque et al., 2021). The authors reported a reduction in fermentation performance in the co-culture compared to the *S. cerevisiae.* This reduction was not observed in our study, which used mixed, simultaneous co-fermentations. This highlights the possibility of employing a mixed starter Sc7/Hv co-culture for brewing, instead of sequential co-fermentation, which is a more complex industrial procedure.

The production of glycerol was significantly higher in the Sc7 monoculture and in the co-fermentations than in the Hv monoculture,

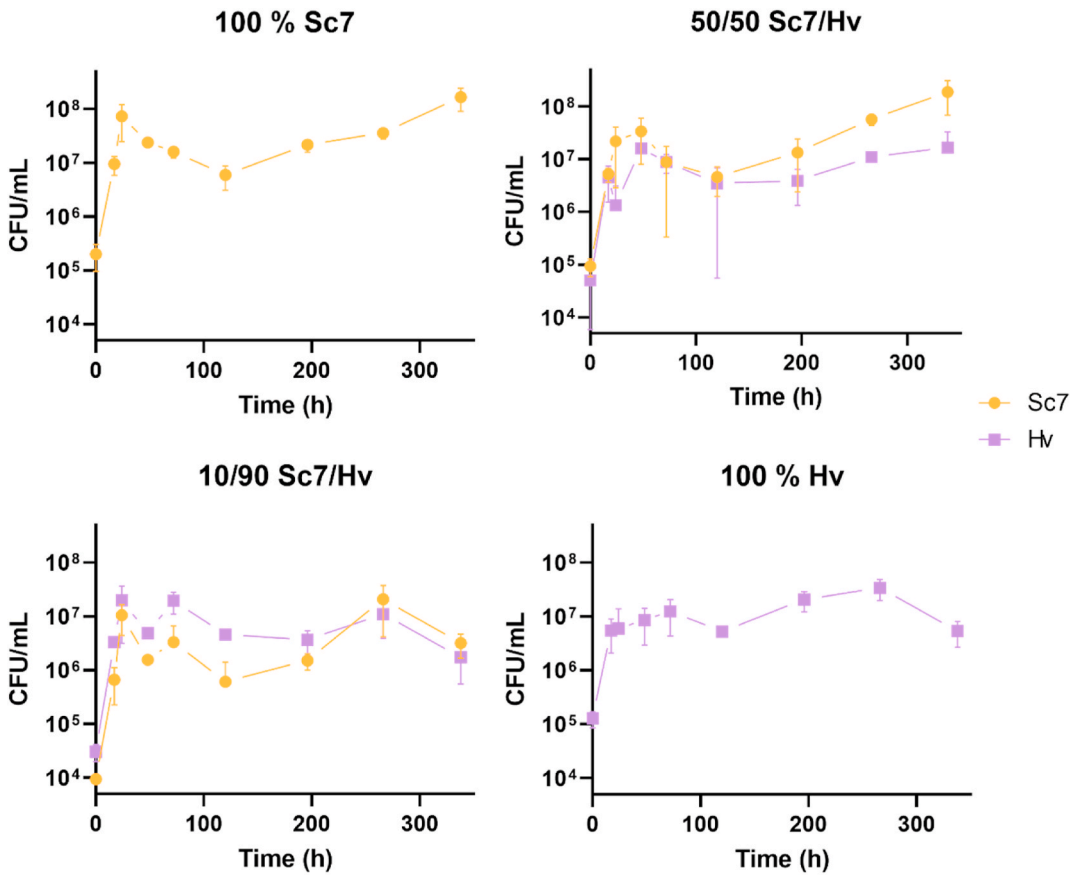

Fig. 4. Population dynamics in the co-fermentations. Sc7 and Hv CFU/mL were tracked during the course of the wort co-fermentations to compare the growth of the co-fermenting yeasts and their monocultures.

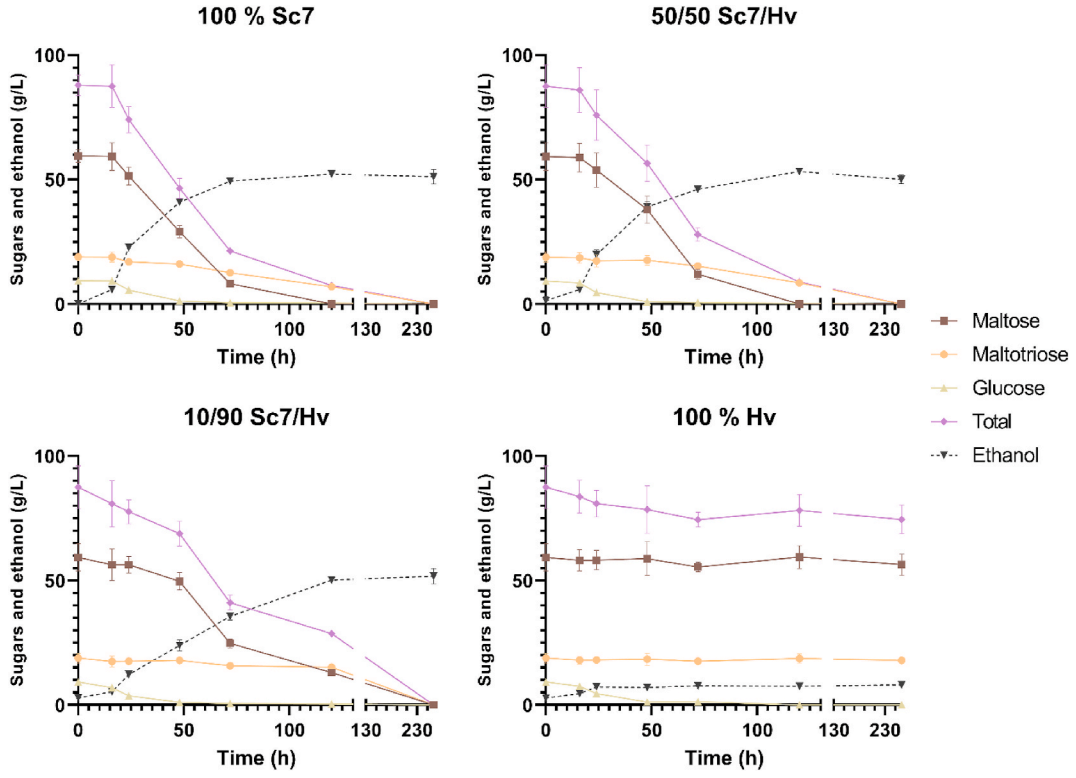

Fig. 5. Sugar consumption and ethanol production during the Sc7 and Hv co-fermentations. Error bars represent the standard deviation of the mean.

while the acetic acid production was positively correlated with the amount of Hv in the inoculum (Fig. 6, Table S5). Glycerol is considered to be a desirable compound that provides body to the product and buffers off-flavours. Typically, in a beer product, its concentration ranges from 1 to 3 g/L (Zhao et al., 2015). In our study, a good amount of glycerol was produced when *S. cerevisiae* was present in the fermentation, even in the ratio 10/90 Sc7/Hv co-fermentation. On the other hand, acetic acid can affect the aroma profile, and a concentration above its detection threshold (0.07–0.20 g/L) (Harrison, 1970; Meilgaard et al., 1982) can negatively impact the quality of commercial ales and lager beers. However, acetic acid concentrations as high as 1.60 g/L are found in other beer styles such as gueuze (Thompson-Witrick et al., 2017). Although we do not know the exact detection threshold of acetic acid in our wort, in our study its production was potentially above or on the upper limit of the detection threshold in the Hv monoculture (0.36 ± 0.02 g/L) and in the 10/90 Sc7/Hv co-fermentation (0.20 ± 0.01 g/L) respectively (Table S5). It is worth highlighting that the odour detection threshold of a volatile compound can vary significantly between different matrices due to interactions within different aroma compounds (Ferreira, 2012).

3.4. Aroma compounds production in the co-fermentations

To assess the aroma profile of the co-fermentations, we carried out semi-quantitative SPME GC-MS on the end-product of the fermentations. We were able to identify twenty-one different flavour compounds relevant for brewing. Five compounds were detected in the Hv and Sc7 first set of fermentations (Table S4, Fig. 3) as well as in the Sc7 and Hv monocultures controls of the co-fermentation experiment (Table 5, Fig. 7). These were 2-phenylethyl acetate, ethyl acetate, isoamyl alcohol, isobutanol and phenylethyl alcohol. Concentrations measured through quantitative GC in the small-scale fermentation experiments match the results of the semi-quantitative GC carried out in the mono-culture controls of the co-fermentation experiments for those compounds, supporting the semi-quantitative data.

To our knowledge, three previous studies have explored the use of Hv/Sc co-fermentation for beer production, through sequential co-inoculation (Larroque et al., 2021; Peces-Pérez et al., 2022; Postigo et al., 2022c). Overall, these have shown that the beers produced through Hv/Sc co-fermentation presented higher concentrations of compounds such as ethyl butyrate, acetoin, diethyl succinate, ethyl acetate, and 2-phenylethyl acetate, compared with pure Sc fermentation Sensory panel testing of the Hv/Sc beers produced in these studies revealed enhanced fruity and floral aroma, as well as bitterness and body.

Our results with simultaneous mixed Hv/Sc fermentation support the mentioned previous evidence in beer, as well as similar evidence for Hv in wine (Carrau et al., 2023; del Fresno et al., 2022; Gallo et al., 2024; Martin et al., 2018; Medina et al., 2013). In our study the presence of Hv increased the concentration of sixteen compounds associated with fruity and sweet descriptors, such as ethyl dodecanoate (waxy, sweet), 2-phenylethyl acetate (floral, rose), ethyl decanoate (waxy, sweet), ethyl 9-decenoate (fruity), ethyl acetate (fruity, ethereal), among others (Fig. 7; Table 5). 2-Phenylethyl acetate showed the highest increase when Hv was present, as observed in the small-scale monoculture experiments.

We classified the aroma compounds into three categories based on the production at the different ratios – *i.* compounds that showed the highest production in the Sc7 monoculture; *ii.* compounds that showed the highest production in the Hv monoculture; *iii.* compounds that showed the highest production in the co-fermentations.

In the first category, we find isoamyl acetate (fruity, banana), a very important aroma in beer (Verstrepen et al., 2003) with commercial *S. cerevisiae* brewing strains producing it in large quantities, likely due to domestication (Gallone et al., 2016). Our wild Hv strain did not produce isoamyl acetate, but a relatively good amount was produced by Sc7, even in the 10/90 Sc7/Hv co-cultures. Ethyl hexanoate (fruity, pineapple) and ethyl octanoate (waxy, fruity) also fall in the first category, both compounds producing pleasant aromas (Gonzalez Viejo et al., 2019; Hong et al., 2021). Isobutanol (ethereal, winey) was also prevalently produced in the Sc7 monoculture, although Hv was also able to produce it to a small degree. This compound has been reported to decrease the aroma quality in wine (de-la-Fuente-Blanco et al., 2016) and could potentially impact the aroma profile of beer as well, although a very low concentration might be desirable. The second category includes 2-phenylethyl propionate, 2-phenylethyl acetate and methyl vinyl ketone. 2-Phenylethyl propionate (floral, sweet) is the result of the esterification of phenethyl alcohol and propionic acid, however, its specific contribution to alcoholic beverages quality has not been extensively explored in the literature. 2-Phenylethyl acetate, which is the result of the esterification of acetic acid and phenylethyl alcohol (Hazelwood et al., 2008), provides a pleasant floral aroma and it is known to be highly produced in the *Hanseniaspora* sp. (Carrau et al., 2023; del Fresno et al., 2022; Zhang et al., 2020). We also detected this compound in the Sc7 monoculture, however, in the Hv monoculture and in the 10/90 Sc7/Hv co-culture its concentration was 2.5-fold and 2.3-fold higher, respectively (Table 5). The overproduction of this acetate ester has been recently attributed to the presence of several putative

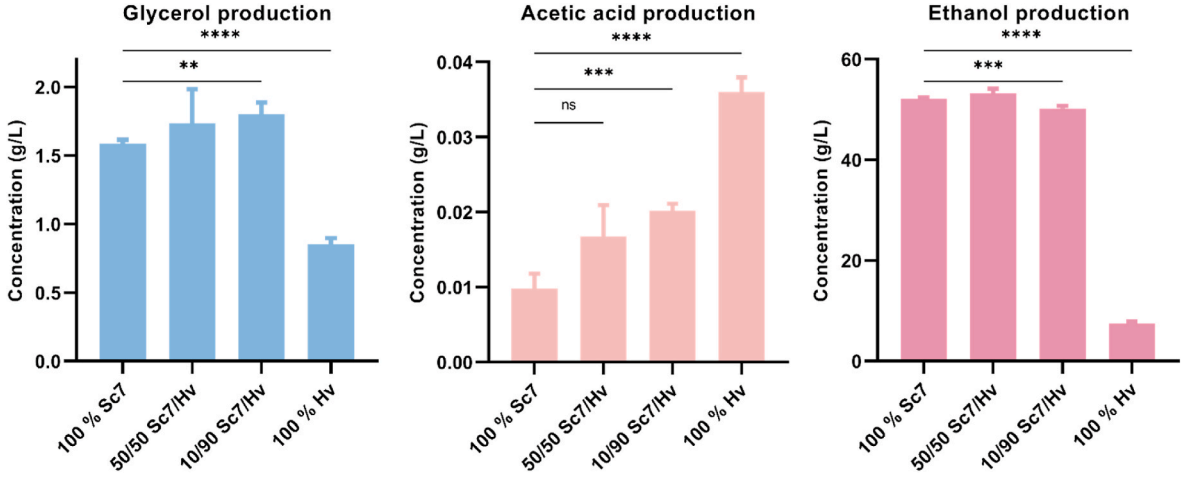

Fig. 6. Glycerol, acetic acid and ethanol production in Sc7 and Hv wort co-fermentations. The graph represents the concentration of the compounds measured at the end of the fermentations. Error bars represent the standard deviation of the mean. Statistical differences were calculated through Tukey one-way ANOVA; **, adjusted p-value <0.01; ***, adjusted p-value <0.001; ****, adjusted p-value <0.0001; ns, not significant.

Table 5
Relative area of the relevant aroma compounds detected in the co-fermentations. The relative areas were calculated through normalisation with an internal standard.

Compound		Relative peak area (x 10^3)			
	Descriptor	**100 % Sc7**	**50/50 Sc7/Hv**	**10/90 Sc7/Hv**	**100 % Hv**
Acids					
2-Ethyl-heptanoic acid	nd[a]	nd	0.09 ± 0.07[b]	nd	nd
Alcohols					
Isoamyl alcohol	Fusel, alcoholic	85.52 ± 45.40	97.51 ± 42.26	100.58 ± 15.19[b]	56.96 ± 10.36
Isobutanol	Ethereal, winey	8.73 ± 0.31[b]	8.72 ± 0.82	6.41 ± 3.06	2.52 ± 0.80
Phenylethyl alcohol	Floral, rose	123.87 ± 27.12	141.99 ± 28.57[b]	82.69 ± 12.55	31.65 ± 12.73
Aldehydes					
Methacrolein	Floral	nd	39.51 ± 0.51	49.55 ± 10.37[b]	nd
Carbonyls					
1,1-Diethoxy ethane	Ethereal, green	1.57 ± 0.16	2.80 ± 0.67	3.20 ± 2.59[b]	nd
Esters					
2-Phenylethyl acetate	Floral, rose	45.98 ± 3.37	386.40 ± 184.43	1051.87 ± 139.03	1195.14 ± 236.37[b]
2-Phenylethyl propionate	Floral, sweet	nd	1.36 ± 0.63	2.31 ± 0.67	4.88 ± 0.97[b]
Ethyl 9-decenoate	Fruity	33.82 ± 2.74	33.98 ± 12.42	35.95 ± 13.74[b]	0.63 ± 0.16
Ethyl 9-hexadecenoate	nd	2.15 ± 0.77	5.03 ± 2.06	13.24 ± 13.24[b]	nd
Ethyl acetate	Ethereal, fruity	4.60 ± 4.60	8.47 ± 2.31	16.08 ± 4.81[b]	4.91 ± 1.13
Ethyl decanoate	Waxy, sweet	31.11 ± 7.69	47.06 ± 16.07[b]	44.43 ± 19.31	0.53 ± 0.13
Ethyl dodecanoate	Waxy, sweet	0.91 ± 0.85	3.19 ± 1.63	4.68 ± 2.27[b]	nd
Ethyl hexanoate	Fruity, pineapple	7.61 ± 0.76[b]	5.17 ± 0.48	4.33 ± 1.06	nd
Ethyl nonanoate	Waxy	0.95 ± 0.38	2.43 ± 1.35[b]	2.30 ± 1.12	nd
Ethyl octanoate	Waxy	93.62 ± 5.68[b]	68.54 ± 4.78	79.48 ± 19.02	0.34 ± 0.17
Ethyl palmitate	Waxy	nd	0.75 ± 0.18	2.95 ± 0.56[b]	nd
Ethyl tetradecanoate	Waxy, sweet	nd	0.22 ± 0.11	0.85 ± 0.81[b]	nd
Isoamyl acetate	Fruity, banana	7.84 ± 1.21[b]	6.20 ± 1.50	5.47 ± 0.68	nd
Isoamyl octanoate	Sweet, fruity	nd	0.35 ± 0.28	0.66 ± 0.26[b]	nd
Ketones					
Methyl vinyl ketone	Sweet	nd	nd	nd	0.23 ± 0.14[b]

[a] nd = non detected compound.
[b] = fermentation with maximum production.

alcohol acetyltransferase genes in Hv, that would boost the conversion of phenylethyl alcohol to 2-phenylethyl acetate (Giorello et al., 2019). Lastly, methyl vinyl ketone (sweet), was only found in the Hv monoculture in relatively low amounts.

Remarkably, the majority of highly abundant volatile compounds fall under the third category (co-fermentations), with the 10/90 Sc7/Hv co-fermentation displaying the highest productions overall (Fig. 7). While this paper was under review, a study on co-fermentations in wine with *S. cerevisiae* and *H. vineae* also showed that a lower Sc/Hv ratio (*i.e.* 20/80) resulted in the highest production of pleasant acetates and esters (Gallo et al., 2024). In our case, the most relevant compounds in this category were ethyl 9-hexadecenoate, ethyl dodecanoate, ethyl nonanoate, ethyl palmitate, isoamyl alcohol, isoamyl octanoate, phenylethyl alcohol and ethyl acetate, the latter displaying a concentration in the 10/90 Sc7/Hv co-culture which was three times higher than the one in the Sc7 monoculture (Table 5). Furthermore, it is interesting that compounds such as ethyl palmitate, isoamyl octanoate and methacrolein, were only found in the co-fermentations. This is intriguing and such an effect has not been reported for this yeast. This could occur due to a synergistic effect between both yeasts where the biosynthetic pathways to produce these metabolites lack one or more substrates, which are produced by the other co-culture yeast. Production of ethyl palmitate has been reported to be higher in wine co-fermented with *Hanseniaspora uvarum*/Sc than with pure Sc, although production was also significant in the latter (Mestre et al., 2019). Therefore, the lack of production of that compound in our Sc monoculture control is likely to be caused by a different genetic background on the Sc of our study, or by differences in the substrate, which are known to dramatically affect aroma profile (Dzialo et al., 2017).

Previous beer co-fermentation studies with Sc and other NYC have highlighted the impact of the inoculation ratio on the final aroma (Canonico et al., 2019, 2016; Irma M. H. van Rijswijck et al., 2017; Wu et al., 2024). In our study with Hv, a more balanced profile was observed in the 50/50 ratio, with a good production of virtually all the measured compounds without having any compound dominating. On the other hand, in the 10/90 ratio, we observed a higher production of 2-phenylethyl acetate and ethyl acetate. It is quite likely that a potential beer created with this Sc7/Hv yeast ratio would have a dominating floral and fruity character due to the high concentration of those two compounds, which can be desirable or not, based on consumer preference and beer style. Considering this, beer producers could tailor the aroma profile of the final beverage by using different inoculum ratios of Sc7 and Hv, creating a strong fruity beverage if using large ratios of Hv, or a more balanced one with a 50/50 ratio.

4. Conclusions

We have confirmed that NCY have the potential to finely adjust the characteristics of beer by adding unique fruity and floral aromas. Our research has identified several NCY strains that display good growth performance as well as an enhanced aroma compound production in wort, Hv standing out as a high producer of ethyl acetate and 2-phenethyl acetate. The low ethanol production of these NCY opens two possibilities for their use in brewing: production of non-alcoholic beers, or co-fermenting with a high ethanol-yielding *Saccharomyces* sp.

We explored the latter approach and found that mixed co-fermentations employing 50/50 and 10/90 Sc7/Hv inoculation ratios significantly increased the overall production of desirable esters without compromising ethanol production compared to single-culture fermentations. Interestingly, certain esters like ethyl palmitate and isoamyl octanoate were only detected in the co-fermentation conditions suggesting a potential synergistic effect between these yeasts that requires further investigation. It's worth noting that higher Hv inoculation ratios may result in increased acetic acid production indicating the importance

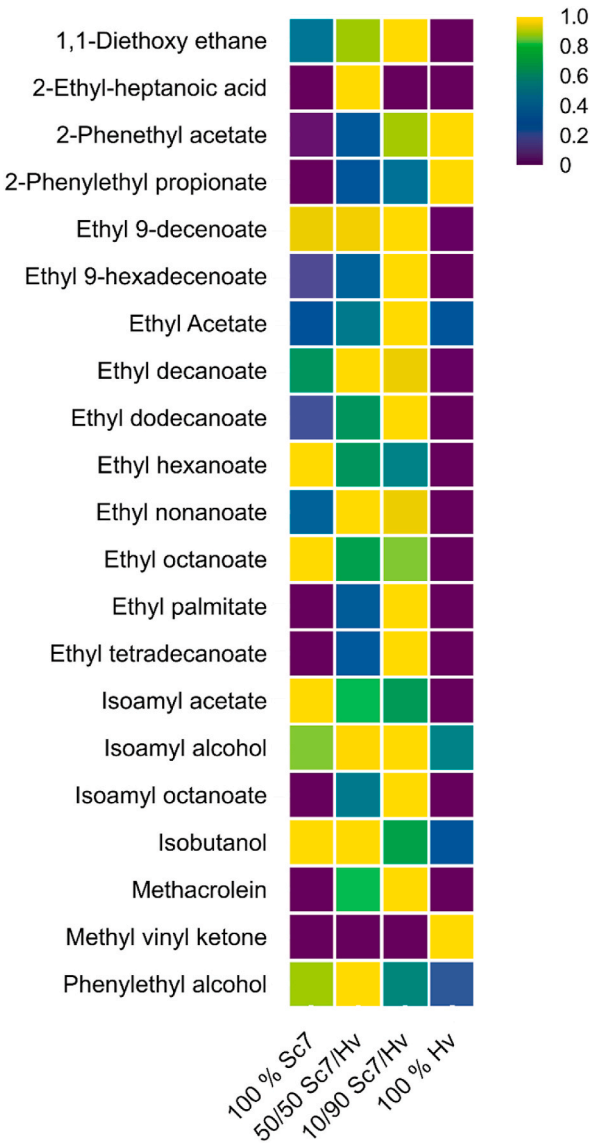

Fig. 7. Heatmap of the aroma compounds measured in the co-fermentation. The plot represents the normalised relative area of the detected aroma compounds in the wort co-fermentations using different ratios of Sc7 and Hv.

of using lower Hv inoculation ratios to balance acetic acid levels in specific beer styles.

In conclusion, our study provides a basis for customising beer aroma profiles by manipulating the ratios of *S. cerevisiae* and *H. vineae* yeast ratios. This method provides brewers with the opportunity to tailor beers according to flavour preferences aligning with consumer demand for more complex and unique beverages. The results highlight the thrilling potential of yeast biotechnology in broadening the flavour range and sensory attractiveness of beers.

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CRediT authorship contribution statement

Jose Aguiar-Cervera: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **Federico Visinoni:** Writing – review & editing, Investigation, Formal analysis. **Penghan Zhang:** Writing – review & editing, Methodology, Formal analysis. **Katherine Hollywood:** Writing – review & editing, Methodology, Supervision. **Urska Vrhovsek:** Writing – review & editing, Supervision, Funding acquisition. **Oliver Severn:** Writing – review & editing, Formal analysis, Conceptualization, Supervision. **Daniela Delneri:** Writing – review & editing, Formal analysis, Conceptualization, Supervision, Funding acquisition.

Declaration of competing interest

All authors declare that there is no conflict of interest related to this article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2024.104585.

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