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RESEARCH

Hanseniaspora uvarum and *Hanseniaspora opuntiae* differ in their growth and in the production of volatile organic compounds in wine

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Abstract

In recent years, *Hanseniaspora uvarum* has been partially replaced in Slovenian vineyards by the thermotolerant and invasive *Hanseniaspora opuntiae*. The fermentation capacity of both *Hanseniaspora* species was evaluated based on CO_2 release in synthetic and natural must. Additionally, co-inoculations of both strains with *S. cerevisiae* were carried out in Sauvignon Blanc must at different ratios (1:10 and 10:1). In all experiments, microbial populations were monitored, and the chemical and volatile profiles of the resulting wines were evaluated. During alcoholic fermentation (AF) with pure cultures, the fermentation kinetics of the two *Hanseniaspora* species were comparable, with CO_2 release in natural and synthetic must being two to five times lower compared to AF with the *S. cerevisiae*. In mixed AF with *Hanseniaspora* species and *S. cerevisiae*, there were no significant differences in fermentation capacity at the end of the fermentation compared to *S. cerevisiae* AF. However, the growth rate appears to be lower in *H. opuntiae* than in *H. uvarum* under oenological conditions. The results also showed that the formation of fermentation products differed between the two *Hanseniaspora* species in pure or mixed cultures, depending on the fermentation medium and inoculum ratio. *H. opuntiae* was a major producer of 2-phenylethyl acetate, and *H. uvarum* was a major producer of glycerol during alcoholic fermentation with natural must. The same tendency was observed for isoamyl acetate and ethyl acetate. We can conclude that *H. opuntiae* could become an important trigger for changes in wine composition if *H. uvarum* is further replaced on grape berries.

Key points

- *In mixed AF, there was no antagonism between Hanseniaspora and Saccharomyces strains*
- *The concentrations of fermentation products differed between Hanseniaspora species.*
- *H. opuntiae could influence changes in wine composition produced by non-inoculated AF.*

Keywords Alcoholic fermentation · Non-*Saccharomyces* yeasts · *Saccharomyces cerevisiae* · Mixed culture · Co-inoculation · Wine quality · Sauvignon Blanc

Introduction

Species of the genus *Hanseniaspora* are among the most widespread yeasts globally found in vineyards on ripe grapes and during the initial stages of wine fermentation, as demonstrated by decades of research (reviewed by Drumonde-Neves et al. 2021). In Slovenian vineyards, yeast populations have been systematically monitored since 1995, with *Hanseniaspora uvarum* consistently identified as the predominant *Hanseniaspora* species (Smole-Možina et al. 1998; Čadež et al. 2010). Notably, a thermotolerant species, *Hanseniaspora opuntiae*, originally isolated from natural habitats in subtropical regions of North America in 1995 (Čadež et al. 2003), was subsequently detected in 2003 in our vineyards in the sub-Mediterranean region (Čadež et al. 2010). Similar observations have been reported in other wine-growing regions, including France (Saubin et al. 2020), Australia (Onetto et al. 2025b), South Africa (Albertin et al. 2016), and South America (Eder et al. 2025). This partial displacement of *H. uvarum*, which shows high resistance to

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low temperatures (Albertin et al. 2016; Onetto et al. 2025b), by the thermotolerant *H. opuntiae* (Fig. S1) is most likely influenced by rising temperatures associated with climate change and could affect the aromatic profile and sensory characteristics of the wines (Fresno et al. 2023; Filippousi et al. 2024).

Most *Hanseniaspora* species are considered weak fermenters and are usually displaced by more competitive and ethanol-tolerant *Saccharomyces* yeasts during the middle phase of spontaneous fermentation and the early phase of inoculated fermentation (Longo et al. 1991; Wyk et al. 2024; Potisek and Čuš 2025). As species such as *H. uvarum* and *H. opuntiae* are typically unable to complete alcoholic fermentation by fully consuming the sugar present in grape must (Rossouw and Bauer 2016), the simultaneous or sequential co-inoculation of these strains with high-fermenting *Saccharomyces* yeasts is a promising strategy to increase wine aroma complexity and provide sensory benefits (Tristezza et al. 2016; Hu et al. 2018; Wang et al. 2024; Wyk et al. 2024). However, studies have shown that the influence of *H. uvarum* and *H. opuntiae* strains on the aromatic profile of wine may differ due to their distinct metabolic activities and their ability to produce volatile compounds with *S. cerevisiae* in mixed fermentations (Rossouw and Bauer 2016).

Numerous studies have shown that the use of *H. uvarum* in the inoculum can improve the sensory perception of wine, particularly its fruitiness, by increasing the formation of acetate and ethyl esters and producing moderate or even lower levels of acetic acid in mixed alcoholic fermentation (AF) with *S. cerevisiae* (Moreira et al. 2008; Tristezza et al. 2016; Zhang et al. 2023; Wang et al. 2024). However, excessive metabolic activity of *H. uvarum* during AF can lead to increased formation of higher alcohols, acetaldehyde, ethyl acetate, and acetic acid (Moreira et al. 2008; Andorrà et al. 2010). The results of individual studies may be contradictory regarding the production of specific metabolites during AF with *H. uvarum*, suggesting intraspecific strain diversity (reviewed by Jolly et al. (2014); Albertin et al. 2016) and the influence of complex metabolic interactions with *S. cerevisiae* (Sun et al. 2025).

Recent studies have demonstrated the positive influence of certain *H. opuntiae* strains, when co-inoculated with *S. cerevisiae*, on the volatile profiles and sensory properties of wines. These strains significantly increase concentrations of acetate esters, mainly 2-phenylethyl acetate, ethyl esters (ethyl decanoate and ethyl dodecanoate), higher alcohols (2-phenylethanol and 3-methyl-1-butanol), and citronellol (Luan et al. 2018; Fresno et al. 2023; Filippousi et al. 2024). Several studies have also confirmed that strains of both *H. uvarum* and *H. opuntiae* can increase glycerol content in pure cultures compared to commercial wine yeast strains of *S. cerevisiae*, contributing to the fullness and sweetness of the wine, and can reduce the final ethanol content in wine in pure culture or in co-inoculation with *S. cerevisiae* strains (Andorrà et al. 2010; Rossouw and Bauer 2016; Tristezza et al. 2016; Mančić et al. 2022).

This study aimed to compare a wild strain of *H. opuntiae* from Hawaii with an indigenous wine strain of *H. uvarum* in terms of their wine fermentative potential and production of volatile organic compounds, as well as to evaluate their use in different proportions in mixed fermentations with *S. cerevisiae* by simultaneous inoculation in Sauvignon Blanc must, and to assess their influence on the aromatic profile of the resulting wine.

Material and methods

Yeast strains

The yeast cultures used for the pure fermentation experiment were obtained from the Slovenian Collection of Industrial Microorganisms (IC ZIM, MRIC UL, Slovenia). *Hanseniaspora uvarum* strain ZIM 0668 was isolated from ripe grapes in Goriška Brda, Slovenia (Čadež et al. 2010), while *Hanseniaspora opuntiae* strain ZIM 2137 (CBS 8733) was isolated from cactus rot affecting *Opuntia ficus-indica* in Hawaii, USA (Čadež et al. 2003). A commercial strain of *Saccharomyces cerevisiae* EC-1118 (Lallemand® SAS, Blagnac, France) was used as the control.

Pure yeast culture alcoholic fermentations

Alcoholic fermentations (AF) were carried out under microaerobic conditions in 500-mL Durham bottles filled with 400 mL of either synthetic must MS300, as described by Bely et al. (1990) and modified by Avbelj et al. (2015), and Sauvignon Blanc must from Ormož, Slovenia, vintage 2022, with a composition of 20.3 Brix (approx. 190 g/L sugar), total acids 5.3 g/L, NH_3/FAN 45/120 mg/L, and pH 3.4. Sauvignon Blanc must was centrifuged at 4000 rpm for 5 min to minimise microbial load. Fermenters were sealed with rubber stoppers equipped with CO_2 outlets. The yeasts for inoculation were grown overnight in YPD broth (yeast extract 10.0 g/L (Biolife, Italy), peptone 20.0 g/L (Biolife, Italy), glucose 20.0 g/L (Merck, Spain)), harvested by centrifugation, and washed in isotonic phosphate saline buffer. The concentration of yeast cells was determined by automatic counting of viable cells using ImageJ software, as described previously (Avbelj et al. 2015). The yeast suspensions were inoculated to reach an initial concentration of 1×10^6 cells/mL. The commercial starter culture was prepared according to the manufacturer's instructions and inoculated at 0.2 g/L of must. AFs were conducted in duplicate at 22 °C, and their progression was monitored daily by measuring weight loss, expressed as the amount of CO_2 released.

Mixed yeast culture alcoholic fermentations

For this part of the experiment, the same must of Sauvignon Blanc was used as described above. Before the fermentation experiments, it was sulphurised with a 5–6% aqueous solution of sulphurous anhydride (Agrolit, Slovenia) to achieve a free SO_2 content of 30 mg/L to prevent oxidation of the must and pasteurised (60 °C, 5 min) to minimise microbial contamination. The inoculum for each yeast strain was prepared in 200 mL of sterile YM liquid medium (yeast extract 3.0 g/L (Biolife, Italy), malt extract 3.0 g/L (Biolife, Italy), peptone 5.0 g/L (Biolife, Italy), glucose 50.0 g/L (Merck, Spain)) in glass Erlenmeyer flasks by incubating overnight at 23 °C with shaking at 200 rpm (Tehtnica, Železniki, Slovenia). After incubation, the concentration of viable yeast was analysed using the CyFlow Cube 6 cytometer (Sysmex Partec GmbH, Goerlitz, Germany) with fluorescein diacetate (FDA) and propidium iodide (PI) staining according to the protocol by Potisek and Čuš (2025), as described in more detail below. Each yeast inoculum was centrifuged in sterile 50 mL Falcon Clarity Conical Centrifuge Tubes (8,500 RCF, 20 °C, 5 min, Heraeus Biofuge Stratos). The resulting pellet was resuspended by vortexing (3000 rpm, IKA, Staufen, Germany) in 50 mL of a mixture of distilled water and must (1:1). The prepared yeast suspensions were then inoculated into the must to achieve the initial concentrations as specified below.

Two independent experiments, A and B, each with three AFs, were carried out in triplicate using 380-mL must in sterile 500-mL fermentation vessels sealed with rubber stoppers and fitted with two-piece airlocks. In the first experiment (A), a pure culture AF of strain *S. cerevisiae* served as a control, followed by two mixed AFs: in the first strains of *S. cerevisiae* and *H. uvarum*, they were inoculated together; in the second strains of *S. cerevisiae* and *H. opuntiae*, they were inoculated together, as this type of inoculation has previously been shown to be superior to sequential inoculation (Tristezza et al. 2016). In all three fermentation trials of experiment A, *S. cerevisiae* was inoculated to reach an initial concentration of 1×10^7 cells/mL, and both *Hansenispora* species to reach an initial concentration of 1×10^6 cells/mL (10:1). In experiment B, the same three AFs trials were performed as in experiment A (AF with pure *S. cerevisiae* as control, AF with *S. cerevisiae* and *H. uvarum*, as well as AF with *S. cerevisiae* and *H. opuntiae*), except that the initial yeast concentrations in the must were reversed: 1×10^6 cells/mL for *S. cerevisiae* in all three fermentation treatments, and 1×10^7 cells/mL for *H. uvarum* and *H. opuntiae* (1:10) in both AFs with *Hanseniaspora* species.

Monitoring AFs

AFs were performed at 22 °C for 10 days and 13 days with once-daily shaking for experiments A and B, respectively. The progression of AF was monitored at 1-day intervals by measuring weight loss, expressed as the amount of CO_2 released. Yeast concentration and viability were assessed by plate counting and flow cytometry at 1 and 4 days after yeast inoculation. Prior to enumeration analysis, samples were filtered through a sterile cell strainer (40 µm, Greiner, Germany) and serially diluted in 1 × phosphate-buffered saline (PBS; pH 7.4).

For flow cytometry (FCM), samples from the appropriate dilution (yeast concentration of approximately 10^5–10^6 cells/mL) were stained with FDA and PI according to the protocol (Potisek and Čuš 2025). After staining, samples were vortexed for 30 s at 2500 rpm (IKA MS 3 basic, Germany) and analysed using the CyFlow Cube 6 cytometer (Sysmex Partec GmbH, Germany) equipped with a blue laser with an excitation wavelength of 488 nm and five detectors: forward light scatter (FSC), side light scatter (SSC), and three fluorescence detectors (FL1-536/40 nm, FL2-590/50 nm, and FL3-RG630 nm). The function and calibration of the device were checked with 3-µm Calibration Beads (Sysmex Partec, Germany) and Count Check Beads (Sysmex Partec, Germany). FCM analysis of yeast counts was performed with a flow rate of 3.0 µL/s and a final analysed sample volume of 100 µL. The following four optical parameters were used: FSC, SSC, green fluorescence signal (FL1), and red fluorescence signal (FL3).

In parallel, the plate counting method was performed according to the method OIV-MA-AS4-01: R2010 (OIV 2025). Three inoculations of two consecutive serial dilutions (30–300 expected colonies grown on one agar plate) of each sample were spread on Wallerstein Laboratory Nutrient agar (Merck, Germany). After 3 days of cultivation at 27 °C, the total number of yeasts–colony forming units (CFU) and the number of colonies of different yeast genera were determined based on colony colour and morphology, and microscopic observation of yeast cells from individual colonies (Nikon Eclipse 80i, Japan). Following the completion of AFs, the wine was sulphurised with 0.1 mL/100 mL SO_2 in the form of a 5–6% aqueous solution of sulphurous anhydride (Agrolit, Slovenia) and decanted into 250-mL glass bottles.

Wine chemical analysis

Standard parameters

The pH value of the wine was measured using a MeterLab PHM 210 (Radiometer Analytical, Lyon, France). Total acids (TA) were determined by sodium hydroxide titration with bromothymol as the indicator for colorimetric modification and expressed as g/L tartaric acid. In accordance with OIV methods (OIV 2025), various parameters were analysed using an enzyme analyser (BS-200, Mindray, Nanshan, Shenzhen, China). Glucose and fructose, total dry matter, and volatile acids (expressed as acetic acid) concentrations were determined using the OIV-MA-AS311-10: R2018, OIV-MA-AS2-03B: R2012, and OIV-MA-AS313-27: 2019 methods. Glycerol content was also analysed directly by enzyme analyser after filtration of wine samples (0.45 µm, Chromafil, RC-45/25 filter). The ethanol content was determined with an Alcolyzer Wine M alcohol meter (Anton Paar, Graz, Austria).

Analysis of esters, 1-hexenol, and (Z)–3-hexenol

The analysis of esters and C6 alcohols in samples from pure yeast culture alcoholic fermentations was carried out according to the method previously described by Bavčar et al. (2011). The procedure involved liquid–liquid extraction with dichloromethane and concentration of the organic phase to a final volume of 1 mL. The samples were then analysed using a GC (Hewlett Packard 6890, Waldbronn, Germany) coupled to a mass spectrometer (Hewlett Packard 5973, Palo Alto, CA, USA). Separation was carried out on a CP-Wax 57CB capillary column (50 m × 0.25 mm, film thickness 0.20 µm) (Varian, Lake Forest, CA, USA) coupled to a deactivated fused silica guard column (2 m × 0.25 mm, Agilent Technologies, Palo Alto, CA, USA). The compounds were identified and quantified using the methods previously described (Bavčar 2011; Bavčar et al. 2011; Bavčar and Česnik 2011).

Esters and C6 alcohols in samples from mixed yeast culture alcoholic fermentations (experiments A and B) were analysed as described in Antalick et al. (2015), Šuklje et al. (2014), and Šuklje et al. (2016). A mixture of isotopically labelled esters—ethyl d11-hexanoate at a concentration of 19.53 mg/L, ethyl d5-cinnamate and ethyl d3-butyrate (all from CDN Isotopes, Ontario, Canada) at 20 mg/L for quantification of esters, and octan-2-ol at a concentration of 68.22 mg/L for C6 compound quantification (Sigma-Aldrich, Schnelldorf, Germany)—was prepared in pure ethanol. In a 20-mL headspace vial, 3 g NaCl was added, followed by 5 mL of wine, 5 mL of MilliQ water, and 10 µL of the internal standards mixture. The vial was immediately capped, vortexed, and placed on a Peltier tray cooler set at + 4 °C. A headspace solid-phase microextraction (HS-SPME) method with a PDMS-CAR-DVB (grey) fibre (Supelco, Bellefonte, USA) was used for sample analysis by gas chromatograph GC2030 (Shimadzu, Kyoto, Japan) coupled to a triple quadrupole mass spectrometer (MS-TQ) TQ8050 NX (Shimadzu, Kyoto, Japan). The MS-TQ was operated to perform simultaneous electron ionisation by SIM (selected ion monitoring) and scan. Compounds were separated on an HP-INNOWAX column (60 m × 0.25 mm × film thickness 0.25 µm) (Agilent Technologies, Santa Clara, CA, USA). Chromatographic and mass operating conditions were kept identical to those outlined in Antalick et al. (2015) and Šuklje et al. (2016). Briefly, the injector was set at 250 °C in splitless mode. The oven temperature programme commenced at 40 °C and was held for 5 min, and then ramped at 3 °C/min to 200 °C, followed by a final increase to 240 °C at a rate of 10 °C/min with a hold time of 10 min, and a total run time of 72.33 min. The flow rate of ultra-high purity helium gas (6.0) was constant at 3 mL/min. The MS source, quadrupole, and transfer line temperatures were set to 230, 150, and 260 °C, respectively. The ions used for quantification of esters and C6 compounds are reported elsewhere (Antalick et al. 2015; Šuklje et al. 2014, 2016).

Analysis of higher alcohols and ethyl acetate

The higher alcohols and ethyl acetate were analysed using gas chromatography (GC) coupled with a flame ionisation detector (FID) (Hewlett Packard 6890, Waldbronn, Germany), without prior extraction. Separation was achieved on a CP-Wax capillary column (57CB, 50 m × 0.25 mm, film thickness 0.20 µm) (Bavčar et al. 2011). For each analysis, 5 mL of wine sample was spiked with 50 µL of the internal standard 4-methyl-2-pentanol (Sigma-Aldrich, Schnelldorf, Germany; 2.78 g dissolved in 100 mL of absolute ethanol) and shaken, and 1 µL was directly injected into the GC-FID system. The method was validated as described previously (Bavčar 2011), and quantification was performed according to established protocols (Bavčar 2011; Bavčar et al. 2011; Bavčar and Česnik 2011).

Statistical analysis

The Shapiro–Wilk normality test was used to assess the normality of the data, while the homogeneity of variances was tested using Levene's test. Significance was determined using parametric analysis of variance (one-way ANOVA) for variables with a normal distribution, and means were separated using the Stats–Fisher's LSD test. For variables with non-homogeneous variances and non-normal distribution, the non-parametric Kruskal–Wallis test was used, and means were separated using the Bonferroni test. When only two groups of samples were compared, significance was tested using the parametric Student's *t*-test. Different letters indicate significant differences at $p \leq 0.05$. Asterisks indicate the significance level: $^{*}p \leq 0.05$, $^{**}p \leq 0.01$, and $^{***}p \leq 0.001$. Unit variation and mean centring were used to create the PCA. Statistical analyses were performed with XLSTAT software version 2023.3.0 (Lumivero 2025).

Results

Fermentation kinetics of pure culture AFs in synthetic and natural musts

The first fermentation experiment with pure cultures of *H. uvarum* and *H. opuntiae* was carried out in both synthetic and natural grape must. The fermentation kinetics and weight loss due to CO_2 release in synthetic must were comparable, regardless of the species used (Fig. S2, left). However, in natural must, *H. uvarum* exhibited a slightly higher rate of CO_2 release than *H. opuntiae*, indicating somewhat greater fermentation activity under these conditions (Fig. S2, right). At the end of alcoholic fermentation, both *Hanseniaspora* species produced similar amounts of CO_2 in synthetic must (1.03 ± 0.01 g/L for *H. uvarum* and 1.23 ± 0.11 g/L for *H. opuntiae*) and in natural must (4.76 ± 0.02 g/L for *H. uvarum* and 4.41 ± 0.14 g/L for *H. opuntiae*). However, in synthetic must, the control fermentation with *S. cerevisiae* produced about five times more CO_2 (5.42 g/L), while in natural must, the amount was about twice as high (10.73 g/L).

Effect of two *Hanseniaspora* species on VOC production during pure culture AF of synthetic and natural must

Table S1 presents the effects of two *Hanseniaspora* species of diverse origin and *S. cerevisiae* as a control on the production of VOCs and other compounds during AF of synthetic and natural must. Despite the low CO_2 release during alcoholic fermentation of synthetic must, there is a clear tendency for higher production of ethyl esters of straight-chain fatty acids (EEFAs) and all acetates by *H. opuntiae* compared to *H. uvarum* (Table S1, left). The opposite is observed for all higher alcohols and ethyl acetate, where *H. uvarum* tends to produce higher amounts. The largest difference in concentration was observed for 2-phenylethyl acetate, which was produced in high concentration by *H. opuntiae*.

In the natural must, these trends were not fully reproducible for the EEFAs, although a tendency towards higher production of ethyl butyrate, ethyl dodecanoate, and ethyl hexanoate was observed in AF with *H. uvarum*, and of ethyl decanoate and ethyl octanoate in AF with *H. opuntiae* (Table S1, right). The same tendency as in synthetic must towards higher production of all acetates by *H. opuntiae* was also observed here, with 2-phenylethyl acetate clearly standing out. The same tendency for higher ethyl acetate production by *H. uvarum* observed in synthetic must also applies to natural must. The differences between the two *Hanseniaspora* species in the production of higher alcohols in natural must were not as apparent as in synthetic must. As the sugar consumption of *S. cerevisiae* was considerably higher in both musts compared to the two *Hanseniaspora* species, comparisons of VOCs production are irrelevant.

Fermentation kinetics and yeast population dynamics in mixed culture AF

In the first experiment (A), strains of *S. cerevisiae* and *H. uvarum*, or *S. cerevisiae* and *H. opuntiae*, were co-inoculated at a ratio of 10:1 in triplicate and a pure culture AF of *S. cerevisiae* was used as a control in the AF of Sauvignon Blanc must. Some minor but significant differences were observed in CO_2 release during AF (Fig. S3, left), where AF co-inoculated with *S. cerevisiae* and *H. opuntiae* showed significantly higher CO_2 release from the second to the fourth day. However, at the end of the AFs, 8.50 ± 0.33 g/100 mL CO_2 was released in the pure Sc AF, 8.59 ± 0.16 g/100 mL in the Sc + Hu AF, and 9.08 ± 0.25 g/100 mL in the Sc + Ho AF. The sum of residual glucose and fructose in the wine was 1.9 ± 0.1 g/L (Sc), 0.9 ± 0.1 g/L (Sc + Hu), and 1.0 ± 0.3 g/L (Sc + Ho), with a significantly higher value in the AF with pure Sc (Table 1).

Yeast concentration determined by plating and yeast viability determined by flow cytometry during AF were measured on the first and fourth days after inoculation (Table 1). Yeast viability on the first day was significantly lower in the AF with Sc + Hu (76.55%) compared to the other two trials, 88.40% (Sc + Ho) and 95.66% (Sc). From the ratio between *S. cerevisiae* and *Hanseniaspora* colony types determined on WL plates, we observed that *S. cerevisiae* dominated the yeast population in both co-inoculated AFs as early as 1 day after inoculation, with 83.61 ± 3.38% in the AF co-inoculated with *H. uvarum* and a significantly higher proportion of 92.65 ± 4.26% in the co-inoculation with *H. opuntiae*. On the fourth day after inoculation (exponential phase), the proportion of *S. cerevisiae* in the viable yeast population in both co-inoculated AFs was already 100%. There were

Table 1 Effects of mixed cultures of *H. uvarum* (Hu) or *H. opuntiae* (Ho) with *S. cerevisiae* (Sc), and pure *S. cerevisiae*, on residual sugar and microbiological parameters (total yeast count (*N*), yeast viability, and percentage of S. *cerevisiae*) on days 1 and 4 of AF of Sauvignon Blanc must, with higher initial populations of *S. cerevisiae* (experiment A) or either *H. uvarum* or *H. opuntiae* (experiment B) ($n = 3$)

Time of AF		End of AF	Day 1			Day 4		
Experiment	Type of inoculum	Residual sugar (g/L)	*N* ($\log_{10}$ CFU/mL)	Yeast viability (%)	Percentage of *S. cerevisiae*	*N* ($\log_{10}$ CFU/mL)	Yeast viability (%)	Percentage of *S. cerevisiae*
A	Sc (pure)	1.9 ± 0.1a[a]	7.63 ± 0.05	95.66 ± 2.12a	100 ± 0.00	7.71 ± 0.04ab	80.62 ± 2.54	100 ± 0.00
	Sc + Hu	0.9 ± 0.1b	7.64 ± 0.08	76.55 ± 3.77b	83.61 ± 3.38b[b]	7.61 ± 0.02b	77.89 ± 0.83	100 ± 0.00
	Sc + Ho	1.0 ± 0.3b	7.64 ± 0.06	88.40 ± 4.98a	92.65 ± 4.26a	7.76 ± 0.10a	79.48 ± 0.23	100 ± 0.00
B	Sc (pure)	1.3 ± 0.4a	7.00 ± 0.01	98.24 ± 0.26a	100 ± 0.00	7.64 ± 0.10a	68.98 ± 6.20	100 ± 0.00
	Sc + Hu	0.5 ± 0.1b	7.07 ± 0.14	96.53 ± 0.63b	25.1 ± 2.4b	7.51 ± 0.10ab	59.25 ± 5.18	88.42 ± 5.11
	Sc + Ho	1.4 ± 0.4a	7.12 ± 0.05	98.53 ± 0.46a	42.5 ± 7.6a	7.36 ± 0.10b	61.76 ± 4.01	96.19 ± 1.35

[a] A one-way ANOVA was used to compare the data. Means followed by different letters and cell colours in a column, separately for each experiment, are significantly different at $p \leq 0.05$ (Fisher's LSD). All stated uncertainties are standard deviations based on three replicates per treatment

[b] The Student *t*-test was used to compare the percentage of *S. cerevisiae* in both mixed AFs

no significant differences in yeast viability at this sampling point, which ranged from 77.89% to 80.62%. The total yeast concentration in the AF co-inoculated with *H. opuntiae* was significantly higher (7.76 ± 0.10 $\log_{10}$ CFU/mL) than in the AF co-inoculated with *H. uvarum* (7.61 ± 0.02 $\log_{10}$ CFU/mL) (Table 1).

In experiment B, with a higher initial concentration of *H. uvarum* or *H. opuntiae* yeasts compared to *S. cerevisiae* yeasts (ratio 10:1), the amount of CO_2 released on the second and third days in the AFs with both *Hanseniaspora* yeasts was significantly lower than in the AF with pure *S. cerevisiae* (Fig. S3, right). On the seventh day, the CO_2 release rates had equalised in all three AFs, and no further significant differences were observed. At the end of experiment B, the total amount of carbon dioxide released was comparable between the AFs: 10.31 ± 0.28 g/100 mL for Sc AF, 10.19 ± 0.31 g/100 mL for Sc + Hu AF, and 10.26 ± 0.04 g/100 mL for Sc + Ho AF. The sum of residual glucose and fructose in the wine was 1.3 ± 0.4 g/L (Sc), 0.5 ± 0.1 g/L (Sc + Hu), and 1.4 ± 0.4 g/L (Sc + Ho) (Table 1), indicating a significantly higher sugar consumption rate in the Sc + Hu AF.

On the first day after inoculation in experiment B, cell viability in co-inoculated AFs with *H. opuntiae* was comparable to that in pure *S. cerevisiae* AF (greater than 98.00%) but was significantly lower in co-inoculated AFs with *H. uvarum* (96.53%) (Table 1). Additionally, in both co-inoculated AFs, *Hanseniaspora* species outnumbered *S. cerevisiae* in the must, with the relative abundance of *S. cerevisiae* at only 25.1 ± 2.4% for Sc + Hu AF and significantly higher at 42.5 ± 7.6% for Sc + Ho AF, indicating a significant difference in the growth kinetics of *Hanseniaspora* species between the two AFs. On the fourth day after inoculation (exponential phase), the highest concentration and cell viability were observed in the *S. cerevisiae* AF (7.64 ± 0.10 $\log_{10}$ CFU/mL, and 68.98 ± 6.20%). However, cell viabilities were lower when *Hanseniaspora* species were co-inoculated, with values of 59.25 ± 5.18% (AF with Sc + Hu) and 61.76 ± 4.01% (AF with Sc + Ho). Notably, in both co-inoculated AFs, *S. cerevisiae* was already the predominant species in the total viable yeast population after 4 days, with proportions of 88.42 ± 5.11% in the Sc + Hu AF and 96.19 ± 1.35% in the Sc + Ho AF.

Impact of *Hanseniaspora* species inoculum type on the chemical composition of wine

Table 2 presents the results of the effects of mixed cultures of two *Hanseniaspora* species and *S. cerevisiae*, and pure cultures of *S. cerevisiae*, on the production of VOCs and other compounds during AF of Sauvignon Blanc must. In experiment A (Table 2, left), *S. cerevisiae* had higher initial populations, while in experiment B (Table 2, right), either *H. uvarum* or *H. opuntiae* had higher initial populations.

In experiment A, as in the pure culture experiment, we observed a clear tendency towards higher production of ethyl esters of EEFAs and all acetates by mixed cultures of *S. cerevisiae* and *H. opuntiae* compared to mixed cultures of *S. cerevisiae* and *H. uvarum* and the pure culture of *S. cerevisiae* (Table 2, left). However, a significant difference was observed for ethyl dodecanoate, with a higher concentration in alcoholic fermentation with mixed cultures of *S. cerevisiae* and *H. uvarum*, followed by mixed cultures of *S. cerevisiae* and *H. opuntiae*, and pure cultures of *S. cerevisiae*. For hexyl acetate, isoamyl acetate, and the sum of acetates, higher concentrations were found in alcoholic

Table 2 Effects of mixed cultures of *H. uvarum* (Hu) or *H. opuntiae* (Ho) with *S. cerevisiae* (Sc), and pure *S. cerevisiae*, on the production of VOCs and other wine compounds in Sauvignon Blanc during AF, with higher initial populations of *S. cerevisiae* (experiment A, left) or either *H. uvarum* or *H. opuntiae* (experiment B, right) ($n = 3$)

	Experiment A—higher initial population of ***S. cerevisiae*** in the must (ratio 10:1)				Experiment B—higher initial population of ***H. uvarum*** or ***H. opuntiae*** in the must (ratio 10:1)			
	Sc + Hu_A	*Sc + Ho_A*	*Sc_A*	*p* values[a]	*Sc + Hu_B*	*Sc + Ho_B*	*Sc_B*	*p* values
Ethyl esters of strain chain fatty acids (EEFAs) (μg/L)								
Ethyl butyrate (20[b])	359.9 ± 30.3	287.3 ± 140.4	308.3 ± 36.8	ns	427.2 ± 88.2	522.3 ± 287.4	616.9 ± 291.0	ns
Ethyl decanoate (200)	785.3 ± 55.3	932.4 ± 200.0	773.9 ± 73.2	ns	817.6 ± 189.1	962.9 ± 451.7	1009.2 ± 439.9	ns
Ethyl octanoate (2)	1007.3 ± 82.2	1189.9 ± 124.5	1073.2 ± 106.6	ns	1006.1 ± 206.8	1235.2 ± 707.7	1541.9 ± 720.3	ns
Ethyl dodecanoate (640)	123.2 ± 10.4a	100.4 ± 1.7b	84.2 ± 7.0c	**[c]	90.5 ± 9.5	116.5 ± 29.8	104.9 ± 29.0	ns
Ethyl hexanoate (5)	719.6 ± 62.8	838.7 ± 112.9	675.0 ± 74.7	ns	739.0 ± 160.9	945.9 ± 526.2	1177.0 ± 548.7	ns
SUM EEFAs	2995.2 ± 232.6	3348.7 ± 302.0	2914.6 ± 283.1	ns	3080.4 ± 652.2	3782.7 ± 2001.1	4450.0 ± 2020.8	ns
Acetates (μg/L)								
Hexyl acetate (670)	99.9 ± 12.3b	149.6 ± 15.2a	109.9 ± 12.9b	**	210.8 ± 56.8	237.7 ± 142.8	191.5 ± 84.8	ns
Isoamyl acetate (30)	2893.0 ± 284.6ab	3079.5 ± 547.8a	2244.1 ± 303.8b	*	5032.0 ± 1114.8	4890.3 ± 2622.6	4488.9 ± 2074.7	ns
2-Phenylethyl acetate (250)	241.2 ± 33.2	260.6 ± 40.9	208.5 ± 30.1	ns	343.9 ± 24.7b	945.0 ± 224.4a	312.4 ± 131.9b	**
SUM acetates	3234.1 ± 319.3ab	3489.8 ± 520.5a	2562.5 ± 310.3b	*	5586.7 ± 1193.0	6073.0 ± 2974.1	4992.8 ± 2239.9	ns
Alcohols (mg/L)								
1-Hexanol (8)	0.23 ± 0.03	0.30 ± 0.06	0.27 ± 0.03	ns	0.29 ± 0.03	0.31 ± 0.12	0.41 ± 0.17	ns
(Z)−3-hexen-1-ol (0.4)	0.017 ± 0.002b	0.025 ± 0.002a	0.020 ± 0.001b	**	0.024 ± 0.002	0.027 ± 0.011	0.032 ± 0.012	ns
2-Methyl-1-propanol (40)	26.0 ± 1.0a	24.6 ± 0.8a	21.8 ± 0.7b	**	29.3 ± 1.1	28.0 ± 0.2	22.0 ± 1.2	ns
2-Methyl-1-butanol (30)	166.2 ± 7.4a	151.2 ± 2.3b	149.1 ± 4.1b	*	120.2 ± 2.4b	111.5 ± 5.0b	159.9 ± 6.3a	***
3-Methyl-1-butanol (30)	26.6 ± 1.1a	24.0 ± 0.4b	23.6 ± 0.6b	**	21.4 ± 0.3b	17.4 ± 0.8c	25.9 ± 2.7a	**
SUM alcohols	218.8 ± 9.5a	199.8 ± 3.4b	194.5 ± 5.3b	**	170.9 ± 2.6b	156.8 ± 5.9c	207.8 ± 10.1a	***
Other compounds								
Ethyl acetate (mg/L) (7.5)	34.6 ± 14.0	30.2 ± 2.4	27.5 ± 7.2	ns	118.0 ± 78.8	60.7 ± 26.1	32.2 ± 20.7	ns
Ethanol (% vol)	12.59 ± 0.08a	12.46 ± 0.04b	12.46 ± 0.04b	*	12.18 ± 0.06b	12.24 ± 0.09b	12.42 ± 0.06a	**
Glycerol (g/L)	10.10 ± 0.36a	5.73 ± 0.38b	6.30 ± 0.87b	***	7.53 ± 0.32a	6.43 ± 0.45b	5.53 ± 0.23c	***
TDM (g/L)	20.80 ± 0.17a	20.47 ± 0.21b	20.50 ± 0.10ab	*	21.00 ± 0.20b	21.57 ± 0.15a	20.90 ± 0.20b	**
VA (g/L acetic acid)	0.21 ± 0.01b	0.25 ± 0.00a	0.25 ± 0.01a	***	0.55 ± 0.04a	0.43 ± 0.01b	0.32 ± 0.03c	***

[a] One-way ANOVA was used to compare the data. Means followed by different letters and cell colours in a row are significantly different at $p \leq 0.05$ (Fisher's LSD). All stated uncertainties are standard deviations based on three replicates per treatment

[b] The olfactory thresholds (OT) for the compounds from ethyl butyrate to ethyl acetate were referenced previously (Šuklje and Čuš 2021) or obtained from other sources (Arias-Pérez et al. 2021; Denat et al. 2021)

[c] The asterisks indicate the level of significance of the one-way ANOVA for type of inoculum: * $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$, whereas ns indicates no significant differences

fermentation with mixed cultures of *S. cerevisiae* and *H. opuntiae*, followed by mixed cultures of *S. cerevisiae* and *H. uvarum*, and pure culture of *S. cerevisiae*. The same tendency as in the pure culture experiment was confirmed here, where the mixed culture of *S. cerevisiae* and *H. uvarum* produced significantly higher amounts of all three higher alcohols (2-methyl-1-propanol, 2-methyl-1-butanol, and 3-methyl-1-butanol) and the sum of the alcohols, followed by the mixed cultures of *S. cerevisiae* and *H. opuntiae* and the pure culture of *S. cerevisiae*. The same trend, though not significant, was observed for ethyl acetate. The mixed culture of *S. cerevisiae* and *H. uvarum* also produced significantly higher amounts of ethanol and glycerol and lower amounts of acetic acid, compared to the mixed culture of *S. cerevisiae* and *H. opuntiae* and the pure culture of *S. cerevisiae*. The wine produced with this combination of starter culture also contained significantly higher amounts of total dry matter (TDM), while the wine produced with the mixed starter culture of *S. cerevisiae* and *H. opuntiae* contains significantly higher amounts of (Z)−3-hexen-1-ol.

In experiment B, where a higher initial concentration of *Hanseniaspora* species was used compared to the *S. cerevisiae* strain, the results of experiment A were not fully replicated. Significantly higher concentrations of 2-methyl-1-butanol, 3-methyl-1-butanol, and the sum of HAs and ethanol were found in wines from the pure culture of *S. cerevisiae* than in wines from mixed cultures (Table 2, right). The wine produced with the mixed culture of *S. cerevisiae* and *H. opuntiae* contains a significantly higher concentration of 2-phenylethyl acetate than the other two treatments. Significantly higher concentrations of 3-methyl-1-butanol and the sum of HAs were obtained with the mixed culture of *S. cerevisiae* and *H. uvarum* compared to the mixed culture of *S. cerevisiae* and *H. opuntiae*. The mixed culture of *S. cerevisiae* and *H. uvarum* produced significantly more glycerol and volatile acidity than the other two yeast combinations, while the highest concentration of TDM was found in the wine produced with the mixed culture of *S. cerevisiae* and *H. opuntiae*. Glycerol and volatile acid production were also significantly higher in the mixed culture of *S. cerevisiae* and *H. opuntiae* compared to the pure *S. cerevisiae* culture.

The PCA analysis was applied to both experiments (A and B) on VOCs and standard parameters analysed in the wines (Fig. 1). In experiment A, the treatments representing different yeast inoculum—namely, the mixed culture of *S. cerevisiae* and *H. uvarum* (Sc + Hu_A), the mixed culture of *S. cerevisiae* and *H. opuntiae* (Sc + Ho_A), and the pure culture of *S. cerevisiae* (Sc_A)—were separated either by principal component (PC) 1, which explained 44.54% of the variation in the data set (Sc + Hu_A vs. Sc + Ho_A and Sc_A), or by (PC) 2, which explained 30.47% of the variation (Sc + Ho_A vs. Sc_A, except for Sc + Ho3_A). The concentrations of EEFAs and acetate esters, except ethyl dodecanoate, ethyl acetate, and 2-phenylethyl acetate, were positively associated with mixed alcoholic fermentation by *S. cerevisiae* and *H. opuntiae*. The same was true for (Z)−3-hexen-1-ol. Conversely, the concentrations of HAs (2-methyl-1-propanol, 2-methyl-1-butanol, and 3-methyl-1-butanol) and their sum, as well as ethanol, glycerol, TDM, and ethyl dodecanoate, were positively associated with mixed alcoholic fermentation by *S. cerevisiae* and *H. uvarum*. In experiment A, only the concentration of VA was positively associated with alcoholic fermentation by the pure *S. cerevisiae* strain.

These results were not reproducible in experiment B, where the treatments were not separated by PC1, which explained 48.40% of the variation, but were separated by PC2, which explained 33.05% of the variation (Sc_B vs. Sc + Ho_B and Sc + Hu_B). As shown, lower reproducibility of the replicates of the same treatment was achieved here

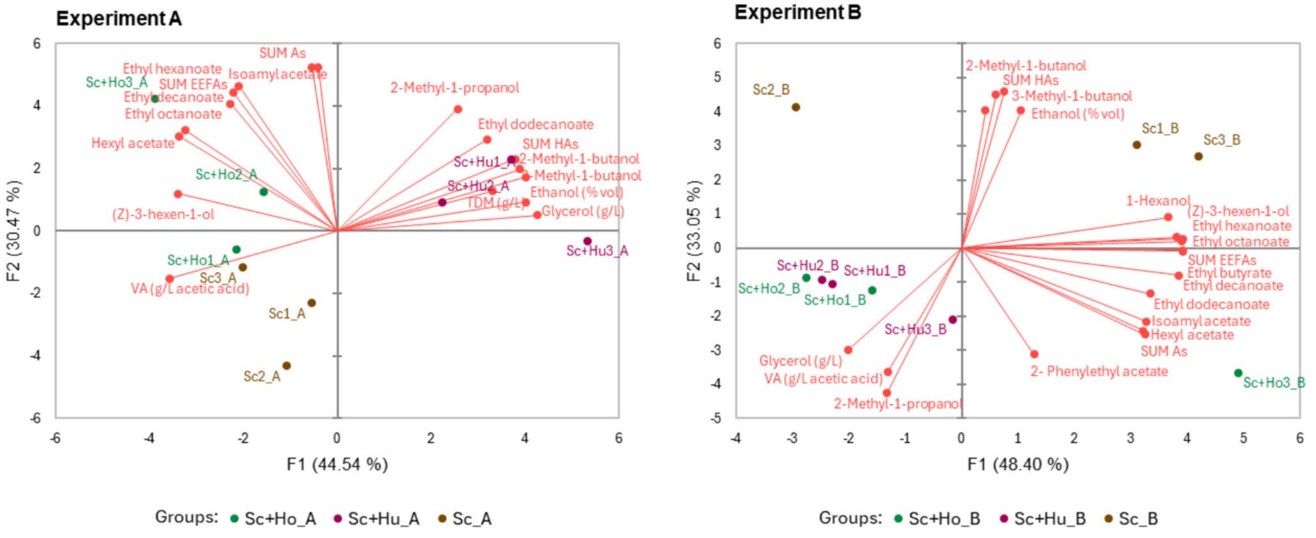

Fig. 1 Loadings for the first two principal components in PCA conducted on the concentrations of VOCs and other wine parameters of Sauvignon Blanc after completed alcoholic fermentations with mixed cultures of *H. uvarum* (Hu) or *H. opuntiae* (Ho) with *S. cerevisiae* (Sc), and pure culture of *S. cerevisiae*. Experiment **A** (left) with a higher initial population of *S. cerevisiae* and experiment **B** (right) with a higher initial population of *H. uvarum* or *H. opuntiae* ($n = 3$)

than in experiment A, as the Sc2_B treatment was separated from the other two replicates of the same treatment by PC1 and the same was true for the Sc + Ho3_B replicate. In this case, the concentrations of glycerol, VA, and 2-methyl-1-propanol were positively associated with AFs by the combinations of *S. cerevisiae* with either *H. uvarum* or *H. opuntiae*, while the concentrations of 2-methyl-1-butanol, 3-methyl-1-butanol, sum of HAs, ethanol, 1-hexanol, (Z)−3-hexen-1-ol, ethyl hexanoate, and ethyl octanoate were positively associated with AF by the pure *S. cerevisiae* strain.

Discussion and conclusions

The aim of this study was to investigate what effects the changes in the yeast population in the must could have on wine composition when the indigenous species of *H. uvarum* is further replaced by thermotolerant *H. opuntiae* on the grape berry (Fig. S1).

The results showed that both strains are weak fermenters, confirming previous findings (Longo et al. 1991; Andorrà et al. 2010; Jolly et al. 2014; Harlé et al. 2020; Wyk et al. 2024; Potisek and Čuš 2025). In the pure culture AFs, both *Hanseniaspora* species exhibited comparable fermentation capacity, which was, however, much lower than that of the pure culture of *S. cerevisiae*. Previous studies have suggested that the low fermentative capacity of *H. uvarum* compared to *S. cerevisiae* can be attributed to reduced pyruvate kinase activity (Langenberg et al. 2017).

In mixed alcoholic fermentation (AF) with co-inoculation of *Hanseniaspora* species and *S. cerevisiae* in Sauvignon Blanc must, there were no significant differences in fermentation ability compared to pure *S. cerevisiae* AF at the end of the process, regardless of the inoculum size of each strain. These results are comparable to those obtained in co-inoculations of *Hanseniaspora vineae* and *S. cerevisiae* (Gallo et al. 2024) and suggest there is no antagonistic activity between the strains used, as previously shown for 30 strains of *H. uvarum* and 10 other *Hanseniaspora* spp. (Albertin et al. 2016). In mixed AFs, the proportion of *S. cerevisiae* was significantly lower in AF with *H. uvarum* compared to AF with *H. opuntiae* during the early phase of AF for both species' ratios in the inoculum, which is a consequence of the faster growth of *H. uvarum* compared to *S. cerevisiae* in the early phases of AF (Onetto et al. 2025a). Also, the growth rate appears to be lower in *H. opuntiae* than in *H. uvarum* under oenological conditions, as previously shown (Harlé et al. 2020). At the same time, we confirmed lower cell viability of mixed AF with *H. uvarum* already in the early phase, which could be due to competitive mechanisms or early cell death of *H. uvarum* yeast (Harlé et al. 2020). These results suggest that *H. opuntiae* is not as competitive with *S. cerevisiae* as *H. uvarum* in the early stage of AF.

The results of using *Hanseniaspora* species in pure AF of synthetic and natural must confirmed that *H. opuntiae* produces higher amounts of 2-phenylethyl acetate during fermentation, supporting previous suggestion to use *H. opuntiae* for the improvement of wine quality (Filippousi et al. 2024). On the other hand, *H. uvarum* produced a higher amount of glycerol and ethyl acetate during alcoholic fermentation in natural must, as previously reported (Moreira et al. 2008; Andorrà et al. 2010; Rossouw and Bauer 2016; Tristezza et al. 2016; Mančić et al. 2022). There was also a trend towards higher production of isoamyl acetate in both mixed AFs with *Hanseniaspora* and *S. cerevisiae* strains, which was more significant in fermentation trials with lower initial concentrations of *Hanseniaspora* yeasts. Increased production of higher alcohols was observed in mixed AF with *H. uvarum* at lower inoculum sizes, and in pure *S. cerevisiae* AF. Given the aforementioned changes, changes in the sensory profile of the wine can also be expected, as glycerol is responsible for the sweet taste of wine (Noble and Bursick 1984), 2-phenylethyl acetate is commonly associated with a rose aroma (Ruiz et al. 2019), while ethyl acetate, isoamyl acetate, and higher alcohols contribute to the fruity and alcoholic aromas of wine (Denat et al. 2021). Therefore, we could assume that wines in which *H. opuntiae* is significantly present during alcoholic fermentation will have a more floral aroma.

The influence of the two *Hanseniaspora* species in natural must depended on the initial yeast concentration and their ratio to *S. cerevisiae*, as already shown by Zhang et al. (2023) and Gallo et al. (2024). This is likely related to significant changes in gene expression of *S. cerevisiae* when used together with *H. uvarum*, and metabolic interactions between both strains that contribute to increased or altered production of volatile compounds (Curiel et al. 2017; Sun et al. 2025). These interactions also include competition between *S. cerevisiae* and *H. uvarum* (Wang et al. 2024) and *H. opuntiae* for resources, although both *Hanseniaspora* species were characterised by high mortality in both mono and mixed fermentations with *S. cerevisiae* (Harlé et al. 2020).

The results indicate that *H. opuntiae* could become a significant factor in altering wine composition if *H. uvarum* continues to be displaced in vineyards, as changes in the volatile profile of wines produced by spontaneous alcoholic fermentation or by late addition of yeast inoculum are likely. However, only one strain of each *Hanseniaspora* yeast was used in this study, so intraspecific variability was not assessed and may play an important role in the influence of VOC production (Wang et al. 2024). However, it was also shown that despite large genetic variation among 115 *H. uvarum* strains, phenotypic variability was very low for the factors investigated (Albertin et al. 2016).

Additionally, the results confirmed some previous findings regarding yeast inoculum size. Specifically, we have shown that a higher inoculation rate of *Hanseniaspora* species compared to *S. cerevisiae* can reduce the ethanol content in wines, supporting previous results (Rossouw and Bauer 2016; Mančić et al. 2022). Furthermore, with a higher inoculum size of the pure *S. cerevisiae* strain, the production of EEFAs and acetates decreases, corroborating earlier observations (Carrau et al. 2010; Eder et al. 2025). Therefore, inoculum size and the proportions of different yeast strains play a significant role in the production of VOCs during alcoholic fermentation.

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Author contributions FČ conceived and designed the research and methodology, wrote the original draft, analysed and curated the data, supervised the experimental work, and acquired funding and resources. MP conducted experiments, analysed and curated the data, wrote the original draft, and reviewed the manuscript. NČ conceived and designed the research and methodology, supervised the experimental work, acquired funding and resources, and reviewed the manuscript. All authors read and approved the manuscript.

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Data availability The original data used in this study are openly available on FigShare at https://doi.org/10.6084/m9.figshare.30455477.

Declarations

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

Consent to participate The research does not use human subjects.

Consent for publication This article does not contain any images that are from other authors.

Competing interests The authors declare no competing interests.

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