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Distinct volatile profiles of *Metschnikowia pulcherrima* L672 and *Hanseniaspora uvarum* L793 determine their antagonistic efficacy against *Aspergillus flavus* in a dried fig agar model

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ABSTRACT

Contamination of dried figs by *Aspergillus flavus* and its carcinogenic aflatoxins poses a significant food safety risk. Volatile organic compounds (VOCs) from biocontrol yeasts offer a promising sustainable alternative to synthetic fungicides. This study aimed to evaluate the effect of VOCs produced by *Metschnikowia pulcherrima* L672 and *Hanseniaspora uvarum* L793 on the growth and aflatoxin production of *A. flavus* in a dried fig agar model. The volatile profiles produced by the yeasts, both individually and during co-culture, were characterized by HS-SPME-GC–MS to correlate them with the observed antagonistic activity. The results showed that VOCs from both yeasts potently inhibited mycelial growth by over 94%. However, significant differences were observed in their anti-aflatoxigenic efficacy. While *M. pulcherrima* L672 significantly reduced aflatoxin levels, *H. uvarum* L793 reduced their synthesis to below the limit of detection. This distinction was strongly correlated with their volatile profiles; *H. uvarum* L793 produced a more complex and abundant blend of VOCs, particularly alcohols and esters, in response to the fungus. In conclusion, our findings demonstrate that while both yeasts are effective fungistatic agents, the superior anti-mycotoxigenic activity of *H. uvarum* L793 is correlated with a more robust and complex volatile response to the pathogen. This highlights the critical role of the specific volatilome composition in determining biocontrol efficacy and positions *H. uvarum* L793 as a prime candidate for controlling *A. flavus* in food systems.

1. Introduction

Volatile organic compounds (VOCs) produced by biocontrol microorganisms are secondary metabolites with low molecular weight, high volatility, high permeability, low toxicity, and a lack of persistent residues, including alcohols, ketones, aldehydes, acids, esters, and sulphur compounds (Zhang et al., 2025; Zhao et al., 2022). VOCs can act as natural antifungal agents, and their study has become increasingly important as a sustainable alternative to synthetic fungicides due to their potential to inhibit the growth of pathogens without causing significant environmental impacts. These VOCs have been reported to effectively inhibit a wide variety of phytopathogenic fungi, including *Botrytis cinerea*, *Fusarium* spp., *Penicillium* spp., *Alternaria* spp., *Colletotrichum* spp., *Monilinia* spp., and *Aspergillus* spp., among others (Calvo et al., 2020; Li et al., 2024; Morita et al., 2019; Tejero et al., 2021; Toral et al., 2021; Zhang et al., 2022). The antifungal mechanisms of microbial VOCs are reported to include disruption of fungal cell membranes, inhibition of spore germination, disruption of fungal metabolism, and modulation of gene expression related to pathogenicity and resistance (Kong et al., 2022; Zhang et al., 2025; Zhao et al., 2022). Furthermore, VOCs have been shown to inhibit the synthesis of mycotoxins, such as aflatoxins, ochratoxin A, and deoxynivalenol (Ballot et al., 2023; Farbo et al., 2018; Tejero et al., 2021).

A variety of microorganisms, including bacteria, filamentous fungi, and yeasts, produce VOCs with antifungal activity capable of inhibiting fruit and plant pathogens without direct contact (Zhao et al., 2022).

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Among bacteria, the genera *Bacillus* and *Pseudomonas* are prominent (Morita et al., 2019; Ulloa et al., 2023), while in filamentous fungi, studies have mainly focused on *Trichoderma* (Joo and Hussein, 2022; Ruangwong et al., 2021). In the case of yeasts, genera such as *Aureobasidium*, *Metschnikowia* and *Hanseniaspora* are also involved in the suppression of moulds, constituting promising alternatives for sustainable biocontrol. The latter two genera are particularly notable for their demonstrated efficacy as biocontrol agents in previous studies. For instance, Ruiz-Moyano et al. (2020) demonstrated that the VOCs produced by the yeast *Hanseniaspora uvarum* L793 reduced the incidence and severity of *B. cinerea* in strawberries and cherries. Tejero et al. (2021) observed that the volatiles produced by the strains *H. uvarum* L793 and *Hanseniaspora opuntiae* L479 inhibited the growth of *Aspergillus flavus* and reduced aflatoxin synthesis on potato dextrose agar (PDA) medium. Ruiz-Moyano et al. (2016) reported that the yeasts *H. opuntiae* L479 and *Metschnikowia pulcherrima* L672 inhibited the growth of *Penicillium expansum*, *Cladosporium cladosporioides*, *B. cinerea*, and *Monilinia laxa* both *in vitro* and *in vivo*. Similarly, in the study by Tejero et al. (2025), the yeast *M. pulcherrima* L672 reduced the incidence and severity of *P. expansum* in stone fruits, along with the synthesis of patulin.

A. flavus is a major fungal contaminant of dried figs and poses a significant risk due to its ability to synthesise aflatoxins, highly toxic secondary metabolites that compromise food safety and the export of this product (EFSA Panel on Contaminants in the Food Chain (CONTAM), Schrenk et al., 2020; Oktay et al., 2011). The main aflatoxins associated with *A. flavus* are aflatoxin B_1 (AFB_1) and aflatoxin B_2 (AFB_2), which are characterized by their blue fluorescence under UV light. While *A. flavus* primarily produces B-type aflatoxins, aflatoxin G_1 (AFG_1) and aflatoxin G_2 (AFG_2), which exhibit green fluorescence, are more commonly produced by *Aspergillus parasiticus*, although they may also occur in contaminated commodities (Pitt and Hocking, 2009). These compounds are classified as Group 1 carcinogens by the International Agency for Research on Cancer (IARC), with AFB_1 being the most prevalent and toxic form (IARC, 2002). The presence of these toxins has been reported in up to 37% of field samples (Heperkan and Karbancioglu-Güler, 2009) and in more than 50% of commercial products with origins in different countries, with generally low levels, although extremely high concentrations have been detected in some cases (Iamanaka et al., 2007). The control of *A. flavus* has historically been based on the use of synthetic fungicides, whose environmental and health impacts have prompted the search for sustainable alternatives. Therefore, there is a growing interest in more environmentally friendly alternatives, such as VOCs produced by yeasts.

To the best of our knowledge, this study provides a novel perspective by evaluating the biocontrol potential of specific yeast strains (*M. pulcherrima* L672 and *H. uvarum* L793) using a fig-based agar medium. This matrix-specific approach is designed to simulate the nutritional and chemical complexity of dried figs, aiming to provide a more accurate approximation of *in vivo* conditions than conventional synthetic media. Furthermore, this work focuses on the differential VOCs profiles produced by these strains and their specific influence on both *A. flavus* growth and aflatoxin production, exploring the hypothesis that substrate-dependent volatile emission may contribute to the inhibition of mycotoxigenic fungi.

The objective of this study was to evaluate the effect of VOCs produced by the biocontrol yeasts *M. pulcherrima* L672 and *H. uvarum* L793 on *A. flavus* M144 growth and its aflatoxin production, and to characterize the associated volatile profiles to explore the potential link between their specific composition and the observed antagonistic activity, in a dried fig agar model system.

2. Material and methods

2.1. Experimental design

For this study, two yeasts with known biological control potential were selected: *M. pulcherrima* L672 (Ruiz-Moyano et al., 2016; De Paiva et al., 2017) and *H. uvarum* L793 (Ruiz-Moyano et al., 2020; Tejero et al., 2021). Both strains were originally isolated from figs and are maintained in the culture collection of the Food Quality and Microbiology Research Group (CAMIALI) at the University of Extremadura (Spain). The inocula were obtained by culturing the strains on potato dextrose agar (PDA, Scharlab, Barcelona, Spain), and incubating them for 4 days at 25 °C.

A. flavus, a common contaminant in dried figs, was used as the pathogen, specifically the strain *A. flavus* M144. This strain was also isolated from dried figs and belongs to CAMIALI collection (previously characterized by Galván et al., 2021). The fungal inoculum was prepared by culturing the mould on PDA plates acidified with 10% tartaric acid, incubated for 7 days at 25 °C. Subsequently, 10 mL of sterile deionised water was added to the plate, and the conidia were dislodged by gently rubbing the surface with a glass rod. Finally, the concentration was adjusted to 5×10^4 conidia mL^{-1} using a microscope (Olympus CX 400, Tokyo, Japan) and a Neubauer chamber (Paul Marienfeld GmbH & Co.KG, Lauda-Königshofen, Germany).

A solid medium based on 10% (*w/v*) dried fig agar was prepared, following the protocol described by Galván et al. (2022). The yeast strains *M. pulcherrima* L672 and *H. uvarum* L793 were inoculated separately, incorporating them directly into the molten and homogeneous medium (maintained at approximately 40 °C in a water bath) before solidification to ensure they were evenly distributed throughout the agar volume. After inoculation, the medium was homogenised using a magnetic stirrer for 1 min to ensure a uniform distribution of the yeasts. Subsequently, the medium was distributed into Petri dishes (90 × 14 mm, high transparency polystyrene), dispensing 20 mL into each one. To ensure consistent gas exchange and avoid the inhibitory effects of CO_2 accumulation or O_2 depletion, the plates were not sealed with Parafilm during the incubation period (Contarino et al., 2019). Furthermore, the plates were arranged in a single layer (not stacked) within the incubator to ensure uniform environmental conditions and VOCs exposure for all replicates. The cell concentration of the yeast inocula was adjusted beforehand by suspending the colonies grown on PDA agar in sterile distilled water, so that the final concentration of yeast incorporated into each dried fig agar plate was 10^3 cells mL^{-1}. This concentration was selected based on preliminary assays to ensure a level of fungal development that allows for the assessment of the antagonistic effect on both growth and aflatoxin production. Additionally, using a moderate yeast concentration is more aligned with industrial requirements, as excessively high microbial loads could lead to organoleptic alterations or undesirable fermentations in the dried figs. Yeast viability and the uniformity of the distribution were verified after solidification by performing microbiological plate counts on representative samples of the prepared medium.

Concurrently, dried-fig agar plates without yeast were inoculated with a 5 μL aliquot of an *A. flavus* M144 suspension at a concentration of 5×10^4 conidia mL^{-1} on sterile cellophane (60 mm diameter, previously sterilized by autoclaving at 121 °C for 15 min) placed on the surface of the medium. All plates, both those containing yeast and those inoculated with the mould, were incubated at 25 °C for 24 h. After this period, the cellophane sheets with the growing mycelium of *A. flavus* M144 were carefully transferred to the yeast-containing plates, or in the case of the pathogen control (AF), to fresh dried-fig agar plates without yeast. The transfer procedure was strictly standardized across all replicates to ensure reproducibility. Subsequently, all plates were incubated under the same conditions for a total of 8 days. After this time, the plates designated for VOCs and aflatoxins analyses were frozen at −80 °C until use. Table 1 summarises the experimental treatments used in this study. All determinations were carried out in triplicate.

Table 1
Description of the experimental treatments used in the study.

Sample code	Description (control or treatment)
BL	Uninoculated dried fig agar (Negative control).
AF	*A. flavus* M144 grown on cellophane and transferred to fresh dried fig agar (Pathogen and transfer control).
672C	Dried fig agar inoculated with *M. pulcherrima* L672 (Yeast control).
672AF	Dried fig agar inoculated with *M. pulcherrima* L672 and cellophane with transferred *A. flavus* M144 (Treatment).
793C	Dried fig agar inoculated with *H. uvarum* L793 (Yeast control).
793AF	Dried fig agar inoculated with *H. uvarum* L793 and cellophane with transferred *A. flavus* M144 (Treatment).

2.2. Mycelial weight determination

After the incubation period, the cellophane sheets containing the mycelium were collected from three replicate plates per treatment to determine the fungal biomass. The fresh weight of the mycelium was calculated as the difference between the total weight (cellophane + mycelium) and the pre-recorded weight of the sterile cellophane sheet. All measurements were performed using an analytical balance and expressed in grams (g). The percentage reduction in mycelial weight for each yeast treatment was calculated relative to the control using the formula: $[(Wc - Wt) / Wc] \times 100$, where Wc is the mean weight of the control and Wt is the weight of the treated sample.

2.3. Instrumental analysis

2.3.1. Preparation of samples

For the analysis of VOCs and aflatoxins, the dried fig agar from each treatment was homogenised. From each homogenised sample, a 1 g aliquot was weighed for subsequent analyses.

2.3.2. Aflatoxin extraction and quantification

The method used for mycotoxin extraction was based on that described by Marin et al. (2024), with minor modifications. Briefly, 1 g of homogenised agar was placed in a 2 mL microcentrifuge tube, and 1 mL of methanol (MeOH) (VWR International, Milan, Italy) was added. The mixture was vortexed for 1 min and then allowed to stand for 60 min. After this time, the extracts were vortexed again and centrifuged at 12,000 $\times g$ for 15 min. The resulting supernatant was filtered through a 0.45 μm syringe filter and transferred to an HPLC vial. Four aflatoxins were analysed: aflatoxin B_1 (AFB_1), aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1), and aflatoxin G_2 (AFG_2) (Fermentek Ltsd, Jerusalem, Israel). The experiments were performed in triplicate ($n = 3$). A matrix-matched calibration curve was constructed for each mycotoxin using blank sample extracts to compensate for potential matrix effects (calibration range: 0.5–50 μg kg^{-1}).

Analyses were performed on an Ultra-High Performance Liquid Chromatography (UHPLC) Dionex Ultimate 3000 system coupled to a triple quadrupole mass spectrometer (TSQ Vantage; Thermo Fisher Scientific Inc., San Jose, CA, USA) equipped with an Electrospray Ionization Source (ESI). The column used for the chromatographic separation was an XSelect® HSS T3 column (150 mm × 2.1, 2.5 μm particle size) (Waters, Wexford, Ireland). The mobile phases used were ultrapure water with 0.2% acetic acid (v/v) (both acquired from VWR International, Milan, Italy) and 5 mM ammonium acetate (Sigma-Aldrich, Milan, Italy) (Phase A) and MeOH (Phase B). The gradient started isocratically at 5% B for 1 min, followed by a linear increase to 90% B over 7 min. Then, the composition was maintained for 3 min before returning to the initial conditions in 1 min. The initial conditions were maintained for 6 min to allow system re-equilibration. The flow rate was set at 0.4 mL min^{-1}, and the column temperature was maintained at 40 °C. The injection volume was 3 μL.

Ionization was performed through an electrospray ionization (ESI) source, operating in the positive mode. The spray voltage was set at +3.5 kV; the capillary and vaporization temperatures were set to 270 and 200 °C, respectively. Argon (Ar) was used as the collision gas. Nitrogen (N_2) was used as both sheath and auxiliary gas, with flow rates set to 50 and 5 arbitrary units, respectively. Regarding the mass spectrometry (MS) detection, the multiple reaction monitoring (MRM) acquisition mode was used, monitoring the selected reaction monitoring (SRM) transitions detailed in Table S1, following the methodology described by Torrijos et al. (2025). Two SRM transitions were monitored for each mycotoxin to ensure their proper detection.

The limits of detection (LOD) and quantification (LOQ) of the analytical method are provided in the Supplementary material (Table S2).

2.3.3. VOCs extraction and quantification

Headspace solid-phase microextraction (HS-SPME) combined with gas chromatography coupled to mass spectrometry (GC–MS) was used to determine the VOCs profile, following the procedure described by Josselin et al. (2024) with minor modifications. Briefly, 1 g of sample was placed into a 20 mL glass vial, and 5 μL of 50 μg mL^{-1} toluene aqueous solution were added as the internal standard (IS). Toluene was selected as the IS due to its high volatility and thermal stability under HS-SPME conditions. Preliminary assays confirmed that endogenous toluene levels in the fig agar matrix were negligible (<5% of the IS signal). To ensure rigorous normalization, for each treatment, a parallel sample without added IS was analysed to determine the background signal, which was subsequently subtracted from the peak area of the spiked samples. The SPME was performed using an SPME fibre coated with 50/30 μm divinylbenzene-carboxene-polydimethylsiloxane (DVB/carboxene/PDMS; Supelco, Bellefonte, PA, USA). After maintaining the sample glass vial at 40 °C for 15 min for headspace equilibration, the fibre was exposed at 40 °C for 30 min for VOCs extraction.

GC–MS analysis was performed using a Thermo Scientific Trace 1300 gas chromatograph coupled to a Thermo Scientific ISQ single quadrupole mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA). Ionization was performed using an electron ionization (EI) source operating at 70 eV. Thermal desorption was carried out for 3 min at 250 °C in the injector port.

The GC separation was carried out using a ZB-WAXplus capillary column (30 m × 0.25 mm, 0.25 μm film thickness) (Agilent Technologies, Santa Clara, CA, USA) and helium (He) as the carrier gas at a flow rate of 1.4 mL min^{-1}. The oven temperature program was as follows: initial temperature of 45 °C held for 7 min; then ramped to 70 °C at 5 °C min^{-1} (held for 3 min); ramped to 120 °C at 3 °C min^{-1} (held for 3 min); and finally ramped to 250 °C at 10 °C min^{-1} (held for 5 min). The injection was performed in splitless mode. MS detection was performed in full-scan mode over the m/z range 35–350.

Identification of the chromatographic peaks was carried out by comparing their acquired mass spectra with those contained in the NIST 14 library. In addition, Linear Retention Indices (LRIs) were calculated based on the retention time of a C8-C20 alkane standard mixture analysed under the same chromatographic conditions. The semi-quantification of the tentatively identified compounds was performed by comparing the peak area of each compound to that of the internal standard (toluene). The experiments were conducted in triplicate ($n = 3$).

2.4. Statistical analysis

The statistical analysis was performed using SPSS for Windows (version 22, IBM Corp., Armonk, NY, USA). Differences between means for mycelium weight and aflatoxin production were evaluated using a one-way analysis of variance (ANOVA) to determine the effect of the different treatments. When significant differences were detected ($p \leq 0.05$), the means were compared using Tukey's HSD (Honestly Significant Difference) post-hoc test. Additionally, to evaluate the effect on

aflatoxin biosynthesis independently of fungal growth, the toxigenic capacity was calculated. This parameter was defined as the ratio of the total aflatoxin concentration to the mycelial fresh weight, expressed as μg of aflatoxin per gram of mycelium (μg/g).

Additionally, multivariate analyses were performed using MetaboAnalyst 5.0 software (Pang et al., 2021) to explore global patterns and relationships within the VOC dataset. To that aim, data was mean-centred and scaled to unit variance (autoscaling). First, Principal Component Analysis (PCA) was applied as an exploratory and non-supervised tool to visualize the inherent variation and natural clustering of the samples without prior knowledge of the treatment groups. Subsequently, Partial Least Squares Regression Discriminant Analysis (PLS-DA) was employed to specifically evaluate the potential differences between the predefined experimental groups. The Variable Importance in Projection (VIP) scores were calculated from this supervised model to identify and rank the most discriminant and characteristic VOCs for each sample group. Finally, a heatmap with hierarchical clustering was generated to visualize the relative abundance of each identified VOC across all samples.

3. Results

3.1. Mycelial fresh weight

The final mycelial fresh weight was determined after eight days of incubation at 25 °C. This endpoint was selected based on preliminary assays to ensure maximum aflatoxin accumulation in the control and to confirm the stability of the inhibitory effect over time. Compared to the control of *A. flavus* M144, a significant reduction in fungal biomass was recorded for *M. pulcherrima* L672 (94.65%) and *H. uvarum* L793 (94.38%), demonstrating the ability of these yeasts to control the growth of *A. flavus* M144 on the dried fig agar model. No significant differences ($p > 0.05$) were observed between the two yeast treatments.

3.2. Quantification of aflatoxins

The results of aflatoxin quantification are presented in Table 2. In the control treatment (*A. flavus* M144 alone), the concentration of AFB_1 was 693.42 ± 436.04 μg kg^{-1}. The presence of the antagonistic yeasts caused a marked reduction in its synthesis. Specifically, the *M. pulcherrima* L672 treatment resulted in a significant decrease to 41.06 ± 10.34 μg kg^{-1}, while in the presence of *H. uvarum* L793, AFB_1 was not detected (<LOD).

Regarding AFB_2, the control treatment exhibited a concentration of 10.67 ± 15.45 μg kg^{-1}. Exposure to *M. pulcherrima* L672 led to a numerically lower concentration (1.24 ± 0.55 μg kg^{-1}), although this reduction was not statistically significant ($p > 0.05$). Similar to AFB_1, AFB_2 was not detected in the *H. uvarum* L793 treatment (<LOD). As expected, no aflatoxins were detected in the control samples (<LOD; uninoculated medium and yeast-only cultures).

Table 2
Quantification of aflatoxins in the different batches studied, after 8 days of incubation at 25 °C.

	AFB_1 (μg kg^{-1})	AFB_2 (μg kg^{-1})
AF	693.42[a] ± 436.04[1]	10.67 ± 15.45
BL	<LOD[2]	<LOD
672C	<LOD	<LOD
672AF	41.06[b] ± 10.34	1.24 ± 0.55
793C	<LOD	<LOD
793AF	<LOD	<LOD
p	*0.02*	*0.298*

[1] Data are expressed as mean value ± standard deviation. Within columns, different letters indicate significant differences between batches ($p < 0.05$).
[2] Below the limit of detection (LOD).

3.3. Identification and quantification of VOCs

The volatile profile generated by both yeasts was characterized using HS-SPME-GC–MS. An example of a chromatogram of the volatile profile of each batch is shown in the Supplementary material (Figs. S1–S6). The identified VOCs are listed in Table 3. A total of 66 compounds were tentatively identified, distributed among several chemical families, including esters, aldehydes, alcohols, alkenes, ketones, carboxylic acids, phenols, aromatic hydrocarbons, and furans. Alcohols were the most abundant chemical family (19 compounds), followed by esters (17) and carboxylic acids (10). Notably, the co-culture of *H. uvarum* L793 with *A. flavus* M144 (793AF) produced the highest levels of most identified compounds related to their abundance, followed by the *H. uvarum* L793 control (793C), and the *M. pulcherrima* L672 co-culture (672AF).

PCA was used to visualize the distribution of samples based on their VOCs profiles. For the *M. pulcherrima* L672 treatments (Fig. 1), the first two principal components (PC1 and PC2) explained 36.6% and 15.7% of the total variance, respectively. The scores plot (Fig. 1A) revealed a clear separation trend between the yeast-containing groups (672AF, 672C) and the control groups (AF, BL), with the 672AF and 672C samples clustering relatively close to each other. The loadings plot (Fig. 1B) indicated that most VOCs were aligned with the distribution direction of the 672AF treatment.

Similarly, the PCA for the *H. uvarum* L793 treatments is shown in Fig. 2. Here, PC1 and PC2 accounted for 54.3% and 13% of the total variance, respectively. In this analysis, the groups showed a clearer separation trend, with a notable separation between the 793AF and 793C treatments, while the AF and BL controls clustered more closely (Fig. 2A). The loadings plot again suggested that many of the volatiles were associated with the 793AF treatment (Fig. 2B).

To further refine the analysis and identify the key discriminant variables, a PLS-DA was performed. For the *M. pulcherrima* L672 experiment, the PLS-DA model revealed a clearer separation trend between the treatment groups compared to the PCA (Fig. 3A). Component 1 and Component 2 described 36% and 11.8% of the variance, respectively. The BL and AF controls were located in different quadrants from the yeast-containing treatments (672AF and 672C), with some distance also observed between the latter two. Fig. 3B shows the top 20 VOCs contributing to group discrimination according to their VIP scores. Most of them (VIP scores >1.2) may represent important contributors to the separation of the 672AF treatment, notably 3-methylbutan-1-ol (VOC30), ethanol (VOC27), benzoic acid (VOC62), 2-methyl-1-propanol (VOC28), and 2-phenylethanol (VOC43). These compounds primarily belonged to the alcohol family, followed by esters, carboxylic acids, and aldehydes (Table 3). Additionally, furan-2-ylmethanol (VOC40) and 3-hydroxybutan-2-one (VOC49) were identified as important discriminators for the 672C treatment.

The PLS-DA model for the *H. uvarum* L793 treatments also showed a clear separation trend between all groups (Fig. 4A), with PC1 describing the 54.2% of the variance and PC2 the 8.3%. The BL and AF controls were also located in different quadrants from the yeast-containing treatments (793AF and 793C), and the distance between the latter two was slightly greater than that observed for the L672 treatments. In this case, all top 20 compounds had VIP scores (Fig. 4B) exceeding the threshold of 1.20, indicating their relevance for group discrimination within the model. As indicated by the colour scale, these 20 VOCs appeared to contribute predominantly to the discrimination of 793AF treatment. The most influential compounds, in descending order of VIP score, included 2-phenylethanol (VOC43), furan-2-ylmethanol (VOC40), benzoic acid (VOC62), 3-methylbutan-1-ol (VOC30), and nonanoic acid (VOC60). The main chemical families represented among these top 20 VOCs were alcohols, followed by esters and carboxylic acids.

The heatmap in Fig. 5 provides a comprehensive visualization of the relative abundance in the volatile profiles across all treatments. The 793AF treatment produced the highest number and abundance of VOCs,

Table 3
Volatile organic compounds identified in the different batches studied after 8 days of incubation at 25 °C.

Peak	RT[1]	Compound	LRI calc.[2]	LRI lit.[3]	ID[4]	*P*[5]	Majority[6]
Ester							
1	2.58	Ethyl Acetate	888	888	MS + LRI	***	793AF
2	7.92	1-Butanol, 3-methyl-, acetate	1118	1115	MS + LRI	***	793AF
3	12.36	Ethyl hexanoate	1227	1227	MS + LRI	*	793AF
4	13.9	Hexyl acetate	1263	1264	MS + LRI	n.s.	793AF
5	16.93	Ethyl heptanoate	1330	1337	MS + LRI	***	793AF
6	21.8	Ethyl octanoate	1432	1433	MS + LRI	***	793AF
7	30.4	Ethyl decanoate	1634	1635	MS + LRI	***	793AF
8	30.9	Ethyl benzoate	1649	1649	MS + LRI	***	793AF
9	36.04	Ethyl 2-phenylacetate	1778	1779	MS + LRI	***	793AF
10	36.9	2-Phenylethyl acetate	1800	1804	MS + LRI	***	793AF
11	37.9	Ethyl dodecanoate	1844	1849	MS + LRI	**	793AF
12	38.31	3-Methylbutyl pentadecanoate	1863	1863	MS + LRI	n.s.	793AF
13	40.96	5-Pentyl-γ-butyrolactone			MS	n.s.	793AF
14	41.35	Ethyl tetradecanoate			MS	***	793AF
15	43.69	Ethyl hexadecanoate			MS	***	793AF
16	43.95	Ethyl 9-hexadecenoate			MS	***	793AF
17	45.31	Methyl stearate			MS	n.s.	AF
Aldehyde							
18	2.12	2-Methylpropanal	816	813	MS + LRI	n.s.	672C
19	2.86	3-Methylbutanal	914	914	MS + LRI	n.s.	672AF
20	6.22	Hexanal	1069	1068	MS + LRI	n.s.	793AF
21	19.69	Nonanal	1386	1385	MS + LRI	n.s.	793C
22	22.19	Methional	1440	1443	MS + LRI	n.s.	672AF
23	22.7	Furfural	1450	1453	MS + LRI	***	BL
24	25	Benzaldehyde	1502	1502	MS + LRI	**	793AF
25	29.86	2-Phenylacetaldehyde	1621	1620	MS + LRI	n.s.	672AF
26	36.78	(2E,4*E*)-Deca-2,4-dienal	1796	1795	MS + LRI	n.s.	793AF
Alcohol							
27	3.16	Ethanol	935	935	MS + LRI	***	793AF
28	7.18	2-Methyl-1-propanol	1100	1110	MS + LRI	***	793AF
29	9.27	Butan-1-ol	1152	1151	MS + LRI	***	793AF
30	11.7	3-Methylbutan-1-ol	1210	1210	MS + LRI	***	793AF
31	18.22	1-Hepten-3-ol	1356	1351	MS + LRI	n.s.	AF
32	18.32	Hexan-1-ol	1358	1360	MS + LRI	n.s.	793AF
33	20.3	Octan-3-ol	1398	1397	MS + LRI	***	AF
34	22.9	Oct-1-en-3-ol	1455	1453	MS + LRI	***	AF
35	23.05	Heptan-1-ol	1459	1459	MS + LRI	***	793AF
36	23.42	6-Methylhept-5-en-2-ol	1468	1469	MS + LRI	n.s.	672C
37	24.6	2-Ethylhexan-1-ol	1494	1495	MS + LRI	n.s.	AF
38	27.5	Octan-1-ol	1562	1561	MS + LRI	n.s.	793AF
39	29.7	(E)-Oct-2-en-1-ol	1617	1616	MS + LRI	**	672AF
40	31.3	Furan-2-ylmethanol	1657	1656	MS + LRI	***	793AF
41	33.43	3-(Methylthio)propan-1-ol	1712	1714	MS + LRI	n.s.	672AF
42	38.5	Benzyl alcohol	1872	1875	MS + LRI	***	793AF
43	39.2	2-Phenylethanol	1904	1906	MS + LRI	***	793AF
44	41.74	4-Phenylbut-3-en-2-ol			MS	n.s.	672AF
Alkene							
45	12.9	Ethenylbenzene	1239	1236	MS + LRI	n.s.	793C
46	21.2	(E)-Oct-2-enal	1418	1416	MS + LRI	n.s.	672AF
Ketone							
47	10.3	Heptan-2-one	1178	1178	MS + LRI	n.s.	793AF
48	13.1	Octan-3-one	1243	1245	MS + LRI	***	AF
49	14.3	3-Hydroxybutan-2-one	1272	1273	MS + LRI	***	793AF
50	16.94	6-Methylhept-5-en-2-one	1330	1333	MS + LRI	***	793C
51	28	3,5,5-Trimethylcyclohex-2-en-1-one	1575	1577	MS + LRI	n.s.	AF
52	28.61	Undecan-2-one	1590	1586	MS + LRI	n.s.	793AF
53	37.1	(E)-1-(2,6,6-Trimethylcyclohexa-1,3-dien-1-yl)but-2-en-1-one	1805	1802	MS + LRI	***	793AF
Carboxylic acid							
54	22.5	Acetic acid	1447	1447	MS + LRI	***	793AF
55	27.59	2-Methylpropanoic acid	1565	1564	MS + LRI	***	793C
56	38	Hexanoic acid	1847	1845	MS + LRI	**	793C
57	39.95	2-Ethylhexanoic acid	1952	1950	MS + LRI	n.s.	672C
58	39.96	Heptanoic acid	1952	1953	MS + LRI	n.s.	793C
59	41.5	Octanoic acid			MS	***	793AF
60	42.8	Nonanoic acid			MS	***	793AF
61	43.9	Decanoic acid			MS	***	793C

(*continued on next page*)

Table 3 (*continued*)

Peak	RT[1]	Compound	LRI calc.[2]	LRI lit.[3]	ID[4]	*P*[5]	Majority[6]
62	45.4	Benzoic acid			MS	***	793AF
63	45.82	Dodecanoic acid			MS	**	793AF
Phenol							
64	44.2	2,4-Di-tert-butylphenol			MS	*	672C
Aromatic hydrocarbon							
65	4.96	Toluene	1028	1028	MS + LRI	n.s.	AF
Furano							
66	41.16	4-Hydroxy-2,5-dimethyl-3(2H)-furanone			MS	n.s.	793AF

[1] RT: Retention time (min).
[2] LRI calc.: Linear Retention Index (calculated).
[3] LRI lit.: Linear Retention Index (literature).
[4] ID: Reliability of identification. MS + LRI: combination of Mass Spectrum (MS) and Linear Retention Index (LRI); MS: identification with Mass Spectrum (MS).
[5] *P*: The *p*-values indicate the level of statistical significance: n.s. = not significant ($p > 0.05$), * = significant ($p < 0.05$), ** = very significant ($p < 0.01$) and *** = highly significant ($p < 0.001$).
[6] Majority: Batch in which the highest value of each compound related to its abundance was quantified.

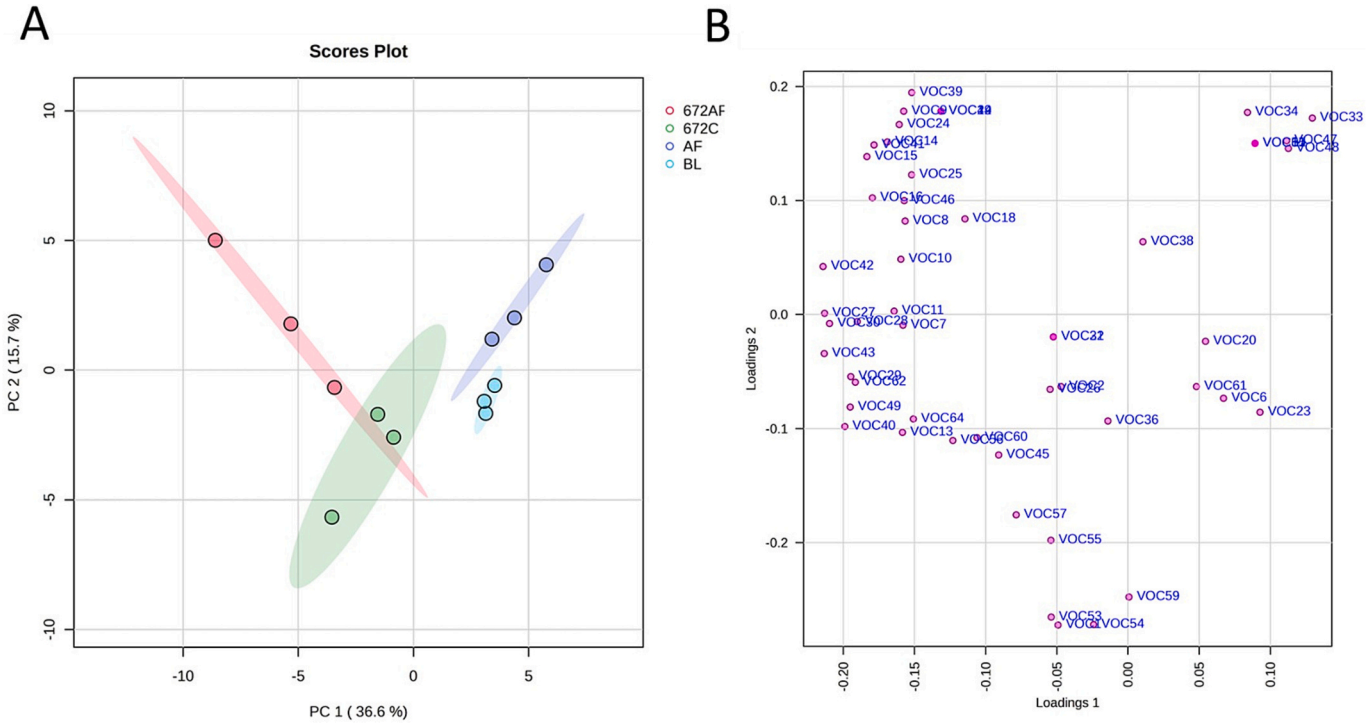

Fig. 1. Principal Component Analysis (PCA) of volatile compounds from the *M. pulcherrima* L672 experiment. (A) Scores plot showing the distribution of samples: *M. pulcherrima* L672 with *A. flavus* M144 (672AF), *M. pulcherrima* L672 control (672C), *A. flavus* M144 control (AF), and dried fig agar blank (BL). (B) Loadings plot showing the contribution of each VOC. The analysis is shown in the plane defined by the first two principal components (PC1 and PC2).

showing a distinct profile pattern compared to the 793C treatment (without *A. flavus* M144), suggesting a shift in the VOCs profile when the yeast is co-cultured with the fungus. The most prominent compounds associated with the 793C treatment were 6-methylhept-5-en-2-one (VOC50), nonanal (VOC21), 2,4-di-tert-butylphenol (VOC64), and hexanoic acid (VOC56).

The *A. flavus* control (AF) showed a distinct VOCs profile pattern, with eight compounds standing out, primarily C8-alcohols and ketones such as oct-1-en-3-ol (VOC34), 2-ethylhexan-1-ol (VOC37), octan-3-ol (VOC33), and octan-3-one (VOC48).

The treatments with *M. pulcherrima* L672 showed a similar trend to *H. uvarum* L793, with a notable shift in the volatile profile in the presence of *A. flavus* M144. The number of volatiles was higher in the 672AF treatment than in 672C, although the overall production was lower than that observed with *H. uvarum* L793. The VOCs associated with the profile of treatment 672AF belonged mostly to the aldehyde and alcohol groups. In contrast, the 672C profile was associated with compounds such as 2-methylpropanal (VOC18), 2-ethylhexanoic acid (VOC57), 6-methylhept-5-en-2-ol (VOC36) and 2,4-di-tert-butylphenol (VOC64).

Finally, the blank control (BL) had the most limited VOCs profile, with furfural (VOC23) being its predominant compound.

4. Discussion

VOCs produced by biocontrol yeasts have emerged as a promising and sustainable alternative to chemical fungicides for inhibiting

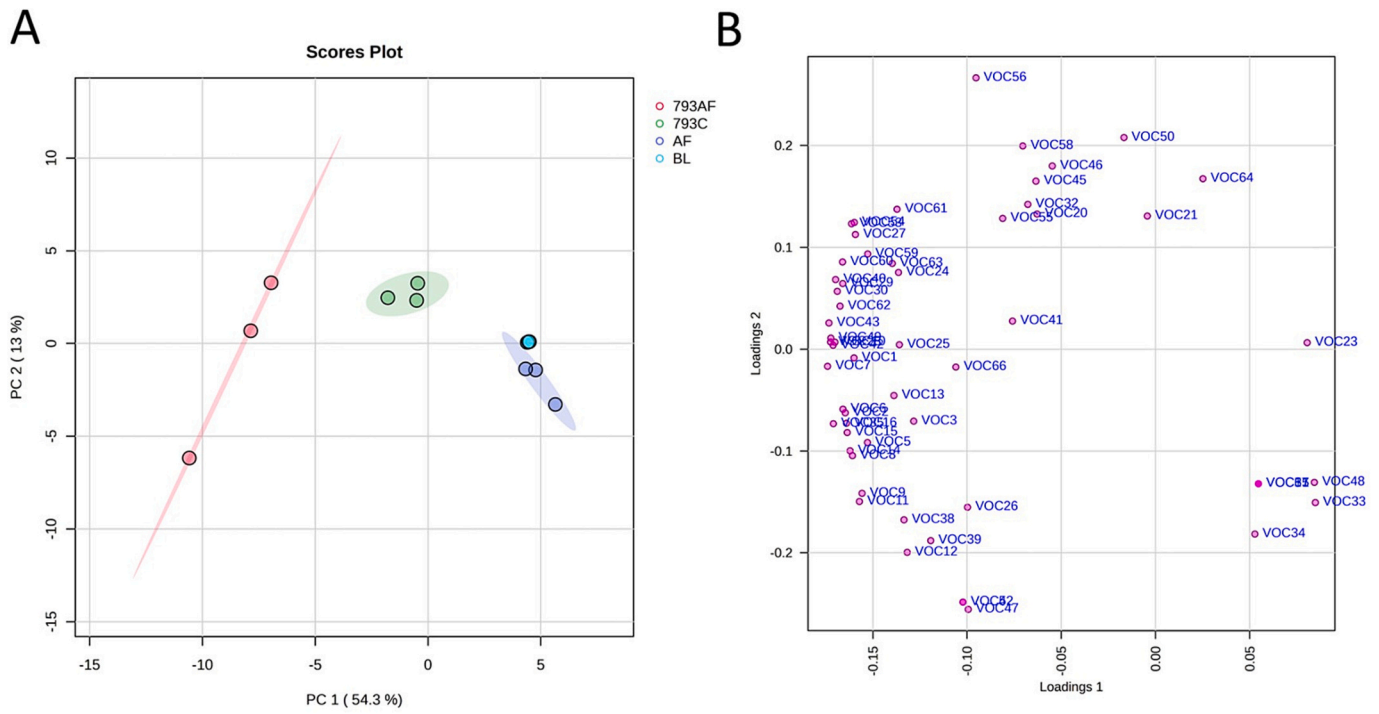

Fig. 2. Principal Component Analysis (PCA) of volatile compounds from the *H. uvarum* L793 experiment. (A) Scores plot showing the distribution of samples: *H. uvarum* L793 with *A. flavus* M144 (793AF), *H. uvarum* L793 control (793C), *A. flavus* M144 control (AF), and dried fig agar blank (BL). (B) Loadings plot showing the contribution of each VOC. The analysis is shown in the plane defined by the first two principal components (PC1 and PC2).

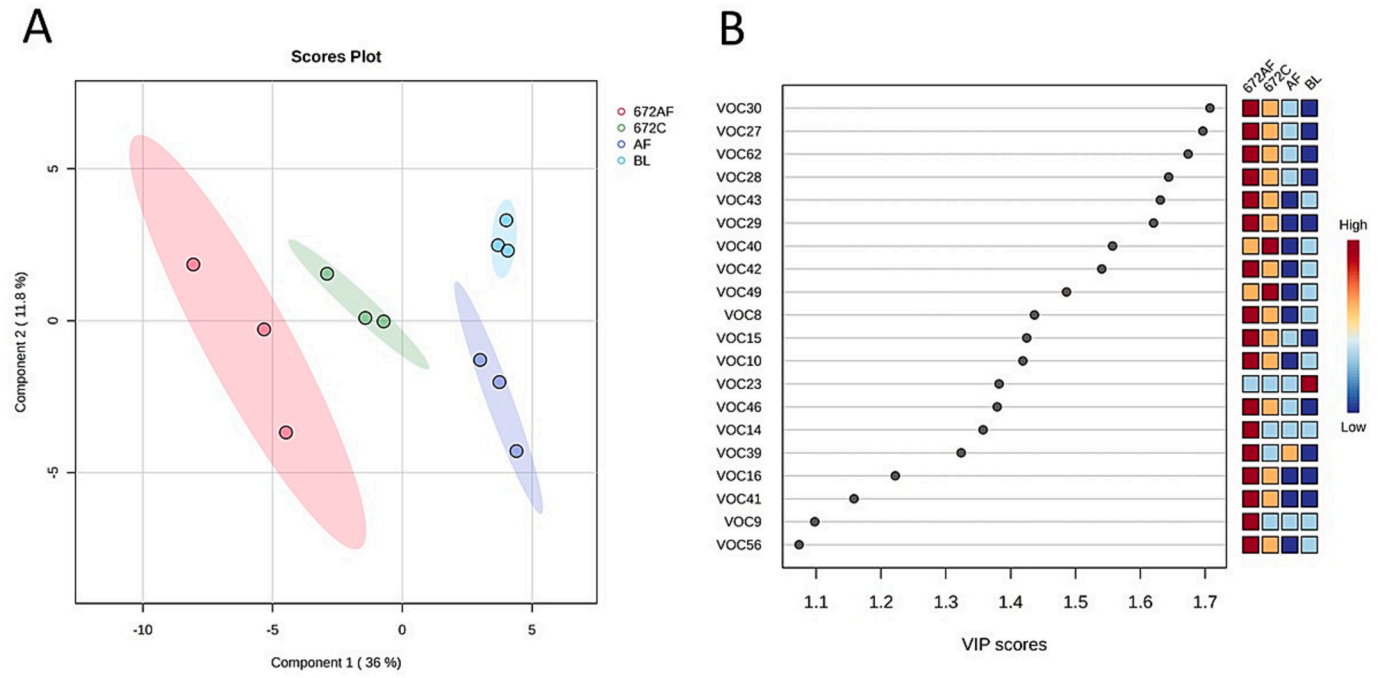

Fig. 3. Partial Least Squares Discriminant Analysis (PLS-DA) of the *M. pulcherrima* L672 experiment. (A) Score plot showing sample distribution across components 1 and 2. The plot shows the distribution of samples: *M. pulcherrima* L672 with *A. flavus* M144 (672AF), *M. pulcherrima* L672 control (672C), *A. flavus* M144 control (AF), and dried fig agar blank (BL). (B) The top 20 most influential variables ranked by their Variable Importance in Projection (VIP) scores.

phytopathogenic fungi. These volatile metabolites can inhibit mycelial growth and mycotoxin production, and their effectiveness depends on the yeast species, the pathogen, and environmental conditions (Oufensou et al., 2023).

Several studies have reported that VOCs produced by biocontrol yeasts can significantly inhibit the mycelial growth and sporulation of *A. flavus*. In particular, species such as *M. pulcherrima*, *Candida nivariensis*, *Saccharomyces cerevisiae* or *H. uvarum* have shown reductions of approximately 60–90% in mycelium growth and a marked decrease in spore formation (Dikmetas et al., 2023; Jaibangyang et al., 2020; Natarajan et al., 2022; Tejero et al., 2021). These findings are consistent with the results obtained in the present study, in which both evaluated

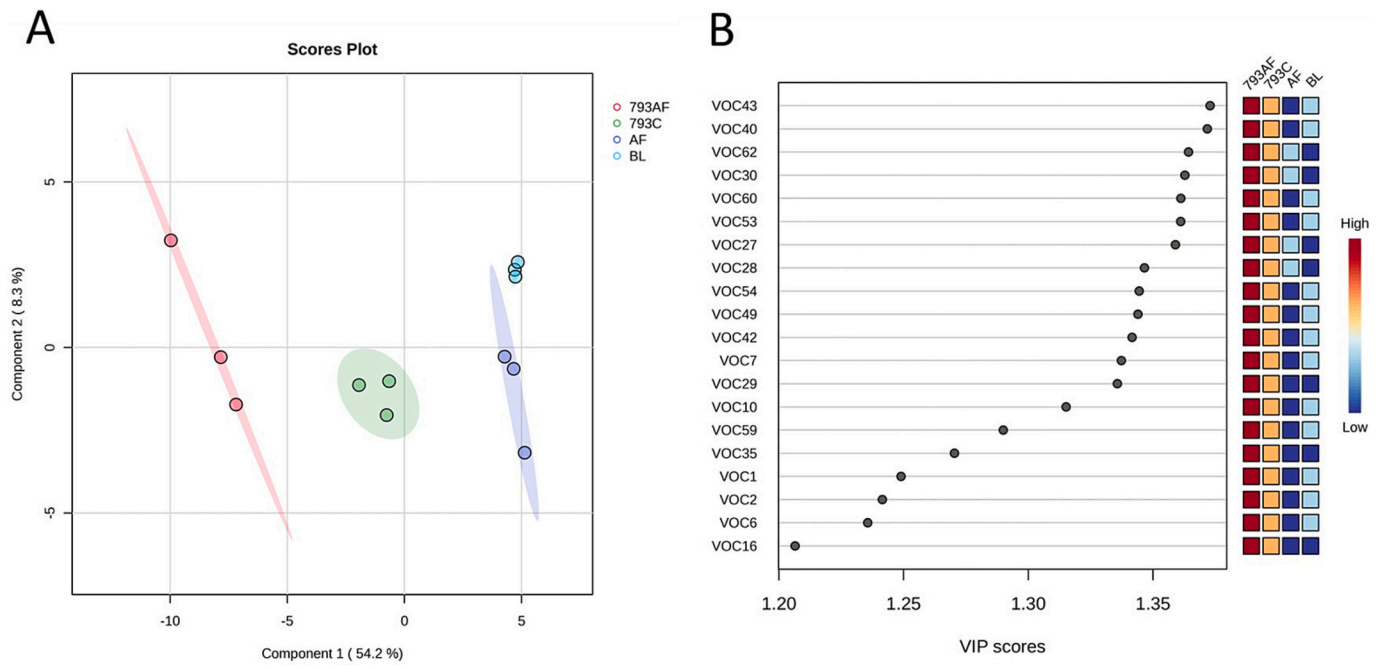

Fig. 4. Partial Least Squares Discriminant Analysis (PLS-DA) of the *H. uvarum* L793 experiment. (A) Score plot showing sample distribution across components 1 and 2. The plot shows the distribution of samples: *M. pulcherrima* L672 with *A. flavus* M144 (672AF), *M. pulcherrima* L672 control (672C), *A. flavus* M144 control (AF), and dried fig agar blank (BL). (B) The top 20 most influential variables ranked by their Variable Importance in Projection (VIP) scores.

yeasts reduced the mycelial biomass of *A. flavus* M144 by approximately 94%, demonstrating a potent inhibitory effect on its growth.

Regarding aflatoxin production, several studies have reported that VOCs emitted by different antagonistic yeasts can significantly reduce the synthesis of these mycotoxins by *A. flavus*. In a study conducted with *C. nivariensis*, a 74.8% decrease in aflatoxin production in corn kernels was observed after exposure of *A. flavus* to its VOCs, where 1-pentanol could be linked to the inhibitory effect (Jaibangyang et al., 2020). Similarly, *S. cerevisiae* and other soil yeasts reduced AFB_1 production by up to 99% under *in vitro* conditions, also causing structural alterations in the fungal mycelium (Natarajan et al., 2022). Furthermore, the genera *Metschnikowia* and *Hanseniaspora* have also demonstrated high efficacy in reducing aflatoxins. Dikmetas et al. (2023) reported that *Metschnikowia* aff. *pulcherrima* DN-HS reduced AFB_1 production from 536.74 μg kg^{-1} to 333.01 μg kg^{-1} *in vivo* in hazelnuts. Similarly, *Hanseniaspora opuntiae* L479 and *H. uvarum* L793 significantly decreased *in vitro* the synthesis of AFB_1 and AFB_2, in addition to inhibiting the expression of the *aflR* gene, which is essential in regulating their biosynthesis (Tejero et al., 2021). These findings are consistent with those obtained in the present study, in which *H. uvarum* L793 reduced the synthesis of AFB_1 and AFB_2 to below the LOD, while *M. pulcherrima* L672 induced a partial reduction of both mycotoxins, although the reduction of AFB_2 was not statistically significant. Regarding the relatively large standard deviations observed in the quantification of AFB_1 and AFB_2 in the control group, it should be noted that such variability is common in mycotoxin production assays. Aflatoxin biosynthesis is a secondary metabolic process highly sensitive to subtle micro-environmental fluctuations and the heterogeneous nature of fungal growth on solid substrates. In particular, the growth and colonization of *A. flavus* on the substrate may be spatially heterogeneous, leading to uneven biomass distribution and localized toxin-producing microenvironments. Additionally, small-scale differences in nutrient availability, oxygen diffusion, and moisture across the substrate may further contribute to variability in aflatoxin production. Consequently, significant variability between replicates is frequently observed even under strictly controlled conditions, a behaviour previously reported in similar studies (Møller et al., 2021; Tejero et al., 2021).

Furthermore, to determine whether the observed reduction in aflatoxins was merely a consequence of growth inhibition or a direct effect on the biosynthetic pathway, the toxigenic capacity was calculated and expressed as μg of aflatoxin per gram of mycelial fresh weight, thereby normalizing toxin production to fungal biomass. In the control treatment, the toxigenic capacity reached 335.03 μg/g for AFB_1 and 5.46 μg/g for AFB_2. In contrast, for the *M. pulcherrima* L672 treatment, these values were drastically reduced to 1.10 μg/g (AFB_1) and 0.03 μg/g (AFB_2). These results suggest that yeast VOCs may affect aflatoxin biosynthesis, as the reduction in toxin levels appeared to be notably higher than the observed 94–95% reduction in fungal growth. This is consistent with previous findings suggesting that microbial VOCs may influence the expression of key genes in the aflatoxin gene cluster, such as *aflR,* as reported by Tejero et al. (2021), although this mechanism was not directly assessed in the present study.

Regarding the volatile profile of *M. pulcherrima* L672, it was observed that the predominant compounds belonged mainly to the alcohol and ester families. This trend is in agreement with the literature, which indicates that these groups are characteristic of the volatile metabolism of this species (Contarino et al., 2019; Coppola et al., 2025). In addition, several compounds also reported in other studies were identified, including 3-methylbutan-1-ol, 2-phenylethanol, ethanol, and benzyl alcohol, which were identified as statistically important variables contributing to the discrimination of the 672AF treatment, and 3-hydroxybutan-2-one, a key discriminator for the 672C treatment (Contarino et al., 2019; Li et al., 2025; Yang et al., 2021). Although the individual activity of these volatiles was not tested using pure standards in this work, some of these compounds have been previously reported to exhibit antifungal properties in other systems (Li et al., 2025; Wu et al., 2024; Zou et al., 2022; Sampaolesi et al., 2024). However, in the present study, their role should be interpreted as contributing to treatment-group discrimination rather than as direct evidence of antifungal or anti-aflatoxigenic activity. In addition, other less abundant volatile compounds were identified in the volatilome of *M. pulcherrima* L672 when co-cultured with *A. flavus* M144 that could contribute to the overall antifungal activity, such as (*E*)-oct-2-enal (Luo et al., 2023). The combined effect of these compounds within the natural volatile blend

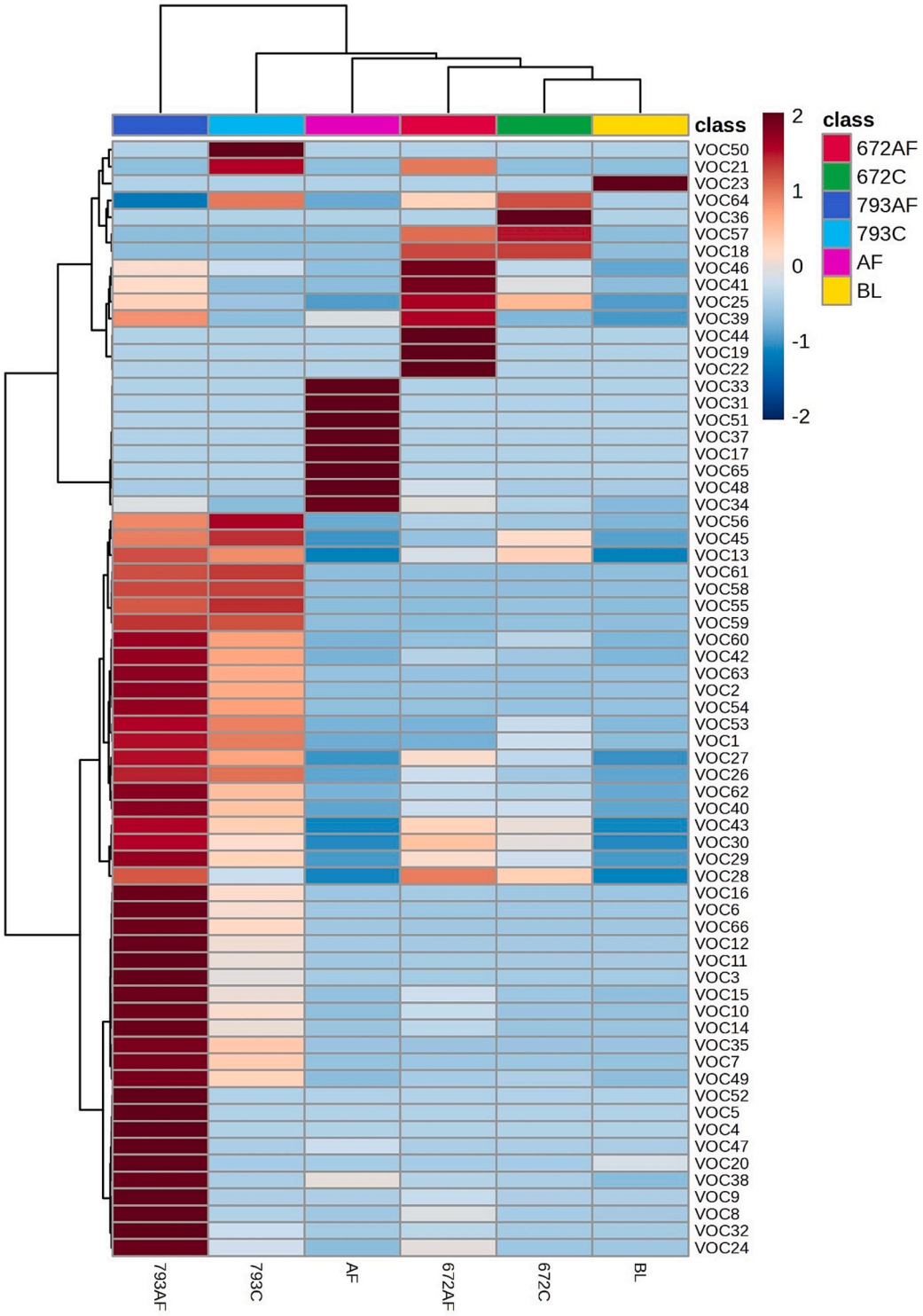

Fig. 5. Hierarchical clustering heatmap of the identified volatile organic compounds. The heatmap visualises the profiles of all experimental treatments: *H. uvarum* L793 with *A. flavus* M144 (793AF), *H. uvarum* L793 control (793C), *M. pulcherrima* L672 with *A. flavus* M144 (672AF), *M. pulcherrima* L672 control (672C), *A. flavus* M144 control (AF), and the dried fig agar blank (BL). The relative abundance of each compound across the samples is represented by the colour scale (blue: low abundance; red: high abundance).

may help explain the high inhibitory rates observed in the fig-based medium.

Similarly, the results obtained in this study confirm that *H. uvarum* L793 is a yeast with a remarkable capacity to produce a wide range of VOCs, mainly esters and alcohols (Table 3). Many of the compounds in this volatile profile are consistent with those described in previous studies, including 2-phenylethanol, 3-methylbutan-1-ol, ethanol, benzyl alcohol, ethyl acetate, 2-phenylethyl acetate, and ethyl octanoate (Qin et al., 2017; Tejero et al., 2021). Among these, 2-phenylethanol was particularly prominent, a compound whose antifungal activity has been previously discussed (Wu et al., 2024; Zou et al., 2022). Other notable compounds included 3-methylbutan-1-ol. Zou et al. (2023) noted that

the pure compound showed antifungal effects against the growth of *B. cinerea* mycelium. This compound, along with benzyl alcohol and 2-phenylethyl acetate, also has known antifungal activity (Sampaolesi et al., 2024). Galván et al. (2022) reported that the pure compound 2-phenylethyl acetate exhibited strong antifungal activity against *A. flavus* and *Aspergillus niger* under both *in vitro* and *in vivo* conditions. All these compounds were detected mainly in the 793AF treatment, where they were identified as discriminatory variables in the PLS-DA model. However, no additional validation (*e.g.*, cross-validation or permutation testing) was performed, and thus these results should be interpreted with caution. It should be noted that VIP scores derived from PLS-DA do not necessarily reflect a functional contribution, as high VIP values indicate statistical relevance for group discrimination rather than biological causality. Accordingly, while these scores identify the compounds most responsible for the differences between treatments, they do not provide direct evidence of their individual antifungal roles. In this study, validation with pure compounds or reconstructed blends was not performed; however, the potential contribution of these markers is discussed based on their well-documented bioactivity in the literature. For instance, some of the compounds included among the 20 most influential VIP variables, such as ethyl 9-hexadecenoate or 3-hydroxybutan-2-one, have not been reported in the literature to exhibit antifungal activity, to our knowledge, suggesting that they may act as metabolic markers of the interaction rather than active inhibitory agents.

A compelling correlation can be drawn between the specific VOC profiles of the co-cultures and the observed biological activities. While both yeasts produced a potent blend of antifungal VOCs sufficient to cause a near-complete inhibition of mycelial growth (~94%), the stark difference in anti-aflatoxigenic efficacy appears to be linked to the specific composition of their volatilomes. *H. uvarum* L793, which reduced aflatoxin synthesis to below the LOD, generated a significantly more complex and abundant volatile profile in the presence of the fungus (Fig. 5). This suggests that a combined effect of multiple compounds, including both high-abundance volatiles and key statistical discriminators like 2-phenylethanol, benzoic acid, and various esters, may be associated with altered aflatoxin biosynthesis, beyond simply inhibiting fungal growth. Conversely, while the VOCs from *M. pulcherrima* L672 were effective at limiting growth, their specific composition and concentration may have been less effective against the molecular mechanisms governing aflatoxin production.

These results demonstrate that *M. pulcherrima* L672 and *H. uvarum* L793 produce antifungal volatile compounds against *A. flavus* M144. The differences observed between the volatile profiles of both yeasts suggest that the nature and quantity of the VOCs emitted are strain-dependent. Furthermore, it was observed that volatile profiles changed depending on the presence or absence of *A. flavus* M144 (Fig. 5). This suggests that the generation of VOCs is a dynamic response to the fungal competitor rather than a static process. This microbial interaction causes the yeasts to undergo a metabolic change, perhaps as a defence or competitive mechanism, which increases the synthesis of specific antifungal compounds. This phenomenon was also observed by Ribeiro et al. (2023), who found that the co-culture of *S. cerevisiae* and *Rhodotorula mucilaginosa* with *Aspergillus carbonarius* led to the production of VOCs that were not detected in individual cultures.

Regarding the specific mechanisms of action, it is important to highlight that the use of a cellophane membrane, while preventing direct physical contact and mycelial penetration between the yeast and the pathogen, allows for the free diffusion of volatile organic compounds (VOCs), as well as secreted enzymes and other soluble metabolites (Liu et al., 2010; Silva-Campos et al., 2022). Therefore, although VOCs are likely the primary drivers of inhibition in this study, other potential complementary processes may be involved. For *H. uvarum* L793, in addition to its complex volatile profile, characterized by a higher diversity of alcohols and esters, frequently associated with fungal growth inhibition and alterations in mycelial morphology (Zhao et al., 2022), the secretion of diffusible non-volatile antifungal metabolites has also been reported (Chen et al., 2025). Such compounds could potentially pass through the cellophane membrane used in our experimental system and may contribute to the observed inhibitory effect (Silva-Campos et al., 2022).

In the case of *M. pulcherrima* L672, its antagonistic activity could also involve multiple hypothetical mechanisms. While not experimentally verified in this work, the potential role of competition for essential nutrients, particularly iron through the production of pulcherrimin, is a well-documented trait of this species (Liu et al., 2025a; Wang et al., 2023). This mechanism could hypothetically limit the availability of iron for *A. flavus* M144, thereby potentially hindering its development and aflatoxin production. Furthermore, the release of extracellular enzymes capable of affecting pathogen development has also been suggested for *M. pulcherrima* (Liu et al., 2025b). These diffusible enzymes or metabolites could potentially migrate across the cellophane membrane and participate in the overall antagonism. Other mechanisms, such as direct parasitism of pathogen hyphae (Ruiz-Moyano et al., 2020), appear less likely under the present experimental conditions due to the physical separation between yeasts and the pathogen. Overall, while VOCs play a major role under the conditions tested, the possible contribution of diffusible metabolites, extracellular enzymes, and nutrient competition, as described in the literature for these species, cannot be entirely ruled out and warrants further investigation.

5. Conclusions

In conclusion, both *M. pulcherrima* L672 and *H. uvarum* L793 demonstrated potent fungistatic activity, effectively inhibiting the mycelial growth of *A. flavus* by over 94%. Both yeasts also exhibited significant anti-aflatoxigenic activity, although *H. uvarum* L793 proved exceptionally effective by reducing aflatoxin synthesis to below the LOD. This distinction in efficacy appeared to be associated with their volatile profiles; *H. uvarum* L793 produced a more complex and abundant blend of VOCs during its interaction with the pathogen. While *H. uvarum* L793 appears to be the superior agent for mycotoxin control in this system, the substantial inhibition achieved by *M. pulcherrima* L672 confirms its potential and warrants further investigation. These findings highlight that the specific composition of the volatilome is critical for determining anti-mycotoxigenic efficacy.

CRediT authorship contribution statement

Paula Tejero: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Raquel Torrijos:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Guillem Campmajó:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Ana Martínez:** Writing – review & editing, Investigation, Formal analysis. **Alejandro Hernández:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization. **Alberto Martín:** Writing – review & editing, Visualization, Funding acquisition. **Alicia Rodríguez:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition, Data curation, Conceptualization. **Chiara Dall'Asta:** Writing – review & editing, Visualization, Supervision, Resources, Project administration, Conceptualization.

Declaration of competing interest

Given her role as Editor of International Journal of Food Microbiology, Dr. Alicia Rodríguez had no involvement in the peer review of this article and had no access to information regarding its peer review. Full responsibility for the editorial process for this article was delegated to another journal editor. The other authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2026.111852.

Data availability

Data will be made available on request.

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