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Bioactive volatiles of brewer's yeasts: Antifungal action of compounds produced during wort fermentation on *Aspergillus* sp.

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ABSTRACT

Previous investigations proved the potential of *Saccharomyces cerevisiae* MBELGA62 and *Pichia kudriavzevii* MBELGA61 as suitable biocontrolling agents against *Aspergillus* sp. through the production of soluble and volatile bioactive antifungal compounds. The present study delves into those finding by means of the identification of the volatile compounds produced by brewer's strains that demonstrated fungistatic and fungicidal effects against *Aspergillus flavus* and *A. parasiticus* when cultured in brewer's wort agar plates. Traditional brewer's yeasts such as *S. cerevisiae* MBELGA62 and *Saccharomyces pastorianus* SAFS235 synthetize volatiles that fully inhibited mycelial development for up to 9 days at 30 °C. The non-conventional brewer's strains *P. kudriavzevii* MBELGA61 and *Meyerozyma guilliermondii* MUS122 increased the lag phase by >100% and significantly reduced the fungal growth rate by 27.5–43.0% and 15.4–31.4%, respectively. In this context, 2-phenylethanol, 2-phenylethyl acetate and benzyl alcohol were identified as the main antifungal agents involved in *Aspergillus* sp.'s inhibition.

1. Introduction

Aspergillus sp. are widespread fungi known for its ability to produce mycotoxins, which are highly detrimental to human health and of concern for food safety. Cereal-based food and beverages are especially prone to mycotoxins contamination. Among these toxins, the aflatoxin B_1 (AFB_1) is classified by the International Agency for Research on Cancer of the World Health Organization within the most harmful human carcinogens of the Group 1 (IARC, 2012). AFB_1 is a thermostable toxin mainly synthetized by *Aspergillus flavus* and *A. parasiticus*, that does not undergo degradation or denaturation by ordinary thermal methods used in the manufacture of food and beverages.

Due to its difficult removal, the strategies to avoid mycotoxins contamination are rather design to prevent the dissemination of *Aspergillus* sp. in food matrices, thus bypassing the production of the detrimental toxins (Muccilli and Restuccia, 2015). Nevertheless, the investigations devoted to the biocontrol of *Aspergillus* sp. and biodetoxification of its metabolites by applying antagonist microorganisms have recently gained attention. A variety of yeast genera are suitable to counteract fungi contamination through different mechanisms, such as the competition for nutrients of the niche; changes in the pH of the medium induced by the secretion of organic acids; production and tolerance to high concentrations of ethanol (Saini et al., 2018); production of antifungal compounds; inhibition of mycotoxins expression (Hua et al., 2014) and degradation or adsorption of mycotoxins (Xu et al., 2022; Nathanail et al., 2016). The production of bioactive compounds that antagonize fungi through different cellular targets is a well-studied property of probiotic yeasts.

Furthermore, the synthesis of enzymes and metabolites with nutritional, sensory, technological and functional impact is one of the greatest potentials of probiotic yeasts to enhance techno-functional characteristics of food and beverages (Sadeghi et al., 2022). An

Abbreviations: VOCs, volatile organic compounds; DDS, double-dish system; PDA, potato-dextrose agar; PD, potato-dextrose broth; GC-FID, gas chromatograph coupled to flame ionization detector; ANOVA, analysis of variance; 2PE, 2-phenyl ethanol; 2PEA, 2-phenylethyl acetate; BA, benzyl alcohol..

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outstanding case is the production of the volatile 2-phenylethanol, that improves the organoleptic side of food with its rose scent, provides antioxidant properties (Wilson et al., 2022) and, in addition, acts as a bioactive compound against fungal and mycotoxin contamination (Hua et al., 2014). The isoamyl alcohol and 2-methylbutyl alcohol were also identified as biocontrol VOCs for the development of *A. flavus* (Tejero et al., 2021), while the ester of the former, the isoamyl acetate, with banana aroma, proved to have a fungistatic effect on *A. brasiliensis* (Ando et al., 2012).

In this sense, brewer's yeasts are a suitable source for the prospection of probiotic strains not deeply investigated. Interestingly, a brewer's *S. cerevisiae* strain was the first one in which mycocins, defined as soluble secreted bioactive compounds that antagonizes fungi and other yeasts, were identified (Sampaolesi et al., 2022). However, research efforts have focused on the technological and organoleptic traits that these microorganisms can supply to fermented food and beverages (Gibson et al., 2017), rather than on their functional properties. Among the few reports on the latter, Campagnollo et al. (2015) and Bovo et al. (2015) demonstrated the capacity of inactivated brewer's yeasts, recently named as postbiotics, to bind different mycotoxins, such as AFB_1. Oliveira et al. (2022) thoroughly reviewed the applications of spent brewer's yeasts as a source of bioactive peptides with antihypertensive, antioxidant and antimicrobial properties. Previous studies of some of us determined that brewer's strains *S. cerevisiae* MBELGA62 and *P. kudriavzevii* MBELGA61 secrete soluble and volatile bioactive substances that antagonizes *Aspergillus* sp. and the germination of its conidia (Sampaolesi et al., 2022).

The present investigation provides further information concerning the chemical nature of the volatile bioactive compounds produced by brewer's yeasts and the minimum amounts at which they are effective against *Aspergillus* sp. Additionally, this contribution introduces the synergic interaction between the volatiles that enhances their antifungal action.

2. Materials and methods

2.1. Microbial strains and culture conditions

Aspergillus flavus CECT 20802 and *Aspergillus parasiticus* CECT 2681 were kindly provided by Professor Pedro Vicente Martínez Culebra (Instituto de Agroquímica y Tecnología de Alimentos, IATA-CSIC) and conserved in potato-dextrose agar plates (PDA, BD Difco™, USA) at 4 °C. Prior to experiments, fungi were cultured in PDA slants for 7 days at 30 °C to induce sporulation.

The yeasts used in this study were isolated from the beer fermentation residue known as yeasts' sludge. They were identified as *Saccharomyces cerevisiae* MBELGA62, *Pichia kudriavzevii* MBELGA61 and *Meyerozyma guilliermondii* MUS122 (Sampaolesi et al., 2023) and preserved in the collection of the Laboratory of Microbiology of the National University of La Plata (Buenos Aires, Argentina). Also, *Saccharomyces pastorianus* SAFS235 was recovered from a dry commercial starter (Fermentis, France), which was re-hydrated in NaCl 0.9% w/v for 30 min at 30 °C before the isolation steps.

The isolates were maintained in YPD agar plates (yeast extract 10 g/L; peptone 20 g/L; dextrose 20 g/L; agar agar 20 g/L) at 4 °C. Overnight pre-cultures of the yeasts were made in YPD broth at 28 °C, in preparation for further assays.

2.2. Wort micro-fermentations at laboratory scale

The brewer's wort was made according to Sampaolesi et al. (2023). Briefly, 90.0 g of powder malt extract for blonde beer styles (Brewferm, Belgium) was solubilized in distilled water to a final volume of 500.0 mL, added with 0.25 mg/mL of $ZnSO_4$ (Sigma Aldrich, Merck) and pH adjusted to 4.5 ± 0.1 with lactic acid before undergo to steam sterilization at 110 °C for 15 min. Later, it was filtrated to eliminate the sedimented protein and the supernatant was fitted to a final sugar concentration of 17.3°Bx. Finally, 417 μL/L of sterile isomerized hop extract was added.

Fermentations were carried out in 100 mL bottles with 70.0 mL of the sterile brewing wort, inoculated with 2×10^6 cells/mL. For accurate seeding, cells in the yeast pre-cultures were quantified in the Muse® Cell Analyzer (Merck Millipore, Germany). The bottles were capped with sterilized Müller valves and incubated at 20 °C under stirring to simulate aeration. The weight loss was monitored until achieving a constant value (Pérez-Través et al., 2014). After the end of fermentation, yeast cells were harvested by centrifugation and the supernatants were analyzed immediately or stored at −20 °C. The experiments were carried out in triplicate. Sterile wort controls and positive fermentation controls inoculated with SafaleT-58 (Fermentis) were included.

2.3. Double-dish system for the study of the antifungal action of yeasts' volatiles

The experiments were carried out following the protocols published by Vero et al. (2013), with modifications. *Aspergillus* sp.'s suspensions of 10^4 conidia/mL were obtained from sporulated PDA slants, dispersed in "spore solution" (sodium lauryl sulfate 0.1 g/L; glucose 10.0 g/L) and quantified using a Neubauer's chamber.

PDA plates were inoculated in the center with 10 μL of the conidia suspension. A brewer's wort agar plate (brewer's wort of 17.3°Bx, pH 4.48 ± 0.01, added with 20.0 g/L of agar agar) was seeded with 100 μL of an overnight YPD broth culture to obtain a yeast grass. Under a sterile environment, the plates were face to each other and sealed with Parafilm®M (Marienfeld Superior, Germany), preventing the loss of the volatile compounds during incubation at 30 °C.

The incubation was carried out placing the double-dish system (DDSs from now on) with the *Aspergillus* sp.'s plate facing up, avoiding conidia dispersion and the direct contact between yeasts and fungi (the DDS' set up is illustrated in the Fig. S1 of the Supplementary Material). In addition, positive fungal growth controls without yeasts and controls with yeasts/without fungi were included. Treatments and controls were made in triplicate.

The diameter of fungal mycelium in all DDSs was monitored during incubation until the positive controls reached the edge of the plates. Curves of mycelia diameter (cm) vs time (hours) were used to calculate the fungal growth parameters. The growth rate, v_{max} (mm/h), was determined as the slope of the linear portion of the curve, and the lag phase (h) was computed as the intersection between the extrapolation of the linear portion of the curve and the abscissa axis.

2.4. Gas chromatography analysis and identification of volatile compounds

The protocol described by Tejero et al. (2021) was followed to identify the volatile organic compounds (VOCs, from now on), with modifications. A 100-μm thick fiber coated with polydimethylsiloxane (Supelco, Bellefonte, USA) was used for the extraction of volatiles by solid-phase microextraction (SPME) from DDSs after 6 days of incubation. The fiber was exposed for 30 min to the micro-atmosphere of the DDSs at 37 °C before injection.

A Thermo Science TRACE GC Ultra gas chromatograph equipped with a flame ionization detector (FID) was used (Thermo Fisher Scientific, Waltham, MA). The analysis of the compounds was carried out on a 30 m × 0.25 mm x 0.25 μm HP-INNOWax capillary column, coated with a layer of cross-linked polyethylene glycol (Agilent, Santa Clara, USA). Carrier gas was helium (flow 1 mL/min), and the oven temperature program was: 5 min at 50 °C, 1.5 °C/min to 100 °C, 3 °C/min to 215 °C and 2 min at 215 °C. The temperatures of the detector and the injector were 280 °C and 250 °C, respectively. The samples were injected in split less conditions. The chromatographic signal was recorded by the ChromQuest programme. The VOCs produced by yeasts were assessed

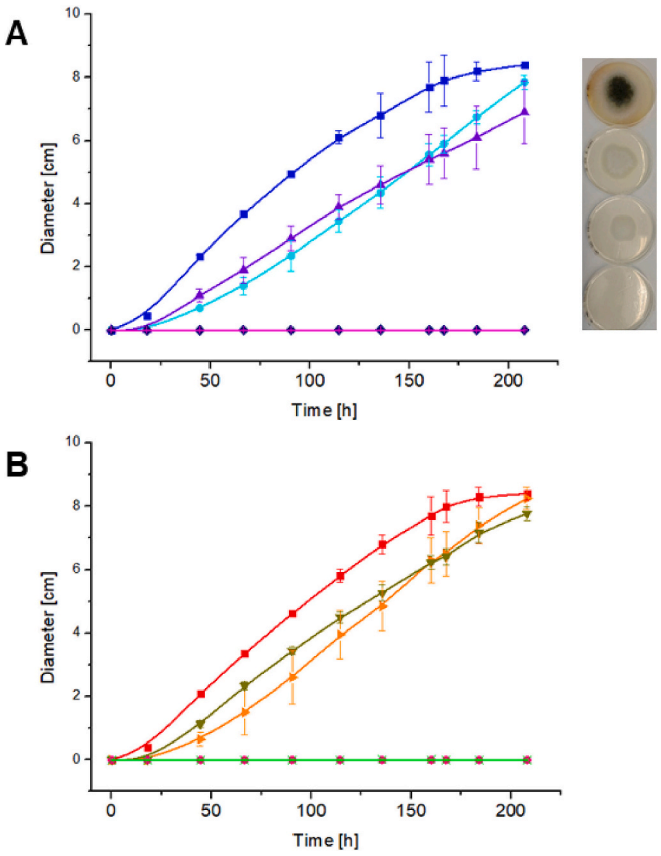

Fig. 1. Fungal mycelium diameter [cm] versus time [h]. A. ■ *A. parasiticus* CECT 2681 (Ap). ● *P. kudriavzevii* MBELGA61 + *Ap*. ▲ *M. guilliermondii* MUS122 + *Ap*. ♦ *S. pastorianus* SAFS235 + *Ap*. + *S. cerevisiae* MBELGA62 + *Ap*. Images of *A. parasiticus* CECT 2681's DDS assays after 9 days of incubation; each plate corresponds to the treatment illustrated on the left panel A. B. ■ *A. flavus* CECT 20802 (Af). ▶ *P. kudriavzevii* MBELGA61 + *Af*. ▼ *M. guilliermondii* MUS122 + *Af*. ● *S. pastorianus* SAFS235 + *Af*. x *S. cerevisiae* MBELGA62 + *Af*.

by comparison with the volatiles quantified in DDSs without yeast (only fungi) and sterile DDSs.

In addition, volatiles produced during wort micro-fermentations were quantified by SPME-GC-FID at 24 h, 48 h, 72 h and 144 h of incubation according to the methodology reported by Ortega et al. (2001). The TRACE GC Ultra gas chromatograph fitted with a TriPlus RSH autosampler (Thermo Fisher Scientific, Waltham, MA) was used. The GC column, carrier gas and flow set up remain the same. For sample preparation, 5.0 mL of supernatant was transferred into a 20.0 mL vial with 5.0 mL of saline solution (300.0 g of NaCl in 1.0 L of deionized water) and tightly closed with a PTFE septum plug. Volatile compounds were extracted via headspace solid phase microextraction technique using a 100 μm polydimethylsiloxane fiber (Supelco). Prior to injection, sample was incubated at 40 °C for 15 min to establish the headspace–liquid equilibrium. Then, the fiber was exposed for 30 min within the headspace of the vial, and desorbed 5 min into the GC-injection port at 250 °C in split less mode. The temperatures were set as described previously.

The identity of the peaks in the chromatograms was ascertained by comparison with chromatograms obtained from standard volatiles analyzed under the same conditions of the samples. The quantitative data were acquired by relating the integrated peak areas and the relative response factor obtained from the areas and concentrations of each standard compound dosed in a suitable matrix.

2.5. Evaluation of the antifungal effect of pure compounds by disc diffusion assay

The compounds identified by GC-FID in the yeasts' cultures were assayed following Hua et al. (2014) protocol, with modifications. PDA plates were surface seeded with suspensions of 10^5 conidia/mL of *Aspergillus* sp. to obtain a mycelium grass. Discs of sterile filter paper with a diameter of 10 mm were soaked with 1, 2 or 3 μL of a compound previously identified in the beer fermentations and placed over the inoculated PDA plates. Three discs were placed in each plate to make the technical replica. Control plates inoculated with conidia and sterile discs were included. All the plates were incubated for 24 h at 30 °C and the presence/absence of mycelium near to the embedded discs was registered.

Additionally, those substances that showed antifungal activity when studied individually, were tested combined by embedding them on a single paper disc to evaluate their interaction and simultaneous effect on *Aspergillus* sp. Controls of each pure compound were included for comparison purposes. For these experiments, the width of the inhibition halos was determined by measuring the distance between the edge of the paper disc and the end of the inhibition zone (absence of mycelium). To determine if there is a synergic, additive or neutralizing effect between the bioactive agents, the sum of the width of halos produced by each pure substance was compared with the measurement of the width of the halo obtained by the combined effect of the compounds applied to the same disc.

Furthermore, to evaluate the potential antifungal properties of beer made with these yeasts, the selected substances were tested at the concentrations quantified by GC-FID in the headspace of micro-fermentations of 144 h (as described in Section 2.3). All the experiments were made in triplicates.

2.6. Effect of the pure compounds on liquid cultures of Aspergillus sp.

Those compounds that showed an inhibitory effect on *Aspergillus* sp. in agar plates were tested in liquid cultures to establish their effect on fungal growth according to Hua et al. (2014). Cultures of the fungi were carried out in 100 mL Erlenmeyer flasks containing 40.0 mL of potato-dextrose broth (BD Difco™) seeded with *Aspergillus* sp.'s suspensions at a final concentration of 10^5 conidia/mL. The treatment flasks were inoculated with 0.5, 1.0, 2.0 or 3.0 μL/mL of the selected bioactive compounds. Positive controls were made without addition of the bioactive compounds. All the treatments were tested in triplicates. The incubation of the flasks was carried out at 30 °C with shaking (150 rpm) for 10 days or until fungal mycelium was observable.

In a next research stage, the bioactive substances were tested at the concentrations quantified by GC-FID in the headspace of micro-fermentations.

2.7. Statistical analysis

The chromatographic data and the kinetic parameters were analyzed with the Infostat software package (Universidad Nacional de Córdoba, Argentina) by one-way ANOVA and Tukey test for means comparison.

3. Results and discussion

This section discusses a sequence of experiments in order to obtain insights in the nature, antifungal properties and concentration-activity relationships of the volatile compounds generated by brewer's yeast. The rationale of the various assays is depicted in the scheme 1 for a better understanding of the ensuing discussions.

Table 1
Growth parameters of *Aspergillus* sp. in the presence of yeast's bioactive VOCs.

Treatment	Lag phase [h]	v_{max} [mm/h][a]	Percentage of reduction of v_{max}
Af	8.58 ± 1.65	0.56 ± 0.02	
Af + *Sc*	–	inc*	∞
Af + *Sp*	–	inc*	∞
Af + *Pk*	21.60 ± 1.54*	0.4 ± 0.1*	27.5 ± 17.8
Af + *Mg*	17.90 ± 1.43*	0.47 ± 0.01	15.4 ± 2.6
Ap	7.10 ± 1.55	0.58 ± 0.02	
Ap + *Sc*	–	inc*	∞
Ap + *Sp*	–	inc*	∞
Ap + *Pk*	19.99 ± 1.68*	0.33 ± 0.09*	43.0 ± 14.8
Ap + *Mg*	18.00 ± 1.64*	0.40 ± 0.04*	31.4 ± 7.6

The results are expressed as the average of three biological replicates ± standard deviation. inc: incomputable. *Af: Aspergillus flavus* CECT 20802. *Ap: Aspergillus parasiticus* CECT 2681. *Sc: S. cerevisiae* MBELGA62. *Sp: S. pastorianus* SAFS235. *Pk: P. kudriavzevii* MBALGA61. *Mg: M. guilliermondii* MUS122.
[a] Calculated as the slope of the curve in the first 114 h of incubation.
* Means that the parameter evaluated in the treatment is significantly different ($P < 0.05$) from the parameter determined in its respective control.

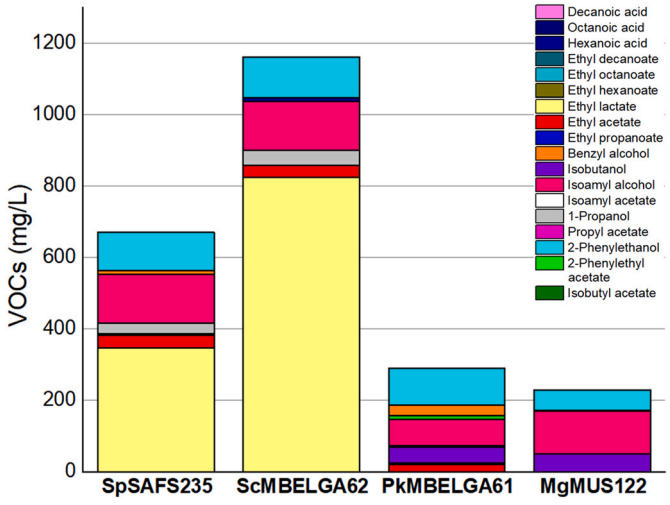

Fig. 2. VOCs detected in the DDSs by SPME-GC-FID analysis. The compounds are expressed in (mg/L) as the difference between the DDSs seeded with yeasts (without *Aspergillus* sp.) and those inoculated with fungi (without yeasts).

Table 2
Antifungal effect of pure organic compounds against *Aspergillus* sp.

Volatile compound	Amount of substance in the disk (μL/disc)					
	A. flavus CECT 20802			*A. parasiticus* CECT 2681		
	1.0	2.0	3.0	1.0	2.0	3.0
2-phenylethanol	–	–	++	–	–	++
2-phenylethyl acetate	+	++	+++	+	++	+++
Isoamyl alcohol	–	–	–	–	–	–
Isobutanol	–	–	–	–	–	–
Benzyl alcohol	–	–	+	–	–	+
1-propanol	–	–	–	–	–	–
Ethyl acetate	–	–	–	–	–	–
Ethyl hexanoate	–	–	–	–	–	–
Ethyl octanoate	–	–	–	–	–	–

(+) The plus sign indicates the observation of an inhibition halo around the embedded disc related to antifungal activity.
(−) The minus sing means that no inhibition zone of mycelium growth was detected.

3.1. Volatile organic compounds produced by brewer's yeast: Evidences of antifungal effect

The effect of the VOCs synthetized by brewer's strains on the growth of *Aspergillus* sp. was tested by exposing the fungi to the micro-atmosphere produced by the yeasts cultured in brewer's wort agar. The Fig. 1A and B shows the evolution of the fungal mycelium diameter of *A. parasiticus* CECT 2681 and *A. flavus* CECT 20802 in time. In addition, the figure contains an image of DDSs of *A. parasiticus* CECT 2681 after 9 days of incubation under the different treatments. The maximum growth rate, v_{max} and the lag phase computed from the curves are presented in the Table 1.

The growth of *Aspergillus* sp. was not detectable in the presence of VOCs produced by *S. cerevisiae* MBELGA62 and *S. pastorianus* SAFS235, meaning that those metabolites fully inhibited *Aspergillus* sp. for 9 days.

Furthermore, the VOCs produced by non-conventional brewer's strains significantly increased the fungal lag phase. In this context, Table 1 shows that the volatiles synthetized by *P. kudriavzevii* MBELGA61 increased 2.5 to 2.8-fold the lag phase. Similarly, *M. guilliermondii* MUS122's VOCs lead to an increment of 2.0 to 2.5-fold of this parameter. Regarding the fungal growth rate, it was significantly lowered by both non-*Saccharomyces* yeasts in the case of *A. parasiticus* CECT 2681, while for *A. flavus* CECT 20802, only *P. kudriavzevii* MBELGA61 displayed a significant reducing effect.

These results are significantly different from the findings published by some of us regarding DDS assays carried out with *S. cerevisiae* MBELGA62 and *P. kudriavzevii* MBELGA61 cultured in PDA (Sampaolesi et al., 2022). In that condition, the inhibitory properties of the VOCs produced by *P. kudriavzevii* MBELGA61 were more effective than those exerted by *S. cerevisiae* MBELGA62's bioactive volatiles, although none of them achieved full inhibition of *Aspergillus* sp. These observations led to the conclusion that the culture medium influences the nature of the antifungal bioactive volatiles produced by the yeasts. This behavior might be attributed to specific metabolic pathways boosted by the composition of each particular medium.

Additionally, all the treatments inhibited the generation of conidia, the reproductive structures of *Aspergillus* sp., preventing the spread of the fungi. The absence of the colorful conidia of *A. parasiticus* CECT 2681 can be verified in the image inside the Fig. 1.

Next, the nature of the VOCs generated in the DDSs was investigated. The Fig. 2 shows the identity and concentration of the VOCs detected in those assays. Throughout DDSs' incubation, the controls of *Aspergillus* sp. produced minimal quantities of 2-phenylethanol (2PE, from now on) (6.1 mg/L), 2-phenylethyl acetate (2PEA, hereinafter) (0.0103 mg/L) and decanoic acid (0.258 mg/L). Therefore, the concentration of VOCs in each treatment has been calculated as the difference between the DDSs seeded with the yeasts plus *Aspergillus* sp. and those inoculated only with fungi (without yeasts).

The results allowed the identification of a variety of substances, some of them already reported as antifungal bioactive compounds, such as 2PE (Hua et al., 2014; Tejero et al., 2021), 2PEA (Belinato et al., 2018), ethyl acetate (Tejero et al., 2021), octanoic acid (Ruiz-Moyano et al., 2020), hexanoic acid, isobutanol, 1-propanol, ethyl lactate, isoamyl alcohol and its acetate (Ando et al., 2012; Tejero et al., 2021) and propionic acid (Garnier et al., 2020), the precursor of ethyl propanoate.

The nature of the volatiles in the micro-atmosphere of the DDSs varied among the yeast species, as can be seen in Fig. 2. All the strains produced a large proportion of 2PE and isoamyl alcohol, both previously reported as antifungal agents (Hua et al., 2014; Tejero et al., 2021). *Saccharomyces* sp. strains synthetized ethyl lactate as a major product while ethyl acetate and 1-propanol were generated to a lesser extent. *S. cerevisiae* MBELGA62 also yielded low quantities of hexanoic acid (see Table S1 of the Supplementary Material), while *S. pastorianus* SAFS235 produced benzyl alcohol (BA, from now on). The highest proportion of BA was detected in *P. kudriavzevii* MBELGA61's micro-atmosphere, as well as isobutanol and a low amount of 2PEA. Under the same

Table 3
Evaluation of the effect of mixtures of bioactive substances against *Aspergillus* sp.

	Treatment	Inhibition zone width [mm]	
A. parasiticus CECT 2681	Control	–	
	2PEA [1 μL/disc]	1.8 ± 0.4	4.6 ± 0.9^{A}*
	2PE [3 μL/disc]	1.5 ± 0.7	
	BA [3 μL/disc]	1.3 ± 0.4	
	Mix[a]	8.5 ± 0.7^{B}	
A. flavus CECT 20802	Control	–	
	2PEA [1 μL/disc]	0.5 ± 0.7	6.3 ± 1.3^{A}*
	2PE [3 μL/disc]	2.8 ± 0.4	
	BA [3 μL/disc]	3.0 ± 1.0	
	Mix[a]	9.5 ± 0.7^{B}	

The results are expressed as the average of three biological replicates ± standard deviation.
2PEA: 2-phenylethyl acetate. 2PE: 2-phenylethanol. BA: benzyl alcohol.
Different superscript capital letters assigned to the result of adding the halos of the three substances and to the halo of the mix indicate that they are significantly different ($P < 0.05$).
[a] Mix: 1 μL of 2-phenylethyl acetate +3 μL of 2-phenylethanol +3 μL of benzyl alcohol per disc.
* The SD of the addition of the three individual measures was calculated as follows: $SD_{addition} = \sqrt{SD1^2 + SD2^2 + SD3^2}$.

conditions, *M. guilliermondii* MUS122 synthetized mostly isoamyl alcohol, 2PE and isobutanol. Other substances were detected in the DDSs in very little quantities, as can be seen in the Table S1 of the Supplementary Material.

3.2. Further insights on the antifungal action of brewer's yeasts volatiles

3.2.1. Identification of bioactive compounds among yeasts' volatiles: fungistatic dose and permanence

The antifungal impact of selected substances described above was further investigated using the disc diffusion assay. The rationale of the selection was based on the compounds which prevail in the volatile atmosphere of the DDSs, where the antifungal impact was endorsed. Besides, other exclusion criteria were applied, ruling out lactic acid for further evaluation. This compound has been discarded as an antifungal agent against *A. flavus* (Gourama and Bullerman, 1995) and *A. parasiticus* (Gamba et al., 2015). The authors reported that its effect on fungal growth would only be related to the reduction of the pH of the medium when the organic acid is solubilized and dissociated. As the present investigation intends to study the impact of the vapor state of the substances, undissociated lactic acid is not a sensible candidate.

Additionally, two ethyl esters of short-chain fatty acids found in trace quantities in the DDSs of *S. cerevisiae* MBELGA62 were included in the analysis, since short-chain fatty acids have been reported as bioactive antifungal agents with the capacity of disrupting fungal membranes (Bhattacharyya et al., 2020).

The Table 2 shows the effect of the selected compounds over the growth of *A. flavus* CECT 20802 and *A. parasiticus* CECT 2681. The results revealed that 2PEA developed the most intense antifungal activity against *Aspergillus* sp. In fact, only 2.0 μL of the pure substance added to the discs induces a clear inhibition halo. Moreover, an incipient antifungal effect was observed with discs poured with 1.0 μL (see Table 2). The 2PE also demonstrated an impact against *Aspergillus* sp., although less than its ester, since it required 3.0 μL/disc to develop a sharp inhibition halo. Both compounds were previously characterized as substances with antifungal properties (Belinato et al., 2018; Tejero et al., 2021). The large proportion of 2PE found in the volatile profile of every brewer's strain partially explain the antifungal impact observed in all the treatments (see Table 1). The higher amounts of the more powerful 2-phenylethyl acetate were quantified in *P. kudriavzevii* MBELGA61's treatments.

Remarkably, the benzyl alcohol, a well-known cell membrane fluidizer, showed a clear inhibition zone near to discs embedded with 3.0 μL of the pure substance, having a similar fungicidal capacity against *Aspergillus* sp. as 2-phenylethanol (see Table 2). This compound had a relevant presence in the micro-atmosphere developed by *P. kudriavzevii* MBELGA61 (Fig. 2).

It is clear that the effect of the bioactive compounds is strongly dependent on its concentration near to *Aspergillus* sp.'s conidia. Besides, the concentration in the gas phase in the micro-atmosphere of the DDSs is directly related with the vapor pressure of the substance at certain temperature. BA is the most volatile (vapor pressure of 12.5 Pa at 25 °C) among the bioactive compounds identified, followed by 2PE (vapor pressure of 11.57 Pa at 25 °C) (Daubert and Danner, 1989) and 2PEA (vapor pressure of 7.5 Pa at 25 °C) (Dykyj et al., 2000). Considering that the vaporized compounds could escape from the disc diffusion assay's plates and consequently reduce its concentration, it is expected that the more volatile the substances are, the shorter and less effective their impact on *Aspergillus* sp. will be. From one side, this observation correlates with the results of the disc diffusion assay presented in the Table 2, since BA is the most volatile and prone to escape of the antifungal VOCs identified during this investigation, meaning a prompt and briefer effect against *Aspergillus* sp.

On the other side, the 2PE is less volatile than BA and could exert a longer-lasting effect over fungi in the disc diffusion assays. Additionally, this volatile was produced in high concentrations by the yeasts, probably maintaining a constant concentration in the micro-atmosphere and a consequent continuous inhibition on fungal growth. Finally, the lower vapor pressure of the ester 2PEA may be related to a more prolonged antifungal effect of this compound. Nevertheless, the molecular mechanisms and fungal cell targets affected by each substance would also play a role on their effect.

Josselin et al. (2022) thoroughly reviewed the inhibition of *A. flavus* by organic volatiles, as well as its effect on aflatoxin synthesis. So far, research focused on the loss of fungal membrane integrity, which in turn affects regulation of the physiological functions of *A. flavus* when is exposed to some volatiles. However, the molecular mechanisms of inhibition and its gene targets remained little explored. Exposure to 2-phenylethanol, farnesol and hexanal, among others, caused shrinkage and detachment of the cell wall in the cytoplasm. Some research reported an alteration of the mitochondrial membrane, which became discontinuous or absent due to changes in their composition when exposed to 2PE, in addition to the down regulation of the mitochondrial dehydrogenases (Josselin et al., 2022). Hua et al. (2014) proved that 2-phenylethanol altered the expression patterns of chromatin and significantly down regulated the cluster of AF biosynthesis, while Galván et al. (2022) found the specific downregulation of *aflR* gene by 2PE. Is worth to notice that those genes are also involved in the regulation of sexual development, sclerotia formation and conidia programming (Caceres et al., 2020), which could explain the absence of conidia synthesis observed in both *Aspergillus* sp. strains challenged with non-conventional brewing yeasts' VOCs. Regarding 2PEA and BA, no research on the mechanisms involved in its effect on fungi has been published.

3.2.2. VOCs' interactions: synergic effect between the bioactive compounds of yeasts

The interactions between the VOCs identified as bioactive agents against *Aspergillus* sp. in the previous studies were thoroughly analyzed by the disc diffusion assay. Table 3 compares the width of the inhibition halos measured after overnight incubation with discs poured with 2-phenyl alcohol, 2-phenylethyl acetate or benzyl alcohol and discs embedded with mixtures of the three compounds together. A direct comparison of the value obtained by the addition of the width of halos produced by each pure substance and the measure of the width of the halo obtained with the mixture of the bioactive agents, led to the conclusion that a synergic interaction occurs between them, since the compounds acting together showed a significant more powerful antifungal activity ($P < 0.05$, Table 3).

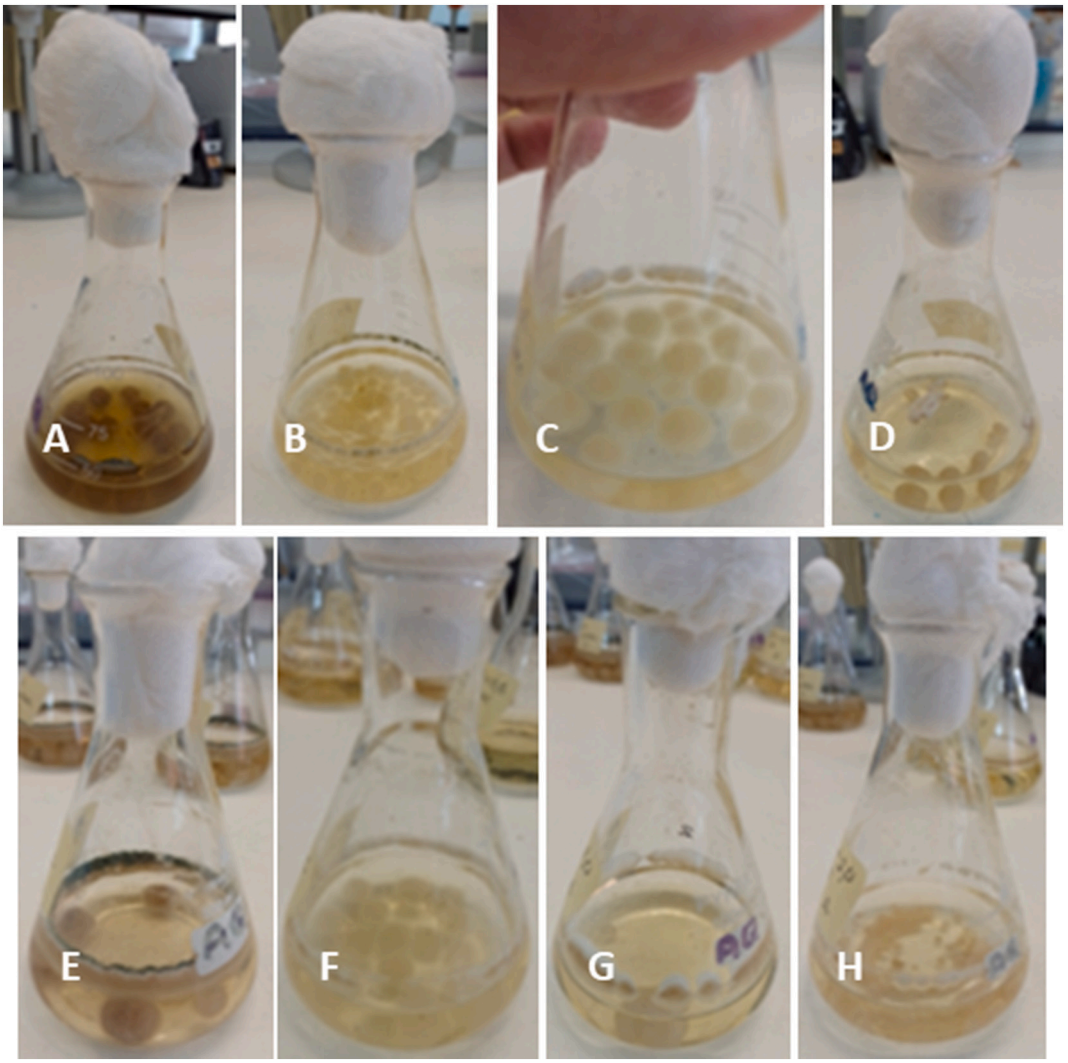

Fig. 3. PD broth cultures after 9 days of incubation. A. Control *A. parasiticus* CECT 2681. B. *A. parasiticus* + 0.5 μL/mL 2-phenylethyl acetate. C. *A. parasiticus* + 2.0 μL/mL benzyl alcohol. D. *A. parasiticus* + 2.0 μL/mL 2-phenylethanol. E. Control *A. flavus* CECT 20802. F. *A. flavus* + 0.5 μL/mL 2-phenylethyl acetate. G. *A. flavus* + 2.0 μL/mL benzyl alcohol. H. *A. flavus* + 2.0 μL/mL 2-phenylethanol.

3.3. Antifungal action of the bioactive compounds in liquid cultures of Aspergillus sp.

The antifungal effect of 2PE, 2PEA and BA in liquid medium was also investigated to gain insights of its action under conditions more similar to the actual beer production process. The Fig. 3 displays the results of the treatments after 9 days of incubation.

As a first step, those bioactive compounds were assayed at the minimal concentrations found effective in the paper discs diffusion assays discussed in the previous section. The pure bioactive substances in liquid cultures proved a fungistatic activity up to 48 h, while the controls developed a visible mycelium after 24 h of incubation. After that period, *Aspergillus* sp. started to grow at different rates depending on the bioactive agent applied. These observations differed from those registered in the DDS assays, in which the fungicidal effect of S*accharomyces* sp.' volatiles remain after 9 days of incubation (see Fig. 1). This difference can be explained by the fact that substances exert a fungistatic effect while they are present in the cultures at certain minimal concentration. Its volatilization and release through the cotton lid of the liquid cultures reduces the impact on *Aspergillus* sp., which in turn start to grow. Instead, in the DDS assays the presence of live yeasts that constantly produces VOCs and the layout of the experiment guarantees the maintenance of adequate concentrations of the bioactive compounds. Moreover, the synergy between the bioactive compounds (see Table 3) produced by the yeasts in the DDSs implies a stronger antifungal effect.

The antifungal effectiveness between the assayed bioactive compounds proved consistent, being the ester the most potent fungistatic agent. The reducing effect on fungal growth compared to the control was observed even after 9 days of incubation at 30 °C, as can be seen in the Figs. 3B and 3F. The experiment reveals that the alcohols exerted no significant reduction in the grow of *Aspergillus* sp. at concentrations bellow 2.0 μL/mL and no inhibition in the first 48 h of incubation at concentrations smaller than 3.0 μL/mL (data not shown). Finally, fungistatic concentrations in liquid medium against both *Aspergillus* strains were determined as 0.5 μL/mL for 2PEA, 3.0 μL/mL for 2PE and 3.0 μL/mL for BA.

3.4. Fermentation's volatiles: towards more complex and functional beer

At the final stage of the investigation, the nature and concentration of the VOCs produced during wort fermentations upon time was determined, to stablish if the antifungal action of the identified bioactive compounds could be a functional trait of beers brewed with these strains.

The Fig. 4 shows the time course of the headspace composition of

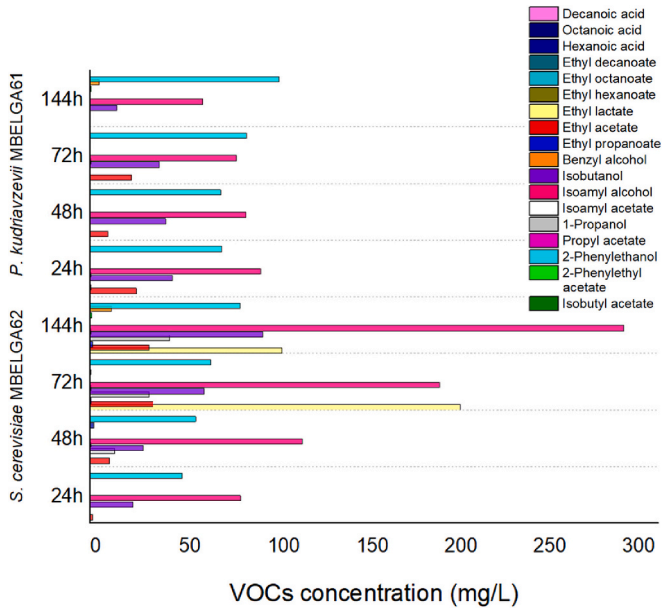

Fig. 4. Time course of VOC's concentration (mg/L) in the headspace of brewing fermentations carried out with *P. kudriavzevii* MBELGA61 and *S. cerevisiae* MBELGA62.

Table 4
Bioactive volatiles quantified in the headspace of 144-h-long wort fermentations.

Fermenting strain	Headspace concentration [mg/L]		
	Benzyl alcohol	2-phenyl ethanol	2-phenylethyl acetate
S. cerevisiae MBELGA62	12.0 ± 0,4	84.6 ± 3.3	0.8 ± 0.4
S. pastorianus SAFS235	10.2 ± 0.3	107.3 ± 5.7	0.5 ± 0.4
P. kudriavzevii MBELGA61	5.2205 ± 0.0007	106.3 ± 25.7	0.5 ± 0.3
M. guilliermondii MUS122	n.d.	58.1 ± 0.8	0.6 ± 0.5

N.d.: not detected.
The results are expressed as the average of two replicates ± standard deviation.

brewing fermentations carried out with the traditional brewer's strain *S. cerevisiae* MBELGA62 and a non-conventional yeast such as *P. kudriavzevii* MBELGA61. Fermentations carried out with *M. guilliermondii* MUS122 and *S. pastorianus* SAFS235 were only analyzed at the end point of incubation (144 h). The concentration in [mg/L] of BA, 2PE and 2PEA are informed in Table 4.

Analysis over time of brewing fermentations revealed that *S. cerevisiae* MBELGA62 produced larger quantities of volatiles than *P. kudriavzevii* MBELGA61 (see Fig. 4). From one side, *Pichia* showed a stable profile of VOCs throughout fermentation, composed mainly by 2PE, isoamyl alcohol, isobutanol and ethyl acetate, all substances previously described as antifungal agents (Ando et al., 2012; Hua et al., 2014; Tejero et al., 2021).

On the other side, the composition of *S. cerevisiae*'s fermentation headspace changed during incubation. In this context, isoamyl alcohol, 2PE, isobutanol and ethyl acetate appeared from the beginning, and the presence of 1-propanol became more important above 48 h. The intense signal of ethyl lactate detected at 72 h of incubation, significantly decreases at 144 h, as observed in Fig. 4. In contrast, the other volatiles increased continuously or maintained its concentration throughout the incubation.

Further, the antifungal effect of 2PEA and BA at the concentrations determined in the fermentations after 144 h of incubation (end point) was investigated by the disc diffusion assay, as can be seen in Fig. 5. It is worth noticing that all the four strains produced levels of 2PE that largely surpasses the minimal antifungal concentration determined previously, so this bioactive compound was not tested at the current research stage.

In this context, the experiments with BA were performed embedding the sterile discs with different volumes of the compound (i.e., 5.0, 9.8 and 11.5 μL), the corresponding volumes (density of BA at 25 °C: 1.044 mg/μL) to the concentrations found in the headspace of *P. kudriavzevii* MBELGA61, *S. pastorianus* SAFS235 and *S. cerevisiae* MBELGA62 fermentations, respectively. Regarding *M. guilliermondii* MUS122 fermentations, no BA was detected (Table 4), as in the DDS micro-atmosphere (see Table S1 of the Supplementary Material).

The 2PEA (density at 25 °C: 1.032 mg/μL), quantified in amounts smaller than the minimal inhibitory concentration ascertained (1.0 μL/disc, see Section 3.2.1), was also included in these experiments, using 0.5 (*P. kudriavzevii* MBELGA61 and *S. pastorianus* SAFS325), 0.6 (*M. guilliermondii* MUS122) and 0.8 μL/disc (*S. cerevisiae* MBELGA62) (see Table 4).

The treatments with 2PEA showed no antifungal activity, as expected (see Table 5 and Fig. 5C). However, the BA in the amounts yielded by *S. cerevisiae* MBELGA62 and *S. pastorianus* SAFS235 produced inhibition halos for both *Aspergillus* strains (see Table 5, Fig. 5A and 5B).

In addition, some of the bioactive compounds quantified in the headspace of brewing wort fermentations after 144 h of incubation were tested in liquid cultures of *Aspergillus* sp. Those concentrations correspond to 0.8 μL/mL for 2PEA, found in *S. cerevisiae* MBELGA62 fermentations; and 5.0 μL/mL or 9.8 μL/mL for BA, all surpassing the minimal fungistatic concentrations in liquid medium determined previously (0.5 μL/mL for the ester and 3.0 μL/mL for the alcohol, see Section 3.3). The Fig. 6 shows cultures of *A. flavus* CECT 20802 and *A. parasiticus* CECT 2681 after 6 days of incubation with those bioactive compounds at 30 °C.

The Fig. 6D to F evidences that none of the fungal species were able to grow in the presence of BA, regardless of the concentration. Similarly, the Fig. 6A shows that *A. flavus* CECT 20802 did not develop a visible mycelium when exposed to 0.8 μL/mL of 2PEA. However, *A. parasiticus* CECT 2681 grew to an observable stage under the action of the ester (see Fig. 6B). This remarkable observation indicates that the concentration of benzyl alcohol, although minimal in terms of its proportion in the total volatile profile of the brewer's yeasts, has a key role in the inhibition of *Aspergillus* sp. achieved by *S. cerevisiae* MBELGA62 and *S. pastorianus* SAFS235.

The fact that brewer's yeasts under actual fermentation conditions produce bioactive compounds in concentrations suitable to inhibit the growth of *Aspergillus* sp. is key evidence of their antifungal properties and the functional traits of the beer produced with them.

4. Conclusion

Traditional brewer's yeasts such as *S. cerevisiae* MBELGA62 and *S. pastorianus* SAFS235, along with the non-conventional brewer's strains *P. kudriavzevii* MBELGA61 and *M. guilliermondii* MUS122, produce a variety of volatile organic compounds during wort fermentation. The fungistatic effect of benzyl alcohol, 2-phenylethanol and 2-phenylethyl acetate (among the VOCs generated by the brewer's yeasts) against *A. flavus* and *A. parasiticus* was proved in this contribution.

The results of the DDS assays demonstrated that the *Saccharomyces* sp. strains cultured in barley wort agar synthetize volatile bioactive compounds that fully inhibit fungal development. Furthermore, *P. kudriavzevii* MBELGA61 and *M. guilliermondii* MUS122 significantly extended the lag phase and reduced the growth rate of *Aspergillus* sp. More important, the bioactive VOCs produced by all the brewer's yeasts prevented the production of conidia, the fungal reproductive structures, thus avoiding *Aspergillus* sp.'s dissemination.

Although a variety of volatiles were synthetized by the yeasts, the

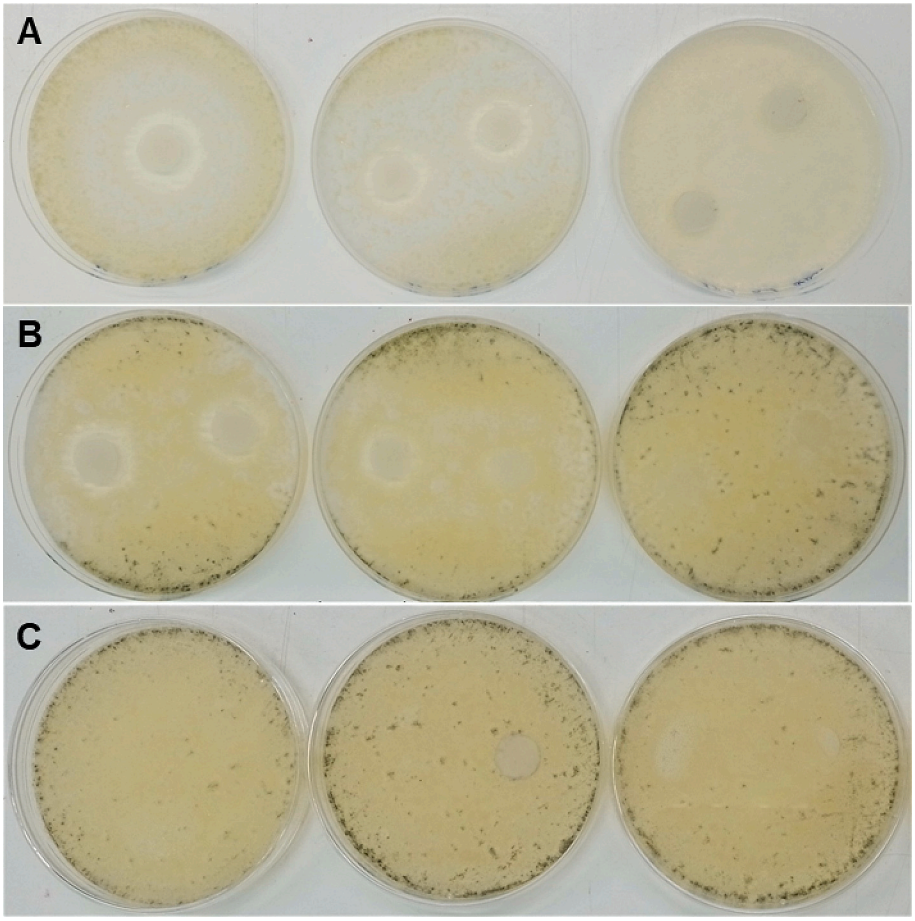

Fig. 5. Evaluation of bioactive agents in the concentrations detected in the headspace of micro-fermentations; disc diffusion assays after 48 h of incubation. A. *A. flavus* CECT 20802 with benzyl alcohol, from left to right: 11.5, 9.8 and 5.0 μL/disc. B. *A. parasiticus* CECT 2681 with benzyl alcohol, from left to right: 11.5, 9.8 and 5.0 μL/disc. C. *A. parasiticus* CECT 2681 with 2-phenylethyl acetate, from left to right: 0.8, 0.6 and 0.5 μL/disc.

Table 5
Disc diffusion assay for concentrations of the bioactive compounds determined in wort micro-fermentations.

Compound	Volume (μL/disc)	Inhibition zone width [mm][a]	
		A. flavus CECT 20802	*A. parasiticus* CECT 2681
2-phenylethyl acetate	0.5	n.o.	n.o.
	0.6	n.o.	n.o.
	0.8	n.o.	n.o.
Benzyl alcohol	5.0	n.o.	n.o.
	9.8	3.5 ± 0.7	3.0 ± 0.0
	11.5	6.0 ± 0.0	4.5 ± 0.7

The results are expressed as the average of three biological replicates ± standard deviation.
n.o.: no observable inhibition zone.
[a] All the inhibition halos were determined after 48 h of incubation.

first conclusion casted by these results is that the 2-phenylethanol is the main responsible of the impact on *Aspergillus* sp.' growth, since this VOC remain a major component of the micro atmosphere of all brewer's strains. Moreover, the amounts of this bioactive volatile quantified in the headspace of wort fermentations far surpasses the minimal antifungal concentration of the alcohol determined in the disc diffusion assays (3.0 μL/disc) and subsequent assays in liquid cultures (2.0 μL/mL).

Interestingly, the concentrations of benzyl alcohol determined in wort fermentations were suitable to inhibit the growth of *Aspergillus* sp. both in disc diffusion assays and in liquid cultures for 6 days, even though it was detected in very low quantities in the headspace of fermentations. These results evidences that benzyl alcohol is a significant antifungal agent in beers brewed with *Saccharomyces* sp. and *P. kudriavzevii* MBELGA61.

The ester 2-phenylethyl acetate, that is produced in very low quantities by the brewer's yeasts, proved effective to inhibit the growth of *A. flavus* but not *A. parasiticus* at those concentrations.

Finally, this investigation provides evidences that the three bioactive volatiles have a synergic impact when acting together. Then, the 2-phenylethyl acetate bioactive effect would be enhanced by the presence of 2-phenylethanol and benzyl alcohol in the volatile profile of brewer's strains.

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CRediT authorship contribution statement

Sofía Sampaolesi: Writing – original draft, Visualization, Investigation, Formal analysis, Conceptualization. **Laura Pérez-Través:** Writing – review & editing, Methodology, Investigation. **Laura E. Briand:** Funding acquisition, Conceptualization, Writing – review & editing. **Amparo Querol:** Funding acquisition, Project administration, Resources, Supervision, Writing – review & editing.

Declaration of competing interest

This manuscript has not been submitted to, nor is under review at,

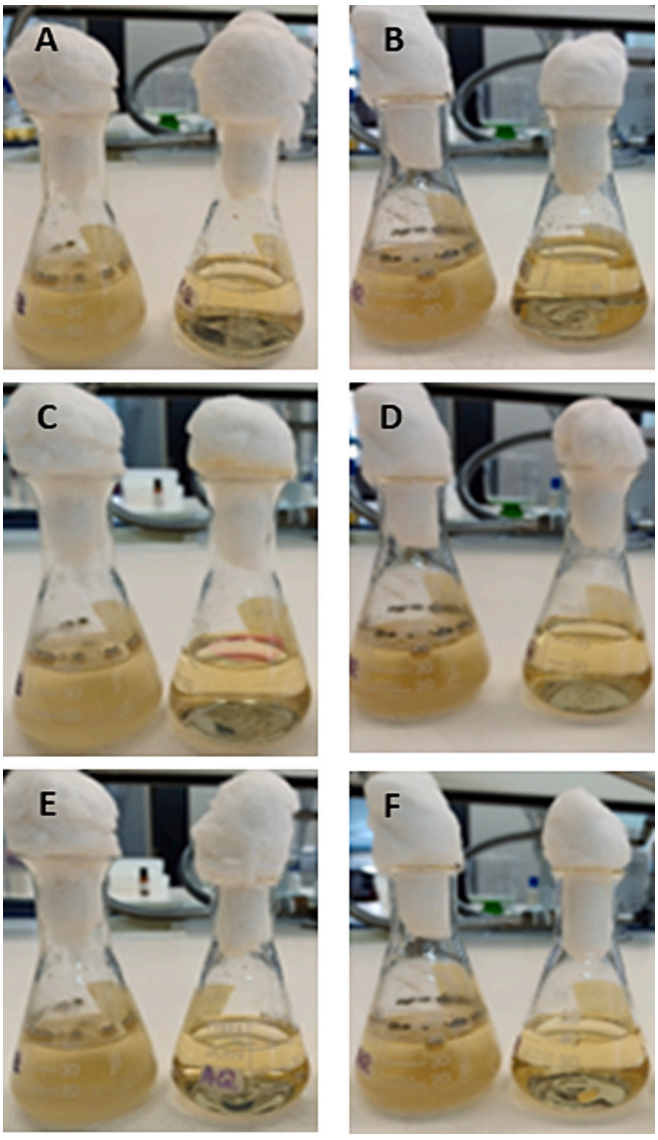

Fig. 6. Cultures of *A. flavus* CECT 20802 (left column) and *A. parasiticus* CECT 2681 (right column) with the bioactive compounds after 6 days of incubation at 30 °C. Each frame is a comparative picture with the respective positive control at the left of the treatment. First row (A, B): + 0.8 μL/mL 2-phenylethyl acetate. Second row (C, D): + 5.0 μL/mL benzyl alcohol. Third row (E, F): + 9.8 μL/mL benzyl alcohol.

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Data availability

Data will be made available on request.

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References

Ando, H., Hatanaka, K., Ohata, I., Yamashita-Kitaguchi, Y., Kurata, A., Kishimoto, N., 2012. Antifungal activities of volatile substances generated by yeast isolated from Iranian commercial cheese. Food Control 26 (2), 472–478. https://doi.org/10.1016/j.foodcont.2012.02.017.

Belinato, J.R., Kupper, K.C., Augusto, F., 2018. *In vivo* investigation of the volatile metabolome of anti-phytopathogenic yeast strains active against *Penicillium digitatum* using comprehensive two-dimensional gas chromatography and multivariate data analysis. Microchem. J. 141, 362–368. https://doi.org/10.1016/j.microc.2018.05.047.

Bhattacharyya, A., Sinha, M., Singh, H., Patel, R.S., Ghosh, S., Sardana, K., Ghosh, S., Sengupta, S., 2020. Mechanistic insight into the antifungal effects of a fatty acid derivative against drug-resistant fungal infections. Front. Microbiol. 11, Article 2116 https://doi.org/10.3389/fmicb.2020.02116.

Bovo, F., Franco, L.T., Rosim, R.E., Barbalho, R., Fernandes Oliveira, C.A., 2015. *In vitro* ability of beer fermentation residue and yeast-based products to bind aflatoxin B1. Braz. J. Microbiol. 46 (2), 577–581. https://doi.org/10.1590/S1517-838246220130400.

Caceres, I., Al Khoury, A., El Khoury, R., Lorber, S., Oswald, I.P., El Khoury, A., Atoui, A., Puel, O., Bailly, J.D., 2020. Aflatoxin biosynthesis and genetic regulation: a review. Toxins 12, 150.

Campagnollo, F.B., Franco, L.T., Rottinghaus, G.E., Kobashigawa, E., Ledoux, D.R., Daković, A., Oliveira, C.A.F., 2015. *In vitro* evaluation of the ability of beer fermentation residue containing *Saccharomyces cerevisiae* to bind mycotoxins. Food Res. Int. 77 (3), 643–648. https://doi.org/10.1016/j.foodres.2015.08.032.

Daubert, T.E., Danner, R.P., 1989. Physical and Thermodynamic Properties of Pure Chemicals Data Compilation. Taylor and Francis, Washington, D.C., USA.

Dykyj, J., Svoboda, J., Wilhoit, R.C., Frenkel, M., Hall, K.R., 2000. Organic compounds, C1 to C57. Part 2. In: Hall, K.R. (Ed.), Landolt-Börnstein – Group IV Physical Chemistry 20B (Vapor Pressure and Antoine Constants for Oxygen Containing Organic Compounds). Springer-Verlag, Heidelberg, Berlin, pp. 111–205. https://materials.springer.com/lb/docs/sm_lbs_978-3-540-49810-0_4 (Accessed on Dec 10, 2023).

Galván, A.I., Hernández, A., de Guía Córdoba, M., Martín, A., Serradilla, M.J., López-Corrales, M., Rodríguez, A., 2022. Control of toxigenic *Aspergillus* spp. in dried figs by volatile organic compounds (VOCs) from antagonistic yeasts. Int. J. Food Microbiol. 376, 109772 https://doi.org/10.1016/j.ijfoodmicro.2022.109772.

Gamba, R.R., Ni Colo, C., Correa, M., Astoreca, A.L., Alconada Magliano, T.M., et al., 2015. Antifungal activity against *Aspergillus parasiticus* of supernatants from whey permeates fermented with kefir grains. Adv. Microbiol. 5 (6), 479–492. https://doi.org/10.4236/aim.2015.56049.

Garnier, L., Penland, M., Thierry, A., Maillard, M.B., Jardin, J., Coton, M., Mounier, J., 2020. Antifungal activity of fermented dairy ingredients: identification of antifungal compounds. Int. J. Food Microbiol. 322, Article 108574 https://doi.org/10.1016/j.ijfoodmicro.2020.108574.

Gibson, B., Geertman, J.-M.A., Hittinger, C.T., Krogerus, K., Libkind, D., Louis, E.J., Magalhaes, F., Sampaio, J.P., 2017. New yeasts – new brews: modern approaches to brewing yeast design and development. FEMS Yeast Res. 17 (4), fox038. https://doi.org/10.1093/femsyr/fox038.

Gourama, H., Bullerman, L.B., 1995. Inhibition of growth and aflatoxin production of *Aspergillus flavus* by *Lactobacillus* species. J. Food Prot. 58 (11), 1249–1256. https://doi.org/10.4315/0362-028X-58.11.1249.

Hua, S.S.T., Beck, J.J., Sarreal, S.B.L., et al., 2014. The major volatile compound 2-phenylethanol from the biocontrol yeast, *Pichia anomala*, inhibits growth and expression of aflatoxin biosynthetic genes of *Aspergillus flavus*. Mycotoxin Res. 30, 71–78. https://doi.org/10.1007/s12550-014-0189-z.

IARC, International Agency for Research on Cancer – World Health Organization, 2012. Review of human carcinogens. In: IARC Monograph on the Evaluation of Carcinogenic Risk to Humans, *100*, 171. IARC Press, Lyon, France (ISBN 13978-92-832-1329-1).

Josselin, L., De Clerck, C., De Boevre, M., Moretti, A., Fauconnier, M.-L., 2022. Impact of volatile organic compounds on the growth of *Aspergillus flavus* and related aflatoxin B1 production: a review. Int. J. Mol. Sci. 23, 15557. https://doi.org/10.3390/ijms232415557.

Muccilli, S., Restuccia, C., 2015. Review: bioprotective role of yeasts. Microorganisms 3 (4), 588–611. https://doi.org/10.3390/microorganisms3040588.

Nathanail, A.V., Gibson, B., Kimmo Peltonen, L.H., Ollilainen, V., Jestoi, M., Laitila, A., 2016. The lager yeast *Saccharomyces pastorianus* removes and transforms *fusarium* trichothecene mycotoxins during fermentation of brewer's wort. Food Chem. 203, 448–455. https://doi.org/10.1016/j.foodchem.2016.02.070.

Oliveira, A.S., Ferreira, C., Pereira, J.O., Pintado, M.E., Carvalho, A.P., 2022. Spent brewer's yeast (*Saccharomyces cerevisiae*) as a potential source of bioactive peptides: an overview. Int. J. Biol. Macromol. 208, 1116–1126. https://doi.org/10.1016/j.ijbiomac.2022.03.094.

Ortega, C., López, R., Cacho, J., Ferreira, V., 2001. Fast analysis of important wine volatile compounds - development and validation of a new method based on gas chromatographic-flame ionization detection analysis of dichloromethane microextracts. J. Chrom. A 923, 205–214. https://doi.org/10.1016/S0021-9673(01)00972-4.

Pérez-Través, L., Lopes, C., Barrio, E., Querol, A., 2014. Stabilization process in *Saccharomyces* intra- and interspecific hybrids in fermentative conditions. Int. Microbiol. 17, 213–224. https://doi.org/10.2436/20.1501.01.224.

Ruiz-Moyano, S., Hernández, A., Galván, A.I., Córdoba, M.G., Casquete, R., Serradilla, M. J., Martín, A., 2020. Selection and application of antifungal VOCs-producing yeasts as biocontrol agents of grey mould in fruits. Food Microbiol. 92, Article 103556 https://doi.org/10.1016/j.fm.2020.103556.

Sadeghi, A., Ebrahimi, M., Shahryari, S., Kharazmi, M.S., Jafari, S.M., 2022. Food applications of probiotic yeasts; focusing on their techno-functional, postbiotic and protective capabilities. Trends Food Sci. Technol. 128, 278–295.

Saini, P., Beniwal, A., Kokkiligadda, A., Vij, S., 2018. Response and tolerance of yeast to changing environmental stress during ethanol fermentation. Process Biochem. 72, 1–12.

Sampaolesi, S., Briand, L.E., De Antoni, G., Peláez, A.L., 2022. The synthesis of soluble and volatile bioactive compounds by selected brewer's yeasts: antagonistic effect against enteropathogenic bacteria and food spoiler–toxigenic *Aspergillus* sp. Food Chem. X, 13, Article 100193 https://doi.org/10.1016/j.fochx.2021.100193.

Sampaolesi, S., Pérez-Través, L., Pérez, D., Roldán-López, D., Briand, L.E., Pérez-Torrado, R., Querol, A., 2023. Identification and assessment of non-conventional yeasts in mixed fermentations for brewing bioflavored beer. Int. J. Food Microbiol. 399, 110254 https://doi.org/10.1016/j.ijfoodmicro.2023.110254.

Tejero, P., Martín, A., Rodríguez, A., Galván, A.I., Ruiz-Moyano, S., Hernández, A., 2021. *In vitro* biological control of *Aspergillus flavus* by *Hanseniaspora opuntiae* L479 and *Hanseniaspora uvarum* L793, producers of antifungal volatile organic compounds. Toxins 13, 663. https://doi.org/10.3390/toxins13090663.

Vero, S., Garmendia, G., González, M.B., Bentancur, O., Wisniewski, M., 2013. Evaluation of yeasts obtained from Antarctic soil samples as biocontrol agents for the management of postharvest diseases of apple (*Malus* × *domestica*). FEMS Yeast Res. 13 (2), 189–199. https://doi.org/10.1111/1567-1364.12021.

Wilson, A., Johnson, J.B., Naiker, M., 2022. Biotechnological modification of cider brewing processes for the enhanced production of 2-phenylethanol. Beverages 8 (4), 64. https://doi.org/10.3390/beverages8040064.

Xu, H., Wang, L., Sun, J., Wang, L., Guo, H., Ye, Y., Sun, X., 2022. Microbial detoxification of mycotoxins in food and feed. Crit. Rev. Food Sci. Nutr. 62 (18), 4951–4969. https://doi.org/10.1080/10408398.2021.1879730.