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Research Articles – Food Microbiology

From hop (*Humulus lupulus* L.) to beer: hop pellets as fungal vectors in dry-hopped craft beers

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Abstract

Dry hopping, a popular technique in modern craft brewing, introduces non-sterile hop material that may act as a source of microorganisms. Although beer is generally considered microbiologically stable, recent findings indicate that hops can harbour viable fungal and bacterial strains with potential effects on beer quality and hop creep enzymes. We investigated the fungal DNA reservoir of commercial hop pellets and its transfer into beer during dry hopping. Using ITS2 metabarcoding, we characterized fungal communities in hop pellets, pre-hopping beer, and dry-hopped beer, complemented by untargeted volatile profiling (HS-SPME-GCMS). Hop pellets contained diverse fungal assemblages dominated by common foliar endophytes. Several yeast genera of fermentative or spoilage relevance were also detected, including *Saccharomyces*, *Wickerhamomyces*, *Rhodotorula*, and *Debaryomyces*. While most taxa were found only in hops, four genera (*Wickerhamomyces*, *Vishniacozyma*, *Bipolaris*, and *Curvularia*) were additionally found in dry-hopped beer but absent from pre-hopping samples, indicating transfer from hops. Metabolomic screening revealed that, besides enrichment of hop-derived metabolites, dry hopping induced shifts in volatile profiles through increases in ethyl esters, higher alcohols, and short-chain fatty acids. Our results demonstrate that commercial hop pellets carry diverse fungal assemblages and that their DNA is detectable in beer after dry hopping, together with aroma shifts consistent with microbial or enzymatic activity.

Keywords: *Humulus lupulus* L.; hop microbiome; fungal communities; dry-hopped beer; metabarcoding; GCMS; HS-SPME

Introduction

The female inflorescences of hop (*Humulus lupulus* L.) have been a defining component of brewing for centuries, valued not only for their bittering and aromatic properties but also for their antimicrobial effects that enhance beer stability (Behre 1999, Almaguer et al. 2014). In recent decades, the craft beer movement and the emergence of hop-forward styles such as New England IPAs have led to the widespread practice of dry hopping, in which elevated hop dosages are introduced without prior boiling, during or after fermentation, to maximize aroma retention (Kirkpatrick and Shellhammer 2018, Young et al. 2023). This practice introduces unsterilized plant tissue into the fermenter, raising questions about whether hops may serve as a vector for microbial entry into the beer matrix.

Beer is widely considered a hostile environment for microbes due to its low pH, ethanol content, high CO_2 levels, and the limited availability of oxygen and nutrients (Sakamoto and Konings 2003). In contrast, studies have shown that hop cones are colonized by diverse microbial communities, including bacteria and fungi, some of which can survive the hop processing steps such as drying and pelletization (Allen et al. 2019, Krofta et al. 2021, Cottrell 2023, Rehorska et al. 2024). Similarly to the above-ground tissues of other plant species, the floral microbiome, i.e. anthosphere, of cultivated hops is dominated by *Pseudomonas* and *Sphingomonas* spp., while *Methylobacterium*, *Rickettsia*, *Xanthomonas*, and members of *Oxalobacteriaceae* also contribute to the microbial reservoir (Allen et al. 2019). In addition, based on a culture-dependent approach, endophytic fungi of *Alternaria*, *Epicoccum*, *Fusarium*, and *Botrytis* have been documented within hop tissues (Riccioni et al. 2025). A recent work has also highlighted that wild-growing hops harbour diverse yeast and yeast-like fungi, including genera such as *Rhodotorula*, *Papiliotrema*, *Hanseniaspora*, and *Wickerhamomyces*, some of which are known for their fermentative or biotransformation potential (Rehorska et al. 2024). These findings suggest that hops may introduce not only metabolites but also potentially viable microbes into beer during dry hopping, with possible implications for its sensory properties. The metabolic activity of hop-associated microorganisms has also been linked to the so-called "hop creep," an industrially relevant refermentation phenomenon occasionally observed in dry-hopped beers and traditionally attributed to hop-derived enzymes (Janicki et al. 1941, Bruner et al. 2020, Stokholm et al. 2020, Cottrell 2023).

Despite the growing recognition of the hop microbiome, most studies so far have focused on bacterial or culture-dependent approaches, while culture-independent profiling of fungal communities remains limited (e.g. Allen et al. 2019, Krofta et al. 2021, Cot-

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trell 2023). This gap is striking considering the extensive diversity of hop cultivars currently used in brewing. Hundreds of varieties are commercially available (Castro et al. 2021), each selected for unique aromatic and chemical profiles, and many are employed in dry hopping (Gomes et al. 2022). Since the plant's secondary metabolite composition can shape its associated microbiota (Jacoby et al. 2021, Pang et al. 2021, Koprivova and Kopriva 2022), varietal differences are likely to influence hop-associated fungal assemblages, with potential implications for beer quality and stability. Nevertheless, how these varietal differences influence the structure and diversity of hop-associated fungal communities remains largely unknown. Here, we investigated whether fungal DNA originating from hop pellets can be detected in beer following dry hopping. Using ITS2 metabarcoding, we compared fungal community profiles of pre-hopping beer, dry-hopped beer, and the hop pellets themselves. To complement the DNA-based approach, we conducted untargeted metabolomic screening (GCMS) to compare pre-hopping beer and dry-hopped beer, aiming to detect not only changes attributable to dry hopping but also shifts suggestive of microbial activity. Together, these analyses provide new insights into the potential role of hop-associated fungi in beer microbiology, particularly in relation to their transfer from hops into beer during dry hopping.

Materials and methods

Beer samples

Beer and hop pellet samples were obtained from two Hungarian craft breweries that regularly produce a range of unfiltered and unpasteurized dry-hopped beers. The first set of samples originated from a top-fermented, unfiltered, and unpasteurized Session IPA produced in a 5000-L fermentation vessel. Dry hopping was carried out using a HopMaster device (Ziptech, Miskolc, Hungary), designed for dry hopping and flavour additions (e.g. fruits, spices). This system ensures an extended contact surface between the beer and hops and can be operated with both pelletized and whole-cone hops. Transfer of the beer between the fermenter and the HopMaster unit was achieved by overpressure circulation. For dry hopping, 10–10 kg commercial hop pellets of *Simcoe* and *Citra* were used in the time of cold crash for 5 days, during which the beer was continuously recirculated through the hop pellets.

The second set of samples derived from a bottom-fermented, unfiltered, and unpasteurized Lager brewed on a 35-L system. Dry hopping in this case was also performed with a HopMaster for 3 days using 35 g of *Citra* at 15°C until cold crash. The two breweries obtained the hop pellets from three different manufacturers. In both breweries, dry hopping was conducted after fermentation.

Sampling was conducted in two phases. Pre-hopping samples were collected from the beer immediately prior to the initiation of dry hopping, and simultaneously from the hop pellets using sterile tools. For both the fermenters and the hop bags, five technical replicates were obtained. At the end of the dry hopping process, the hops were removed, and dry-hopped samples were collected after 2 days, before bottling. All samples were stored at −80°C until DNA extraction.

Metagenomic DNA extraction and sequencing

For DNA extraction, hop pellets were lyophilized and ground into a fine powder. Beer samples were centrifuged at 12 000 rpm for 20 min, the resulting supernatant was carefully discarded, and DNA was extracted from the resulting pellet. DNA from both hops and beer samples was isolated using the DNeasy Plant Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. DNA concentration was estimated for each sample using a NanoDrop 2000 spectrophotometer, and extracts were stored at −20°C prior to sequencing.

For fungal community profiling, the fungal nrDNA internal transcribed spacer 2 (ITS2) region was amplified using the primer pair of fITS7 (Ihrmark et al. 2012) and ITS4 (White et al. 1989), each appended with Illumina adapters (Illumina, San Diego, CA, USA). Polymerase chain reactions were performed in 25 μL volumes and contained 2× KAPA HiFi HotStart ReadyMix, 0.5 µM of each primer, and ∼10 ng of template DNA, with cycling conditions of 95°C for 3 min, followed by 25–32 cycles of 95°C for 30 s, 56°C for 30 s, and 72°C for 30 s, and a final extension at 72°C for 5 min. Amplicons were indexed with Nextera™ DNA CD Indexes (Illumina, San Diego, CA, USA), purified with AMPure XP beads (Beckman Coulter, Brea, CA, USA), quantified by Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA), and pooled equimolarly. Library quality was verified on an Agilent 2100 Bioanalyzer with a High Sensitivity DNA Kit (Agilent Technologies, Santa Clara, CA, USA). Sequencing was performed on an Illumina MiSeq platform with MiSeq Reagent Kit v2 generating 250 bp paired-end reads (Illumina, San Diego, CA, USA). All procedures were conducted at Eurofins Biomi Kft. (Gödöllő, Hungary) according to Illumina standard amplicon protocols.

HS-SPME-GCMS analysis

Volatile organic constituents of the beer samples were analysed by headspace solid-phase microextraction coupled with gas chromatography–mass spectrometry (HS-SPME-GCMS). Measurements were carried out on a QP2010s GCMS system (SHIMADZU Corp., Kyoto, Japan), equipped with a Gerstel Multi-Purpose Sampler (Gerstel, Mülheim an der Ruhr, Germany), thermal desorption unit (TDU), and cryogenically cooled injector (CIS4). For each sample, 10 mL of beer and 3 g NaCl were sealed in a 20 mL screw-cap headspace vial. Samples were incubated at 40°C with agitation at 500 rpm for 20 min, followed by HS-SPME extraction under identical conditions for 20 min. The fibre used (Supelco, Bellefonte, PA, USA) was coated with divinylbenzene-carboxen-polydimethylsiloxane 50/30 µm (DVB/CAR/PDMS) which was thermally desorbed in the TDU at 250°C for 240 s in splitless mode with a transfer time of 4 min. The injector temperature was initially maintained at –30°C for 4 min, then increased to 300°C at 12°C/s and held for 3 min in splitless mode. Chromatographic separation was performed on an Agilent VF-WAXms column (60 m × 0.25 mm i.d., 0.25 μm film thickness) using helium (6.0) as the carrier gas at a constant flow of 1.0 mL/min. The oven temperature program was as follows: 40°C for 12 min, ramped to 70°C at 3°C/min, and further increased to 240°C at 5°C/min. Mass spectra were acquired in scan mode over an m/z range of 50–300.

Data processing

Raw sequence data were processed using the dada2 pipeline (version 1.26.0) implemented in R (Callahan et al. 2016), constructing amplicon sequence variants (ASVs). Based on quality score profiles, forward and reverse reads were truncated at 240 and 210 bp, respectively. Reads exceeding a maximum expected error (maxEE) threshold of two were discarded. Subsequent steps included denoizing of individual reads, merging of paired-end reads, removal of chimeric sequences, and generation of an ASV abundance table. Taxonomic classification of representative ASVs was performed using vsearch (version 2.29.9) (Rognes et al. 2016) against the

UNITE fungal ITS reference database [25], retaining only assignments exceeding a threshold of 97%.

Data files resulted from GCMS analysis were converted to a non-vendor specific NetCDF format by the GCMS's data processing software (GCMSSolution, Shimadzu) and resulting .CDF files were converted to an Agilent file format .D to analyse in the software Profinder 10 (Agilent Technologies, Santa Clara, CA, USA). Profinders Batch Recursive Feature Extraction (small molecules/peptides) module was used as a deconvolution, feature extraction and alignment workflow. Compound features were kept only if they were present in all three replicates in at least one sample. The feature files generated by Profinder (PFA files) were imported into Agilent Mass Profiler Professional 15 (MPP), a chemometrics platform used in identification of the features. Feature intensities were exported as compound peak areas. The identification of compounds was performed by comparison of measured compound mass spectra to mass spectra obtained by the injection of standards and by comparison of measured compound mass spectra to mass spectra stored in Wiley 7, NIST08 or FFNCS mass spectral libraries. Resulting identified feature lists were exported as .CSV files for further statistical analysis in R.

Statistical analyses

All statistical analyses were conducted in R. The ASV abundance table was normalized by rarefying to the smallest library size (50 881 sequences) using the *rrarefy* function of the vegan package (Oksanen et al. 2015). Shared and unique ASVs among hops, pre-hopping beer, and dry-hopped beer were visualized with a Venn diagram using the VennDiagram package (Chen and Boutros 2011), based on presence/absence data. To assess fungal community composition in hop pellets, we performed Principal Coordinate Analysis (PCoA) on Bray–Curtis dissimilarities calculated from Hellinger-transformed ASV counts using the vegan package. Negative eigenvalues were corrected with the Cailliez method (Cailliez 1983, Legendre and Legendre 1998). The ordination was used to visualize patterns among samples according to hop variety (*Citra* and *Simcoe*) and hop pellet manufacturer (1; 2; 3). To test for significant effects of these factors, we conducted permutational multivariate analysis of variance (PERMANOVA) with the *adonis2* function in vegan, based on 9999 permutations. In addition, homogeneity of group dispersions was evaluated with the *betadisper* function. Relative abundances of fungal genera were visualized using the ggplot2 package (Wickham 2016) as pie charts, displaying the ten most abundant taxa within each sample type (hops, pre-hopping beer, dry-hopped beer). Relative abundances of fungal genera were visualized using the ggplot2 package (Wickham 2016) as scatter plots. To ensure that relative-abundance comparisons reflected only taxa that co-occur in both samples of a given pair, we restricted each comparison to shared genera, defined as genera with non-zero counts in both samples being evaluated. For each pairwise comparison, the abundance matrix consisting of the shared genera was subjected to a centered log-ratio (CLR) transformation (Aitchison 1982). CLR values were computed as:

$$CLR\,(x_i) = \ln\left(\frac{x_i}{g}\right).$$

where x_i is the count of genus i, and g is the geometric mean of all genera included in that comparison. By applying CLR only to the shared genera, both samples were centered using the same denominator, thereby ensuring direct, ratio-based comparability between samples. Pairwise scatterplots were generated by plotting the CLR abundance of each genus in the dry-hopped beer against its CLR abundance in either hops or pre-hopping beer. The 1:1 line represents equal CLR abundance between the two samples. Points above or below the line reflect enrichment in the corresponding axis sample. Genera that were present in both samples but absent in the Pre-hopping beer were highlighted (red) to indicate potential introduction through hop material. All analyses were performed in R (version 4.4.3) using the ggplot2 and ggrepel packages. ASVs assigned to *Saccharomyces* were excluded from this analysis, as this genus accounted for the majority of reads (~99%) in beer samples, representing 10 out of 75 ASVs (~13%).

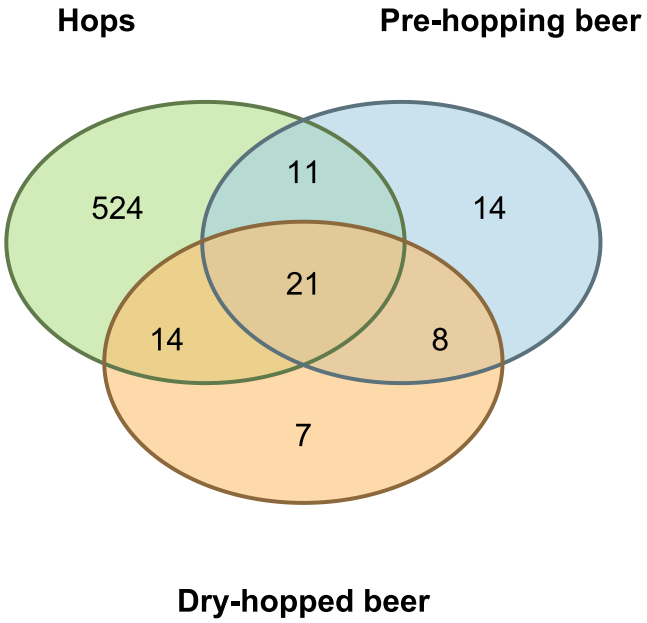

Figure 1. Venn diagram showing the distribution of fungal ASVs detected in hops (green), pre-hopped beer (blue), and dry-hopped beer (orange). The numbers in each section indicate the numbers of ASVs detected.

For the GCMS data analysis the feature intensity data were centred and scaled to achieve zero means and unit variances., then a Principal Component Analysis (PCA) was performed as a preliminary analysis using the package mixOmics (Lê Cao et al. 2009, Rohart et al. 2017), followed by a cluster heatmap (Eisen et al. 1998, Wilkinson and Friendly 2009) using the ComplexHeatmap package. Hierarchical clustering of both rows (samples) and columns (compounds) was performed using Canberra distance as the dissimilarity metric and Ward's minimum variance method (Ward.D2) for agglomeration. To improve stability, the k-means clustering option with four clusters and 20 repetitions was applied for row partitioning.

Results

Fungal communities in hop pellets, pre-hopping beer and dry-hopped beer

Following the quality filtering of the sequences the average ITS2 read number was 59 570 for hop samples and 85 701 for beer samples. Across the three sample types (hop pellets, pre-hopping beer, and dry-hopped beer), a total of 599 fungal ASVs were detected, representing 181 genera. As expected, hop pellet samples contained the highest richness, with 570 ASVs assigned to 172 genera (Fig. 1).

PERMANOVA revealed that fungal community composition in hop pellets differed significantly among manufacturers

(R^2=0.694, $P = 0.0001$) and also between hop varieties (R^2=0.301, $P = 0.0009$) (Fig. 2). However, *betadisper* analysis indicated significant differences in dispersion within hop varieties ($P = 0.0001$), while no such effect was observed within manufacturers ($P = 0.4003$). These results suggest that although PERMANOVA detected a significant effect of hop variety, the observed variation was most likely driven by heterogeneity among manufacturers; therefore, the observed differences cannot be conclusively attributed solely to hop variety.

Of the 172 fungal genera detected in hops, the most abundant based on rarefied abundance were predominantly common foliar endophytes, with *Cladosporium* being the most abundant, followed by *Alternaria*, *Vishniacozyma*, *Filobasidium*, *Sporobolomyces*, *Stemphylium*, *Aureobasidium*, *Fusarium*, *Calephoma*, and *Gibellulopsis* (Fig. 3). In addition, ASVs identified as members of the *Saccharomyces* genus were also detected in hop pellets (2 ASVs; ~0,12% of the total ITS2 read count in hop pellets), all of which were likewise present in the beer samples. Several other genera were also identified in the hop samples that are known to include yeasts and to produce volatile organic compounds (VOCs), such as *Rhodotorula*, *Papiliotrema*, *Malassezia*, *Debaryomyces*, *Wickerhamomyces*, *Nakaseomyces*, *Candida*, *Cryptococcus*, *Sporobolomyces*, *Cystobasidium*, *Cystofilobasidium*, *Filobasidium*, *Moesziomyces*, *Bullera*, *Pseudozyma*, *Vishniacozyma*, *Naganishia*, *Solicoccozyma*, *Rhodosporidiobolus*, *Symmetrospora*, and *Cyberlindnera* (Hassan et al. 1994, Lee et al. 1995, Suh et al. 1996, Barnett 2000, Johnson 2013, Aung et al. 2015, Bagheri et al. 2015, Esmaeili et al. 2015, Wang et al. 2015, Basso et al. 2016, Bellut et al. 2019, Nawaz et al. 2019, Stosiek et al. 2019, Haelewaters et al. 2020, Poontawee and Limtong 2020, Sathiyamoorthi et al. 2020, Rios-Navarro et al. 2021, de Almeida et al. 2022, Dikmetas et al. 2023, Ametefe et al. 2025, Malassigné et al. 2025).

As anticipated, both types of beer samples contained far fewer ASVs than the hop pellets. In pre-hopping beer, 54 ASVs representing 23 genera were detected, while in dry-hopped beer, 50 ASVs representing 22 genera were identified (Fig. 1). In both cases, *Saccharomyces* was the most abundant genus (10 ASVs, ~99% of the total ITS2 read count in beer samples). In addition, dominant genera in pre-hopping beer included *Alternaria*, *Cladosporium*, *Malassezia*, *Aureobasidium*, *Botrytis*, *Cystofilobasidium*, *Seimatosporium*, *Diplodia*, and *Pseudopithomyces*, whereas in dry-hopped beer the most abundant genera were *Cladosporium*, *Alternaria*, *Aureobasidium*, *Malassezia*, *Filobasidium*, *Calophora*, *Candida*, *Vishniacozyma*, *Pseudopithomyces*, and *Wickerhamomyces* (Fig. 3). Notably, 14 ASVs assigned to four fungal genera were detected in dry-hopped beer that were absent from pre-hopping beer but present in hop pellets, specifically *Wickerhamomyces*, *Vishniacozyma*, *Bipolaris*, and *Curvularia* (Fig. 1).

Untargeted metabolomic screening of beer samples

Analysis of VOCs revealed clear differences between beer styles and hopping. In untargeted GC-MS data processing, a total of 33 compounds were consistently detected across all samples, spanning major chemical classes such as higher alcohols, esters, fatty acids, and terpenoids, yet only one of the 33 detected compounds was not identified with GCMS libraries. PCA indicated that beer style was the dominant source of variation, with the first principal component (PC1) explaining 88.7% of the variance and clearly separating Lager from Session IPA samples (Fig. 4).

Within each style, dry hopping also influenced the volatile profile, as evidenced by a consistent shift along PC2 (6.1% variance), although the magnitude of this effect was smaller compared to the style-related differences. Sample replicates grouped tightly, confirming the reproducibility of the analytical approach. Hierarchical clustering of all identified VOCs further supported these findings (Fig. 5).

The heatmap showed distinct clustering of pre-hopping and dry-hopped beers within each style, with characteristic groups of compounds enriched after hopping. Among the 33 detected volatiles, 18 differed significantly ($P < 0.05$, FC > 2) between pre-hopping and dry-hopped samples. Increases were particularly notable for hop-derived terpenes such as β-myrcene, linalool, β-citronellol, p-menth-1-en-8-ol, and the sesquiterpene humulene, which are known constituents of hop essential oils and contribute directly to the aroma of dry-hopped beers.

Interestingly, several metabolites not typically associated with hop oils were also elevated after dry hopping (Fig. 5). These included higher alcohols (3-methyl-1-butanol, methionol), short-chain fatty acids (3-methyl-butanoic acid, 3-methyl-pentanoic acid), medium-chain ethyl esters (ethyl heptanoate, ethyl nonanoate), and ketones (2-nonanone, 2-undecanone). The presence and enrichment of these compounds suggest metabolic activity beyond simple extraction from hop material.

Discussion

Fungal communities of commercial hop pellets

Dry hopping has long been recognized for its impact on beer aroma and chemistry, but its microbiological dimensions have received far less attention. Based on ITS2 metabarcoding we provide molecular evidence that several fungal genera present in hops can be detected at the DNA level in beer after dry hopping, suggesting that hop material contributes to the microbial complexity of beer beyond its aromatic role. In the two dry-hopped beers examined (Session IPA and Lager), breweries used *Citra* (two samples) and *Simcoe* (one sample) hops, supplied by three different manufacturers. Fungal community composition differed by both variety and manufacturer (Fig. 2), although dispersion values indicated that variation was primarily driven by manufacturer. This suggests that differences in cultivation environment and processing may override varietal effects. Previous studies have shown that abiotic and biotic factors, such as geographical location, soil and cultivation method strongly shape hop metabolite and aroma profiles even within a single cultivar, with *Cascade* and *Mosaic* hops exhibiting distinct volatile compositions depending on growing region (Van Holle et al. 2021, Féchir et al. 2023). Such variation in bioactive metabolites may in turn select for distinct microbial assemblages, consistent with evidence that plant metabolite composition influences the associated microbiota (Pang et al. 2021). Furthermore, hop health status has been shown to alter rhizosphere microbial communities within a variety (Gallego-Clemente et al. 2023).

Sequence data confirmed the presence of DNA from common phyllosphere-associated endophytes such as *Alternaria*, *Epicoccum*, *Aureobasidium*, *Cladosporium*, and *Stemphylium* in hop pellets (Riccioni et al. 2025) (Fig. 3). In addition, we detected fungal genera previously reported as yeasts or VOC producers—*Saccharomyces*, *Rhodotorula*, *Papiliotrema*, *Malassezia*, *Debaryomyces*, *Candida*, *Filobasidium*, *Wickerhamomyces*, *Nakaseomyces*, *Cryptococcus*, *Sporobolomyces*, *Cystobasidium*, *Cystofilobasidium*, *Moesziomyces*, *Bullera*, *Pseudozyma*, *Vishniacozyma*, *Naganishia*, *Solicoccozyma*, *Rhodosporidiobolus*, *Symmetrospora*, and *Cyberlindnera*, many of which have been described in alcoholic beverages (Hassan et al. 1994, Lee

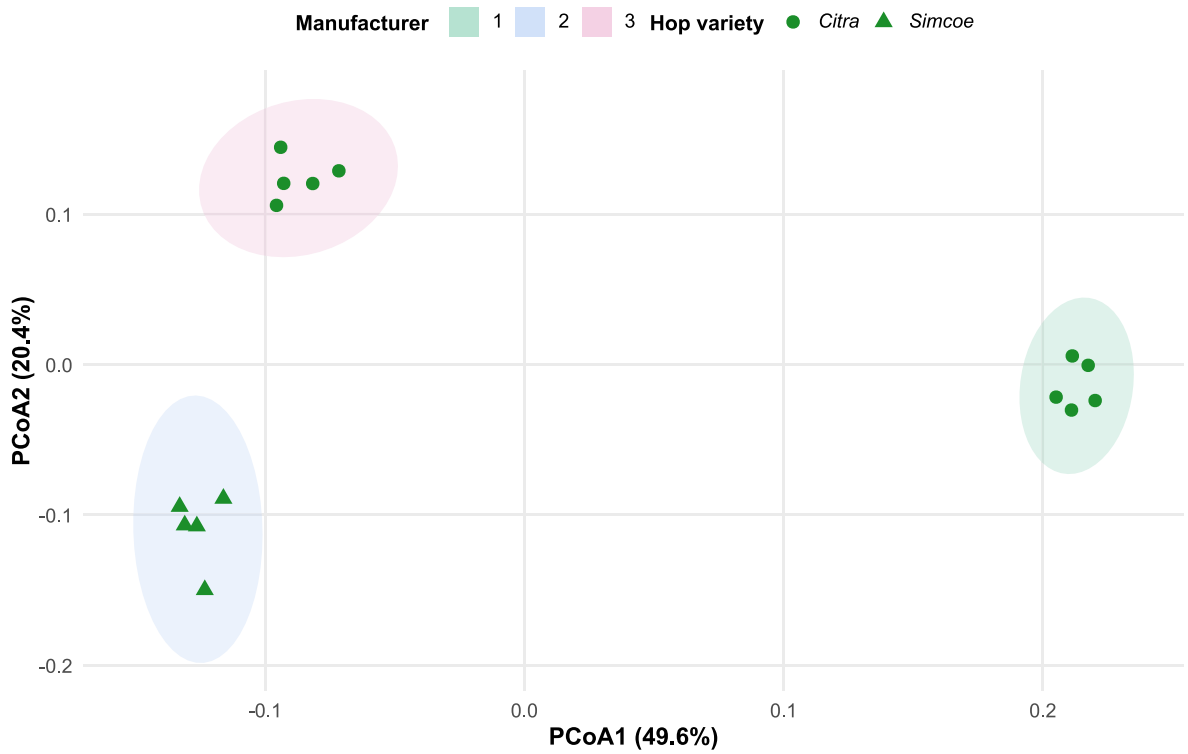

Figure 2. Fungal community composition in hop pellets based on nrDNA ITS2 amplicon sequencing with PCoA using Bray–Curtis dissimilarities calculated from Hellinger-transformed ASV counts. Ellipses represent hop pellet manufacturers (green = 1, blue = 2, pink = 3), while symbols denote hop varieties (circle = *Citra*, triangle = *Simcoe*). Ellipses represent 95% confidence intervals for each group.

et al. 1995, Barnett 2000, Johnson 2013, Aung et al. 2015, Bagheri et al. 2015, Esmaeili et al. 2015, Basso et al. 2016, Bellut et al. 2019, Nawaz et al. 2019, Haelewaters et al. 2020, de Almeida et al. 2022, Dikmetas et al. 2023, Ametefe et al. 2025, Malassigné et al. 2025; Suh et al. 1996, Wang et al. 2015, Stosiek et al. 2019, Poontawee and Limtong 2020, Sathiyamoorthi et al. 2020, Rios-Navarro et al. 2021).

The detection of *Saccharomyces* species in hop pellets is noteworthy, although only ASVs that were also detected in pre-hopping beer samples were identified. This genus includes not only the traditional brewer's yeasts *Saccharomyces cerevisiae* and *Saccharomyces pastorianus*, but also other species relevant for beer production (Nikulin et al. 2020, Hutzler et al. 2021, Iturritxa et al. 2023). Industrial strains of *S. cerevisiae* and *S. pastorianus* have undergone long-term domestication and selection, yielding traits such as efficient sugar utilization, ethanol and osmotic stress tolerance, and desirable flavour production (Stewart 2016, Gallone et al. 2018). Wild strains, by contrast, may lack these optimizations but possess wide genetic and phenotypic diversity that can contribute novel traits to fermentation outcomes (Fay and Benavides 2005, Gallone et al. 2018, Molinet and Cubillos 2020).

Beyond *Saccharomyces*, non-*Saccharomyces* yeasts were identified in hop pellets. *Rhodotorula* spp., classified as oxidative yeasts, can dominate early stages of fruit wine fermentation (Bagheri et al. 2015, Borren and Tian 2021). Recent studies show that *Rhodotorula mucilaginosa* modulates fermentation when co-cultured with *S. cerevisiae*, increasing 1-butanol and ethyl lactate in cider (S. Liu et al. 2025) and enhancing aroma complexity in wine (Calabretti et al. 2012, Wang et al. 2017). The presence of wild yeasts like *Candida*, *Filobasidium*, and *Debaryomyces* is often linked to off-flavours (phenolic, acidic, fatty acid, and estery), as well as haze and turbidity (Esmaeili et al. 2015). Poor hygiene during beer maturation and conditioning may allow these taxa to cause spoilage (van der Aa Kühle and Jespersen 1998). Although most of the hop-associated fungal genera were detected only in hop pellets and not in beer, the possibility remains that hops act as a vector for fungi capable of persisting under adverse conditions. This could be particularly relevant when dry hopping is performed during fermentation rather than afterward. Moreover, the detection of rare taxa in large fermentation volumes (e.g. 5000 L) can be limited by sampling scale and sequencing depth, underscoring the need for more intensive sampling strategies and RNA-based assays to determine microbial viability in future studies.Members of the *Wickerhamomyces* genus are biotechnologically relevant yeasts across multiple industries as food, environmental, biofuel, agricultural, and medical sectors (Nundaeng et al. 2021). *Wickerhamomyces anomalus* is frequently isolated from grapes and wine, where it enhances fermentation complexity, though its high ethyl acetate production has limited its use in brewing (Padilla et al. 2018). Nevertheless, recent studies highlight its potential benefits for craft brewing applications (Basso et al. 2016, Chen et al. 2025). *Cyberlindnera* species are notable for high ester production (*Cyberlindnera saturnus*, *Cyberlindnera mrakii*, and *Cyberlindnera subsufficiens*) (Inoue et al. 1994, Yilmaztekin et al. 2008, Aung et al. 2015, Liu and Quek 2016). In a study *C. subsufficiens* was selected for pilot-scale brewing, where optimized fermentation conditions enhanced fruitiness and reduced the typical wort-like off-flavour. The resulting non-alcoholic beer outperformed commercial counterparts in sensory evaluation, highlighting the potential of non-*Saccharomyces* yeasts as viable alternatives for non-alcoholic beer production (Bellut et al. 2019).

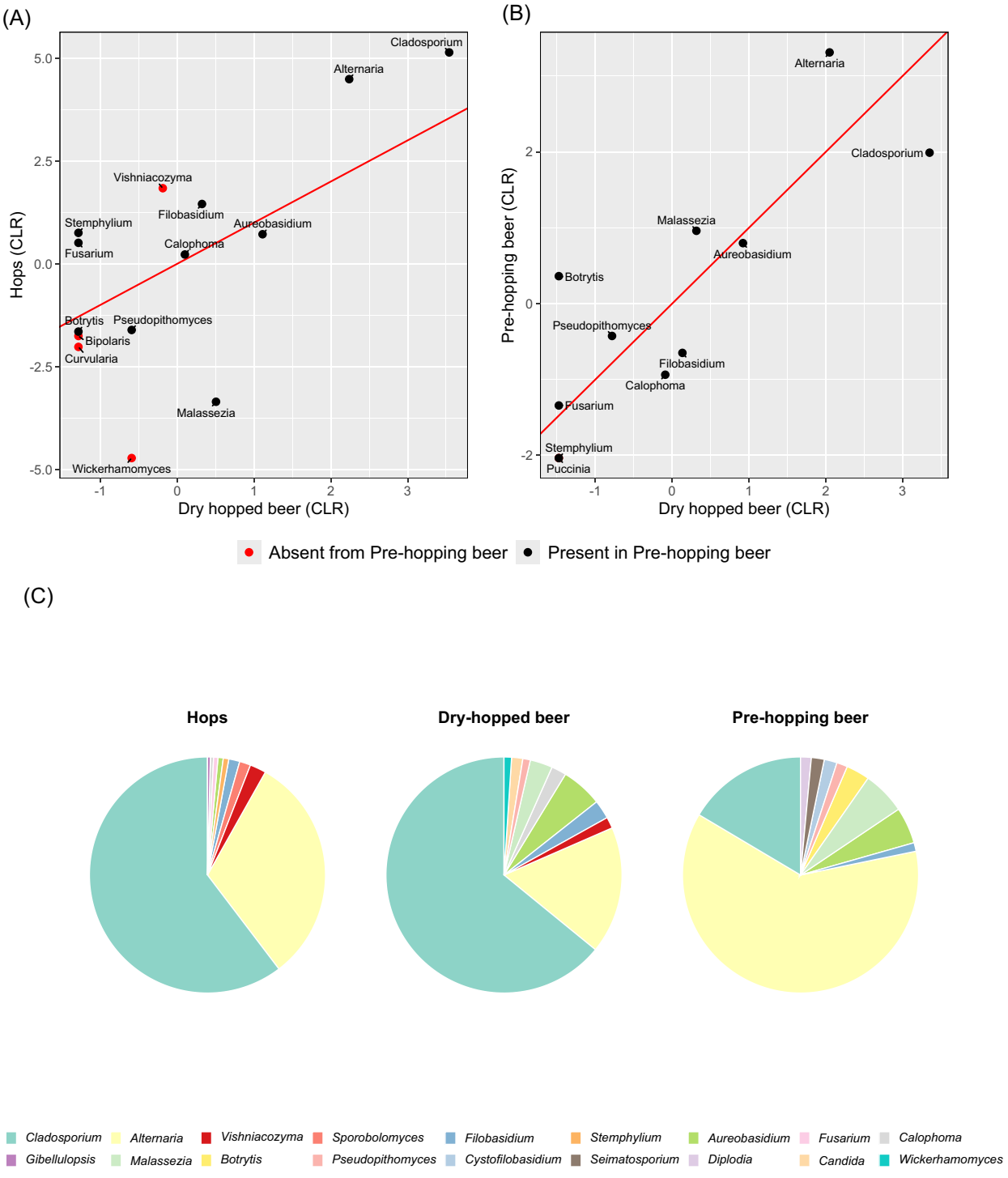

Figure 3. Scatter plot of genera detected in both compared samples (a–b). CLR-transformed abundances were plotted pairwise, with the red 1:1 line denoting equal relative abundance. Black points represent genera present in Pre-hopping beer samples, while red points mark genera absent from pre-hopping beer but detected after hop addition. Points above or below the line indicate relative enrichment in the corresponding sample. Ratio of fungal genera based on rarefied abundance, visualized as pie charts (c). For each sample type (hops, pre-hopping beer, and dry-hopped beer), the ten most abundant genera are shown without the genus *Saccharomyces* that dominated the beer samples representing ~99% of the total ITS2 read count.

Common fungal genera of hop pellets and dry-hopped beer

We identified four fungal genera, *Wickerhamomyces*, *Vishniacozyma*, *Bipolaris*, and *Curvularia* that were detected in both hop pellets and dry-hopped beer but were absent from pre-hopping beer. Among these, *Wickerhamomyces* is particularly noteworthy due to its documented role in both wine and beer fermentations and its association with the production of ethyl acetate and other volatile esters that can influence aroma complexity (Padilla et al. 2018). The detection of *Vishniacozyma*, *Bipolaris*, and *Curvularia* is also intriguing, as these genera have not commonly been reported from beer and may reflect the microbial reservoir carried by hops. Although some of these taxa are less likely to directly affect beer

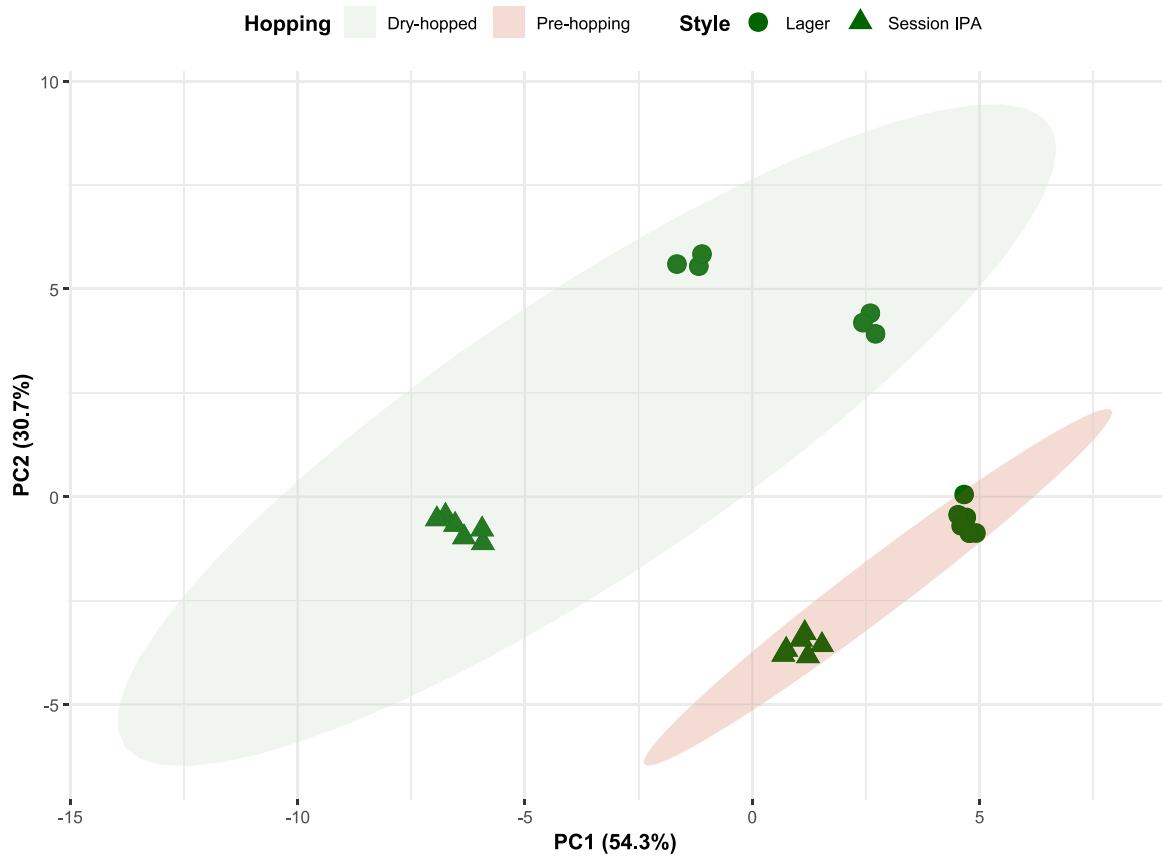

Figure 4. PCA score plot based on the relative abundances of 33 volatile compounds. Pre (orange)- and dry-hopped (green) states are indicated with ellipses. Symbols represent Lager (circle) and Session IPA (triangle) styles. Ellipses represent 95% confidence intervals for each group.

flavour, their persistence in the beer matrix after hop removal is remarkable and suggests that hop-derived fungi can withstand, at least transiently, the physicochemical stress of the brewing environment.The genus *Vishniacozyma*, established by Liu et al. (2015), is generally non-fermentative (Zhu et al. 2023) but encompasses metabolically versatile species with diverse biotechnological relevance. Several members, such as *Vishniacozyma foliicola* and *Vishniacozyma victoriae*, have been described as endophytes with biocontrol potential (Gorordo et al. 2022, Nian et al. 2023), while others, including *Vishniacozyma psychrotolerans* and *V. victoriae*, are capable of lipid accumulation and the production of fatty acids and ergosterol for industrial use (Deeba et al. 2018, Villarreal et al. 2018). To our knowledge, *Vishniacozyma* has not previously been reported from beer, although it is known to occur abundantly on malting barley. Notably, *V. victoriae* has been associated with pink kernel discoloration of barley and has occasionally been discussed in connection with beer gushing (over-foaming), although it is not regarded as a primary cause (Pettersson 2023).Similarly, *Bipolaris* and *Curvularia* have not been directly reported from beer. Both genera are well-known pathogens of cereals, including malting barley, where their presence can reduce grain quality (Lugo-Torres 2020). *Bipolaris* species typically cause leaf spots, blights, and rots on barley, rice, maize, wheat, and sorghum (Manamgoda et al. 2014). In contrast, *Curvularia* species are dematiaceous fungi with facultative pathogenic and endophytic lifestyles, capable of producing a wide array of secondary metabolites—such as alkaloids, terpenes, polyketides, and quinones—some of which exhibit antimicrobial or anti-inflammatory properties (Mehta et al. 2022).

Impact of dry hopping on hop and microbial volatiles

Untargeted GCMS analysis revealed that dry hopping not only enriched hop-derived volatiles such as terpenes but also altered the overall aroma profile through increases in esters, higher alcohols, and fatty acids, changes that may reflect microbial activity associated with hop material. Our analysis confirmed that the majority of volatile changes observed after dry hopping could be attributed to hop-derived compounds. Among these, the monoterpene β-myrcene and the sesquiterpene humulene were the most abundant, consistent with previous reports identifying these terpenes as major constituents across diverse hop cultivars (Aberl and Coelhan 2012, Klimczak et al. 2023). Likewise, ketones such as 2-nonanone, 2-decanone, and 2-undecanone, which were detected at elevated levels in our dry-hopped samples, have long been recognized as common hop volatiles (Sharpe and Laws 1981). The detection of geranyl formate, another compound repeatedly associated with hop essential oils (Sharpe and Laws 1981), further supports the dominant contribution of hop metabolites to the aroma profile of dry-hopped beers.

Beyond these expected hop volatiles, our results highlight the enrichment of compounds that are not classical constituents of hop oils but rather align with microbial or enzymatically mediated processes. Interestingly, this observation is closely connected to the phenomenon of hop creep, where refermentation in dry-hopped beers is driven not only by hop-derived enzymes but also by microbial contributions, but the origin of these esters is not straightforward: although ethyl esters in beer may arise from

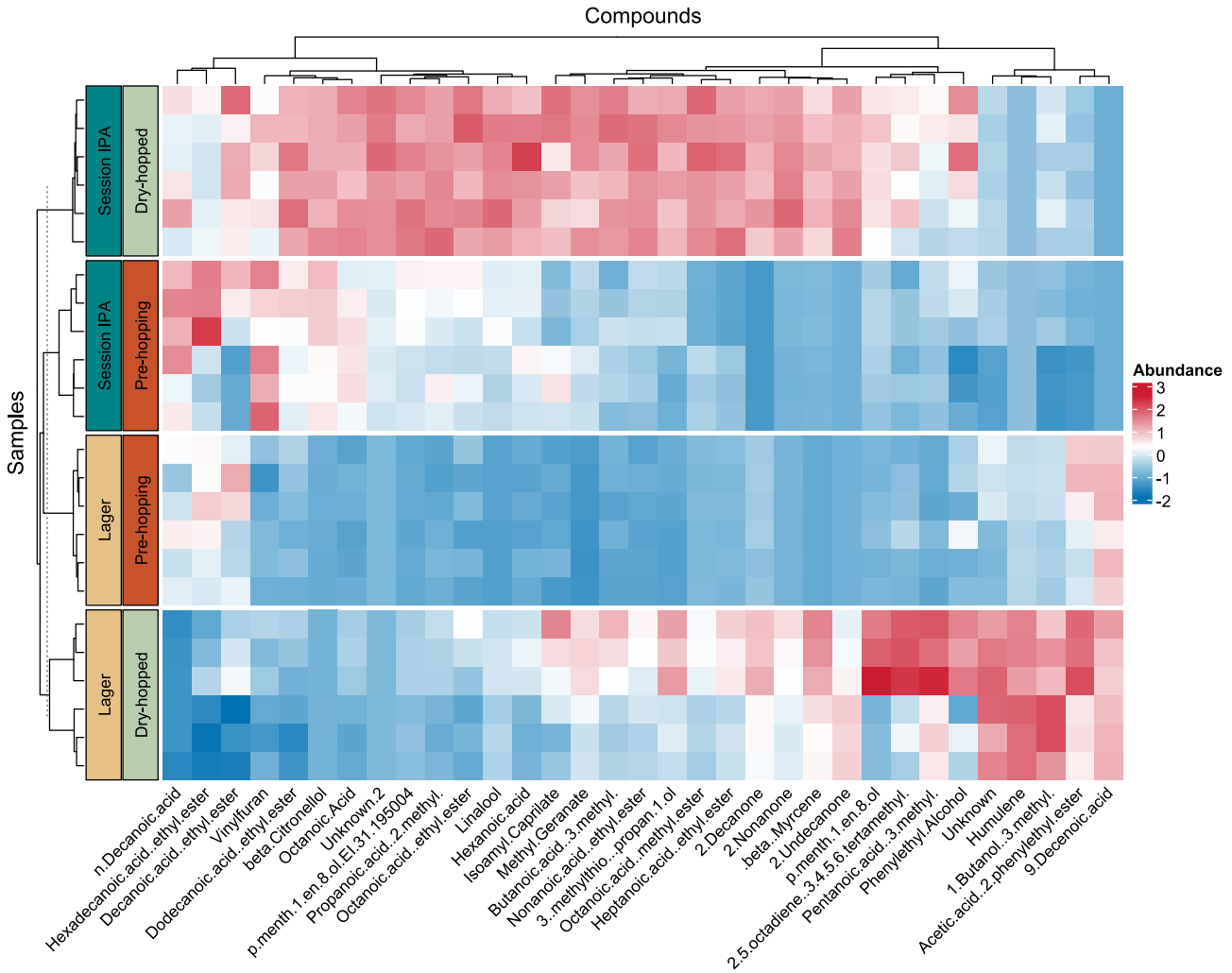

Figure 5. Heatmap of the relative abundances of 33 volatile compounds identified in beer samples before and after dry hopping. Data were normalized by z-score, and hierarchical clustering was applied to both compounds and samples.

non-enzymatic esterification during storage (Williams and Wagner 1978, 1979; Vanderhaegen et al. 2003, 2007), recent work by Brendel et al. (2020a) demonstrated that such esters accumulate predominantly in dry-hopped beers, even when precursor acid concentrations are comparable to those in un-hopped controls. Since both sample types were stored under identical conditions, the authors concluded that non-enzymatic reactions alone could not explain the pattern. Yeast metabolism was also ruled out, as the same yeast was present in both conditions (Brendel et al. 2020a). Instead, enzymatic activity associated with hops was proposed as a plausible mechanism, supported by evidence that ester formation could be inhibited by pH adjustment, heat treatment, or salt concentration (Brendel et al. 2020b). Our results are consistent with this hypothesis, as we also observed an increase in ethyl esters of 2- and 3-methylbutanoic acid, suggesting an enzymatic or microbially facilitated pathway induced by dry hopping.

In addition to esters, we found elevated levels of higher alcohols such as 3-methyl-1-butanol (isoamyl alcohol) and methionol, as well as short-chain fatty acids including 3-methylbutanoic and 3-methylpentanoic acids. These compounds are well-established products of yeast amino acid catabolism via the Ehrlich pathway and can also result from microbial interactions in beer (Olaniran et al. 2017). The fact that the ITS2 metabarcoding analysis of our samples confirmed the presence of microbial DNA in hops and its persistence after hopping supports the view that at least part of the observed volatile changes may stem from microbial metabolism, either through direct transformation of hop-derived precursors or through stimulation of yeast secondary pathways.

Taken together, our results demonstrate that the aroma modulation caused by dry hopping cannot be explained solely by the transfer of hop oil constituents into beer. While monoterpenes and sesquiterpenes provide the most immediate sensory impact, additional compounds of microbial or enzymatic origin—particularly ethyl esters, higher alcohols, and fatty acids—contribute to the final volatile profile. The style-dependent magnitude of these effects, with Session IPA showing stronger shifts than Lager, suggests that matrix effects or differences in fermentation conditions may further modulate these processes. Overall, these findings support the growing recognition that dry hopping is not only an extraction step but also a biochemical intervention that introduces both hop-derived volatiles and enzymatic or microbial activities shaping beer aroma.

Conclusion

Our findings demonstrate that commercial hop pellets harbour diverse fungal communities, including yeasts of potential fermentative and spoilage relevance. The detection of their DNA in beer

after dry hopping supports the idea that fungi associated with hops can be transferred into the beer matrix during this process. Untargeted metabolomic profiling further indicated that, beyond hop oil constituents, volatile shifts consistent with microbial or enzymatic activity occur after dry hopping. Overall, the introduction of hop-associated fungi during dry hopping may influence beer quality in both beneficial and detrimental ways—enhancing aroma complexity while potentially affecting stability and sensory balance.

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Conflicts of interest: The authors declare no conflict of interest regarding this manuscript.

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Data availability

DNA sequences used in this study were deposited in GenBank (Accession numbers: PX421699–PX422298).

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