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Nutritional Profile and Ecological Interactions of Yeast Symbionts Associated with North American Spruce Beetle (*Dendroctonus rufipennis*)

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Abstract

To better understand functional ecology of bark beetle-microbial symbioses, we characterized yeast associates of North American spruce beetle (*Dendroctous rufipennis* Kirby) across populations. Seven yeast species were detected; *Wickerhamomyces canadensis* (Wickerham) Kurtzman et al. (Sachharomycetales: Saccharomycetaceae) was the most common (74% of isolates) and found in all populations. Isolates of *W. canadensis* were subsequently tested for competitive interactions with symbiotic (*Leptographium abietinum*, = *Grosmannia abietina*) and pathogenic (*Beauvaria bassiana*) filamentous fungi, and isolates were nutritionally profiled (protein and P content). Exposure to yeast headspace emissions had isolate-dependent effects on colony growth of symbiotic and pathogenic fungi; most isolates of *W. canadensis* slightly inhibited growth rates of symbiotic (*L. abietinum*, mean effect: − 4%) and entomopathogenic (*B. bassiana*, mean effect: − 6%) fungi. However, overall variation was high (range: − 35.4 to + 88.6%) and some yeasts enhanced growth of filamentous fungi whereas others were consistently inhibitory. The volatile 2-phenylethanol was produced by *W. canadensis* and synthetic 2-phenylethanol reduced growth rates of both *L. abietinum* and *B. bassiana* by 36% on average. Mean protein and P content of *Wickerhamomyces canadensis* cultures were 0.8% and 7.2%, respectively, but isolates varied in nutritional content and protein content was similar to that of host tree phloem. We conclude that *W. canadensis* is a primary yeast symbiont of *D. rufipennis* in the Rocky Mountains and emits volatiles that can affect growth of associated microbes. *Wickerhamomyces canadensis* isolates vary substantially in limiting nutrients (protein and P), but concentrations are less than reported for the symbiotic filamentous fungus *L. abietinum*.

Keywords Insect · Microbial ecology · Nutrition · Competition · Symbiosis · Yeast

Introduction

Yeasts are frequently associated with insects and play a variety of important functional roles in these associations [22]. Yeasts can provide nutritional benefits [39], produce volatile compounds that act as insect infochemicals [7, 27] or fumigants with antibiotic properties [14], and yeasts may alter habitat suitability by regulating microbial interactions, substrate decomposition, and substrate chemistry [21], Foster and Fogleman 1989). Through these effects, insect-symbiotic yeasts have consequences for insect demography and may influence vitality rates including fecundity, lifespan, and survivorship [6, 34]. Although insects and their feeding behaviors contribute to the dispersal and diversity of yeast communities [45], many of these associations are somewhat incidental and related to the abundance of particular yeast species in the local environment. However, for some plant-feeding insect taxa that utilize nutritionally poor substrates (e.g., Homoptera, many Coleoptera), specific yeast taxa are associated with specialized anatomical structures, generally "mycetocytes" (internal cells in the digestive tract; [16] or "mycetangia" (external secretory glands; [46], underscoring

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their important role in the survival of insect species with limited or poor diets. Yet, most insect-yeast associations remain uncharacterized, which may ultimately constrain our ability to understand the mechanisms driving important plant–insect interactions.

In temperate forests of western North America, widespread tree mortality resulting from interactions between phloeomycetophagous bark beetles (Coleoptera: Curculionidae) in the genus *Dendroctonus* and foundation conifer species are among the most conspicuous natural disturbances on the landscape. *Dendroctonus* beetles are keystone insects that periodically outbreak across large areas, with substantial consequences for ecosystem forest structure, function, and composition [28]. *Dendroctonus* beetles have a unique life history [43],most species feed on and reproduce within the subcortical phloem environment, but beetles also rely on associations with a variety of filamentous fungal and bacterial symbionts to obtain nutrition [5, 8, 9], provide defense against pathogens [1, 40], and reduce concentrations of toxic secondary chemicals in the tree phloem environment [3]. Although yeasts are well-known as ubiquitous associates of *Dendroctonus* beetles [11] and were among the earliest fungal symbionts to be consistently isolated from bark beetles and their substrates (e.g., [10, 23], their ecological functions have not been investigated to the same extent as filamentous fungi and bacteria, and remain mostly untested.

Here, our goal is to characterize the yeast species associated with *Dendroctonus rufipennis* Kirby, the North American spruce beetle, in the southern Rocky Mountains and test some possible ecological functions of beetle-associated yeasts. Over the last decade, an ongoing outbreak of *D. rufipennis* caused extensive mortality of alpine spruce forest in the southern Rocky Mountains, with millions of hectares of collective forest mortality occurring from 2010–2020 in the states of Colorado, Utah, and Wyoming [31]. Consequently, *D. rufipennis* populations are a significant concern for ecosystem managers, and insight into factors impacting beetle success could potentially be exploited for integrated pest management.

Earlier studies aimed at describing symbiont communities associated with *D. rufipennis* reported that yeasts were isolated with ~ 100% frequency [42], but yeasts were not further considered during identification of fungal taxa. Here, we expand this body of work by isolating and identifying yeast associates. Our specific objectives are to (1) isolate and describe the primary yeast species associated with the *D. rufipennis* in the Southern Rocky Mountain region; (2) evaluate interactions between yeasts and beetle-associated filamentous fungi (including symbionts and pathogens); and (3) characterize the nutritional profiles of yeasts isolated from *D. rufipennis*. These findings expand our knowledge of holobiome function in an important forest insect, with potential consequences for understanding complex interactions among tree-killing bark beetles, microbial symbionts, and forest ecosystems.

Materials and Methods

Isolation of Yeasts from *D. rufipennis*

We collected *Dendroctonus rufipennis* from four populations in the southern Rocky Mountain region in 2016, including (1) Guanella Pass (Colorado, hereafter CO), (2) Cameron Pass (SB), (3) Togowotee Pass (Wyoming; WY), and (4) Soapstone Basin (Utah, UT; Fig. 1). In each population the primary host tree of *D. rufipennis* is Engelmann spruce (*Picea engelmannii* Parry ex. Engelm.), though in some locations Colorado blue spruce (*Picea pungens* Engelm.) is also present. Blue spruce is a resistant host [35] that often occurs in different micro-locations than Engelmann spruce (blue spruce is found mostly in drainages and mesic sites and is rarely colonized by *D. rufipennis*, whereas Engelmann spruce is more often found in mountain pass regions, at more xeric sites, and in contiguous stands). Accordingly,

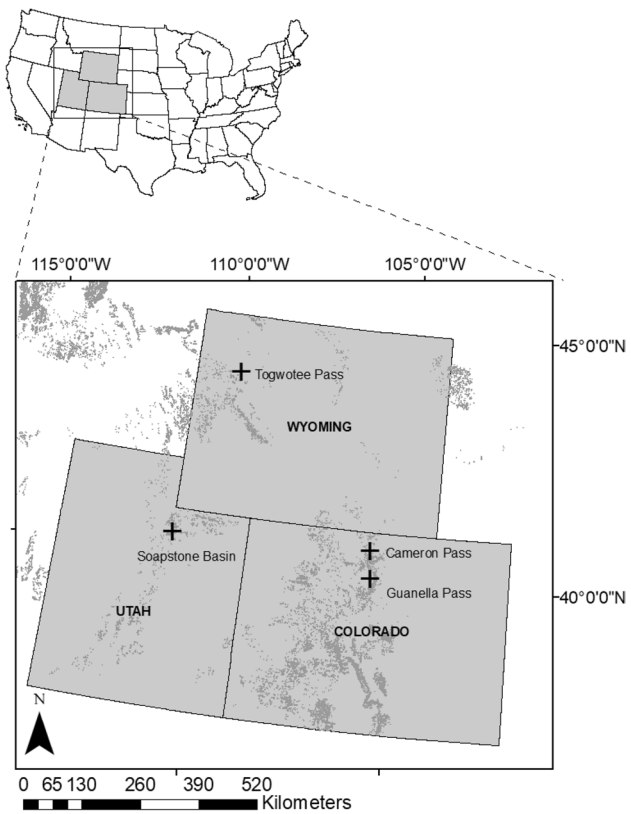

Fig. 1 A map of the locations in the western United States where *Dendroctonus rufipennis* samples were collected for yeast isolation. Sampled populations are denoted with crosshatches; dark gray polygons denote areas of mapped forest mortality from *D. rufipennis* from 2012 to 2016

we only sampled *D. rufipennis* from Engelmann spruce in the present study.

We located trees with outward evidence of natural infestation, including visible pitch tubes, sawdust/frass, woodpecker flaking, and/or evidence of crown decline. We used a hatchet to remove bark and expose egg gallery sections; we collected live adult *D. rufipennis* from galleries and immediately placed them into individual 10 ml glass sample vials using sterile forceps [4]. We cleaned forceps with ethanol between uses. Vials containing *D. rufipennis* were placed on ice and returned to the lab for isolation. We made all collections during the late summer (July and August), collecting adult beetles actively constructing egg galleries (i.e., post-flight and mated individuals); in each population we collected beetles from multiple gallery systems and trees.

We isolated yeasts from the *D. rufipennis* integument onto 2% malt extract agar (MEA) amended with gentamicin sulfate (MilliporeSigma, St. Louis, MO, USA) in a sterile laminar flow hood by pressing individual beetles into growth media, pressing each beetle 5–10 times in a dish. Beetle sex was not differentiated during isolations due to external similarity between males and females; therefore, our samples presumably represent yeasts isolated from both sexes. We inverted the dishes and incubated them in the dark at 23 °C for 72 h. After 72 h, a sterile 10 µl inoculating loop was used to collect yeast growth from the pressings and transfer to a new dish. All (100%) of sampled *D. rufipennis* yielded yeasts. Morphologically similar representative isolates were chosen at random from each population ($n = 58$ isolates total: Guanella Pass, CO, $n = 13$; Cameron Pass, SB, $n = 15$; Soapstone Basin, UT, $n = 10$; Togwotee Pass, WY, $n = 20$) for subsequent identification using molecular methods.

Identification of Yeast Isolates

We extracted Yeast DNA using ZR Fungal/Bacterial DNA MiniPreps (Zymo Research Corporation, Irvine, CA). Extractions were performed following manufacturer's protocols. We quantified DNA using a Nanodrop 2000 (ThermoFischer, Inc., Waltham, MA), and DNA was stored at − 20 °C until use. The D1/D2 domains of the rDNA large subunit were amplified with the primers NL-1 (forward) and NL-4 (reverse) following the protocols of O'Donnell (1993). We ran PCR products on 1.5% agarose gels with 0.5 × TBE buffer and stained with GelRed (Biotium, Fremont, CA), and visualized bands using UV light to confirm amplification. PCR products were purified using ExoSAP-IT® PCR Product Cleanup (Affymetrix, Santa Clara, CA) following manufactures instructions and sequenced at Eurofins (MWG Operon USA, Louisville, KY).

Sequences were aligned and manually edited in Geneious Prime (Biomatters, Inc.). We generated a single alignment with all 58 sequences using MUSCLE 3.8.425 [17] and identified unique haplotypes using DnaSP v5 [37]. Yeast species of these unique haplotypes were identified with the BLAST function in the NCBI blastn database (http://blast.ncbi.nlm.nih.gov/Blast.cgi). For phylogeny generation, matching sequences were added as references, and sequences of *Sakaguchia meli* (Genbank # NG 058,080) and *Naohidea sebacea* (Genbank # NG 042,442) were used as outgroups in a maximum likelihood phylogeny generated using PhyML [24] and a Bayesian phylogeny was generated using MrBayes 3.2.6 (Heulsenbeck and Ronquist 2001), both implemented in Geneious Prime using the GTR substitution model. We assessed support for clades in the maximum likelihood phylogeny using 1,000 bootstrap resamples. Bayesian inferences phylogeny was performed whereby the Markov chain Monte Carlo (MCMC) search was run with four chains for 3,000,000 generations from which 30,001 trees were drawn; the first 6,000 trees were discarded as "burn-in."

Ecological Profiling of Yeast Isolates

We (1) tested effects of exposure to yeast headspace volatiles, and direct contact with yeast cells, on growth of both symbiotic and pathogenic filamentous fungi (*L. abietinum* and *B. bassiana*), (2) analyzed the headspace volatiles produced by yeast isolates, and (3) subsequently tested effects of exposure to synthetic volatiles identified from yeast headspace on growth of filamentous fungi. Only yeasts identified as matching the sequence profile of *W. canadensis* were used, and only a subset of these isolates were tested to manage the size of experiments.

Test 1: Test of Yeast Headspace on Growth of Filamentous Fungi

We tested in vitro effects of exposure to headspace volatiles of five *W. canadensis* isolates (CO1 and CO3 [Guanella Pass, CO], SB 13 [Cameron Pass, CO], and WY 8 and WT 15 [Togwotee Pass, WY]) on radial growth rates of five isolates of the symbiotic blue stain fungus *L. abietinum* (isolates: CO1, CO7, and CO11 [Guanella Pass, CO], and WY5 and WY7 [Togwotee Pass, WY]; [15] and five isolates of the entomopathogen *B. bassiana* (isolates: 14B, 50C, 429DA, GHA, and Spruce 1; [33].

Partitioned petri dishes containing 2% MEA with a shared headspace were used for the experiment. On one side of the dish, 10 µl of yeast cell culture was streaked onto media using an inoculating loop; the other side of the dish was inoculated with a 1 × 1-mm plug of agar containing actively growing mycelia of *L. abietinum* or *B. bassiana* in the exponential growth phase. *Wickerhamomyces canadensis* isolates were allowed to establish for 48 h prior to inoculating dishes with filamentous fungi, and yeast cells completely occupied the portion of the dish where they were streaked

prior to the test. Each *W. canadensis* isolate × filamentous fungal isolate combination was replicated three times (5 W. *canadensis* isolates × 5 fungal isolates × 2 species × 3 replications = 150 experimental units). Each isolate of *L. abietinum* and *B. bassiana* was also grown in partitioned dishes with no yeast as a control; controls were also replicated three times. Dishes containing *W. canadensis* + fungi (treatments) or fungi alone (controls) were placed in the dark at 23 °C and new growth of fungal colonies was traced daily for 10 d or until hyphae reached the end of the dish. Radial growth of fungal colonies in response to yeast headspace was computed by dividing total growth distance by the number of days (mm/day). Growth rates of replicates in the "treatment" category were standardized to the mean growth rate of isolates in the 'control' category such that colony growth was analyzed as a percent change relative to the control growth rate for each *L. abietinum* and *B. bassiana* isolate.

Test 2: Test of Contact Between Yeast and Filamentous Fungi

We also tested (1) whether exposure to *W. canadensis* cultures arrest growth of *L. abietinum* via inhibition, and (2) whether *L. abietinum* was able to grow over media occupied by *W. canadensis*. In this test, the same *W. canadensis* × *L. abietinum* combinations were used and replicated in triplicate, but the following conditions differed (1) both microorganisms were plated at the same time rather than allowing *W. canadensis* to establish first, and (2) petri dishes with no partition were used to allow physical contact among isolates as well as a shared headspace. *Wickerhamomyces canadensis* isolates were inoculated onto dishes by streaking 1/3rd of the dish, and *L. abietinum* isolates were inoculated as in the first test. Growth of *L. abietinum* was traced daily for 10 d, and the following categorial responses were recorded: (1) whether *L. abietinum* cultures reached *W. canadensis* cultures during to test period (scored as "yes" or "no"), and (2) whether *L. abietinum* cultures grew over media occupied by W. canadensis (scored as "yes," "no," or "inhibited" in the case that the first condition was scored as a "no").

Test 3: Collection and Analysis of Yeast Headspace Volatiles

We used a modified dynamic headspace system to sample [26] volatile emissions from ten isolates, as previously described for yeast-like microbial species [12]. All isolates used in this analysis were from the same population (Guanella Pass, Colorado, USA). Briefly, isolates were prepared by streaking 10 µl of pure cell culture onto 8.5-cm diam petri dishes containing 2% MEA and incubated for 72 h in the dark. To trap yeast headspace volatiles, individual petri dishes containing cultures were placed, open, inside of a 3-L glass chamber with an entry and exit port and charcoal-filtered nitrogen was passed through the chamber at a rate of 50 ml/min for 100 min. Nitrogen exiting the chamber passed through a collection trap consisting of a glass tube containing 10 mg of an adsorbent polymer (HayeSep Q). Traps were eluted with 250 µl of GC-grade hexane (> 99% purity) and concentrated to a final volume of ~ 10 µl under a stream of nitrogen. System blanks were also collected using petri dishes containing 2% MEA only (no yeast culture) as a control.

A 1-µl aliquot of each ($n = 10$) concentrated eluant was manually injected onto a gas chromatograph (Agilent 7820A) coupled to a mass spectrometer (Agilent 5977B Mass Selective Detector) equipped with an HP5 ultra-inert column (dimensions: 30 m × 250 µm × 0.25 µm film thickness). The GC was operated in split mode (1:1) with helium as the carrier gas at a flow rate of 1.2 ml/min, and a front inlet temperature of 250 °C and foreline pressure of 6.90 kPa. The temperature program was as follows: initial temperature 40 °C then increasing by 10 °C/min until 250 °C and a final temperature hold time of 5 min. Total ion chromatograms were integrated using the ChemStation software (Agilent Technologies, Santa Clara, CA, USA) and compounds in extracts were putatively identified by comparing mass spectra to those in the National Institute of Standards and Technology (NIST 2017) mass spectral library. Compounds of interest were subsequently confirmed and quantified by comparing peak areas in samples to authentic standards at known concentrations. Abundances of emitted volatiles were standardized to yeast culture mass by dividing total compound abundances by the mass of yeast cultures (weighed to the nearest 0.1 mg). On average, yeast culture biomass was 170 mg ± 10 mg at the time of sampling.

Test 4: Test of Synthetic Volatiles Isolated from Yeast Headspace on Growth of Filamentous Fungi

A second experiment was performed to evaluate effects of the primary volatile identified in yeast headspace (2-phenylethanol). Instead of inoculation with yeast, one side of the partitioned dish was left empty and a 2 ml vial containing 1 ml of 2-phenylethanol stoppered with ~ 0.1 g cotton (concentration in test arena: 1% by volume); radial growth of each *L. abietinum* and *B. bassiana* isolate was tested in triplicate in response to 2-phenylethanol.

Test 5: Nutritional Profiling of Yeast Isolates

We selected a total of nine isolates for nutritional profiling based on species identifications. We only used yeasts identified as matching the sequence profile of *Wickerhamomyces canadensis* (the dominant yeast in our sample) in nutritional analyses. The rationale was that this approach would yield

information on interspecific variation in a common yeast symbiont, relative to beetle population phase. We also evaluated the protein content of host tree (*Picea engelmannii*) phloem for comparison.

As above, isolates were prepared by streaking 10 µl of pure cell culture onto 8.5-cm diam petri dishes containing 2% MEA and incubated for 72 h in the dark. Selected isolates were then prepared for analysis by lysing. Yeast cells were collected from media and suspended in Y-PER (Thermo Fisher, Waltham, MA, USA) at a ratio of 5 µL reagent per mg culture. Lysate was collected per reagent protocol- suspensions were agitated at room temperature for 20 min before centrifugation at 14,000 × *g* for 10 min. Supernatant was reserved for analysis.

Phosphorous Content of Yeast Cells Total phosphorus was measured using an adaptation of the ascorbic acid method [36]. In brief, reagent solutions of ammonium molybdate (6.00 g ammonium molybdate tetrahydrate, 0.24 g antimonyl potassium tartrate, 5.00 g ammonium sulfamate, 120 mL sulfuric acid diluted to 500 mL with deionized H_2O), and ascorbic acid (7.2 g ascorbic acid in 100 mL dH2O) were combined at a ratio of 5:1. Samples were prepared by combining 7 µL of ammonium molybdate-ascorbic acid with 93 µL of sample. A standard curve was generated using a 2.0 mg/L phosphate ion solution. Samples were incubated for 15 min at room temperature before measurement of absorbance at 880 nm using a UV–Vis spectrophotometer (Jasco V-730, JASCO, Inc., Easton, MS, USA).

Protein Content of Yeast Cells Total protein content of collected lysates was measured using a commercially available bicinchoninic acid assay (Pierce™ BCA Protein Assay Kit, Thermo Fisher, Waltham, MA, USA) according to manufacturer's instructions.

Protein Content of Spruce Phloem Phloem was collected from 27 Engelmann spruce trees at the Cameron Pass (CO) site using a 2-cm punch; phloem samples were taken at 1.3 m height at a random aspect from the bole of randomly selected trees that were > 10 cm in diameter at breast height (1.3 m).

Phloem samples were pulverized in liquid N using mortar and pestle and the resulting pulp was analyzed following pretreatment with dilute acid hydrolysis, as described in Shi et al. [41]. Thirty milligrams of crushed phloem pulp was added to 1 ml of 3% H_2SO_4 aqueous solution and heated to 80 °C for 1 h. The resulting mixture was sonicated for 15 min and centrifuged at 5000 rpm for 5 min. Samples were then treated with 1.5 M NaOH to neutralize the acid, resulting in a final pH of 8. Protein measurements were made on the resulting mixture using a Bradford assay kit and the microplate protocol (Pierce™ Detergent Compatible Bradford Assay Kit: ThermoFisher Scientific). Photospectrometer measurements were conducted at 595 nm in triplicate using 10 µL of each standard or sample, 300 µL of Bradford Assay Reagent, and incubating for 10 min. Phosphorous content was not analyzed for spruce phloem.

Data Analysis

Test 1: Test of Yeast Headspace on Growth of Filamentous Fungi

A two-way ANOVA model was used to analyze the partitioned petri dish experiment testing the fixed effects of 'treatment' (*W. canadensis* isolate), fungal isolate (*L. abietinum* or *B. bassiana*) and a treatment × fungal isolate interaction on the response of mean percent change in *L. abietinum* and *B. bassiana* radial growth rates.

Test 2: Test of Contact Between Yeast and Filamentous Fungi

A chi-square test was used to analyze the conditional outcomes of direct physical exposure to *W. canadensis* colonies on the ability of *L. abietinum* to (1) reach yeast colonies during the growth period (yes or no) and (2) grow over yeast colonies (yes, no, or inhibited).

Test 3: Analysis of Yeast Headspace Volatiles

The mean and standard error of volatile compounds collected from yeast isolates are reported in units of µg compound per mg yeast per minute ($\mu g^{-1}\ mg^{-1}\ min^{-1}$).

Test 4: Test of Synthetic Volatiles Isolated from Yeast Headspace on Growth of Filamentous Fungi

To test effects of exposure to yeast headspace volatiles, the percent change in mean relative growth rates of *L. abietinum* and *B. bassiana* (i.e., relative to control growth rates) was pooled across replicates within each species and compared to zero using a two-tailed Student's *t*-test.

Test 5: Nutritional Profiling of Yeast Isolates

Two-way ANOVA models were used to test the fixed effects of isolate identity and source population on variation in the response of mean phosphorous and protein content of yeast tissues. Post-hoc tests (Tukey's HSD test) were applied to make all pairwise comparisons of means. Phloem protein concentration was plotted alongside yeast isolates for visual

comparison, but was not statistically tested in the same model.

Results

Identification of Yeast Isolates

Seven unique haplotypes were identified out of the total 58 isolates that were sequenced at the rDNA large subunit. Each of the 7 haplotypes were identified as different species using NCBI BLAST and this was verified in the phylogenetic analyses (Table 1). Some BLAST results highlighted two species within the top 5 hits, therefore these two species were included in the phylogeny (Table 1; Fig. 2). Most sequences had 100% identity to a single yeast species which also clustered together in the phylogeny. However, from the combined BLAST and phylogenetic analyses, we found one haplotype that may represent an undescribed species (Haplotype 3). Sequence identity of Haplotype 3 was a close match to both *Peterozyma toletana* and *P. xylosa*, which are closely related species (Fig. 2).

Wickerhamomyces canadensis was the most commonly identified species and represented 43/58 of total isolates (74%). Only two species were found in all locations, *W. canadensis* and *Candida piceae*, though *C. piceae* only represented 10% (6/58) of the total isolates. The five additional yeast species were found infrequently. One isolate of *Yamadazyma tenuis* was collected each from Utah and Wyoming.

Table 1 Genbank matches, haplotypes, and deposit numbers for yeasts identified in the present study

Yeast isolate IDs	D1/D2 haplotype	Blast Results (species: query coverage: e-value: percent identity)	GenBank #
CO1, CO2, CO3, CO4, CO7, CO8, CO9, CO10, CO11, CO12, CO13, CO16, SB1, SB2, SB3, SB7, SB8, SB9, SB10, SB11, SB13, SB14, SB15, UT3, UT7, UT8, UT13, UT15, UT16, UT18, UT23, WY1, WY3, WY6, WY8, WY9, WY11, WY12, WY15, WY16, WY17, WY18, WY19, WY22, WY23	Hap1	*Wickerhamomyces canadensis*: 100%: 7e-153: 98.12%	ON783877
CO5, SB5, SB6, SB12, UT17, WY9	Hap2	*Candida piceae*: 100%: 9e-148: 100% *Ogataea cecidiorum*: 100%: 5e-159: 98.77%	ON783878
WY22	Hap3	*Peterozyma toletana*: 100%: 6e-161: 100% *Peterozyma xylosa*: 100%: 5e-159: 99.83%	ON783879
WY14	Hap4	*Kuraishia capsulata*: 100%: 9e-157: 99.12%	ON783880
UT2, WY9.2, WY13	Hap5	*Yamadazyma tenuis*: 99%: 5e-159: 100%	ON783881
WY5	Hap6	*Candida oregonensis*: 100%: 4e-180: 100%	ON783882
WY15.2, WY16.2	Hap7	*Cystobasidium minutum*: 100%: 5e-159: 100%	ON783883

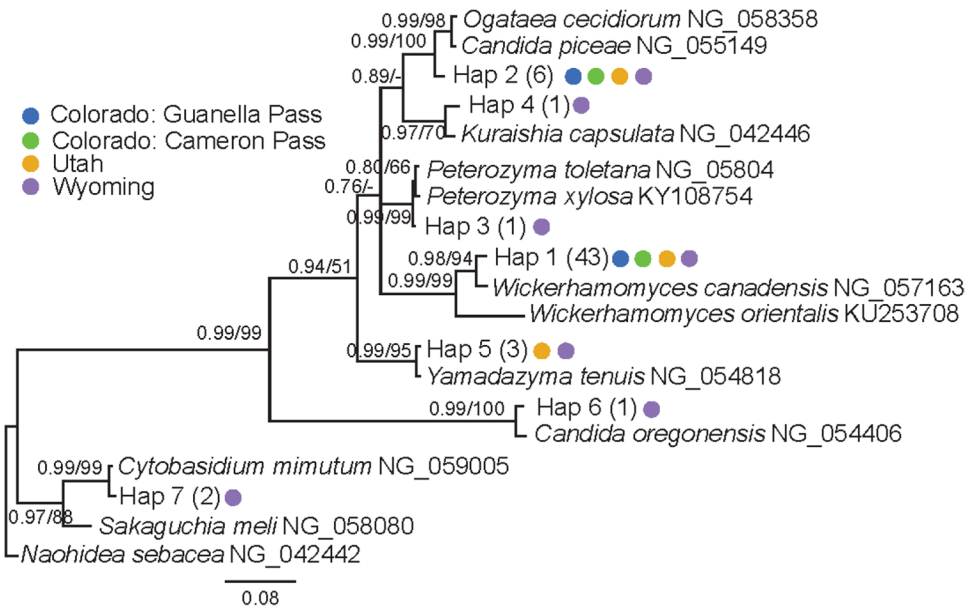

Fig. 2 Bayesian inference phylogeny based on the D1/D2 large subunit rDNA region of representative yeast haplotypes from cultures isolated from *Dendroctonus rufipennis*. All isolates are listed in Table 1 with corresponding haplotype identification numbers and/or reference accession numbers. Branch support includes posterior probability (PP) and bootstrap support (BS) and indicated as (PP/BS). Colored circles denote location of where species were collected

Wyoming beetles has the greatest diversity of yeast species that included all 7 yeast species (Table 2).

Test 1: Test of Yeast Headspace on Growth of Filamentous Fungi

Exposure to yeast headspace had significant effects on the radial growth rate of *L. abietinum* colonies (whole model: $F_{24,\ 50} = 3.703$, $P < 0.001$). There was evidence that *W. canadensis* isolate identity ($F_{4,50} = 4.626$, $P = 0.003$) and *L. abietinum* isolate identity explained variation in *L. abietinum* radial growth rates ($F_{4,50} = 10.845$, $P < 0.001$). However, there was no evidence of a *W. canadensis* isolate × *L. abietinum* isolate interaction ($F_{16,\ 50} = 1.678$, $P = 0.080$). On average, exposure to *W. canadensis* volatiles reduced *L. abietinum* colony growth by -4.2% (range: − 29.8% to + 88.6%), and this reduction differed from zero ($t_{74} = -1.687$, $P = 0.047$). Headspace of two *W. canadensis* isolates (CO1 and WY15.1) had slight positive effects on mean radial growth of *L. abietinum*, whereas two had slight negative effects (CO13 and WY8), and one isolate had strong negative effects on mean radial growth rates (SB 13; Table 3).

Exposure to yeast headspace also had significant effects on the radial growth rate of *B. bassiana* colonies (whole model: $F_{24,\ 50} = 8.280$, $P < 0.001$). There was no evidence that *W. canadensis* isolates differentially affected *B. bassiana* growth rates ($F_{4,50} = 2.003$, $P = 0.108$); however, *B. bassiana* isolates varied substantially in their responses to *W. canadensis* headspace ($F_{4,50} = 45.510$, $P < 0.001$). There was no evidence of a *W. canadensis* isolate × *B. bassiana* isolate interaction ($F_{16,50} = 0.542$, $P = 0.910$). On average, exposure to *W. canadensis* volatiles reduced *B. bassiana* colony growth rate by − 6.1% (range: − 35.4 to + 16.5%), and this reduction differed from zero ($t_{74} = -4.263$, $P < 0.001$). Exposure to *W. canadensis* volatiles had slight positive effects on growth response of three *B. bassiana* isolates (14B, 429DA, and GHA), while two isolates (50C and Spruce 1) were strongly negatively affected (Table 4).

Test 2: Test of Contact Between Yeast and Filamentous Fungi

In 84% of trials, *L. abietinum* cultures were able to grow to make contact with *W. canadensis* cultures, and there was no difference in the frequency with which fungal cultures grew to contact with yeast across the yeast isolates ($\chi^2 = 7.540$, $P = 0.110$, df = 4, N = 75), indicating no evidence for a zone of inhibition among *W. canadensis* isolates. However, there was evidence that yeast isolates varied in their ability to maintain resource space free from *L. abietinum* colonies ($\chi^2 = 26.379$, $P < 0.001$, df = 8, $N = 75$): *L. abietinum* grew over yeast cells in 49% of trials was prevented from growing

Table 2 The frequency of seven yeast species isolated from four southern Rocky Mountain populations of *Dendroctonus rufipennis*

Population	n	*Candida oregonensis*	*Candida piceae*	*Yamadazyma tenuis*	*Cystobasidium minutum*	*Kuraishia capsulata*	*Peterozyma* sp.	*Wickerhamomyces canadensis*
Guanella Pass, CO	13	0	1	0	0	0	0	12
Cameron Pass, CO	15	0	3	0	0	0	0	12
Soapstone Basin, UT	10	0	1	1	0	0	0	8
Togwotee Pass, WY	20	1	1	2	1	1	1	13
total	58	1	6	3	2	1	1	43

Table 3 Mean percent change (± standard error) in radial growth rate of *L. abietinum* isolates (blue stain fungi) in response to exposure to volatiles from *W. canadensis* (yeast) headspace in a partitioned petri dish experiment. Percent changes are relative to the mean control growth rate for each *L. abietinum* isolate (i.e., grown in the absence of yeast volatiles). Lettering in the 'mean' row and column denotes Tukey's HSD test for main effects of *W. canadensis* isolate and *L. abietinum* isolate, respectively

Treatment	*Leptographium abietinum* isolate (blue stain fungi)					Mean
	CO 1	CO 7	CO 11	WY 5	WY 7	
W. canadensis, CO 1	− 8.66 ± 4.09	12.80 ± 4.43	4.63 ± 6.02	− 1.08 ± 2.06	− 4.97 ± 5.54	0.54 ± 2.67 **A**
W. canadensis, CO 3	− 9.18 ± 3.38	5.19 ± 2.28	13.20 ± 9.90	− 6.80 ± 3.47	− 15.92 ± 0.99	− 2.70 ± 3.39 **AB**
W. canadensis, SB 13	− 22.08 ± 3.72	− 6.84 ± 6.76	− 3.37 ± 3.74	− 8.51 ± 2.85	− 28.35 ± 0.86	− 13.83 ± 2.99 **B**
W. canadensis, WY 8	− 2.99 ± 6.76	4.56 ± 1.09	− 2.80 ± 3.74	− 3.37 ± 2.06	− 3.98 ± 3.58	− 1.71 ± 1.71 **A**
W. canadensis, WY 15	− 7.63 ± 4.92	2.02 ± 1.67	12.34 ± 4.28	− 4.51 ± 1.51	− 15.42 ± 3.48	− 3.71 ± 2.73 **A**
Mean	− 15.25 ± 3.50 **C**	− 2.67 ± 3.86 **AB**	5.55 ± 6.13 **A**	− 9.17 ± 2.55 **BC**	− 21.18 ± 4.63 **C**	

Table 4 Mean percent change (±standard error) in radial growth rate of *B. bassiana* isolates (entomopathogen; white muscardine disease) in response to volatiles from *W. canadensis* (yeast) headspace in a partitioned petri dish experiment. Percent changes are relative to the mean control growth rate for each *B. bassina* isolate (i.e., grown in the absence of yeast volatiles). Lettering in the 'mean' row denotes Tukey's HSD test for main effects of *B. bassiana* isolate

Treatment	*Beauvaria bassiana* isolate (entomopathogen)					Mean
	14 B	50 C	429 DA	GHA [a]	Spruce 1	
W. canadensis, CO 1	2.90 ± 1.45	− 4.12 ± 1.47	2.88 ± 8.56	0.34 ± 3.40	− 22.58 ± 4.92	− 4.11 ± 3.13
W. canadensis, CO 3	0.00 ± 2.51	− 11.50 ± 2.55	− 2.60 ± 1.37	2.04 ± 2.94	− 27.95 ± 2.84	− 8.00 ± 3.08
W. canadensis, SB 13	4.34 ± 4.34	− 8.55 ± 2.95	5.62 ± 3.62	5.44 ± 1.70	− 24.73 ± 5.98	− 3.57 ± 3.50
W. canadensis, WY 8	6.12 ± 5.38	− 14.45 ± 1.47	− 2.60 ± 4.94	5.44 ± 1.70	− 22.58 ± 5.88	− 5.61 ± 3.41
W. canadensis, WY 15	1.45 ± 1.41	− 15.93 ± 5.10	− 6.72 ± 2.74	0.34 ± 1.70	− 25.80 ± 5.85	− 9.33 ± 3.09
Mean	2.96 ± 1.41 **A**	− 10.91 ± 1.60 **B**	− 0.68 ± 2.19 **A**	2.72 ± 1.09 **A**	− 24.73 ± 2.00 **C**	

[a]Commercially available strain

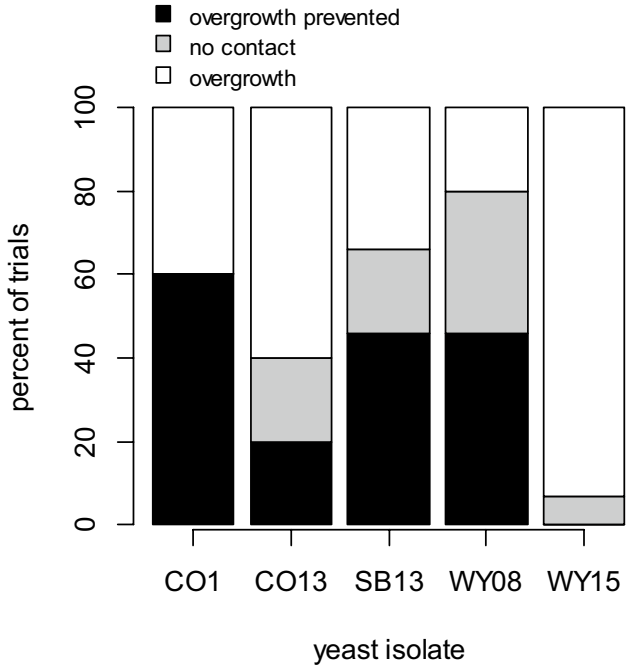

Fig. 3 The proportion of trials in which *W. canadensis* isolates prevented overgrowth, prevented contact with evidence of zone inhibition, or were overgrown by the symbiotic filamentous fungus *L. abietinum* in tests allowing physical contact between the two species

over yeast cells in 34% of trials and never made contact with yeast cells in the remaining 16% of trials (Fig. 3).

Test 3: Analysis of Yeast Headspace Volatiles

The single primary component of yeast headspace was 2-phenylethanol and was emitted by yeast isolates at an average rate of 1.47 ± 71 SE μg^{-1} per mg^{-1} yeast per min^{-1} (Fig. 4). Other minor components identified in headspace included styrene and butane, but comparison with system blanks revealed these products were off gasses from petri dishes or growth media. Accordingly, abundances of these compounds are not reported and were not directly tested.

Test 4: Test of Synthetic Volatiles Isolated from Yeast Headspace on Growth of Filamentous Fungi

Exposure to 2-phenylethanol at a concentration of 1% by volume uniformly reduced growth rate of *L. abietinum* by 36% on average, and this difference was different from zero (t_{14} = -7.728, $P < 0.001$). Similarly, exposure to 2-phenylethanol uniformly reduced growth rate of *B. bassiana* by 36% on average, and this difference was also different from zero ($t_{14} = -10.199$, $P < 0.001$; Fig. 5).

Test 5: Nutritional Profiling of Yeast Isolates

Mean phosphorous and protein content of *W. canadensis* was 7.2 and 0.8% per unit mass, respectively, and mean protein concentration of Engelmann spruce phloem was 1.1% per unit mass. There was evidence that mean phosphorous content of *W. canadensis* tissues varied significantly by isolate identity ($F_{8,95} = 71.457$, $P < 0.001$) and population ($F_{2,101} = 24.355$, $P < 0.001$), with isolates from Togwotee Pass (WY) and Cameron Pass (CO) exhibiting higher mean phosphorous concentrations than isolates from the Guanella Pass (CO) region (Fig. 6a). Similarly, there was evidence that mean protein content of *W. canadensis* tissues varied significantly by isolate identity ($F_{8,97} = 36.491$, $P < 0.001$) and population ($F_{2,103} = 51.019$, $P < 0.001$). All three populations differed from one another in terms of protein concentration, and isolates from Togwotee Pass (WY) had more protein on average than isolates from Cameron Pass (CO) and Guanella Pass (CO), respectively (Fig. 6b). Subsequent correlation analysis indicated that phosphorous and protein content were not correlated in *W. canadensis* isolates (Pearson's $r = 0.416$, $P = 0.264$).

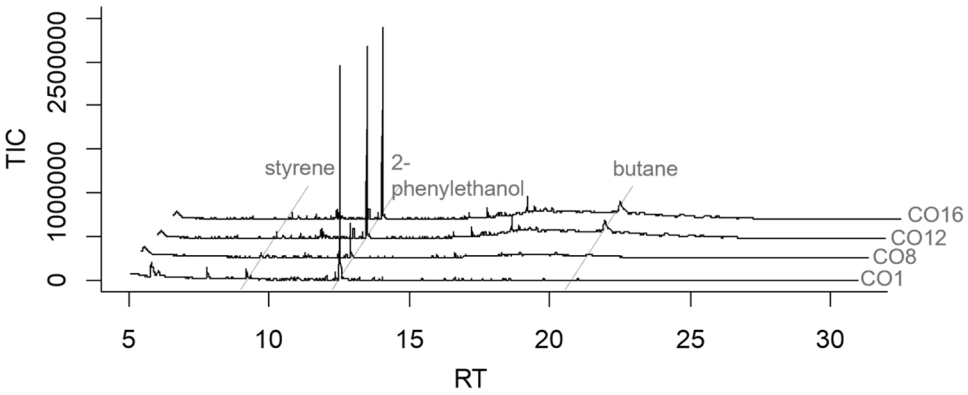

Fig. 4 Gas chromatograph traces comparing headspace volatiles collected from four yeast isolates (*Wickerhamomyces canadensis*) associated with *Dendroctonus rufipennis*. The x-axis shows retention time (RT) in minutes, and the y-axis shows total ion chromatograms (TIC). Traces are offset for ease of visual comparison

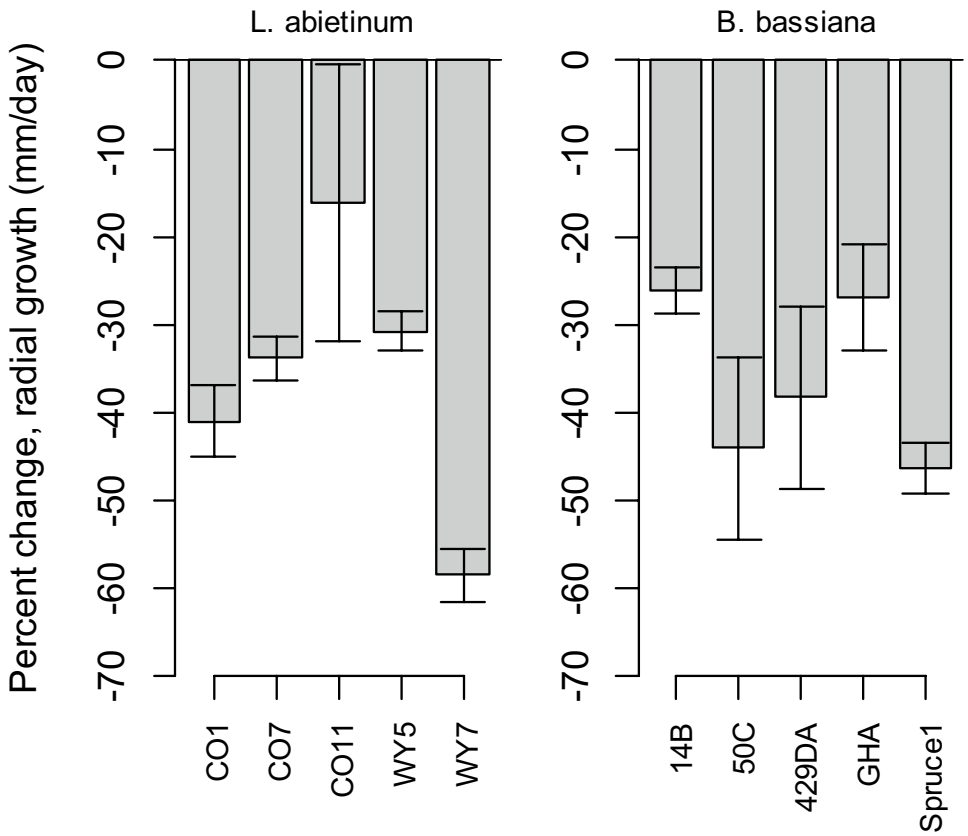

Fig. 5 Effects of in vitro exposure to vapors of synthetic 2-phenylethanol on change in mean radial growth rate (± SE) of isolates of *Leptographium abietinum* (symbiont) and *Beauvaria bassiana* (entomopathogen), relative to control growth rates. Isolate identity is shown on the x-axis

Discussion

Yeasts are extremely common associates of tree-feeding bark beetles; most yeast assemblages in the bark beetle holobiome are poorly understood but may have important ecological effects on beetles or microbial communities with which they interact [11]. Earlier reports estimated that nearly 100% of sampled *D. rufipennis* were associated with yeasts and yeasts were even more common than symbiotic filamentous fungi (i.e., *L. abietinum*, [42], but until now these yeasts have not been further characterized. Here, we isolated seven yeast species from the integument of *D. rufipennis*. *Wickerhamomyces canadensis* was the most frequently encountered species (74% of isolates) and was collected from all sampled populations, indicating that *W. canadensis* is the most abundant yeast on the beetle exoskeleton in the southern Rocky Mountains. Given the ubiquity of yeast-bark beetle associations and the prevalence of *W. canadensis* in the microbial assemblage of *D. rufipennis*, a better understanding of *W. canadensis* ecology could provide insight into the interactions that impact spruce beetle performance.

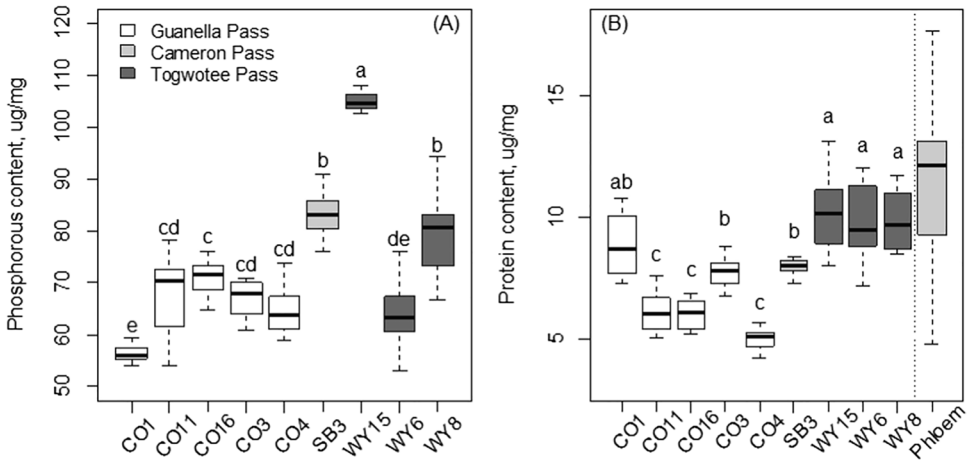

Fig. 6 The distribution of (**A**) phosphorous and (**B**) protein concentration for nine yeast isolates (*Wickerhamomyces canadensis*) associated with *Dendroctonus rufipennis* across three regions of Colorado and Wyoming. Lettering represents Tukey's HSD test; boxplots in each panel not connected by the same letter differ significantly. In (B), the protein content of host tree phloem (*Picea engelmannii*, $n = 27$ samples) is plotted alongside yeast isolates

Headspace volatiles produced by cultures of *W. canadensis* had inhibitory effects on both symbiotic (*L. abietinum*) and pathogenic (*B. bassiana*) filamentous fungi, but on average these effects were relatively minor (4 and 6% reduction to mean colony growth rates, respectively). However, some isolates of *L. abietinum* and *B. bassiana* grew faster when exposed to *W. canadensis* volatiles (Tables 3 and 4), indicating substantial variation in *W. canadensis* × filamentous fungi interactions. *Wickerhamomyces canadensis* isolates collected from beetle populations in Cameron Pass (CO) had the most inhibitory effects on growth of symbiotic fungi (Table 3), though there was no evidence of variation among isolates in terms of their inhibitory effects on pathogenic fungi (Table 4). However, there was variation among isolates of pathogenic fungi in their responses to *W. canadensis*, and an isolate collected from Engelmann spruce (Spruce 1; [33] was strongly negatively affected by *W. canadensis* headspace volatiles. In a subsequent assay testing contact between *W. canadensis* and *L. abietinum*, we found little evidence for 'contact zone inhibition' by *W. canadensis*, but cultures were able to exclude *L. abietinum* from colonizing resource space in more than half of trials (Fig. 3). This outcome contrasts with the findings from earlier studies (e.g., [14], where yeasts (*Ogataea pini*, formerly *Pichia pini*) associated with the southwestern pine beetle (*Dendroctonus barberi* Hopkins) were strongly inhibitory to *B. bassiana* but readily grew in mixed culture with beetle-symbiotic fungi (*Entomocorticium* sp.).

A single compound (2-phenylethanol) was identified as the primary volatile metabolite produced by *W. canadensis* and was consistently emitted by all tested cultures and at a relatively high rate (Fig. 4). 2-phenylethanol is a common fermentation byproduct and is described from the headspace of other ubiquitous yeasts and yeast-like fungi growing on carbon-rich substrates including *Saccharomyces cerevisae*, *Pichia anomala*, and *Aureobasidium pullalans* [12, 18, 29], and others). Consistent with other studies (e.g., [29], we report that exposure to 2-phenylethanol is inhibitory to filamentous fungi,here, isolates of both *L. abietinum* and *B. bassiana* were strongly negatively affected by synthetic 2-phenylethanol (~ 36% reduction in growth rate for both filamentous fungal species). In the subcortical gallery environment of a tree colonized by spruce beetles and associated microbes, both symbiotic and pathogenic fungi would also be exposed to a suite of tree secondary metabolites, particularly terpenoids, in addition to yeast metabolites. Terpenoids are extremely toxic to *B. bassiana* and strongly reduce radial growth [33], while *L. abietinum* tolerates much higher concentrations [15], suggesting that tree secondary metabolites likely mediate *D. rufipennis*-pathogen and *D. rufipennis*-symbiont interactions in addition to volatiles produced by *W. canadensis*. For example, studies of interactions among microbial symbionts in the mountain pine beetle (*Dendroctonus ponderosae* Hopkins) demonstrate that interactions between symbiotic fungi and bacteria can be amplified or even reversed in the presence of a single monoterpene found in most conifers (α-pinene,[2], underscoring the need for future studies to interpret beetle-microbe and microbe-microbe interactions on substrates containing phytochemicals.

2-phenylethanol produced by *W. canadensis* could have a variety of important ecological effects in addition to their impacts on associated filamentous fungi. For example, 2-phenylethanol is a broadly bioactive compound with important behavioral effects on many insect taxa, including wasps (Hymenoptera), flies (Diptera), and beetles (Coleoptera) [13]. Accordingly, 2-phenylethanol may be an important semiochemical within the North American spruce beetle system and could potentially serve as an attractant for secondary beetle species or parasitic taxa that take advantage of 2-phenylethanol as a signal of available host material (e.g., [44]. Alternatively, 2-phenylethanol may also be an important deterrent to *D. rufipennis* if high concentrations signal a host

tree in which they would likely experience high rates of intraspecific competition. These functions have not yet been tested but could improve potential integrated pest management applications for *D. rufipennis*. 2-phenylethanol could also directly impact the success of *W. canadensis*; for example, studies on the biotechnological production of 2-phenylethanol by yeasts demonstrate strong inhibition to growth of some yeasts due to cytotoxic effects [19], which could have negative feedbacks to *W. canadensis* proliferation within the gallery environment. Therefore, the sensitivity of various microbes, including *W. canadensis*, to 2-phenylethanol may be important for understanding dynamics of symbiotic interactions in this system.

Aside from their interactions within the microbial assemblage of bark beetles, earlier studies report that associations with yeast broadens the diet breadth of bark beetles by providing access to limiting nutrients that are uncommon in tree phloem, and access to yeast may be a requirement for development [38]. Specifically, nitrogen is a minor component of conifer phloem and in general limits the development of insects in many habitats,in some cases, nitrogen limitations faced by herbivorous insects are overcome via symbiotic associations [25]. We report that *W. canadensis* cultures had slightly lower mean nitrogen (protein) content (0.8%) than was found in the phloem of host trees (1.1%), indicating that the yeast is not a particularly rich source of N in comparison to phloem alone. However, protein content of spruce phloem was highly variable (Fig. 6B; range: 1.2 – 17.6 µg/g), and there could be instances where the yeast is a source of nutrition under the condition that a specific tree is nutritionally poor. The highest mean protein concentrations were found in *W. canadensis* isolated from *D. rufipennis* populations in Togwotee Pass, WY (Fig. 6). Moreover, protein content of *W. canadensis* was about fourfold lower than reported for the symbiotic filamentous fungus *L. abietinum* (Davis et al. 2019), indicating that association with *L. abietinum* is likely more nutritionally beneficial to *D. rufipennis* than association with *W. canadensis*.

We also detected isolate-to-isolate variation in all measured phenotypic traits of *W. canadensis* including production of 2-phenylethanol (Fig. 4), interactions with filamentous fungi (Table 3 and Fig. 3), and nutritional content (Fig. 6). The level of phylogenetic resolution in our analysis was insufficient to determine whether these differences were associated with genetic variation (Fig. 2), but it seems probable since all tests were done under nearly identical, controlled laboratory conditions (i.e., a constant environment). This phenotypic variation underscores the importance of analyzing microbial traits and interactions at an isolate level, and we expect that associations with specific genetic groups of *W. canadensis* could have consequences for beetle performance by mediating interactions with other symbionts or providing differing nutritional benefits. Although it is difficult to experimentally assess beetle-microbe associations under controlled or repeatable settings, such an approach would be extremely valuable for disentangling the relative contributions of symbionts from their genetic variation to bark beetle population dynamics and could advance basic theory in insect-symbiont ecology.

In summary, multiple saccharomycetous yeasts inhabit the integument of the North American spruce beetle (*D. rufipennis*), but *W. canadensis* is by far the most frequent. *Wickerhamomcyes canadensis* is likely also present in other substrates (e.g., frass, beetle galleries) and life stages (larvae, pupae), but only adults (the primary life stage associated with movement of microorganisms across habitats) were evaluated here. It is unknown whether yeast communities contained in the intestinal tract of *D. rufipennis* are similar to those identified here from the integument, but this may have consequences for beetle nutrition, and it is possible that *D. rufipennis* harbors symbiotic yeast and filamentous fungi internally. In addition, yeast species composition and phenotypic variation in yeast isolates could occur across beetle population phases (i.e., endemic low-density populations, incipient outbreaks, or epidemic high-density populations). Future studies would do well to design sampling of bark beetle microbial communities relative to beetle population phase in order to evaluate whether and how symbiotic associations contribute to rapid changes in herbivore population densities. *Wickerhamomcyes canadensis* had isolate-dependent interactions with the spruce beetle symbiont *L. abietinum* and the entomopathogen *B. bassiana*, and these interactions were likely mediated by production of the volatile compound 2-phenylethanol, a common fermentation byproduct. In addition, isolates could provide access to nutritional resources (protein and phosphorous) that are extremely limiting in the phloem of coniferous trees, and there is within-haplotype diversity in nutritional benefits provided by *W. canadensis*. Collectively, these findings add to our knowledge of the factors affecting interactions within the bark beetle microbiome and may help to elucidate mechanisms contributing to beetle population dynamics across landscapes.

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Author Contribution TSD and JES designed the study. TSD collected isolates and performed interaction experiments. JES performed genetic identification of isolates. CVB and CC performed the nutritional analyses. TSD, JES, and CVB analyzed the data. TSD wrote the first draft of the manuscript and all authors contributed to editing the manuscript.

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Data Availability Data will be made available for research purposes upon reasonable request to the authors.

Declarations

Ethics Approval This work does not contain any studies with human participants or animals performed by any of the authors.

Conflict of Interest The authors declare no competing interests.

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