Effects of live yeasts and their metabolic products on bumble bee microcolony development

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Abstract

Bumble bees can benefit from fungi, though the mechanisms underlying these benefits remain unknown and could include nutrition, resource supplementation, or pathogen protection. We tested how adding living yeasts or their metabolic products to *Bombus impatiens* diets in a factorial experiment affects microcolony performance, including survival, reproduction, and pathogen presence. We additionally assessed effects of yeast treatments on diet (nectar and pollen) chemical composition using untargeted metabolomics. Yeasts impacted microcolony reproduction and survival, but effects depended on source colony. Colonies containing the putative pathogen *Aspergillus* showed reduced reproduction, but yeast treatments reduced *Aspergillus* prevalence. Yeast treatments altered chemical composition of nectar and pollen, but most distinguishing compounds were unidentified. Our results suggest limited direct effects of yeasts via nutrition, resource supplementation, or modification of diets, instead suggesting that yeasts may benefit bees through interactions with the pathogens including *Aspergillus*. Overall, the effects of yeast supplementation are context-dependent, and more research is necessary to better understand the factors important in determining their impacts on bee hosts.

Keywords: bee microbiome, metabolomics, *Bombus*, *Aspergillus,* mycobiome

Introduction

Insect-microbe associations are common and widely recognized for the role that microbes play in disease as well as the many beneficial functions they provide to their insect hosts. Most research has focused on the interactions between insects and bacteria, which are widespread and obligate in some insect systems (Feldhaar 2011, Kwong et al. 2017, Moran et al. 2005). However, microbial communities associated with insects are diverse, containing bacteria, fungi, viruses, and other taxa (Bessette & Williams 2022, Kaufman et al. 2000, Leigh et al. 2018, Scully et al. 2013). For some insect species, the importance of beneficial fungi has been recognized (Biedermann & Vega 2020), with fungi playing crucial roles in host biology, including acting as food, modifying host diet, or contributing compounds important to insect development. For example, several species of ants, termites, and beetles grow fungi in their nests as a food source (Mehdiabadi & Schultz 2009, Mueller & Gerardo 2002, Vanderpool et al. 2017). In addition, cactophilic *Drosophila* depend on fungi that inhabit the cactus wounds in which these flies breed and feed. These fungi detoxify toxic cactus compounds and produce sterols and vitamins essential to fly development (Starmer et al. 1986, Vega & Dowd 2005). Certain species of planthoppers, beetles, and bees also rely on associated fungi for the production of developmentally required sterols (Nasir & Noda 2003, Noda & Koizumi 2003, Paludo et al. 2018). Insect-associated fungi may also play a role in breakdown of complex food resources, including polysaccharides like cellulose, hemicellulose, or pectin (Li et al. 2012, Li et al. 2021, Vega & Dowd 2005).

Many bee species also host fungi, although the nature of these associations are just beginning to be understood (Rutkowski et al. 2023). A subset of these fungi are documented bee pathogens, including species in the genera *Aspergillus*, *Ascosphaera*, and *Vairimorpha*.

However, these groups represent only a fraction of the fungal diversity associated with bee nests. Diverse groups of fungi are commonly isolated from the nests and guts of many bee groups, including honeybees, bumble bees, stingless bees, and solitary bees (Christensen et al. 2024, Dharampal et al. 2020, Echeverrigaray et al. 2021, Gilliam 1997, Rutkowski et al. 2023, Yun et al. 2018), many with unknown or beneficial impacts on bee health. Emerging evidence suggests that some bee species within the genus *Melipona* have lost bacterial symbionts and gained the yeast *Starmerella* within their gut instead (Cerqueira et al 2021) although the function of these yeasts remains unknown. For bumble bees (genus *Bombus*), yeasts have notable impacts on bee behavior and health. Foraging *Bombus* workers prefer to visit flowers inoculated with nectar yeasts over those without microbes (Schaeffer & Irwin 2014, Herrera et al. 2013, Yang et al. 2019), though these nectar yeasts were not found to impact colony development in *B. impatiens* (Schaeffer et al. 2017). Previous work in *Bombus impatiens* and *B. vosnesenskii* has found that adding nest-associated fungi to bee diets can improve bee survival and reproductive output, and help bees recover from negative effects of fungicide exposure (Rutkowski et al. 2022). Other work has found similar positive effects of adding flower or bee-associated fungi to the diets of *B. terrestris*, though these positive effects were dependent on the fungal species added (Pozo et al. 2020, Pozo et al. 2021).

The mechanisms behind these positive effects are unknown, but a few mechanisms are hypothesized (Steffan et al 2023). First, bees could derive nutrients from ingesting fungal cells, as has been implicated in the solitary bee genus *Osmia* (Dharampal et al. 2019, Steffan et al. 2019). Fungi may also produce vitamins and other metabolites important in bee diets or aid in the breakdown of dietary compounds. In the stingless bee *Scaptotrigona depilis*, a *Zygosaccharomyces* yeast provides sterols necessary for bee development, greatly improving

74 larval survival (Paludo et al. 2018). In addition to nutritional impacts, fungi may directly
75 suppress the growth of pathogens or improve bee immune function (Christensen et al. 2024,
76 Pozo et al. 2020). A previous test of nutritional hypotheses using flower- and bee-associated
77 fungal species found that different fungal species varied in their effects on *B. terrestris*, with
78 some yeasts benefitting colony reproduction. However, the mode through which these fungi were
79 provided had no impact (Pozo et al. 2020).
80 Here, we leverage a fungal community previously shown to benefit bumble bee fitness
81 (Rutkowski et al. 2022) to test possible nutritional mechanisms through which fungi impact bee
82 health. We hypothesize that fungi influence bumble bees through a combination of nutritional
83 input from ingestion of fungal cells and fungal metabolites, and modification of nest provisions
84 (stored nectar and pollen). To test this hypothesis, we factorially manipulated the presence of live
85 fungal cells and fungal metabolites. We fed these solutions to microcolonies of the bumble bee
86 *Bombus impatiens* and measured microcolony survival and reproductive success over the course
87 of four weeks. If yeast effects are primarily chemically-mediated, the presence of live fungi or
88 their metabolites (all yeast treatments) should benefit bumble bee survival and reproduction. If
89 yeast effects are mediated via interactions between live yeasts and bees, only live yeasts (not
90 metabolites alone) should benefit bees.
91
92 **Methods**
93 *Bumble bee rearing conditions and experimental setup*
94 We created 104 microcolonies of the commercially-reared bumble bee *Bombus impatiens*
95 from 7 separate source colonies (Koppert, USA). Microcolonies are queenless colonies of
96 bumble bee workers. In the absence of a queen, workers will lay eggs that develop into adult

male offspring (Cnaani et al. 2002, Lopez-Vaamonde et al. 2007), allowing measures of microcolony survival as well as reproduction. *B. impatiens* is native to the eastern half of the United States, but commercial colonies are used throughout the US for pollination of greenhouse crops such as tomato and pepper (Velthuis et al. 2006). *Bombus impatiens* is often used in research as a model bumble bee species, and has been shown to benefit from addition of fungi in a previous study (Rutkowski et al. 2022). For this study, source colonies of *B. impatiens* were reared with nectar solutions provided by Koppert and sterilized honeybee-collected pollen (Koppert, USA). Pollen was sterilized using ethylene oxide to ensure that it was not contaminated with the bee pathogen *Ascosphaera apis*, which has previously been found in honeybee-collected pollen (Dharampal et al. 2020, Rutkowski et al. 2022) and can infect adult bumble bees (Maxfield-Taylor et al. 2015). Ethylene oxide gas was used due to the effectiveness of this sterilization method against fungi as well as its minimal side effects on bees compared to other common pollen sterilization methods (Strange et al. 2023). Although efforts were made to limit pathogen introduction via contaminated honeybee pollen, commercially reared colonies vary in the presence of pathogens and parasites (Strange et al. 2021).

Each microcolony consisted of five workers from a single source colony. Microcolonies were initially provided with a pollen ball, nectar, and wax pellets (Geelywax, China) to encourage reproduction. Pollen balls were created by mixing equal parts finely ground and sterilized honeybee-collected pollen (Koppert, USA) and sterile nectar. Nectar was created using a 2:2:1 ratio of glucose:fructose:sucrose, to a final total sugar concentration of 30%. Nectar was autoclaved prior to use to ensure sterility, after which non-essential amino acids (Cytiva, USA) were added to a final concentration of 5%. All microcolonies were initially fed with nectar

inoculated with 7.5 ppm of the fungicide propiconazole (QualiPro, USA) for one week to reduce existing fungal abundance associated with each microcolony (Rutkowski et al. 2022).

Each microcolony was then assigned to a diet treatment consisting of 1) the presence or absence of live yeast cells and 2) the presence or absence of yeast metabolites. This created a total of four treatment groups (n=26 microcolonies/treatment). Treatment 1 included live yeast cells grown in artificial nectar for 5 days before being given to microcolonies (live yeasts + metabolites). Treatment 2 consisted of live yeast cells grown in artificial nectar for 5 days before cells were filtered out using a 0.2µm filter, leaving behind only modified nectar (metabolites only). Treatment 3 consisted of yeast cells newly suspended in artificial nectar (live yeasts only), and Treatment 4 consisted of sterile control nectar without yeast (no live yeasts, no metabolites). Yeasts *Debaryomyces hansenii, Starmerella sorbosivorans,* and *Zygosaccharomyces rouxii* were isolated from the honeypots of commercial *B. impatiens* colonies and were beneficial to bees in previous studies (Rutkowski et al 2022). These yeasts were grown separately and then added to the nectar of Treatments 1, 2, and 3 at a concentration of $1x10^4$ cells/mL for each species.

Microcolonies were kept on their treatments for four weeks, and nectar was replaced every three days (except in the case of Treatment 2, which was replaced every day to minimize yeast metabolism and modification of nectar). Pollen balls were made using these same treatment nectars and were replaced every three days until the start of reproduction, after which point colonies were given supplemental pollen as needed. Pollen balls were made once a week and stored at 4^oC between replacements. Survival of workers in each microcolony was recorded daily, and time to emergence of the first adult offspring was tracked. To determine the impact of treatment on bumble bee feeding, nectar consumption over a three day period was measured for a subset of microcolonies (n=12/treatment) by weighing nectar cups before and after providing

them to colonies. At the end of four weeks on their treatments, all surviving colonies were frozen and dissected to remove, count, and weigh all offspring (eggs, larvae, pupae, and adult males).

After microcolony creation, we noticed that microcolonies across all treatments that were created from source colonies that were older than one month (n=28) rarely produced offspring and died much sooner than other microcolonies. As many of these microcolonies died early into application of treatments, we did not include microcolonies sourced from old colonies in our analysis below. Treatments were equally represented in the dropped colonies, so that for all treatments, final sample size was equal (n=19 microcolonies/treatment).

Pathogen presence and abundance

Because symptomatic fungal infection was observed in the larvae of some microcolonies, all microcolonies were screened for the presence and abundance of two common fungal pathogens (Strange et al. 2021). Diseased larvae from two microcolonies that exhibited fungal growth over their brood were plated on yeast media and resulting fungal growth was sampled and boiled in sterile water to extract DNA. DNA was amplified using PCR with the primers ITS86F (5' GTGAATCATCGAATCTTTGAA 3') and ITS4 (5' TCCTCCGCTTATTGATATGC 3') and the following run conditions: an initial denaturation at 95^{o}C for 2mins, followed by 40 cycles of 95^{o}C for 30s, 55^{o}C for 30s and 72^{o}C for 1min, and final extension step at 72^{o}C for 10mins. Amplified DNA was sent to the UCDNA Sequencing Facility at UC Davis for Sanger sequencing using the same primers. The resulting sequences were identified using NCBI BLAST and both matched to *Aspergillus flavus* at 98.89% and 98.99% identity (Table S1). Therefore, all microcolonies were screened for pathogens using three primer sets targeting *Aspergillus flavus*, the *Aspergillus* genus, as well as the bee-specialist

genus *Ascosphaera* (Evison & Jensen 2018), which has previously been detected in commercial *B. impatiens* colonies (Dharampal et al. 2020, Rutkowski et al. 2022).

To screen for pathogens, three workers in each microcolony were dissected to remove the midgut and hindgut. All three gut samples were pooled together and DNA was extracted using a PowerSoil Pro Kit (QIAGEN). To screen for the presence of *Aspergillus flavus*, we used the *A. flavus*-specific primers FLAVIQ1 (5' GTCGTCCCCTCTCCGG 3') and FLAVQ2 (5' CTGGAAAAAGATTGATTTGCG 3'), using run conditions as in Sardiñas et al. 2011. To screen for the presence of the genus *Aspergillus,* we performed PCR using the genus-specific primers ASAP1 (5' CAGCGAGTACATCACCTTGG 3') and ASAP2 (5' CCATTGTTGAAAGTTTTAACTGATT 3'), using run conditions as in Sugita et al. 2004. To screen for *Ascosphaera*, the genus-specific primers AscoALL-1 (5' GCACTCCCACCCTTGTCTA 3') and AscoALL-2 (5' GAWCACGACGCCGTCACT 3') were used, using run conditions as in James & Skinner 2005.

Multiple colonies tested positive for the presence of *A. flavus*, and abundance was quantified using qPCR on samples using the same primers and qPCR cycle conditions specified in Sardiñas et al. 2011. We used a custom plasmid (Eurofins Genomics Blue Heron) containing a known concentration of *A. flavus* amplicon DNA to convert from Cq values to total abundance (Table S2).

Chemical analysis of yeast modifications to diet

To understand effects of yeast growth on the chemical composition of diet, nectar and pollen were collected from each microcolony three days after treatment application and a subset of these samples (n=5 per treatment, from two source colonies) were submitted to the West Coast

Metabolomics Center at UC Davis for untargeted metabolomics using GC-TOF MS. To prepare nectar samples, 30uL of nectar was extracted using 1mL of a 5:2:2 methanol:chloroform:water solution. Samples were then vortexed for 10s, shaken for 5 mins, and centrifuged for 2 mins at 14,000rcf. Each 50uL aliquot of the resulting supernatants was dried. For pollen samples, 4.0±0.3mg of pollen was extracted using 1mL of the same solution as for nectar. Samples were then sonicated for 1hr with 1.6mm steel balls, shaken for 5mins, and centrifuged for 2 mins at 14,000rcf. From the resulting supernatant, 450uL aliquots were dried.

Aliquots of 0.5uL of prepared samples, resuspended in MSTFA, were injected into a 7890A GC coupled with a LECO Pegasus IV TOF MS, onto a RESTEK RTX-5SIL MS column with an Intergra-Guard at 275^{o}C with a helium flow of 1 mL/min using a splitless method. Samples were held in the GC at 50^{o}C for 1 min before heating to 330^{o}C at a rate of 20^{o}C/min, followed by sample holding for an additional 5 mins. The transfer line was heated to 280^{o}C and the EI ion source to 250^{o}C. The MS collected data from 85m/z to 500m/z at an acquisition rate of 17 spectra/s. Data preprocessing was performed using ChromaTOF version 2.32, with automatic mass spectral deconvolution and peak detection using a 5:1 signal:noise ratio. Preprocessed chromatograms were then identified using the BinBase database, using the associated BinBase algorithm. Peak height was used to report compound quantification and used in statistical analysis (below).

Statistical analysis

All data analysis was carried out in R version 4.2 (R Core Team 2022). We tested if the presence of live yeasts (presence/absence), metabolites (presence/absence), or source colony influenced microcolony parameters. We tested for differences in the likelihood that a microcolony produced any offspring across treatments using a generalized linear model with a

binomial distribution. Offspring abundance (total and for each life stage) between treatments was analyzed using a generalized linear model with a negative binomial distribution ('MASS' package, Venables & Ripley 2022). The effect of worker survival on total offspring abundance was analyzed using a generalized linear model with a negative binomial distribution. This survival term was calculated as the average number of workers alive during the entire duration of the microcolony life span. We compared the average mass of offspring (separately for each life stage) across treatments using ANOVA. Larval mass was not normally distributed and was square-root transformed prior to analysis. As few microcolonies produced pupae and adult males, the three way interaction between treatments and source colony was not included in these analyses. Differences in offspring composition (proportion of developmental stages) between treatments was analyzed using a chi-square test, run on a contingency table of proportions of each offspring stage (eggs, larvae, pupae, and adults) by treatment.

Survival of microcolony workers was analyzed using a Cox proportional hazards model for recurrent events ('survival' package, Therneau 2022), with live yeast presence, metabolite presence, and source colony as predictors and microcolony ID as a random effect. Per-bee nectar consumption was compared between treatments using ANOVA.

To test if the presence and abundance of presumed pathogens differed between yeast treatments, *Aspergillus* or *A. flavus* presence across treatments and source colonies was analyzed using a generalized linear model with a binomial distribution, while abundance of *A. flavus* was analyzed using an ANOVA. For these models, live yeast presence, metabolite presence, source colony, and all interactions between factors were included as predictors. Prior to all subsequent analyses of *Aspergillus* impacts on offspring production, data was subset to only include source colonies that tested positive for *Aspergillus*. The impact of *Aspergillus* presence on offspring

presence was analyzed using a generalized linear model with a binomial distribution, with *Aspergillus* presence in a microcolony, live yeast presence, metabolite presence, and their interaction as predictors. The impact of *Aspergillus* on offspring abundance was analyzed using a generalized linear model with a negative binomial distribution, with *Aspergillus* presence in a microcolony, live yeast presence, metabolite presence, and their interaction as predictors. The impact of *Aspergillus* on survival was analyzed using a Cox-proportional hazards model, with live yeast presence, metabolite presence, *Aspergillus* presence within a microcolony, and their interactions as predictors, and microcolony ID as a random effect. Only *Aspergillus* impacts on offspring production and worker survival were analyzed, as *A. flavus* was present in appreciable amounts from only five microcolonies, and *Ascosphaera* was not detected in any microcolonies.

Metabolomics data was filtered and normalized using MetaboAnalyst 6.0 (Pang et al. 2021). Nectar and pollen samples were processed and analyzed separately, with live yeast presence and metabolite presence as main and interactive factors. According to standard MetaboAnalyst filtering recommendations, features were first filtered using median to remove low-value features, and then using interquartile range to remove features that remained constant throughout the dataset. Raw data contained 564 features, and after filtering contained 423 features. Data were normalized by median and auto scaled (mean-centered and divided by the standard deviation of each variable) before analysis to achieve a more normal distribution. The ANOVA2 function in MetaboAnalyst was used to test for differently abundant compounds between treatment groups, with the false discovery rate used for multiple testing p-value correction. For subsequent analyses, normalized data was imported into R. We ran a redundancy analysis ('vegan' package, Oksanen et al. 2022) to test for differences in metabolite profile between treatment groups, followed by permutation tests to determine significance (999

permutations). Random forest analysis ('randomForest' package, Liaw & Wiener 2002) was run to determine accuracy in classifying samples to treatments based on metabolomic profiles, using 10,001 trees per model. Results were visualized using the packages 'ggplot2' (Wickham 2016) and 'viridis' (Garnier et al. 2023).

Results

Yeast effects on microcolony reproduction vary with source colony

80% of microcolonies produced at least one offspring, with a mean of 14.4 offspring per microcolony (range 0-60 offspring). The probability that a microcolony produced at least one offspring was impacted by source colony ($X^2 = 12.9$, df = 6, p = 0.045), but was unaffected by metabolite presence ($X^2 = 0.16$, df = 1, p = 0.69), live yeast presence ($X^2 = 0.59$, df = 1, p = 0.44), or any interactions between factors (p > 0.05 for all comparisons). Microcolonies that survived longer were more likely to produce offspring ($X^2 = 7.1$, df = 1, p = 0.008).

Total offspring abundance (eggs, larvae, pupae, and adult males) was affected by both live yeast presence and metabolite presence, but these effects were dependent on source colony (live yeast presence * source colony interaction: $X^2 = 26.8$, df = 6, p < 0.001, metabolite presence * source colony interaction: $X^2 = 19.2$, df = 6, p = 0.004, Figure 1). For four of the seven source colonies, fungal treatment in some form was beneficial to offspring production, while for the other three colonies, treatments were either detrimental or had no effect. Offspring abundance was also higher in microcolonies that lived longer ($X^2 = 22$, df = 1, p < 0.001).

Considering each life stage separately, the abundance of eggs, larvae, pupae, and adult males was also dependent on the interactions between treatments and source colonies (Table 1). The mass of most life stages was unaffected by treatments or source colony, except for larval

mass, which was lower in microcolonies given yeast metabolites (Table S3). Offspring composition was significantly different between treatments ($X^2 = 19.2$, df = 9, p = 0.02, Figure 2), with microcolonies receiving only live yeast cells containing compositionally more larvae and males than other treatments.

Effects of treatments on worker survival varies by source colony

Worker survival was affected by the three-way interaction between live yeast presence, metabolite presence, and source colony ($X^2 = 25.8$, df = 5, p < 0.001, Table 2). Workers in microcolonies from four source colonies survived longer when given fungal treatments in some form, though the most beneficial mode of application varied across source colonies. For the other three source colonies, fungal treatments had either neutral or negative effects on worker survival compared to the control.

Nectar consumption was unaffected by treatments, but differed across source colonies

Microcolonies consumed similar amounts of nectar regardless of live yeast presence ($F_{1,29} = 2.21$, p = 0.15) or metabolite presence ($F_{1,29} = 0.001$, p = 0.98). However, microcolonies from different source colonies consumed different amounts of nectar ($F_{4,29} = 16.2$, p < 0.001). There were no significant interactions between any of the factors (p > 0.05 for all comparisons).

Fungal treatments reduce Aspergillus *prevalence, but may exacerbate negative pathogen effects*

Because fungal pathogen infection was observed in a subset of microcolonies, colonies were screened for pathogens using PCR and qPCR. *Ascosphaera* was not detected in any microcolony, but *Aspergillus* was detected in 25 of 76 (33%) of microcolonies, and *A. flavus* was

304 detected in 5 of 76 (6.6%) microcolonies using PCR. We further investigated *A. flavus*
305 abundance using qPCR, and found that most microcolonies contained very low amounts, while a
306 few microcolonies exhibiting symptomatic infection contained very high levels (range 0.09 -
307 3,533,021 copies, median = 8.5 copies).

308 Live yeast presence and metabolite presence interacted to impact *Aspergillus* presence,
309 with control microcolonies exhibiting the greatest incidence ($X^2 = 4.13$, df = 1, p = 0.04, Figure
310 3A). Source colonies also differed in *Aspergillus* presence ($X^2 = 24.6$, df = 6, p < 0.001).
311 However, the presence and abundance of known pathogen *A. flavus* (detected with much lower
312 incidence) were not significantly affected by live yeast presence, metabolite presence, or their
313 interaction (Table S4), though source colonies differed in both presence ($X^2 = 17.3$, df = 6, p =
314 0.008) and abundance ($F_{6,66} = 8.11$, p < 0.001) of *A. flavus*.

315 Considering only source colonies that tested positive for *Aspergillus* (all source colonies
316 except SS4), *Aspergillus* presence reduced the probability that a microcolony would produce
317 offspring, although this effect was dependent on live yeast and metabolite presence and was most
318 pronounced for microcolonies receiving live yeast cells or metabolites alone (*Aspergillus*
319 presence * live yeast presence * metabolite presence interaction, $X^2 = 10.05$, df = 1, p = 0.002,
320 Figure 3B). *Aspergillus* presence also interacted with live yeast presence and metabolite presence
321 to impact total offspring number; microcolonies receiving no fungi or both live cells and
322 metabolites did not respond as negatively to *Aspergillus* presence as those receiving either live
323 cells or metabolites singly. However, this result was only marginally significant (three-way
324 interaction, $X^2 = 3.19$, df = 1, p = 0.074). The presence of *Aspergillus* within a microcolony had
325 no impact on adult bumble bee survival, and did not interact with any yeast treatments to impact
326 survival (Logrank test, global p = 0.5).

The presence of yeast cells alters chemical composition of bee diets

To determine how yeast treatments impacted the chemical composition of diets provided to bees, GC-MS metabolomics was performed on nectar and pollen taken from microcolonies three days after treatment application. Qualitatively, nectar contained a lower diversity of compounds than pollen (Figure S1) and contained mostly glucose, fructose, and sucrose present at high relative abundance. Pollen (here, a combination of nectar and pollen) contained mainly proline, glucose, fructose, gluconic acid lactone, and sorbitol. The diversity of compounds in nectar and pollen was unaffected by treatments (Table S5).

The presence of live yeast cells altered the chemical composition of nectar (RDA, $F_{1,16}$ = 1.60, R^2 = 0.06, p = 0.032, Figure 4A), while metabolite presence ($F_{1,16}$ = 1.31, p = 0.10) and the interaction between treatments ($F_{1,16}$ = 1.27, p = 0.12) had no effect. For pollen provisions, the interaction of live yeast presence and metabolite presence impacted compound composition ($F_{1,16}$ = 1.51, R^2 = 0.025, p = 0.042, Figure 4B).

Using live yeast presence as a response, random forest was able to successfully classify nectar samples to treatment based on chemical compound composition 90% of the time (10% OOB error). Using metabolite presence as a response, the classification accuracy was 75% (25% OOB error). For pollen samples, random forest correctly classified samples to live yeast treatments 60% of the time (40% OOB error) but only 25% of the time (75% OOB error) when metabolite presence was used as a predictor. Chemical features that were most important in distinguishing treatment groups differed by sample type, and were largely unidentified compounds (Figure S2).

In nectar, four compounds differed between metabolite presence treatments. Two unidentified compounds, 403585 ($F_{1,16}$ = 103, $p < 0.001$) and 403592 ($F_{1,16}$ = 120, $p < 0.001$) were lower in metabolite treatments, while unidentified compound 121482 was higher in metabolite treatments ($F_{1,16}$ = 19.7, p = 0.03, Figure S3A). Adenosine was marginally lower in metabolite treatment groups ($F_{1,16}$ = 15.5, p = 0.05). For pollen, only one unidentified compound, 112602, differed between treatments, and was lower in metabolite treatments ($F_{1,16}$ = 57.3, $p < 0.001$, Figure S3B). Mass spectra of these unidentified compounds is provided in Table S6.

Discussion

The experiment performed here tested several possible mechanisms through which symbiotic fungi might benefit bumble bees. Specifically, we tested if bees benefit from living fungal cells or from fungal metabolites and/or from alteration of food resources by fungi. We found no benefit of fungi across all microcolonies, either as living cells, their metabolites, or their effects on food, but we did observe treatment effects that were dependent on source colony identity, suggesting context-specific benefits of yeasts.

Colonies may have responded differently to fungal treatments for a variety of reasons, including differences in colony age, queen and worker health, genetic background, and variation in pre-existing microbial communities associated with each bee colony. Some studies document strain-level differences in bacterial gut microbiome composition between commercial *B. impatiens* source colonies (Hammer et al. 2022, Meeus et al. 2015; but see Rutkowski et al. 2022). In addition, microbial communities of stored provisions can vary between colonies, particularly in the presence and abundance of pathogens (Dharampal et al. 2020, Graystock et al. 2013), including viruses, *Vairimorpha, Crithidia*, and *Ascosphaera*, among others (Pereria et al.

2019, Strange et al. 2022). Previous work has documented the presence of *Ascosphaera* in the pollen provided to colonies (Dharampal et al. 2020, Rutkowski et al. 2022), which can infect adult bumble bees (Maxfield-Taylor et al. 2015). For this reason, we sterilized the pollen provided to microcolonies in the current experiment. Despite this, we observed offspring of microcolonies from one source colony (SS5) infected by the fungal pathogen *Aspergillus flavus*, and upon further investigation, observed variation in the presence of *Aspergillus* among source colonies. Although we did not manipulate the presence of this pathogen, variation in its presence allowed for a natural experiment testing yeast effects on bee-*Aspergillus* interactions. Interestingly, microcolonies derived from the source colony exhibiting symptomatic infection were the only ones that benefitted from a combination of live fungal cells and metabolites, though the presence and abundance of *A. flavus* was unaffected by fungal treatment (perhaps due to low replication within this source colony).

Results of other studies suggest that bee-associated fungi can suppress the growth of multiple bee pathogens and parasites: the presence of yeasts in *B. impatiens* colonies was associated with the absence of certain pathogenic fungi including *Ascosphaera apis* (Dharampal et al. 2020) and multiple yeasts suppress the growth of *Crithidia bombi* in vitro (Pozo et al. 2019). Our results add further experimental *in vivo* evidence of anti-pathogen effects of yeasts: here, any microcolonies treated with live yeasts or their metabolites had reduced incidence of *Aspergillus* compared to the control, suggesting possible suppression by symbiotic yeasts and their chemical byproducts. However, when *Aspergillus* was present in colonies, yeasts seemed to exacerbate negative effects of the pathogen, leading to lower rates of offspring production. Honeybees stressed with pesticides or pathogens more frequently hosted yeasts, suggesting that yeasts may exploit weakened bee hosts (Gilliam 1973, Gilliam et al. 1974). This may also be the

case for bumble bees experiencing pressure from pathogens, but as this yeast-pathogen interaction was not the focus of this experiment, the conclusions we can draw from these data are limited, and further experiments explicitly testing the interactions of bee-associated yeasts with *Aspergillus* and other bee pathogens are necessary.

The current results add to a body of literature documenting variable and context-dependent impacts of fungi on bee health. Previous yeast addition experiments have found impacts of yeasts that vary based on bee and yeast species used. A previous study of *B. terrestris* used a similar study design to differentiate the effects of yeast cells and yeast metabolites/yeast-modified media on bee colonies (Pozo et al. 2020). They found that the nectar-associated yeast species *Metschnikowia gruessii* and *Rhodotorula mucilaginosa* as well as the bee-associated *Candida bombiphila* (=*Wickerhamiella bombiphila*) led to the production of more offspring, but this effect was independent of how the yeasts were administered. *M. gruessii, M. reukaufii*, and *C. bombiphila* also reduced larval mortality in these colonies. *Candida bombi* (=*Starmerella bombi*) was found to have no impact on colony health, as we found here for the related species *Starmerella sorbosivorans*. Another study that added the nectar yeast *Metschnikowia reukaufii* to *B. impatiens* microcolonies found it had no impact on colony development, though it was attractive to bees in foraging trials (Schaeffer et al. 2017). Finally, previous results using the same yeast community as in this study showed that fungal addition was beneficial to *B. impatiens* survival and reproduction (Rutkowski et al. 2022). In all, these results suggest that responses of bees to fungal supplementation are dependent on both bee and fungal identity, and that even if these are held constant, outcomes may still vary based on other underlying factors.

Given the known impacts of yeast metabolism on floral nectar traits, such as the concentration of amino acids and secondary metabolites, sugar concentration and composition,

418 and volatile profile (Herrera et al. 2008, Rering et al. 2021, Vannette & Fukami 2016, Vannette
419 & Fukami 2018), it is surprising that metabolomics analysis did not reveal large differences in
420 compound composition between treatment groups for nectar or pollen. Most of the compounds
421 that were detected as significantly different between treatments were unidentified, limiting the
422 interpretation of our results. It is possible that yeasts alter metabolite composition in additional
423 ways that were not detected using the untargeted primary metabolism method we used, which
424 generated relative abundance measures of most compounds but not absolute abundances.
425 Additionally, GC-MS mainly targets carbohydrates, amino acids, hydroxyl acids, free fatty acids,
426 purines, pyrimidines, and aromatics. Other types of compounds, such as higher molecular weight
427 lipids and complex carbohydrates, are not detected using this method and may be particularly
428 important when considering yeast metabolism in this system. Two genera of yeasts used in this
429 experiment, *Starmerella* and *Zygosaccharomyces*, produce lipids that have the potential to
430 impact bee health. *Zygosaccharomyces* yeasts produce ergosterols necessary for development in
431 certain stingless bee species (Paludo et al. 2018, Paula et al. 2023), while *Starmerella* yeasts
432 produce sophorolipids that have broad antimicrobial properties (De Clercq et al. 2021, de O
433 Caretta et al. 2022). Research investigating whether bee-associated yeasts produce these
434 compounds in bee diets, and the resulting impacts on bee health, is necessary to further
435 understand this bee-yeast association.

436 Overall, we found that impacts of yeast addition on *Bombus impatiens* microcolonies
437 were inconsistent, and instead depended on source colony identity. These differences across
438 source colonies may have been due to multiple factors, including variation in pathogen
439 communities associated with each colony. We find partial support for the hypothesis that bee-
440 associated yeasts may influence bee health through suppression of pathogens, as *Aspergillus* was

441 more prevalent in control microcolonies. However, additional experiments explicitly testing this
442 interaction are necessary to draw clear conclusions about the role of these yeasts in pathogen
443 suppression and bee health. Taken together, we suggest that yeasts are common and perhaps
444 ubiquitous symbionts of bumble bees and their stored food, but are likely not obligate mutualists,
445 offering context-specific benefits as well as costs.

446

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450

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Data availability statement: Bee response data and metabolomics data, along with all associated R code, is publicly available on Dryad (https://doi.org/10.5061/dryad.f1vhhmh5t)

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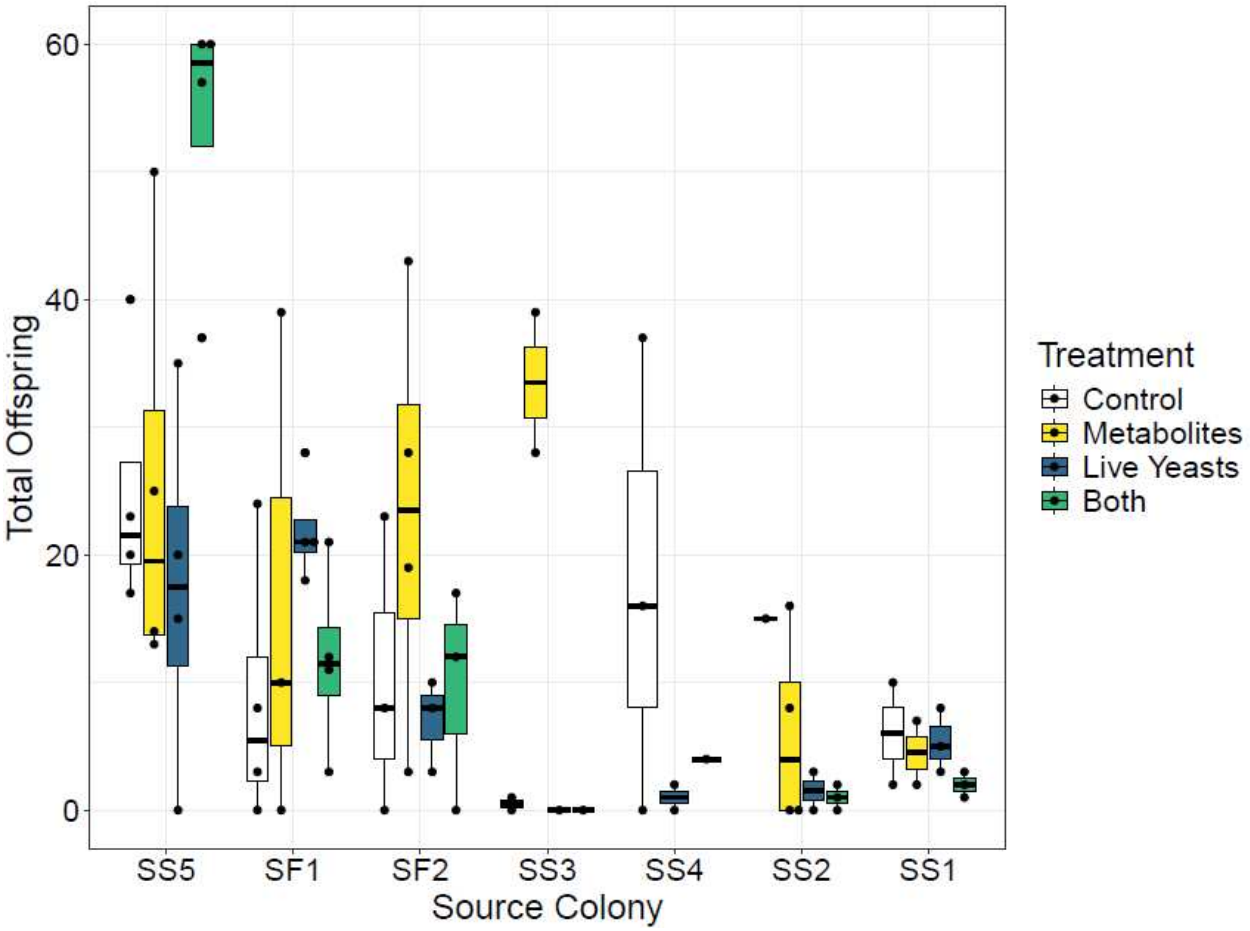

674

675 Figure 1. Total number of offspring (eggs, larvae, pupae, and adult males) produced by *B.*

676 *impatiens* microcolonies exposed to different fungal treatments, faceted by source colony.

677 Treatment effects were dependent on the source colony the microcolony was from (live yeast

678 presence * source colony interaction: $X^2 = 26.8$, df = 6, $p < 0.001$, metabolite presence * source

679 colony interaction: $X^2 = 19.2$, df = 6, $p = 0.004$).

680 Table 1. Effects of yeast treatments and source colony on microcolony offspring abundance,
681 separated by life stage.

Response variable	Predictor variable	X^2 value	p-value
Egg number	Live yeast presence	2.96	0.086
	Metabolite presence	0.01	0.92
	Source colony	**47.7**	**< 0.001**
	Live yeast presence * Metabolite presence	0.01	0.92
	Live yeast presence * Source colony	**17.1**	**0.009**
	Metabolite presence * Source colony	**18.7**	**0.005**
	Live yeast presence * Metabolite presence * Source colony	9.62	0.087
Larvae number	Live yeast presence	0.53	0.47
	Metabolite presence	1.64	0.20
	Source colony	**61.5**	**< 0.001**
	Live yeast presence * Metabolite presence	0.39	0.53
	Live yeast presence * Source colony	**17.2**	**0.009**
	Metabolite presence * Source colony	11.5	0.075
	Live yeast presence * Metabolite presence * Source colony	2.32	0.80
Pupae number	Live yeast presence	2.00	0.16
	Metabolite presence	2.29	0.13
	Source colony	**24.2**	**< 0.001**
	Live yeast presence * Metabolite presence	0.12	0.73
	Live yeast presence * Source colony	**12.7**	**0.049**
	Metabolite presence * Source colony	12.4	0.054
Male number	Live yeast presence	0.0002	0.99

	Metabolite presence	0.97	0.33
	Source colony	**23.5**	**< 0.001**
	Live yeast presence * Metabolite presence	3.65	0.056
	Live yeast presence * Source colony	**15.7**	**0.015**
	Metabolite presence * Source colony	**16.8**	**0.01**

682

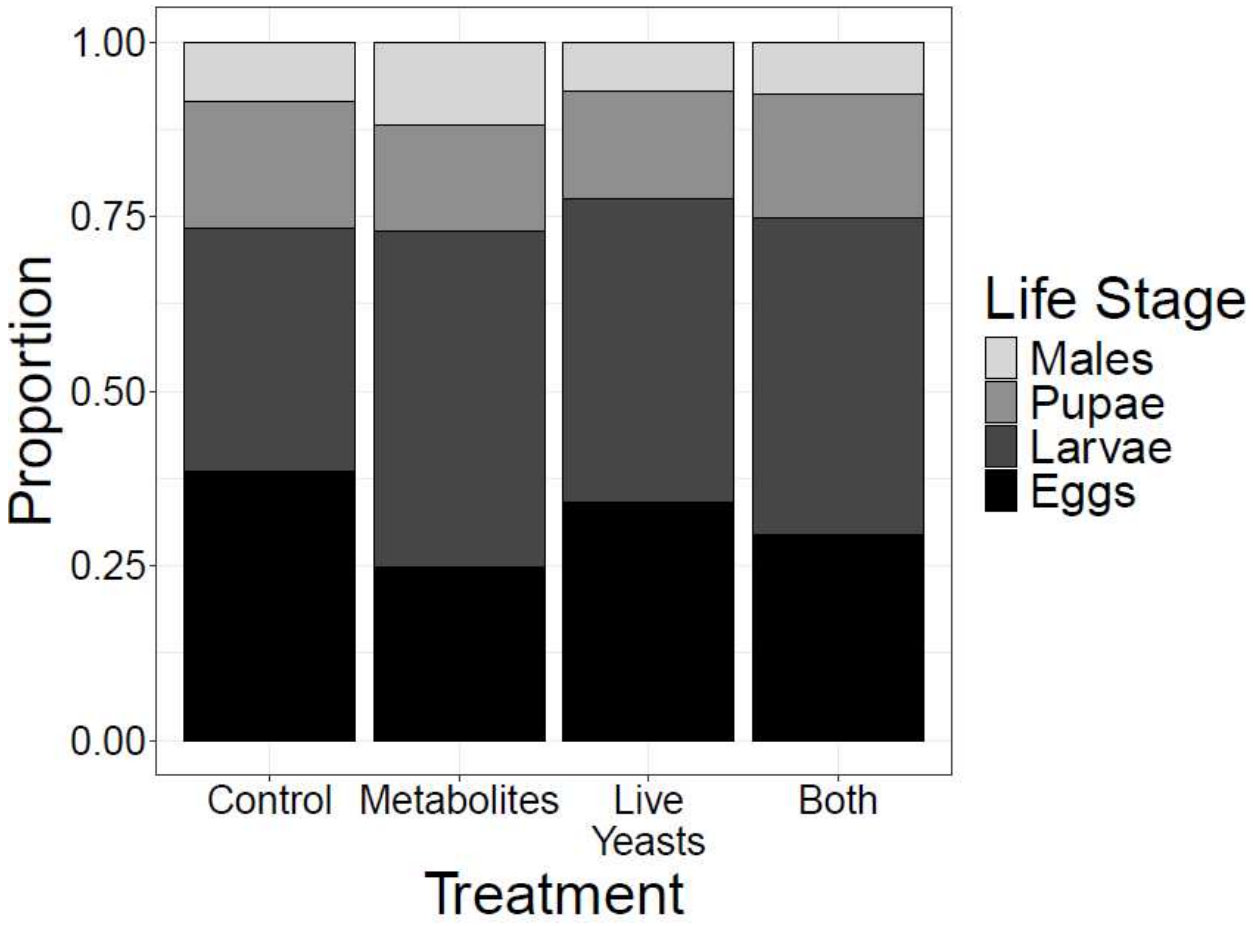

Figure 2. The life-stage makeup of the offspring produced by *B. impatiens* microcolonies exposed to different fungal treatments. Offspring composition was different between treatments, with the live yeasts only treatment having a higher proportion of larvae and males (X^2 = 19.2, df = 9, p = 0.02).

688 Table 2. Effects of yeast treatments and source colony on survival of *B. impatiens* workers.

689 Results are from a Cox proportional hazards model with microcolony ID as a random effect.

Predictor	X^2 value	p-value
Live yeast presence	**4.64**	**0.03**
Metabolite presence	**59**	**< 0.001**
Source colony	**72.6**	**< 0.001**
Live yeast presence * Metabolite presence	2.73	0.10
Live yeast presence * Source colony	9.81	0.13
Metabolite presence * Source colony	11.1	0.09
Live yeast presence * Metabolite presence * Source colony	**25.8**	**< 0.001**

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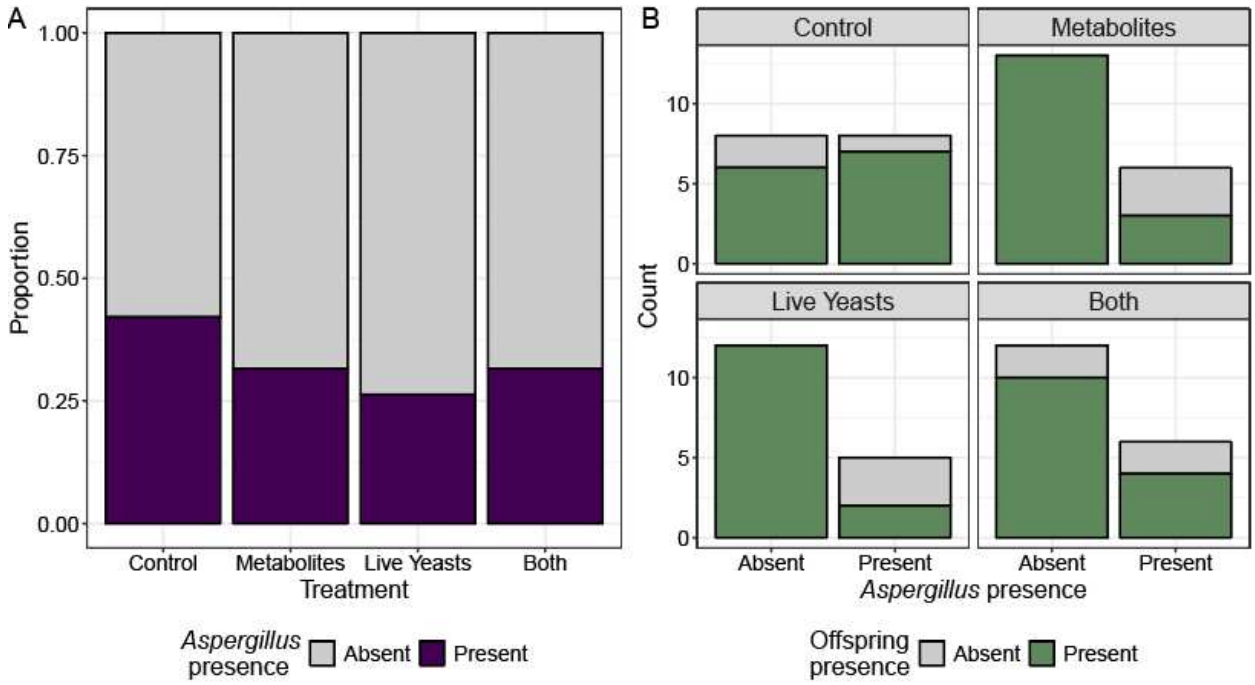

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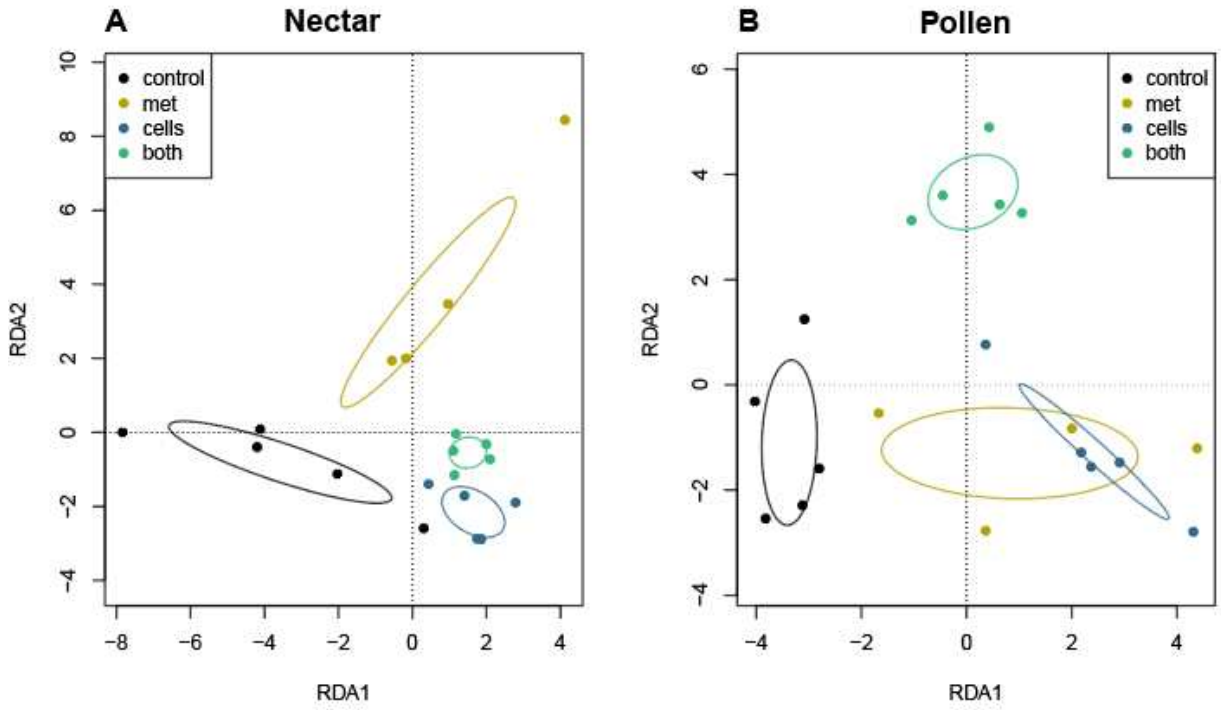
692 Figure 3. The prevalence of *Aspergillus* in microcolonies exposed to yeast treatments, and its
693 impact on microcolony offspring production. A) Yeast treatments interacted to impact
694 *Aspergillus* prevalence, with control microcolonies exhibiting the highest incidence ($X^2 = 4.13$,
695 df = 1, p = 0.04). B) *Aspergillus* negatively impacted offspring production ($X^2 = 7.71$, df = 1, p =
696 0.006), though this effect also depended on yeast treatments ($X^2 = 10.05$, df = 1, p = 0.002).
697 Panel A includes microcolonies from all source colonies, while Panel B includes microcolonies
698 from source colonies that tested positive for *Aspergillus* (70 microcolonies).



699

700 Figure 4. Ordination plots of metabolomics profiles of A) nectar and B) pollen provisions

701 provided to *B. impatiens* microcolonies across different fungal treatments. Living yeast presence

702 impacted the metabolomic composition of nectar samples ($F_{1,16} = 1.60$, $R^2 = 0.06$, $p = 0.032$),

703 while living yeast and metabolite presence interacted to impact pollen metabolites ($F_{1,16} = 1.27$,

704 $R^2 = 0.025$, $p = 0.12$).