Gut transplants from bees fed an antipathogenic pollen diet do not confer pathogen resistance to recipients

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Abstract

Pollinators are threatened by diverse stressors, including microbial pathogens such as *Crithidia bombi.* Consuming sunflower pollen dramatically reduces *C. bombi* infection in the bumble bee *Bombus impatiens,* but the mechanism behind this medicinal effect is unclear. We asked whether diet mediates resistance to *C. bombi* through changes in the gut microbiome. We hypothesized that sunflower pollen changes the gut microbiome, which in turn reduces *Crithidia* infection. To test this, we performed a gut transplant experiment. We fed donor bees either a sunflower pollen treatment or buckwheat pollen as a control treatment, and then inoculated recipient bees with homogenized guts from either sunflower-fed or buckwheat-fed donor bees. All recipient bees were then fed a wildflower pollen diet. Two days after the transplant, we infected recipients with *C. bombi,* and two days later, we provided another donor gut transplant. To quantify infection, we performed both fecal screens and dissections of the recipient bees. We found no significant differences in *C. bombi* infection intensity or presence between bees that received sunflower-fed microbiomes versus buckwheat-fed microbiomes. This suggests that sunflower pollen's effects on pathogen resistance are not mediated by gut microbiota.

Main text

Pollinators have widespread impacts on our environment and economy [1, 2], but they are susceptible to microbial pathogens, some of which are implicated in their declines [3]. One such pathogen is *Crithidia bombi ('Crithidia'* hereafter), which commonly infects bumble bees (*Bombus* spp.). *Crithidia* negatively impacts bumble bee fitness; it reduces learning and foraging ability [4], and under stressed conditions increases worker mortality [5] and reduces colony-founding by nearly 40% [6]. It is transmitted fecal-orally, on flowers or within colonies [7], [8].

The gut microbiome can play a significant role in bee health; variation in the bumble bee gut microbiome predicts susceptibility to *Crithidia* infection [9]. Low *Crithidia* counts are associated with high microbial diversity, large gut bacterial load, and the presence of certain bacterial taxa such as *Apibacter*, *Lactobacillus* Firm-5, and *Gilliamella* spp. [10]. Furthermore, gut microbial communities are a stronger predictor of host susceptibility to *Crithidia* than host genetics [9].

In addition to the gut microbiome, diet can play an important role in bee resistance to pathogens. For example, sunflower pollen (*Helianthus annuus*) has consistently and dramatically reduced *Crithidia* infection in *Bombus impatiens* workers [11], and sunflower abundance on farms was associated with reduced worker infection and increased colony reproduction [12]. Currently, two studies have linked diet to changes in bee gut microbiomes [13, 14], but none have connected these changes to pathogen resistance. We asked if the anti-pathogenic effects of a sunflower pollen diet are mediated by changes in the gut microbiome. Our study aims to shed light on the interactions between diet, pathogens, and the gut microbiome, which will improve our understanding of how floral resources affect bee health and may inform conservation strategies for these important pollinators.

Feeding bees different diets and observing effects on pathogen resistance does not distinguish whether resistance was due to direct effects of diet, or mediated by the diet-induced changes in the gut microbiome. To disentangle these effects we used a gut transplant experiment, inoculating a recipient host with the whole dissected gut contents of a donor host. This approach is a relatively inexpensive and straightforward way to assess effects of the gut microbiome on host phenotype, without requiring sequencing or culturing. Previous studies have used gut

transplants to determine the effects of gut microbial communities on *Crithidia* infections [9, 10, 15], but have not addressed the potential role of diet in these interactions.

Experiments took place at the University of Massachusetts Amherst campus from May to September 2022. We fed "donor" *B. impatiens* workers either sunflower pollen or buckwheat pollen and then dissected out their guts and fed them to experimental "recipient" bees (Fig. 1) that were then inoculated with *Crithidia*. We hypothesized that recipients that received gut

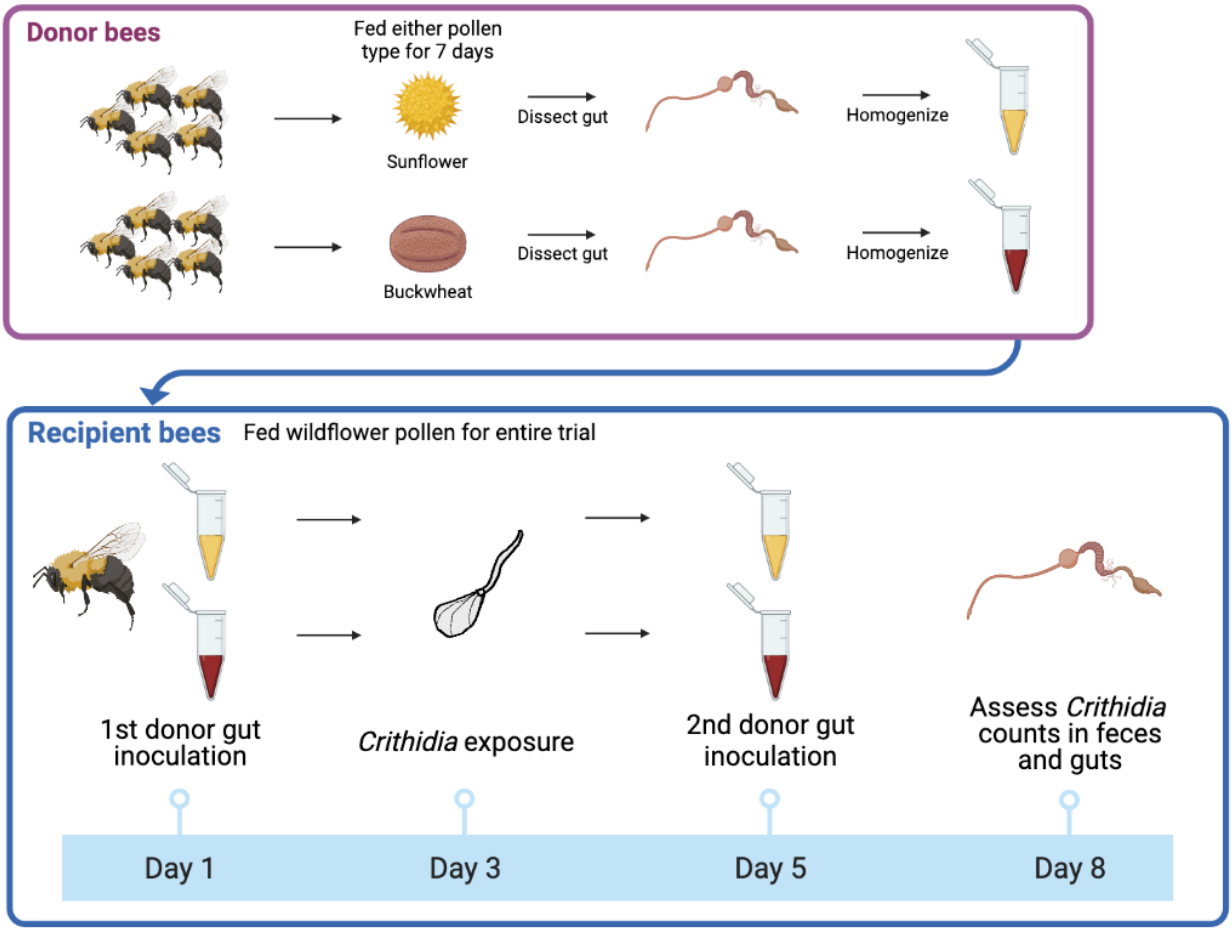

Fig. 1 Diagram of the experimental procedure. We treated recipient bees with a gut solution from the dissected donor bees. On day 3 we inoculated them with *Crithidia* and on day 5 we gave them a second gut treatment. We performed fecal screens and gut dissections on day 8 to assess infection

microbes from sunflower-fed bees would exhibit lower *Crithidia* infections compared to those that received gut microbes from buckwheat-fed bees. We conducted two rounds of the experiment, using 36 recipient bees in the first round and 48 in the second, for a total of 84 bees

(42 per microbiome treatment; bees were maintained individually and are the unit of replication). We outline our basic methods and statistical analysis below; additional details can be found in the supplemental material.

We removed donor bees from their natal colony and assigned them to sunflower or buckwheat pollen diets. We placed groups of 5-10 bees (with the same diet and natal colony) in separate containers (15 cm x 15 cm x 9 cm; Biobest LTD., Ontario, Canada) in a dark incubator at 27 ℃, and fed them their respective diet for 7 d. Then, we removed three bees per group, dissected their guts, pooled together the supernatant solution, and mixed it with equal parts 50% sucrose to create the microbiome solution.

Next, we removed recipient bees from the same natal colonies and randomly assigned them to a "buckwheat-fed microbiome" or "sunflower-fed microbiome" treatment. We used non-sterile adult workers who were presumably already inoculated with gut microbes from their nestmates, so that our treatments are an augmentation of the existing gut microbiome. We fed recipient bees 15 µl of microbiome solution of their assigned treatment from donors originating from the same colony, observing until they consumed all the solution. We then isolated each bee in a 16 oz. deli cup and fed them a wildflower pollen ball and 15 ml of 30% sucrose solution, replaced every 2-3 days. Two days after the initial gut transplant treatment, we inoculated recipient bees with 15 µl of *Crithidia* inoculum (600 cells/µl; 9000 cells total). Two days later, we administered a second dose of sunflower and buckwheat microbiome treatments to the recipients.

Five days after *Crithidia* inoculation, we quantified infection via both fecal screens and gut dissection. Since volumes of fecal samples were often too small to assess cell counts on a hemocytometer, we diluted feces with Ringer's solution (Sigma-Aldrich). We counted moving *Crithidia* cells in 0.02 µl aliquots of both diluted fecal samples and homogenized gut solution at 400X using a compound light microscope. After counting cells in 0.02 µl diluted feces, we calculated cells/µl feces after accounting for dilution, while in guts we retained counts per 0.02 µl as our response. We collected the right forewing of each bee and measured marginal cell length using ImageJ to estimate bee size [16].

We used R version 4.2.1 [17] for all analyses and the glmmTMB function [18] for all models. We analyzed four responses in separate models: *Crithidia* presence and cell counts in 1 µl of feces and *Crithidia* presence and cell counts in 0.02 µl of gut solution. We used

microbiome treatment and bee size (estimated as marginal cell length) as fixed effects, with *Crithidia* inoculation date and colony as random effects in all initial models, including fecal volume as an additional fixed effect in the fecal models. We used the DHARMa package [19] to assess model fit, and AIC for model selection (AICcmodavg package [20]). Microbiome treatment was always retained in the final models since that was our variable of interest. Colony was retained as a random effect in the models for cells per µl feces and probability of cell presence in 0.02 µl of gut solution. Sample sizes for sunflower and buckwheat microbiome treatments were 24 and 32 respectively for the fecal models, and 30 and 34 for the gut models.

We found no significant difference in *Crithidia* cell counts between buckwheat-microbiome and sunflower-microbiome bees in feces or gut solutions (Fig. 2a and 2c, Table 1a and 1c). There was also no significant difference in probability of *Crithidia* presence in feces or gut solution (Fig. 2b and 2d, Table 1b and 1d). Surprisingly, higher fecal volume corresponded with a higher probability of cells in feces (Table 1d), but lower cell counts (Table 1c). Larger

Terms	**a.** Cells/0.02 µl gut		**b.** Probability of cells in gut		**c.** Cells/µl feces		**d.** Probability of cells in feces	
	χ^2	p	χ^2	p	χ^2	p	χ^2	p
Treatment	0.069	0.793	0.602	0.438	0.078	0.780	0.262	0.609
Bee size	2.962	0.085	5.024	**0.025**	0.098	0.754	7.182	**0.007**
Fecal Volume	n/a	n/a	n/a	n/a	5.536	**0.019**	6.482	**0.011**

Table 1. X^2 and p values from final models predicting **(a)** *Crithidia* cells per 0.02 µl of gut solution, **(b)** the probability of *Crithidia* cells in 0.02 µl of gut, **(c)** *Crithidia* cells per µl of feces, and **(d)** the probability of *Crithidia* cells in 1 µl of feces. Bold text indicates $p < 0.05$

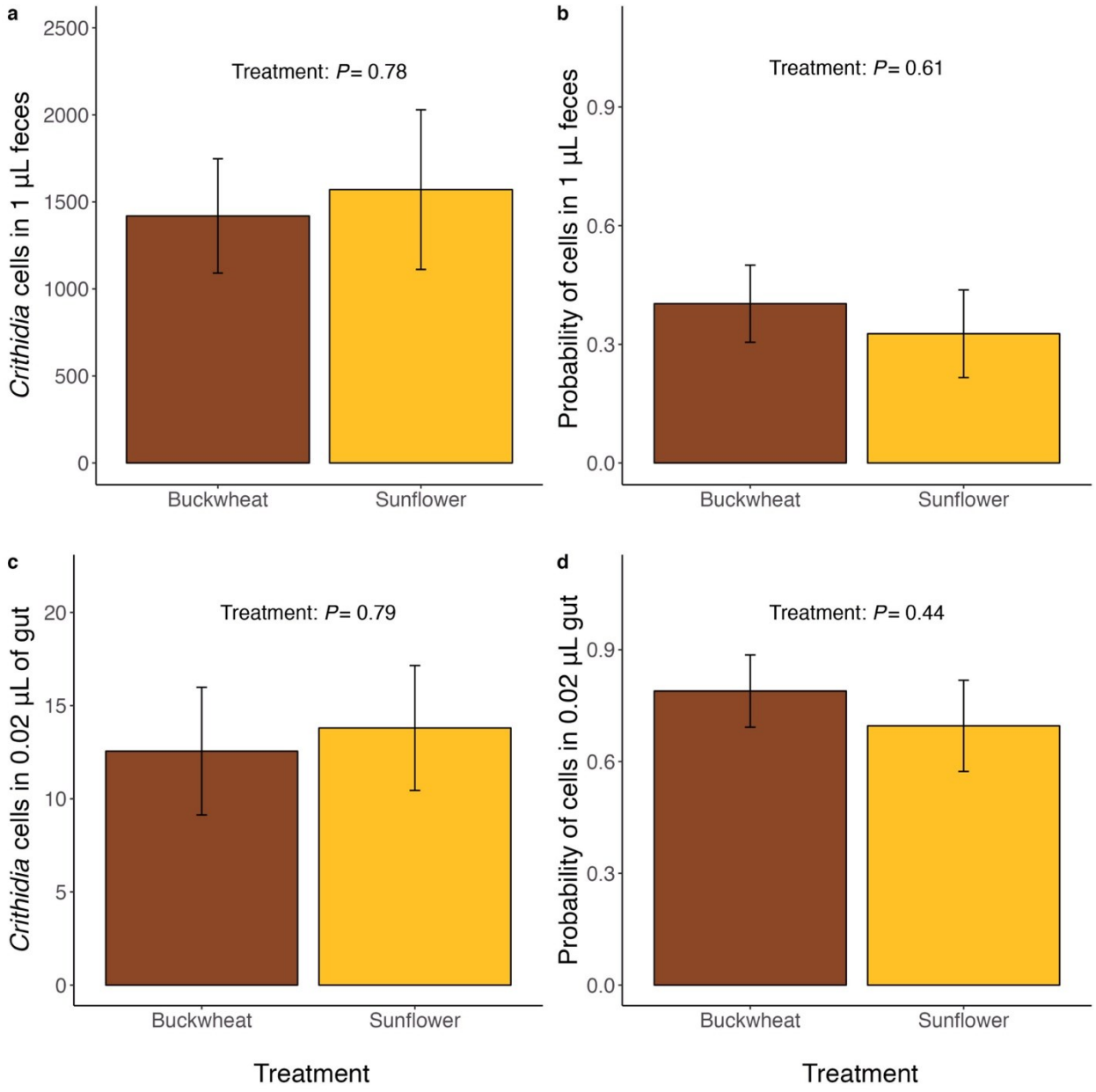

Fig. 2 Effect of sunflower pollen on **(a)** *Crithidia* cells per µl of feces, (**b**) the probability of *Crithidia* cells in 1 µl feces, (**c**) *Crithidia* cells per 0.02 µl of gut solution, and (**d**) the probability of *Crithidia* cells in 0.02 µl of gut solution in *Bombus impatiens* workers. Means estimated by a generalized linear model; error bars indicate standard error back-transformed by emmeans

bees also had lower probability of *Crithidia* presence in feces (Table 1d) and a non-significant trend for lower counts in gut solution (Table 1a).

Our results suggest that sunflower pollen's ability to reduce *Crithidia* infection in *B. impatiens* is not due to changes in the gut microbiome. There may have been diet-induced

changes in the gut microbiome, but they did not significantly reduce *Crithidia* infection. We used workers with already established gut microbial communities, due to high mortality of newly emerged bees in prior versions of the experiment. This may have made it more difficult for the microbiome treatments to alter the existing microbial community. Additional experiments using newly emerged bees without an established microbiome may show stronger treatment effects. However, recent data show that *Bombus impatiens* workers fed sunflower pollen exhibited similar gut bacterial communities, in both composition and diversity, to those fed a wildflower pollen mix (Fowler et al., in prep). Compared to the control, bees fed sunflower pollen did not have higher prevalence of the bacterial taxa associated with lower *Crithidia* infections found in previous research [10]. This finding is consistent with our results that gut bacterial communities transplanted from sunflower-fed bees had no effect on *Crithidia* infection in recipients. Together, these results suggest that sunflower pollen does not alter the bee gut microbial community in ways that reduce *Crithidia* infection.

The mechanism underlying sunflower pollen's dramatic effect on *Crithidia* infection is still not established. However, new research suggests that the spiny sunflower pollen exine is responsible [21], which may mean the effect is mechanical rather than chemical. Uncovering the mechanism behind sunflower pollen's medicinal properties will help further our knowledge of how certain plants may benefit declining pollinator populations.

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Statements and Declarations

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Competing Interests
The authors have no competing interests.

Author contributions
LSA and AEF conceived and designed the experiment. RTY collected the data and carried out the research. AEF analyzed the data. RTY wrote the first draft of the manuscript and all authors contributed to subsequent drafts. All authors read and approved the final manuscript.

Data availability
All data and code will be made publicly available at Scholarworks@UMass upon acceptance for publication.