

ORIGINAL ARTICLE

Microbiome-mediated immunity: Limited support from the gazelle dung beetle

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Funding information

National Science Foundation, Grant/Award Numbers: 2243725, 1901680; National Science Foundation Graduate Research Fellowship Program, Grant/Award Number: 2141416

Associate Editor: Sheena Cotter

Abstract

1. Insects commonly live in environments rich in opportunistically pathogenic microbes and thus have evolved traits that decrease the likelihood of infection and subsequent loss of fitness. In many cases, these adaptations involve other microbes, which aid in preventing pathogen colonization via the production of antimicrobial compounds, pathogen blocking or immune priming, thus modulating the reactivity of the host immune system once challenged.
2. Here, we use the gazelle dung beetle *Digitonthophagus gazella* to assess the role of the host's complex microbiome in supporting host immune functions, including basal protein concentration, phenoloxidase activity and lysozyme-like activity in the larval hemolymph and the host's ability to overcome an experimentally induced and standardized immune challenge.
3. We find that the inherited microbiome mediates an increase in larval mass and basal hemolymph protein concentration throughout development yet does not affect basal hemolymph phenoloxidase or lysozyme-like activity. Further, the presence/absence of a microbiome did not affect the host's ability to survive experimentally induced exposure to *Serratia marcescens*.
4. These results are discussed in light of both dung beetle ecology and insect-microbiome interactions broadly. Taken together, results suggest that the *D. gazella* microbiome may function primarily to enhance development rather than immunity.

KEYWORDS

constitutive immunity, *Digitonthophagus*, immune priming, Scarabaeinae, symbiont

INTRODUCTION

Insects have evolved to inhabit a wide range of complex and diverse environments. Many such environments, such as soil, water, and dung, tend to be rich in microbial pathogens (Cui et al., 2017; Delgado-Baquerizo et al., 2020; Ledón-Rettig et al., 2018), thus influencing the evolution of traits to prevent or overcome possible infections (Siva-Jothy et al., 2005). These traits can be behavioural [such as ants

removing and avoiding corpses (Cremer, 2019) or bees cleaning individuals (Foose et al., 2022; Simone-Finstrom, 2017)], morphological [such as the exoskeletal cuticular layers or the peritrophic membrane acting as physical barriers (Ortiz-Urquiza & Keyhani, 2013; Terra, 2001)], or immunological [such as antimicrobial production (Brey et al., 1993) or hemocyte activity (Gillespie et al., 1997)] in nature. Traits preventing the onset of infection are particularly important as research has shown that immunological responses – initiated

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after an infection manifests – can be expensive. This is evident both genetically, where evolution of increased immunity may result in negative pleiotropy on other traits (Boots & Begon, 1993; Schwenke et al., 2016; Stearns, 1998), and phenotypically, where immunological responses reduce overall fitness (Long & Nanthakumar, 2004; Rauw, 2012).

Immunological interactions can also vary as a result of communities of insect-associated microbes, known as microbiota. In several study organisms, the microbiota has been shown to directly decrease infection risk by conferring increased pathogen resistance to their host. Perhaps the simplest method of conferring this benefit is via competitive exclusion, whereby the mere presence of microbial species decreases both free space and resources required for pathogenic microbes to colonize the host (McLaren & Callahan, 2020). Alternatively, microbiota may produce and deploy antimicrobial compounds against potential pathogens. For example, the beewolf-associated *Streptomyces* produce compounds that prevent pathogen growth in the host's subterranean chamber (Kroiss et al., 2010) and the Lagriinae beetle-associated *Burkholderia* produce antifungal compounds preventing fungal growth on host eggs (Flórez et al., 2018). Similarly, intracellular bacteria such as *Wolbachia* are capable of *pathogen blocking*, limiting occurrence or severity of infections through mechanisms such as altering host gene expression patterns (Bhattacharya et al., 2020; Lindsey et al., 2018; Terradas & McGraw, 2017). However, few studies have explored whether the host-associated microbiota may be involved in immune priming. Immune priming occurs when the immune state of an organism is changed by some previous encounter (Kurtz et al., 2025), as is common in vertebrates where host baseline immune defence and the reactivity of the immune system can be shaped by interactions with their microbiome (Henrick et al., 2021; Seloosse et al., 2014). Several studies have demonstrated that the insect immune system, too, may be primed by past interactions, in particular interactions with pathogens (Contreras-Garduño et al., 2016; Critchlow et al., 2019; Kurtz et al., 2025) – but the role played by commensal or beneficial microbiome members in immune system priming remains to be fully explored.

Lastly, in host insects which rely on their microbial community for digestive or nutrient supplementation, microbiota may provide an *indirect* benefit to host immunity. For instance, many insects subsist on resources that require symbiotic microbes to break down indigestible resources (Beza-Beza et al., 2024) or biosynthesize essential nutrients that insects cannot produce (Douglas, 1998). Because host energy and nutritional states can influence the strength and nature of immune responses (Cotter et al., 2011) and the outcome of infections (Ponton et al., 2023), microbiome-mediated nutrient supplementation may therefore be an important, albeit indirect, driver of insect immunity (Cotter et al., 2011).

Here, we assess the degree to which the heritable and developmentally relevant microbiota of the gazelle dung beetle *Digitonthophagus gazella* influences host immune potential. *D. gazella* are common and native to South Africa and were secondarily introduced to North and Central America as well as Australia due to their ecosystem services in the context of dung- and nuisance fly management (Doubé

et al., 1991). Adult beetles reproduce underneath dung pads of herbivores, such as cattle, by constructing subterranean brood balls out of dung. Adult females oviposit a single egg per brood ball which they position on top of a faecal pellet consisting of the adult female's own excrement (commonly referred to as the *pedestal*) which is then consumed by larvae upon hatching (Estes et al., 2013). The consumption of this pedestal maintains the association between these beetles and their maternally inherited microbiota which in recent years has been shown to play a major role in host development (Parker et al., 2018, 2021; Rohner & Moczek, 2024; Schwab et al., 2016), likely by contributing metabolic pathways required to subsist on their recalcitrant diet (Holter, 2016; Jones, Moczek et al., 2025; Muller, 1980). However, recent work also indicates that microbial communities passed in pedestals of the bull-headed dung beetle, *Onthophagus taurus*, contain a significant portion of environmentally acquired microbes including several commonly pathogenic fungi, such as *Ascomycota*, *Basidiomycota*, and *Chytridiomycota* (Jones, Moczek et al., 2025). Similarly, *Desulfovibrio* was found natively in the dung used by *O. taurus* (Ledón-Rettig et al., 2018) and *Serratia* occurs in high abundance within eggs and adults of *Copris incertus* (Suárez-Moo et al., 2020). The consistent observation of potential pathogens in the dung beetle microbiome suggests that they may be common facets of the dung environment and that beetles may possess mechanisms able to prevent subsequent infections.

Here, we therefore sought to assess whether the maternally inherited microbial community of *D. gazella* may influence the immunological potential of larval *D. gazella*. Specifically, we ask if the presence of the pedestal affects host innate immune metrics, including protein concentration, phenoloxidase activity, and lysozyme-like activity in the larval hemolymph, as well as the host's ability to overcome a laboratory challenge with *Serratia marcesens*, an opportunistic entomopathogen. Our results suggest that dung beetle microbiota, despite consistently affecting host development, may do so largely without any major impacts on host immunity.

MATERIALS AND METHODS

Beetle husbandry

Digitonthophagus gazella were collected near Wichita, Kansas, U.S.A., in September 2023 and maintained in a colony at 28°C, 45% humidity, with a 16:8 h light: dark cycle as described in Schwab et al. (2017). The colony housed an initial population of around 250 individuals and were fed twice a week with homogenized dung collected at Marble Hill Farm, Bloomington, Indiana. The individuals used for this experiment were the first or second generation offspring of these wild caught adults produced between April and October 2024 following methods described in Shafiei et al. (2001). In brief, male and female adults were selected haphazardly from the colony and added to containers of compacted, moist soil with ample dung for 3 days to produce brood balls (Shafiei et al., 2001). Eggs extracted from these brood balls were then assigned to develop either in the presence of their pedestal microbiome (M+) or without it (M–). Individuals in the

M+ group were transferred into 12-well plates with their pedestals while individuals in the M− group were separated from their pedestal, surface sterilized with 200 µL sterilization solution [1% bleach, 0.1% Triton-X 100 in double-distilled water (Schwab et al., 2016)] and rinsed with 200 µL sterile PBS before transferal. 12-well plates were provisioned with sterilized dung from hay-fed cows, following methods described in Schwab et al. (2016). Beetles were checked daily for hatching before being left to develop until the timepoint specified for a given assay. Trials were set up to include 120 individuals for the immune potential assay (60 per treatment group), 60 individuals for the protein across development assay (different 10 per age group), 144 individuals for the brood chamber inoculation assay (36 per treatment group), and 96 for the hemolymph inoculation assay (24 per treatment group), for a total of 420 individuals. Final, realized sample sizes are lower due to mortality ranging from 15 to 30%, depending on treatment group and approach, as well as failures during sample preparation.

Immune potential assays

To determine if developing in the presence of the pedestal microbiota had any effect on baseline immune potential of the host, we divided individuals into M+ and M− groups, as described above, and allowed them to develop for 10 days after hatching before extracting hemolymph to measure their protein concentration (PC), phenoloxidase activity (PO), and lysozyme-like activity (LL). To extract hemolymph, larvae were surface sterilized, using the sterilization solution described above, before a Hamilton syringe was inserted along the side of the abdomen and used to extract 2 µL of hemolymph. This hemolymph was diluted into 78 µL of sterile PBS to rupture any hemocytes and provide adequate volume for subsequent assays. PC, PO, and LL were all measured in batches on the day of extraction in clear bottom 96-well cell culture plates. PC was measured using a Protein Quantification Assay kit (Takara Bio; REF: 740967.250) and following the microplate assay procedure described therein.

PO and LL activities were measured following methods modified from Adamo (2004). For PO, 25 µL hemolymph-PBS solution was added to 75 µL of alpha-chymotrypsin and left to incubate for 20 min at room temperature, then 100 µL of 0.2M L-Dopa solution (Cayman Chemical Company; item 13,248) was added, and the change in absorbance across 1 h was measured at 490 nm. Ten microlitre phenylthio-carbamide solution was added to one additional assay well on each plate as a negative control, alongside another well with chymotrypsin in the absence of hemolymph, to confirm that absorption changes could be attributed to PO and not spontaneous degradation of L-Dopa or degradation caused by other chemicals. None of these controls showed any colour change across our assays, confirming that observed absorption change resulted from phenoloxidase originating in the beetles. Lysozyme-like activity in turn was measured using a turbidity assay. In short, we combined 30 µL of hemolymph-PBS solution, 70 µL of PBS, and 100 µL of heat-killed *Serratia marcescens* in a well and measured the change in OD600 continually across 5 h. The

measurements for LL assays were analysed as lysozyme-like activity per protein concentration.

Protein across development assay

We measured protein concentration across a range of larval ages to determine if the microbiome modulates changes in protein concentration across a short developmental window. Specifically, we first reared M+ and M− larvae, as described above, and allowed them to age to 9, 10 or 11 days old. When an individual larva reached their assigned age, they were weighed, their hemolymph was extracted, and the protein concentration in their hemolymph was measured, following the methods described above.

Serratia inoculation into the brood chamber assay

To measure the effect of microbiota on outcomes resulting from exposure to a potential pathogen, we established a protocol for culturing *Serratia marcescens*. The strain of *S. marcescens* we used was isolated from a population of *D. gazella* maintained in the lab and originally collected from Queensland, Australia the year prior. We identified this bacterium first by its red colonies then by comparing its 16S rRNA gene sequence with BLAST (Madden, 2013) against NCBI's Nucleotide database (National Center for Biotechnology Information [NCBI], 1988). This sequence was amplified using the 27F and 1492R primers (27F: AGA GTT TGA TCM TGG CTC AG; 1492R: GGT TAC CTT GTT ACG ACT T; M represents A or C; sequence included in the Dryad repository). This microbe was selected for its opportunistic pathogenic role in other systems and based on a short pilot study which suggested it may decrease larval survival of *O. taurus*. *Serratia marcescens* was maintained in a 50:50 glycerol:LB broth solution in a −80°C freezer. Prior to use in inoculations, we performed a growth curve, calculating both optical density (OD) and colony-forming units (CFUs). To do so, 100 µL of *S. marcescens* was transferred from overnight culture into 200 mL LB broth and aerated (300 rpm) in an incubator at 28°C. Both OD600 and CFU counts were taken once per hour until a decrease in OD was observed. It was determined that the exponential growth phase occurred between three and 7 h after beginning a culture, thus all inoculations using *S. marcescens* were made within this timing window. For each round of culturing, *S. marcescens* abundance was estimated based on OD600. Once the intended abundance was reached, *S. marcescens* was pelleted via centrifugation, LB broth was removed, and the pellet rinsed twice with sterile PBS, followed by centrifuging and removing the supernatant between each rinse, before being diluted in sterile PBS to the appropriate concentration.

Serratia inoculation of hemolymph assay

Finally, to determine whether maternally inherited microbiota affect hosts' ability to survive exposure to *S. marcescens*, we first measured

if the presence of the microbiota changed the impact of *S. marcescens* exposure on survival when the bacteria is enriched in the brood chamber. To accomplish this, we split eggs into a full factorial design where individuals were either M+ or M−, as described above, and received either an egg-surface inoculation of 10^5 CFUs of *S. marcescens* resuspended in PBS or sterile PBS alone. Inoculation was administered by pipetting 10 μ L of solution onto the egg and surrounding dung prior to hatching. After inoculation, individuals were monitored for survival to adulthood. Finally, the impact of direct hemolymph exposure on survival was measured. Beetles were reared into M+ and M− groups, as described above, and allowed to age 10 days. On day 10, individuals were weighed and injected, near the middle of their left side, with either 10^5 CFUs of *S. marcescens* per gram weight of host suspended in 1–10 μ L sterilized PBS or 5 μ L sterile PBS using a 10 μ L Hamilton syringe. Prior to inoculation, hemolymph was sampled to measure PC, as described above. Following inoculation, individuals were monitored for survival. Metadata for all assays are included in the Dryad repository.

Statistical testing

All statistical tests were conducted in R v4.5.2 (R Core Team, 2025). The effect of microbiota presence on larval mass, protein concentration, phenoloxidase activity and lysozyme-like activity were analysed with individual linear mixed models using *lmer* from *lme4* v1.1.38 (Bates et al., 2015) and *Anova* from *car* v3.1.5 (Fox & Weisberg, 2019). Batch, plate, and well were initially included in the aforementioned models, with microbiota as the sole fixed effect. The final models only included batch and plate as random effects because that model had the lowest AIC measurements and well did not have a significant effect on the data. The influence of microbiota presence and age on protein concentration was also analysed using *lmer* from *lme4* v1.1.38. Here, microbiota, age, weight, and the interaction between microbiota and age were all treated as fixed effects. Weight was included as a fixed effect because it correlates with both age and protein concentration. Plate and well were treated as random effects with both being kept in the final model since AIC was similar across alternate models. Finally, the effect of *S. marcescens* on survival was analysed using *glm/glm*, from *lme4* v1.1.38, with a binomial error distribution. The effect of pre-hatching inoculation and microbiome on survival was measured with a model including microbiome, inoculation, and their interaction. Batch, plate, and well were initially included in the model but were dropped because they had no effect on the data and increased the AIC value. The impact of subcutaneous inoculation was measured with a similar model, but without batch since this assay was completed in a single batch. Similarly, all random effects had no impact on the data, so they were dropped from the final model. Tables giving detailed results for all statistical tests are provided in the supplemental data file (Tables S1–S4). All plots were produced in R using *ggplot2* (Wickham, 2016) with multiple plots being combined into single figures using *cowplot* (Wilke, 2025).

RESULTS

Microbiota presence enhances larval mass and protein concentration but does not affect phenoloxidase or lysozyme-like activity

To determine if the microbiome influences immune potential, we first measured if disturbing microbial inheritance had any effect on common metrics of baseline immunity. More specifically, we measured if developing without the pedestal present influenced protein concentration, phenoloxidase activity or lysozyme-like activity in the larval hemolymph. We compared larval mass to confirm that the pedestal microbiome was adequately removed in the M− treatment, as mass is expected to decrease when individuals develop without their pedestal

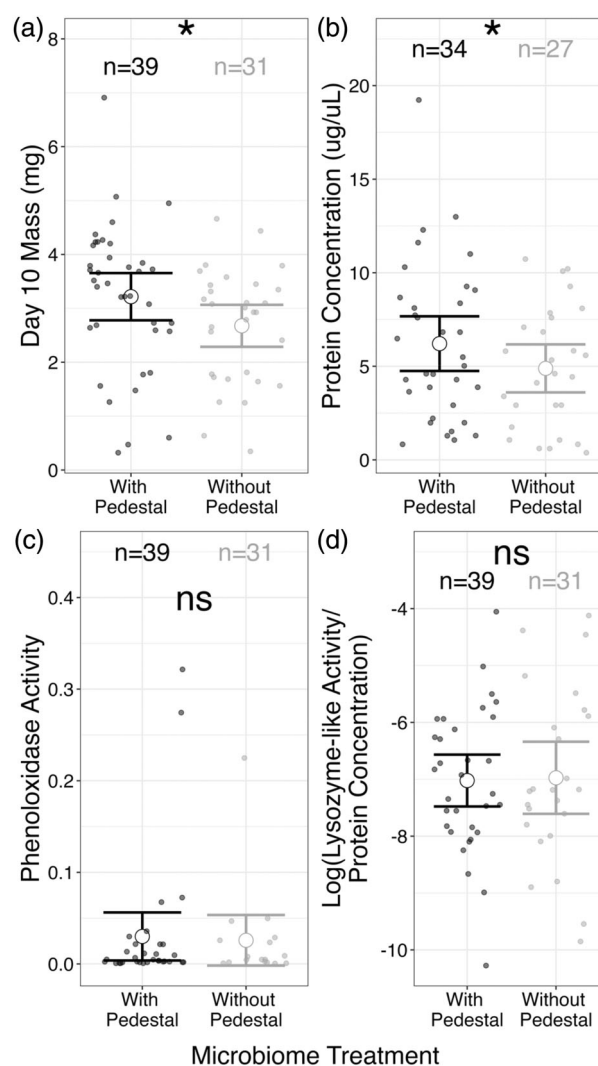


FIGURE 1 Depriving *Digitonthophagus gazella* of their pedestal influences (a) larval mass and (b) basal hemolymph protein concentration but not basal larval hemolymph (c) phenoloxidase or (d) lysozyme-like activity. Large points represent means, bars show the upper and lower 95% confidence intervals, and smaller points are individuals. Individuals with access to their pedestal (M+) are in black and individuals deprived of their pedestal (M−) are in grey.

microbial community (Rohner & Moczek, 2024; Schwab et al., 2016). We found that the average mass of M− individuals was indeed significantly lower than that of M+ individuals (ANOVA: $\chi^2(1,70) = 5.592$, $p = 0.01804$, Figure 1a), suggesting effective pedestal removal. Protein concentration was similarly affected by microbiome treatment with M+ individuals exhibiting significantly higher protein concentration compared to M− individuals (ANOVA: $\chi^2(1,61) = 4.9436$, $p = 0.02619$, Figure 1b). Conversely, microbiota treatment had no effect on either lysozyme-like activity (ANOVA: $\chi^2(1,57) = 0.0395$, $p = 0.8424$, Figure 1d) or phenoloxidase activity (ANOVA: $\chi^2(1,49) = 0.2003$, $p = 0.6545$, Figure 1c). Overall, this data suggests that pedestal removal negatively influences hemolymph protein concentrations but has little to no effect on our other measures of insect immunity.

Host age and microbiota interact to influence protein concentration

The *D. gazella* microbiome is also capable of accelerating the developmental rate of its host (Rohner & Moczek, 2024; Schwab et al., 2016), therefore leading us to hypothesize that this effect may alter fluctuations of the host's immune system, which typically vary throughout development (Cotter et al., 2008). We surveyed protein concentration across a short window of rapid larval development (Rohner & Moczek, 2024) to determine if the microbiota has the potential to shift changes in protein content over time. Weight, which is known to vary with both age and microbiota treatment and correlates with

protein concentration (ANOVA: $\chi^2(1,37) = 8.472$, $p = 0.0036$), was added as a covariate to account for its effects. An ANOVA factoring in age, treatment and the interactions between them revealed that the interaction between age and microbiota treatment had the largest effect ($\chi^2(2,37) = 10.113$, $p = 0.0064$), while age alone had a significant but weaker effect ($\chi^2(2,37) = 6.532$, $p = 0.0382$, Figure 2), and microbiota alone had no significant effect at all ($\chi^2(1,37) = 0.981$, $p = 0.322$). Thus, our data supports the hypothesis that the maternally inherited microbiota alters developmental shifts in protein concentration, possibly accelerating natural shifts throughout development.

Microbiota presence does not affect survival in response to *Serratia* exposure

Lastly, we tested if the microbial community was capable of mitigating the potentially negative effects of *S. marcescens* exposure. We tested this in two contexts. First, we tested if inheritance of the maternal microbiota would alter host reaction to an increase of *S. marcescens* in the brood ball. Despite a considerable addition of *S. marcescens* into the brood ball, there was no detectable effect of inoculation on beetle survival (ANOVA: $\chi^2(1122) = 0.461$, $p = 0.497$, Figure 3a). Presence of the maternal microbiota (ANOVA: $\chi^2(1122) = 0.057$, $p = 0.811$) and the interaction between microbiota presence and *S. marcescens* addition also had no significant effect (ANOVA: $\chi^2(1122) = 0.875$, $p = 0.350$). This result suggests that abundance of this strain of *S. marcescens* in the developmental environment did not affect host survival. To test if the microbiota could influence the outcomes of

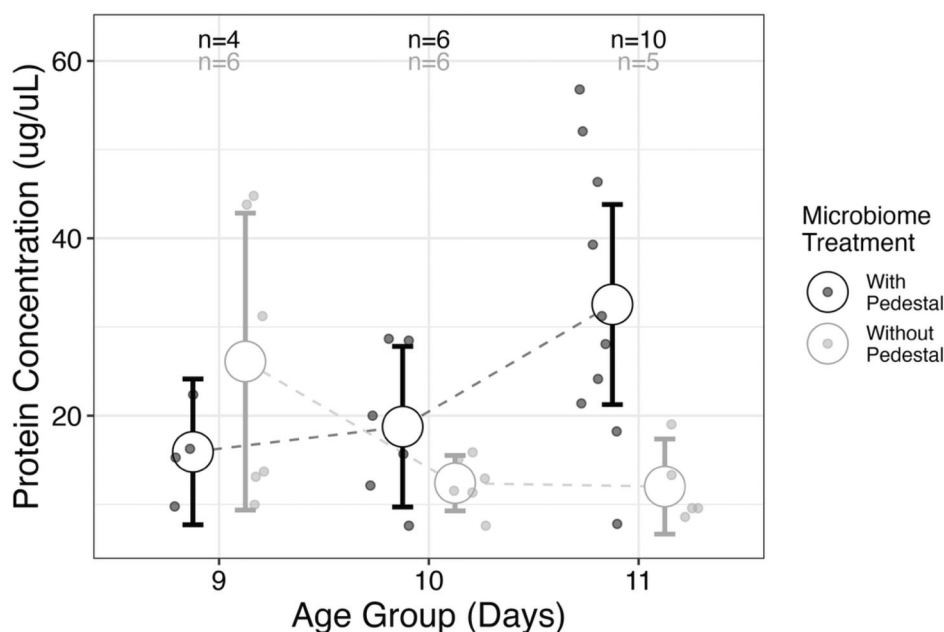


FIGURE 2 Presence or absence of the pedestal influences the interaction between basal protein concentration in the larval hemolymph and age within a window of peak larval development. Large points represent means, bars show the upper and lower 95% confidence intervals, and smaller points are individuals. Individuals with access to their pedestal (M+) are in black and individuals deprived of their pedestal (M−) are in grey.

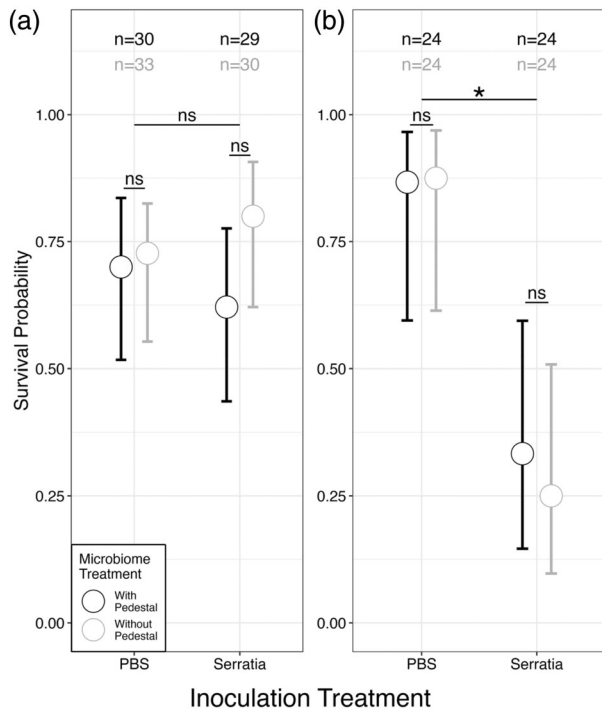


FIGURE 3 Influence of *Serratia* exposure on survival probability. (a) Addition of *Serratia* to the brood chamber prior to hatching has no effect on survival probability while addition directly into the hemolymph does (b). Presence of the pedestal had no effect on survival regardless of exposure route. Points represent estimated marginal means and bars show the upper and lower 95% confidence intervals. Individuals with access to their pedestal (M+) are in black and individuals deprived of their pedestal (M-) are in grey.

entry of *S. marcescens* into the hemolymph, and if *S. marcescens* could negatively influence the beetle through direct infection, the experiment was repeated with *S. marcescens* being introduced internally via injection into the larval hemolymph. We found that *S. marcescens* injections did reduce *O. taurus* survival (ANOVA: $\chi^2(1,62) = 9.5050$, $p = 0.002$, Figure 3b), microbiota presence had an insignificant effect (ANOVA: $\chi^2(1,62) = 0.0048$, $p = 0.945$), and the interaction between them was also statistically insignificant (ANOVA: $\chi^2(1,62) = 0.129$, $p = 0.719$). This demonstrates that, at least for this particular *Serratia* strain, the maternally transmitted microbial community does not alter infection outcomes.

DISCUSSION

The microbiome has been implicated as a key factor influencing insect health and development across a range of study systems. Such studies have revealed that the microbiome can shield insects from pathogenic infections of bacteria, fungi, and viruses (Bhattacharya et al., 2020; Flórez et al., 2018; Kroiss et al., 2010; Lindsey et al., 2018; McLaren & Callahan, 2020; Terradas & McGraw, 2017). Yet our understanding of the mechanisms underlying microbiome-mediated immunity and their limitations remains rather limited. Work to date suggests that

microbiomes may enhance immunity *indirectly*, such as providing the resources required for stronger host immune reactions by supplementing challenging diets (Beza-Beza et al., 2024; Cotter et al., 2011; Douglas, 1998), or *directly*, such as alleviating the need for host immune reactions by preventing pathogen colonization or pathogen proliferation via several forms of competitive exclusion (Bhattacharya et al., 2020; Lindsey et al., 2018; McLaren & Callahan, 2020; Terradas & McGraw, 2017). Finally, recent research raises the potential for microbiome-mediated immune priming in insects, a component of the complex immune system previously thought to be unique to vertebrates (Contreras-Garduño et al., 2016; Critchlow et al., 2019; Henrick et al., 2021; Seloosse et al., 2014). To broaden our understanding of how microbial communities influence immunity in insects, we sought to assess whether the presence of the heritable microbiota of *D. gazella* influences their innate immune potential or their potential to overcome immune challenges.

Overall, our results show, at best, modest support for the role of the microbiota in either priming or enhancing host immunity. Individuals retaining their microbial communities incurred only modest differences in immune potential, as assessed by hemolymph protein concentration, which in some taxa does predict ability to overcome an infection (Adamo, 2004). However, we failed to detect differences in other metrics, including hemolymph phenoloxidase or lysozyme-like activity or survival across our treatment groups. Interestingly, past results in *Onthophagus taurus*, another dung beetle, diverge from our own in several ways (Cotter et al., 2008). Specifically, this study found that immune potential throughout larval development significantly correlates with overall host state, i.e., larger males had both higher protein concentrations and more phenoloxidase activity than smaller males (Cotter et al., 2008), as well as exhibited a stronger difference in protein concentration across body sizes. This raises the possibility that either host condition and immunity are more decoupled in *D. gazella* than *O. taurus* or that host condition itself was not affected enough by the removal of the pedestal microbiota in *D. gazella* to influence immunity.

Our study utilized individuals bred from a population recently obtained from the field, therefore allowing us to minimize the effects of lab rearing on the microbiota but preventing us from controlling genetic variation. This may have influenced our results as the impact of the microbiota on insect immune response can be influenced by symbiont genotypes (Koch & Schmid-Hempel, 2012) or a combination of symbiont and host genotypes (Johnston & Rolff, 2015). More in-depth, quantitative genetic studies also reveal that the relative influence of host, symbiont and parasite genotype on immune outcomes can be influenced by each party, creating even more complex interactions (Purkiss et al., 2022). Importantly, Rohner and Moczek (2024) found that *D. gazella* itself exhibits significant among-family variation in how microbiome inheritance influences development, showing that host genetic effects are important in this species, and thus may have influenced our results. A more comprehensive study should disentangle the impacts of host genetic and microbiome compositional variation on insect immunity to better understand potential impacts of the microbiome on dung beetle immunity and immune priming.

Our study also identified an unexpectedly weak effect of *Serratia* on beetle survival. Specifically, we introduced the commonly pathogenic bacterium *Serratia* at high abundance in the egg environment and found that this did not significantly affect beetle survival, even though it was introduced in abundances far higher than those observed naturally (Jones, Newton et al., 2025). Yet, direct inoculation into the hemolymph did significantly decrease survival, suggesting that the strain of *Serratia* used in our study is capable of infecting and killing *D. gazella*, and even under these conditions we failed to identify a measurable effect of the maternally inherited microbiota on survival. We did not test the survival impacts of other bacteria so it is possible that any, even a mutualistic, microbe may decrease survival when injected into the hemolymph. This is important to note because even though *S. marcescens* is commonly pathogenic across other insects (Akhtar et al., 2026; Petersen & Tisa, 2013), including scarab beetles (Klein & Kaya, 1995; Pineda-Castellanos et al., 2015), it was observed in high abundances in other dung beetles (Suárez-Moo et al., 2020) and is even beneficial to some insects (Petersen & Tisa, 2013; Scarsia et al., 2016). Regardless of the precise role played by *S. marcescens* in dung beetle ecology, the absence of an immunological benefit documented here contrasts with work in other insects which suggests that the microbiome can improve infection outcomes (Flórez et al., 2018; Kroiss et al., 2010). Further measurements of how verified *D. gazella* pathogens interact with the microbiota to influence survival is therefore needed to confirm the role of the microbial community in immunity as well as the conditions (i.e., environmental vs. internal) through which the microbiome may exert its impact.

Finally, we discovered an interaction between age and microbiota affecting protein concentration. Across the time window studied, we observed protein concentrations increasing in individuals with intact microbial communities yet decreasing in individuals without. However, even though protein concentration is generally considered a predictor of immunity in other insects (Adamo, 2004), this difference across treatment group did not correlate with survival. While this microbially-influenced change in protein concentration therefore does not appear to improve immunity, it may still influence developmental outcomes (Cotter et al., 2008). Research into the causes and effects of these changes may elucidate the exact mechanisms by which the microbiome benefits host development.

In conclusion, our results suggest that *D. gazella* does not rely heavily on its inherited microbial community to form an effective immune defence, at least not throughout the timepoints we studied. These results, along with past research, call into question if commonly pathogenic interactions between insects and microbes are maintained in dung beetles. Finally, despite the lack of strong interactions between the microbiome and *D. gazella*'s innate immunity, we believe that dung beetles broadly, given their species richness, diverse host-microbiome interactions and microbe-rich ecology, may provide a potentially powerful model to further our understanding of how the microbiome and nutrition feedback on insect immunity.

AUTHOR CONTRIBUTIONS

Joshua A. Jones: Conceptualization; data curation; formal analysis; investigation; visualization; writing – original draft; writing – review

and editing; methodology; validation. **Irene L. G. Newton:** Conceptualization; methodology; resources; supervision; writing – review and editing; writing – original draft; project administration; funding acquisition. **Armin P. Moczek:** Methodology; conceptualization; writing – original draft; writing – review and editing; supervision; resources; project administration.

ACKNOWLEDGEMENTS

We would like to thank Max Proctor for collecting *D. gazella* in the field, Eve Pieri, Isabel Manley and Sadie Kidd for expert help with beetle husbandry and the members of the Moczek and Newton Labs for constructive comments. This work was supported in part through generous funding from the National Science Foundation (2243725 and 1901680 to APM) and a National Science Foundation Graduate Research Fellowship Program (2141416 to JAJ). The opinions, interpretations, conclusions and recommendations are ours and are not necessarily endorsed by the National Science Foundation.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

Data used for these analyses and figures are included in a Dryad repository (<https://doi.org/10.5061/dryad.r4xgxd2v7>) (Jones et al., 2026).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

Table S1. Effect of microbiome treatment on basic immune metrics. Model was run four times independently, one for each trait, and included microbiota (whether an individual received their pedestal or not) as a fixed effect and batch (round of breeding individuals came from) and plate (which 12-well plate an individual were raised in) as random effects.

Table S2. Output of an ANOVA determining the variables influencing protein concentration. Model contained protein concentration as the dependent variable and microbiota, age (number of days between hatching and collecting hemolymph), weight (mass at time of hemolymph collection), and the interaction between microbiota and age as fixed effects. Plate and well (the specific well within the 12-well plate an individual was raised in) were included as random effects.

Table S3. Influence of environmental exposure to *Serratia* on survival to adulthood. Model included survival probability as the dependent variable and *Serratia* inoculation (whether *serratia* was added to their well prior to hatching or not), microbiota, and the interaction between *Serratia* inoculation and microbiome as fixed effects.

Table S4. Influence of hemolymph infection of *Serratia* and microbiome on survival probability. Model included survival probability as the dependent variable and *Serratia* inoculation (whether *Serratia* was inoculated into the hemolymph at 10 days post hatching), microbiota, and the interaction between *Serratia* inoculation and microbiota as fixed effects.

How to cite this article: Jones, J.A., Newton, I.L.G. & Moczek, A.P. (2026) Microbiome-mediated immunity: Limited support from the gazelle dung beetle. *Ecological Entomology*, 1–9. Available from: <https://doi.org/10.1111/een.70146>