

Gut bacteria prime and reinforce mature leaf oviposition preference in beetles via plant mediated feedback

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

This paper presents a lucidly-described investigation with advanced genetic techniques and microbial manipulation experiments that greatly enhances our understanding of the interactions between the microbiome, insect behavior, and plant-insect ecology. The authors clearly demonstrate multiple biologically interesting phenomena, including (1) the positive relationship between natural *Plagioderia versicolora* gut microbial flora (*Pseudomonas*) and mature leaf oviposition preference, (2) the positive chemotaxis of *Pseudomonas putida* toward indole-3-acetic acid (IAA), (3) the accumulation of IAA and *Pseudomonas* on newly-produced willow leaves following *P. versicolora* feeding, (4) the elevated expression of regulatory gene TO1 in *P. versicolora* in response to *Pseudomonas* colonization, and (5) the positive relationship between TO1 upregulation and mature leaf oviposition preference. From these lines of evidence, the authors construct a plausible mechanism that links *P. versicolora* oviposition behavior, *Pseudomonas* transmission, and host plant compensatory growth. The evidence and analysis presented in this study is of high value to the field of plant-insect interactions, however major concerns are listed below.

Some aspects of the study stand out for their clarity and strength of presentation. Notably, the study demonstrated that oviposition choice is microbiota-dependent, identified *Pseudomonas putida* as a key strain correlated of this behavior, and associated it with host genes implicated in behavioral regulation. The behavioral component and the host gene regulation by the bacteria alone could have formed a compelling manuscript by itself.

In contrast, the study of the bacterial horizontal and vertical transmission is substantially weaker. In its current form, it obscures rather than clarifies the core findings. The manuscript conflates oviposition preference with adult-to-adult transmission; these processes must be conceptually and experimentally separated in the manuscript. The mode of transmission is a significant unresolved issue that introduces ambiguity. In several places, the inclusion of additional results increases confusion rather than providing mechanistic insight. Notably, the occurrence and abundance of bacteria, including *P. putida*, are not systematically characterized across relevant beetle compartments, such as mouthparts, gut, frass, eggs, larvae, and newly emerged adults and finally leaf tissue. Consequently, the relative contributions of horizontal versus vertical transmission remain unclear.

In addition, the mechanism underlying IAA induction is puzzling. The data suggest that bacteria induce the signal (IAA) that subsequently attracts them. This scenario is unusual in chemical ecology (and would be the first report) and requires validation through experimentation. More generally, the bacterial transmission route remains unclear, leaving open the fundamental question of whether *P. putida* functions as an environmental bacterium or an obligate (or semi-obligate) symbiont. Clarifying these points could substantially alter the interpretation of the study and therefore requires careful attention.

Finally, the inconsistency of the proposed TO1-mediated mechanism is problematic and must be explicitly addressed in the discussion. The results suggest that TO1 is not strictly necessary for the behavioral phenotype or that other, parallel pathways are involved.

More broadly, several key claims, including the existence of a positive feedback loop, adaptive behavioral reinforcement, and microbial 'priming' are not yet supported by the results. These claims rely heavily on correlations and rescue experiments that do not fully exclude alternative explanations.

In summary, although there are many impressive pieces of work that build on the authors' previous publications on this system and present intriguing and potentially important observations linking gut bacteria, plant responses, and insect oviposition behavior, several core claims, particularly those concerning mechanistic mediation, adaptive significance, and positive feedback dynamics, are not sufficiently supported for publication in their current form.

Despite the substantial corrections listed above and below, I do not want to discourage the authors, and I would like to emphasize that I was very impressed by the quality, ambition, and technical execution of this study. I therefore congratulate the authors and I strongly encourage the authors to submit a revised version because I truly believe it will be a remarkable contribution to the field.

Major Comments:

P. putida migration to new leaves and IAA-inducibility

Fig. 3A does not make it clear whether beetle feeding occurred on new or mature leaves, even though adults are expected to preferentially feed on new leaves. The temporal sequence is also unclear: how long after feeding do new shoots appear, and how quickly does *P. putida* migrate to the upper leaves? Furthermore, it is unclear whether the increased abundance of *P. putida* in the upper leaves is due to beetle-mediated deposition followed by migration or reflects the enrichment and accumulation of pre-existing environmental populations within or on the surface of the plant leaves.

The physical route of migration is also unclear. Does *P. putida* move along the leaf surface, through vascular tissue, or within internal plant tissues? Distinguishing between these possibilities would substantially strengthen the proposed mechanism.

The interpretation of Fig. 3B is particularly puzzling. The authors do not mention the mechanism here. The data suggest that *P. putida* induces indole-3-acetic acid (IAA) accumulation, which generates a chemotactic signal that attracts *P. putida* itself, which is unheard of in any system. If this is correct, it would represent an unusual (first?) mechanism in chemical ecology and would require direct experimental validation. A straightforward experiment involving the application of cultured *P. putida* to unwounded and mechanically wounded leaves (simulating insect feeding) could determine whether the bacteria alone induce IAA accumulation. It is surprising that bacteria, rather than chewing damage, appear to drive this effect, given that insect herbivory typically elicits phytohormone responses. Additionally, removing gut microbiota often affects insect physiology i.e. making them unwell especially if the gut microbiota is important for the host health. Potential differences in feeding behavior between CONV and ABX beetles should therefore be explicitly addressed.

Leaf and insect bacterial load and diversity

A clearer characterization of bacterial diversity and load across insect and plant would improve the manuscript. It would have been useful (and expected in such study) to report a bar plot of bacterial community composition (16S rRNA sequencing) and absolute bacterial quantification (qPCR) for gut and leaf tissues to clarify whether *P. putida* behaves as a transient environmental bacterium or a persistent symbiont.

Additionally, it is unclear how *P. putida* was distinguished from other potentially present *Pseudomonas* taxa in the gut. Information on the number of operational taxonomic units (OTUs or ASVs) per genus, their geographic consistency, and the diversity of *Pseudomonas* strains isolated would help determine if *P. putida* is a dominant, recurrent associate or one of many closely related taxa.

Horizontal versus vertical transmission

The mode of *P. putida* transmission is still unclear. Fig. 3F shows that it is unclear whether transmission from beetles to leaves occurs via saliva during feeding, frass deposition, or another route. Quantifying the eGFP-tagged *P. putida* strain in the mouthparts, gut, frass, and leaf tissue over time would clarify this. Such experiment is necessary and should be easy to set up.

More broadly, the relative contributions of vertical and horizontal transmission are ambiguous. If *P. putida* is transmitted to eggs, this must be demonstrated directly. Conversely, if pupation eliminates gut bacteria, as is typical in holometabolous insects, then adult reacquisition of *P. putida* must occur via the environment. In that case, horizontal transmission would be obligatory, and the proposed 'feedback loop' would operate differently than implied. Therefore, clarifying when and where *P. putida* is present across life stages (eggs, larvae, and newly emerged adults) is essential. The difference between horizontal and vertical transmission needs to be clarified.

Role of *Pseudomonas*

Quantifying *P. putida* in the eggs is necessary because it may provide protection against entomopathogenic fungi, a hypothesis that I suggest to the authors. There are many examples of such defensive symbionts in insect-microbe symbiosis, whether they are obligate or not. The presence of *P. putida* on the eggs could provide support for this hypothesis. Additionally, the author could inoculate germ-free eggs with *P. putida*, followed by an entomopathogen, to demonstrate antifungal protection. Ultimately, *P. putida* whole genome mining with antiSMASH software could provide a list of antimicrobial metabolite candidates. Bioassay and genome mining are suggestions, whereas DNA barcoding of the eggs is necessary to reveal *P. putida*'s presence and confirm vertical transmission.

Positive feedback loop and adaptive interpretation

Although the title and discussion repeatedly invoke a 'positive feedback loop', no evidence is presented for generational

reinforcement, increasing *Pseudomonas* prevalence across generations, or population-level amplification. The data support directional interactions, but they do not demonstrate a self-reinforcing feedback loop in an ecological or evolutionary sense. Similarly, statements such as those in L277–279, which frame mature-leaf oviposition as an adaptive strategy to protect offspring, are speculative. Reduced larval performance on upper leaves alone does not demonstrate adaptive maternal behavior unless oviposition choice is linked to net reproductive success.

Use of 'Dietary Specialization'

The phrase 'dietary specialization' is used multiple times throughout the paper in contradictory ways. 'Dietary specialization' is invoked in the introduction and discussion (L69, L220) to signal the different tissue feeding habits between adult and larval *P. versicolora* on the same host plant, which is at odds with the usual meaning of the term to describe general host species monophagy in an herbivore species without reference to lifestages or plant tissues. However, in the term's first instance (L64), the words following the comma 'which reduces both inter- and intraspecific competition' seems to indicate both the usual meaning of 'dietary specialization', which reduces interspecific competition, as well as this unique meaning that distinguishes adult and larval habits, which reduces intraspecific competition. I recommend the addition of clarifying language to indicate this dual meaning of 'dietary specialization' in this first instance (L64), because all later instances of the phrase in this paper refer exclusively to the unique, adult-versus-larval-feeding-habits meaning.

Inconsistency in the proposed TO1-mediated mechanism

The author proposes that *Pseudomonas putida* modulates oviposition preference via regulation of the takeout gene TO1. However, this model is inconsistent since while TO1 knockdown abolishes the mature-leaf oviposition preference (Fig. 5H), *P. putida* reintroduction nevertheless restores this preference even under TO1 knockdown (Fig. 6D). This result contradicts the proposed causal role of TO1 as a required downstream mediator of *P. putida*'s effect on behavior. At minimum, these data suggest that TO1 is not strictly necessary, or that parallel pathways exist. What was the level of TO1 expression after the KO? Probably not 100% which means that it could interfere with the experiment's results. As currently presented, the mechanistic model is therefore not supported by the data and clarification are required to carefully address the limitations of the results for understanding the mechanism.

Miscellaneous

L277-279 'This suggests that the adult's preference for laying eggs on mature leaves, away from the *P. putida*-enriched new leaves, serves as a strategy to avoid the negative impact of the bacterium on their offspring.' This is purely speculative.

L280 'While *P. putida* negatively affects larval fitness, we observed no significant impact on adult survival or clutch size of eggs.' None of the experiments in this work test and demonstrate this hypothesis. The negative effect could come from the chemical defense induced by IAA production for example. How would the *P. putida* have a negative effect on larvae but not on adult?

Minor Comments:

Introduction:

L64: L70-73: Omit final sentence. Repetition from the end of the previous paragraph (L59-61) L84: Replace "*P. putida*" with "one *Pseudomonas* species, *P. putida*". Since both *Plagioderia* and *Pseudomonas* abbreviate to "*P.*", further clarification would better signal to readers that *P. putida* is a microbial symbiont, rather than a related beetle species.

Results:

L139-141: Edit to clarify that the "significant increase in IAA concentration" was specifically observed in the upper leaves.

Discussion:

L250-251: Elaboration needed. In what sense is leveraging the plant environment more effective at transmitting *Pseudomonas* relative to leaf surface acquisition? This notion seems to depend on an unwritten premise that *P. versicolora* adults prefer compensatory growth-produced leaves relative to adult beetle-damaged leaves, which is not suggested by the data, and different from a preference for new over mature leaves indicated in other sections. It's perfectly reasonable to hypothesize such a preference for compensatory growth leaves, but it should be stated explicitly here.

L279: Soften language. The data presented does not demonstrate that *P. putida* specifically reduces the fitness of larvae fed upper leaves from CONV-damaged plants, as opposed to any other potential chemical or microbial factors that distinguish those leaves from those of the other treatments.

Materials and Methods:

L342: Replace "*S. babylonica*" with "*Salix babylonica*".

Figures and Tables

Figure 1C, L740: Since the pie charts contain the same information as the bar chart, the pie charts should be omitted for simplicity.

Figure 3, L781-784: Mislabelled caption. Should be "(C) Relative abundance of *P. putida* ..." and "(D,E) Chemotactic response of *P. putida* (D) and *E. aerogenes* (E)..." or the labels in the figure should be switched.

Figure 4D,E L802-803: Clarify that the different shades in the bar graphs denote the larval instars

(Remarks to the Author)

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Reviewer #3

(Remarks to the Author)

The manuscript by Li et al combines field studies of the leaf beetle *Plagioderma versicolora* with oviposition bioassays and the molecular characterization (RNAi knock-down) of a candidate gene. This is an interesting manuscript and the suggested link between specific gut bacteria and behavioral adaptation sounds fascinating. One can appreciate many aspects of this work, and the general system of the beetle herbivore-gut microbiome association can be considered to be of potential broad interest.

The overall impression of this manuscript is that it has lots of experiments and data analysis and is mostly written well. However, the interpretation of the data goes beyond what is justified, with quite some level of speculation based on rather scarce or unclear evidence. Unfortunately, it is therefore not a major advance in research on interactions between gut microbial partners and symbiont-driven behavioral adaptation (i.e. oviposition). More specifically, the data presented is not sufficient to support the proposed role of the takeout gene TO1 as a link between *Pseudomonas putida* and the suggested manipulation/induction of *P. versicolora* female oviposition preference.

In general, one does get the impression that the authors seem to approach the whole system in a somewhat biased way. For example, they seem to neglect several issues related to the methods and approaches used, e.g. the problems associated with disrupting the normal gut microbiota-host insect interface and the possible subsequent alterations in response to pathogen encounters. In addition, they overlook the link between the potential pathogenicity of *P. putida* in the larvae and the adult immune response observed in the transcriptomic (RNAseq) data.

Originally, avoidance of intraspecific competition was suggested as the main argument for female oviposition preference for old leaves. This now clashes with the new argument of female avoidance of elevated *P. putida* levels found in new leaves. Several of the main questions remaining are: where does *P. putida* come from, who transfers what amounts to the plant and who picks up *P. putida* from the ingested plant material again? This is important to know, since *P. putida* is an environmental bacterium, frequently found in the rhizosphere as well as on/in plants, but obviously also in adult (and larval?) *Plagioderma versicolora* beetles. If eggs are laid on old leaves, eggs and newly hatched larvae will potentially be exposed to lower (?) *P. putida* levels before later larval instars will eventually have to move to newer leaves. Since adult beetles seem to even prefer young (upper) leaves, one could ask why those adults seem to not be negatively impacted by the elevated levels of the opportunistic entomopathogen *P. putida*. Instead of proposing a direct link between the differences in relative abundance of *P. putida* in mature and young leaves and larval performance, one could also argue for other differences between the leaves (i.e. unrelated to *P. putida* loads), including plant defense metabolites. Differential induction of plant metabolites as a result of tissue damage exerted by beetles with and without gut microbiomes could be the reason for the variable performance observed in subsequent larval feeding assays. In general, it needs to be noted that young and mature leaves can respond very differently to the same wounding/feeding/microbe stimulus.

In general, after looking at the many different experiments performed by the authors in more detail, the reader is left with more questions in mind, instead of the conclusion of the authors that they show "...gut bacteria ... ultimately sustaining a symbiont-driven behavioral adaptation".

Below several of the abovementioned and other issues are listed in more detail.

Gut microbiota and oviposition preference:

The authors have shown that germ-free females shifted their oviposition preference towards new leaves, whereas the re-introduction of gut microbiota restored their preference for mature leaves. This could have multiple reasons, e.g. it could be the result of a shift in gut nutrient sensing, changing the female oviposition choice to more nutrient-rich plant material. Alternatively, depleting the gut microbial community could also result in the avoidance of mature leaves with potentially altered microbial (not only *Pseudomonas*) loads. It is noteworthy that the oviposition rate on mature leaves dropped considerably in germ-free females, to about one third of the rate observed in both conventional and females where bacteria were re-introduced (Figure 1E, right panel). However, information on overall oviposition differences (i.e. how many of the germ-free females actually laid any eggs) is missing.

Pseudomonas abundance in mature leaves:

There seems to be decent evidence supporting a higher abundance of *Pseudomonas*-like gut microbes in beetles feeding on mature leaves. However, when having a closer look at Figure 2G, the absolute abundance of *Pseudomonas* does not seem to differ much between beetles feeding on young or mature leaves. Furthermore, the significant results in Figure 2G (top panel) seem to be driven by two samples showing very high relative abundance levels of *Pseudomonas*. Considering the above, evidence that *Pseudomonas* is really highly correlated with "mature-leaf oviposition preference" of the females is not really convincing. It would have been important to also perform 16S rRNA sequencing and bacterial community assessments of the mature and young leaves – without beetle feeding. Multiple dietary factors can alter the gut microbial composition of herbivorous insects, including plant defense metabolites and leaf nutritional content differences. In this specific context, one of the major questions is whether there is a shift in relative abundances of permanent or transient (i.e. obtained from the diet) bacteria in the beetles' gut.

Furthermore, what remains to be shown is the identity of the *Pseudomonas* species grouped as *Pseudomonas* in all the microbiome analysis. It is unclear why the authors subsequently only focus on *Pseudomonas putida* for all of the other assays. Important questions remain unanswered:

- What is the evidence supporting the notion that only a single *Pseudomonas* species is found among the gut bacteria?
- What is the prevalence of *P. putida* in the leaves of host plants and/or beetles from the field?
- Have *P. putida* levels increased in larvae fed on the new leaves damaged by conventional beetles?
- How much variation in *Pseudomonas* levels can be found among individual females and how would this translate to differences in oviposition preferences?

Chemotactic response of *P. putida*:

Whether *P. putida* is actively migrating towards younger leaves or whether bacterial cells are transported by the plant remains unclear. Although the authors state that "These results (Figure 3D) demonstrated that *P. putida* exhibited positive, concentration-dependent chemotaxis towards IAA ..", the supposed "migration upwards from the feeding site to new leaves by sensing IAA .." could also be due to other compounds (i.e. defense metabolites) produced by the plant during beetle feeding. In other words, whether IAA itself mobilizes *P. putida* to migrate away from the feeding site is not well supported, even if demonstrated with IAA in isolation. Leaf damage by e.g. feeding herbivores induces a multitude of plant responses, including defense responses mainly orchestrated by jasmonic acid and its derivatives. Plant wounding and additional herbivore-derived factors usually result in local responses, but also systemic spreading of the response to other parts of the plant (e.g. other leaves). Considering the multitude of changes in damaged versus undamaged leaves, it remains unclear why the authors specifically focus on IAA only.

RNAseq analysis:

In the RNAseq analysis, 63 differentially expressed genes were identified, predominantly associated with structural proteins, but also immune response factors. However, the authors solely focus on two genes supposedly related to neural signaling, the takeout homologs TO1 and TO2.

Among the differentially expressed genes, there are several genes potentially related to immune signaling, pattern recognition and/or regulation, i.e. a lectin, an amidase and a proteinase inhibitor. Furthermore and even more importantly, three different attacins, a defensin and a coleopteracin are differentially expressed, all of which are immune effectors (or more precisely antimicrobial peptides) utilized by insects to counter pathogen attacks and to sometimes control the uncontrolled spread of mutualists.

Given that the two takeout genes identified in the RNAseq analysis are only moderately differentially expressed, one does wonder why exactly those two genes have been selected for further analysis. In general, the Supplemental Material does not provide any information on fold-change levels and the direction of change (increased or decreased transcript levels) for the genes listed in Table S3.

Pseudomonas is known to have insecticidal toxins similar to nematode-vectorized insect pathogens. Considering the role of *P. putida* as an opportunistic pathogen (which was e.g. shown in another beetle species, *Leptinotarsa*) one does wonder about the differential expression of such a high number of AMPs. Are these AMPs more abundant in the presence of *P. putida* specifically - or are AMP expression levels linked to the presence/absence of a complex gut microbiome in general?

Takeout genes and RNAi:

Takeout proteins, part of the Takeout/juvenile hormone-binding protein (JHBP) family, are implicated in regulating feeding behavior, circadian rhythms, and potentially social interactions in insects. In general, takeout gene(s) encode a secreted protein related to circulating carrier proteins of lipophilic factors, such as juvenile hormone. It would be important to know whether the candidate takeout gene is regulated by JH signaling, similar to other takeout genes. Expression of takeout genes is not limited to neuronal tissues, since members of this gene family are also expressed in e.g. gut or fat body tissue. In addition, it has been shown that the expressions of takeout genes is regulated by age and nutritional status, and in some Lepidoptera even by the application of insecticides. In *Drosophila*, the takeout mutant dies rapidly in response to starvation, indicating a link between survival and food status sensing. Since the above suggests multiple roles of takeout genes in insects, the authors should refrain from linking the role of TO1 and TO2 to neural signaling only. It is interesting to note that both TO1 and TO2 (for which overall expression profiles look almost identical) are expressed also in the body, not only in the head. Any knock-down approach would thus also impact TO gene expression/function in the body.

Although the authors only mention 2 takeout-like genes, in insects the takeout gene family can be quite large, with usually 10-20 and in some cases even more (e.g. 25 in termites) genes. With these numbers in mind and given the sometimes very high level of sequence conservation among takeout genes, controlling for off-target effects of gene knock-down experiments is crucial. This could even be done using a transcriptome only. However, since a reference genome exists for this species, the authors need to clarify this shortcoming by applying an off-target search across the complete genome. For the RNAi experiment, only a single dsRNA fragment was generated for the TO1 candidate gene, but at least two different regions of the gene should be considered for dsRNA generation. There is also no information about the length of the dsRNA fragment. Screening for off-targets using all possible short interfering RNAs deduced from the long dsRNA fragment, allowing for mismatches would have been important to exclude the untargeted knock-down of other takeout-like (or completely unrelated) genes.

Tissue material used for RNAseq:

In their RNAseq experiment, the authors used complete heads, but mentioned that "the chitinous cuticle was removed from the heads prior to sample preparation". I am not really sure why the authors have performed this step, since this approach can certainly introduce a bias in some or even all of the samples. Removing the cuticle without affecting any other, sometimes rather brittle, easy-to-fragment tissues such as fat body can be quite tricky. Gene expression levels could be affected by the length of the dissection procedure as such and by how much of other tissue material remains on the cuticle which was removed after beetle decapitation. Furthermore, it remains unclear whether - together with the cuticle - the

complete antennae were also removed. The authors need to address the issues mentioned above, and should also provide information about biological variability of the RNAseq samples, by e.g. providing a Multidimensional Scaling plot displaying all biological replicates of the treatments.

Minor comments:

- The references are formatted inconsistently
- Line 37ff: In the introduction, the authors state that "Among these decisions, ..." – but fail to explain which decisions they actually mean.
- Line 44: what is "fundamental insect ecology" supposed to mean?
- In general, the authors list almost exclusively mutualistic interactions between insects and microbes in the introduction. They seem to completely ignore the multitude of antagonistic and pathogenic interactions.
- Lines 55ff: Only in very few cases (if at all) is there clear evidence for real co-evolution between insects and microbes in general. The authors should therefore refrain from stating co-evolution in the context of gut-resident microbes and their hosts.
- Lines 59-61 and 71-73 are the exact same sentence. Consider rephrasing one of them.
- Line 150: were the bacteria used in this assay also isolated from the beetles as stated in line 158? If so, please state it here.
- Line 364: this looks like a header that wasn't properly formatted.
- The change in nomenclature of the leaves halfway through from new and mature to upper and lower seems unnecessary and adds confusion.
- Throughout the results, the mentioning of some data regarding the observed effects for main results such as the differences in oviposition preference, larval and pupal weight is missing.
- Gene expression patterns of TO1 and TO2 seem to be almost identical. However, except for briefly mentioning some differences between TO1 and TO2, no direct comparison of TO1 and TO2 sequences and domains are shown (neither in Figure 5 nor as a supplement). If TO1 plays the suggested central role in the interaction between *P. putida* and the host, what role might TO2 have, given its highly similar expression pattern?
- In the discussion a figure is cited in the first paragraph and then never again. I think having figure citations in the discussion helps tie it to the results.
- For the cultivated of the bacteria from beetle guts, did the authors know beforehand that the kind of cultivation medium they used for the experiments would select for the most representative members of the microbiota? If so, it would be nice to state that somewhere. Otherwise it could be nice to show that the post-incubation microbes match the natural composition.

Reviewer #4

(Remarks to the Author)

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

I deeply appreciate all the edits, adjustments, responses, and additional experiments that significantly improve the manuscript, clarify the mechanisms, and broaden the impact of this remarkable work.

I am very happy with all the changes, and in my humble opinion, the manuscript is now ready for publication in Nature Communications.

Reviewer #2

(Remarks to the Author)

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Reviewer #3

(Remarks to the Author)

The authors have done a thorough job of responding to the reviewer comments and have addressed most major concerns. The manuscript was greatly improved as the authors went to great lengths to address the comments not only by changing phrasing throughout the manuscript and providing a better contextual framework, but also by reanalyzing existing data and performing additional experiments. However, some issues remain or were added in this process.

Takeout genes:

The authors' re-analysis of the genomic and transcriptomic data is appreciated and very valuable. The process of

streamlining the 2 takeout genes into a single gene model which was explained in the rebuttal should be described in the methods and mentioned in the results section. This would prevent future efforts to characterize this gene coming to the same initial conclusion of the existence of 2 highly similar takeout genes.

Defensin knockdown:

It is appreciated that the authors placed more relevance into the differences in immune regulation between conditions and tried to address the comments.

However, since immune responses are usually triggered by signalling cascades leading to transcriptional regulation, the knockdown of a single immune effector, even if this is the most highly differentially expressed one, cannot provide information on whether or not differences in immune regulation lead to differences in behavior. Especially in insects there is a large amount of overlap between activities of individual antimicrobial peptides. Furthermore, we did not intend to imply that differential expression of a single AMP would result in behavioral differences. I am afraid to say that the defensin knock-down experiment is essentially useless and would be misleading to the readers.

The recommendation would be that, rather than including this experiment in the final manuscript, the authors try to discuss a bit better what the immune upregulation in GF conditions may mean in this context.

P. putida's ecological relevance in larvae compared to adults:

The authors argue in line 219 that the fact that newly emerged larvae are more susceptible to *P. putida* compared to newly eclosed adults represents an "ontogenic shift in susceptibility, indicating that *P. putida* may function as a stage-specific conditional pathogen".

This sentence should be moved to the discussion where further context should be added on whether this is known for other insect-pathogen interactions.

Minor comment:

- Please add the mean development times and weight of the control group as well in line 210.

Reviewer #4

(Remarks to the Author)

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Reviewer #1 (Remarks to the Author):

General Comment: This paper presents a lucidly-described investigation with advanced genetic techniques and microbial manipulation experiments that greatly enhances our understanding of the interactions between the microbiome, insect behavior, and plant-insect ecology. The authors clearly demonstrate multiple biologically interesting phenomena, including (1) the positive relationship between natural *Plagioderia versicolora* gut microbial flora (*Pseudomonas*) and mature leaf oviposition preference, (2) the positive chemotaxis of *Pseudomonas putida* toward indole-3-acetic acid (IAA), (3) the accumulation of IAA and *Pseudomonas* on newly-produced willow leaves following *P. versicolora* feeding, (4) the elevated expression of regulatory gene *TO1* in *P. versicolora* in response to *Pseudomonas* colonization, and (5) the positive relationship between *TO1* upregulation and mature leaf oviposition preference. From these lines of evidence, the authors construct a plausible mechanism that links *P. versicolora* oviposition behavior, *Pseudomonas* transmission, and host plant compensatory growth. The evidence and analysis presented in this study is of high value to the field of plant-insect interactions, however major concerns are listed below.

Some aspects of the study stand out for their clarity and strength of presentation. Notably, the study demonstrated that oviposition choice is microbiota-dependent, identified *Pseudomonas putida* as a key strain correlated of this behavior, and associated it with host genes implicated in behavioral regulation. The behavioral component and the host gene regulation by the bacteria alone could have formed a compelling manuscript by itself. In contrast, the study of the bacterial horizontal and vertical transmission is substantially weaker. In its current form, it obscures rather than clarifies the core findings. The manuscript conflates oviposition preference with adult-to-adult transmission; these processes must be conceptually and experimentally separated in the manuscript. The mode of transmission is a significant unresolved issue that introduces ambiguity. In several places, the inclusion of additional results increases confusion rather than providing mechanistic insight. Notably, the occurrence and abundance of bacteria, including *P. putida*, are not systematically characterized across relevant beetle compartments, such as mouthparts, gut, frass, eggs, larvae, and newly emerged adults and finally leaf tissue. Consequently, the relative contributions of horizontal versus vertical transmission remain unclear.

In addition, the mechanism underlying IAA induction is puzzling. The data suggest that bacteria induce the signal (IAA) that subsequently attracts them. This scenario is unusual in chemical ecology (and would be the first report) and requires validation through experimentation. More generally, the bacterial transmission route remains unclear, leaving open the fundamental question of whether *P. putida* functions as an environmental bacterium or an obligate (or semi-obligate) symbiont. Clarifying these points could substantially alter the interpretation of the study and therefore requires careful attention.

Finally, the inconsistency of the proposed *TO1*-mediated mechanism is problematic and must be explicitly addressed in the discussion. The results suggest that *TO1* is not strictly necessary for the behavioral phenotype or that other, parallel pathways are involved.

More broadly, several key claims, including the existence of a positive feedback loop, adaptive behavioral reinforcement, and microbial ‘priming’ are not yet supported by the results. These claims rely heavily on correlations and rescue experiments that do not fully exclude alternative explanations.

In summary, although there are many impressive pieces of work that build on the authors' previous publications on this system and present intriguing and potentially important observations linking

gut bacteria, plant responses, and insect oviposition behavior, several core claims, particularly those concerning mechanistic mediation, adaptive significance, and positive feedback dynamics, are not sufficiently supported for publication in their current form.

Despite the substantial corrections listed above and below, I do not want to discourage the authors, and I would like to emphasize that I was very impressed by the quality, ambition, and technical execution of this study. I therefore congratulate the authors and I strongly encourage the authors to submit a revised version because I truly believe it will be a remarkable contribution to the field.

Response: We are deeply encouraged by your recognition of our study's ambition, technical execution, and its value to the field of plant-insect-microbe interactions. Your critical insights into the transmission dynamics, the "puzzling" nature of the IAA induction, and the regulatory complexity of the *TOI* pathway have been instrumental in refining our mechanistic model and elevating the rigor of our manuscript. Our responses to your primary concerns are summarized below:

1. Systematic characterization and mixed transmission strategy: To resolve the ambiguity regarding horizontal versus vertical transmission, we systematically quantified *P. putida* abundance across all relevant biological compartments: eggs, larvae (1st–3rd instars), pupae, specific adult tissues (mouthparts, gut, and frass), and leaf tissues (Revised Fig. 3 & S3). Our data now support a mixed transmission strategy that ensures stability:

- **Vertical transmission:** We confirmed that *P. putida* is present on the egg surface (maternal egg-smearing). Crucially, we observed that neonates consume their eggshells upon hatching (RL Video 1), a behavior that ensures the stable vertical transfer of the symbiont to the next generation. Our eGFP-tagged *P. putida* assays (Revised Fig. 3I-L) demonstrate that once colonized in the adult gut, *P. putida* persists in the F1 generation even without additional environmental supplementation.
- **Horizontal dynamics:** In addition to the results of "adult-to-adult transmission" you mentioned, we showed *P. putida* is significantly enriched in adult mouthparts and frass (Revised Fig. 3L). This suggests that adult beetles act as active vectors, redistributing the bacterium to new feeding sites (horizontal recruitment).
- **Geographic Ubiquity:** To address whether *P. putida* represents a core associate or a mere environmental transient, we characterized phyllosphere microbial communities across geographically distant populations using 16S rRNA high-throughput sequencing and qPCR (Wuhan, Qinhuangdao, and Guiyang). Together with its stable presence in the gut of beetles feeding on both new and mature leaves, the consistent dominance of *P. putida* across these sites supports a stable, non-transient association with *P. versicolora* (Revised Fig. 3 & S3).

2. Clarifying the IAA induction and translocation route: You rightfully noted the "puzzling" nature of the IAA induction. Our new experiments clarify that the bacterium itself does not trigger the IAA elevation; rather, the elevation is a specific host plant response to beetle feeding.

- **The "Follower" mechanism:** By comparing IAA and *P. putida* abundance in ABX, CONV, and CONV+*P. putida* groups, we show that the plant-derived IAA burst precedes and drives the bacterial enrichment. *P. putida* is not "calling itself" but is instead exploiting the plant's compensatory growth signals (IAA) via positive chemotaxis (Revised Fig. 3C & F). We simultaneously monitored Jasmonic Acid (JA) and Chlorogenic Acid (CGA); the results confirm that *P. putida* recruitment is specifically coupled with the IAA-mediated growth response rather than a generalized defense or wounding response.

- **Endophytic migration:** Using fluorescence microscopy and eGFP-tracking, we successfully visualized *P. putida* migrating through internal plant tissues, including vascular bundles (Revised Fig. S5). This confirms that the enrichment in upper leaves results from active translocation within the plant tissue, rather than simple surface contamination.
- 3. Refining the *TOI*-mediated mechanism:** We agree that *TOI* should be viewed as a key regulatory hub rather than a sole, indispensable driver. We now characterize the *TOI* pathway as part of a robust, potentially redundant neuroendocrine network. The ability of *P. putida* to rescue the phenotype even under *TOI*-suppression suggests a "functional threshold" effect or the existence of parallel regulatory pathways (e.g., other bacterial metabolites) that ensure behavioral stability. To address the "inconsistency" noted (where behavior persists despite RNAi), we have revised our Discussion (Lines 377-387).
- 4. Validating "Priming" and feedback loops:** To move beyond correlation and support our claims of "behavioral reinforcement":
- **Causal Priming:** We conducted a dietary enrichment assay where supplementing conventional (CONV) beetles with *P. putida* intensified the mature-leaf preference, providing causal evidence for microbial "priming" (Revised Fig. 6F-H).
 - **Adaptive Trade-off:** Comparative immunity assays (Revised Fig. 4I) demonstrate that adults possess significantly higher immune tolerance to *P. putida* than larvae. This explains the adaptive significance: females selectively oviposit on mature leaves to distance their susceptible offspring from bacterial hotspots, while still functioning as resilient vectors for symbiont transmission.
 - **Revised Terminology:** Given the lack of long-term field fitness data, we have conservatively renamed the "positive feedback loop" to a "plant-mediated feedback loop" to more accurately reflect the observed ecological dynamics.

We have addressed your specific major and minor comments in details in the point-by-point section below. We believe these revisions have clarified the core findings and substantially strengthened the mechanistic insights of our study.

Major Comments:

Comment 1. *P. putida* migration to new leaves and IAA-inducibility

Fig. 3A does not make it clear whether beetle feeding occurred on new or mature leaves, even though adults are expected to preferentially feed on new leaves. The temporal sequence is also unclear: how long after feeding do new shoots appear, and how quickly does *P. putida* migrate to the upper leaves? Furthermore, it is unclear whether the increased abundance of *P. putida* in the upper leaves is due to beetle-mediated deposition followed by migration or reflects the enrichment and accumulation of pre-existing environmental populations within or on the surface on the plant leaves.

The physical route of migration is also unclear. Does *P. putida* move along the leaf surface, through vascular tissue, or within internal plant tissues? Distinguishing between these possibilities would substantially strengthen the proposed mechanism.

The interpretation of Fig. 3B is particularly puzzling. The authors do not mention the mechanism here. The data suggest that *P. putida* induces indole-3-acetic acid (IAA) accumulation, which generates a chemotactic signal that attracts *P. putida* itself, which is unheard in any system. If this is correct, it would represent an unusual (first?) mechanism in chemical ecology and would require

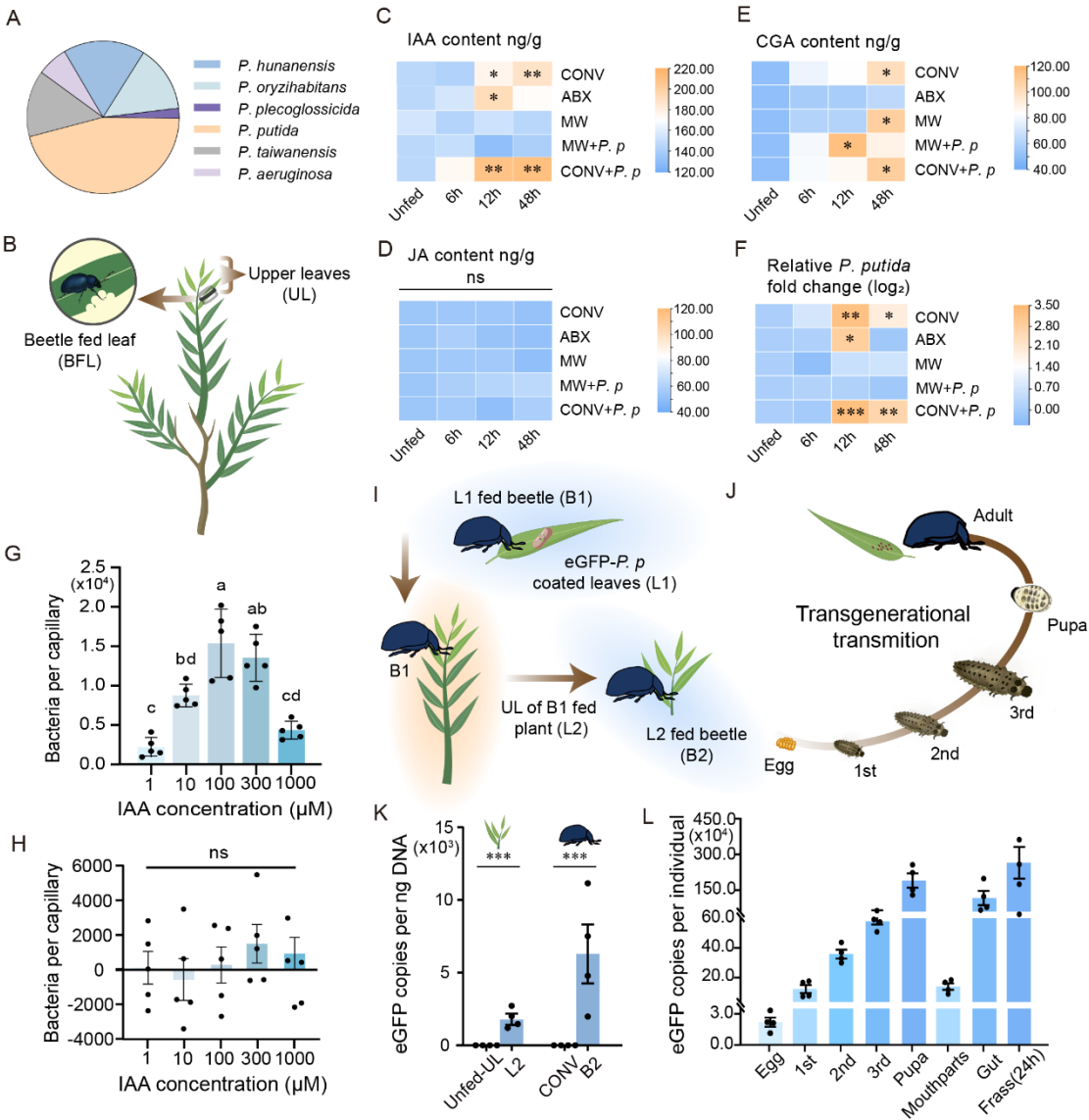
direct experimental validation. A straightforward experiment involving the application of cultured *P. putida* to unwounded and mechanically wounded leaves (simulating insect feeding) could determine whether the bacteria alone induce IAA accumulation. It is surprising that bacteria, rather than chewing damage, appear to drive this effect, given that insect herbivory typically elicits phytohormone responses. Additionally, removing gut microbiota often affects insect physiology i.e. making them unwell especially if the gut microbiota is important for the host health. Potential differences in feeding behavior between CONV and ABX beetles should therefore be explicitly addressed.

Response: We thank the reviewer for these critical questions. We also sincerely appreciate the constructive suggestions. In response, we have conducted additional experiments to address these issues accordingly:

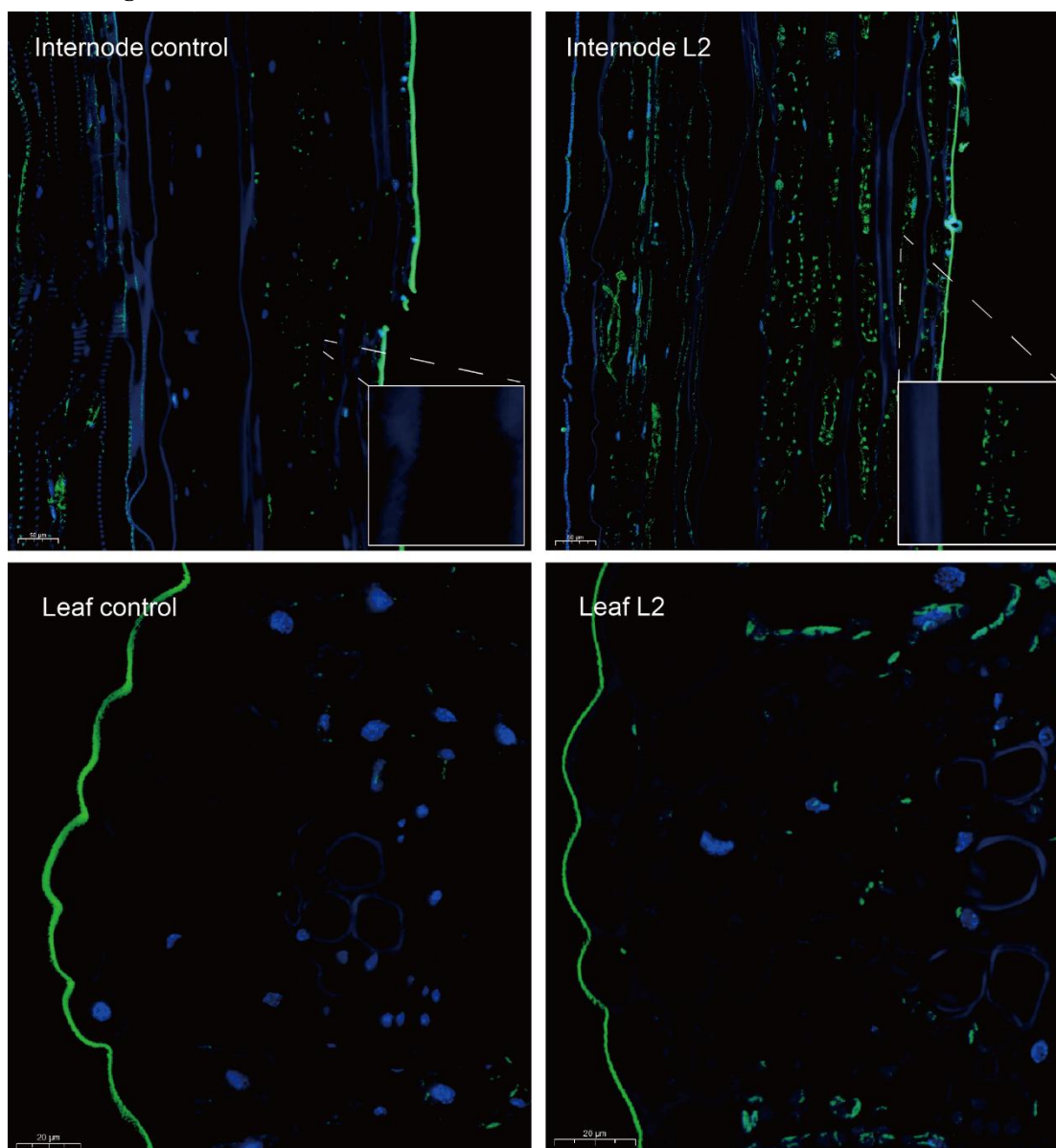
- 1. Clarification of Experimental Setup and Temporal Dynamics:** In our assays, adults were restricted to feeding on a single set of pre-existing new leaves (as now specified in the Methods lines 560-561). The timing of new shoot emergence on willow branches is influenced by the physiological state of the plant, its stability, and light conditions. Under our experimental conditions, new leaves typically appear in about three days. It should be clarified that the “new leaves” in our experiment were already present before the treatment and do not refer to leaves that emerged during the experiment. We conducted a time-course analysis (6h, 12h, and 48h post-feeding). Our results demonstrate that *P. putida* recruitment to distal upper leaves (UL) is a rapid, systemic process that peaks at 12h post-infestation (Revised Fig. 3F).
- 2. Distinction between Enrichment and Accumulation:** To determine the source of the bacteria in distal tissues, we employed eGFP-tagged *P. putida* as a traceable marker. The successful detection of eGFP-tagged *P. putida* in the upper leaves following feeding by colonized beetles (Fig. 3K) confirms that the primary inoculum originates from the beetle (gut/mouthparts) and is actively translocated, rather than merely reflecting the enrichment of pre-existing phyllosphere populations. Besides, while ABX-treated (microbiota-free) beetle damage can trigger a transient mobilization of indigenous endophytic *Pseudomonas*, this population returned to baseline by 48h. In contrast, feeding by CONV beetles resulted in significant and sustained colonization at 48h. This divergence suggests that while the plant provides the “pathway,” the beetle serves as the essential “reservoir” for establishing high-density *P. putida* populations in new growth.
- 3. Physical Route of Migration:** We used fluorescence microscopy to track the migration of eGFP-tagged *P. putida*. We observed eGFP signals localized within the vascular tissues and near the vascular bundles of stems and leaves (Revised Fig. S5). This histological evidence, combined with our qPCR data, confirms that *P. putida* utilizes an internal endophytic route (likely vascular translocation) to move from the primary feeding site to distal tissues.
- 4. Validating the IAA Induction Mechanism:** Our new experiments confirm that *P. putida* is a “follower” of host signaling. Mechanical wounding (MW) followed by the direct application of cultured *P. putida* failed to trigger either an IAA burst or bacterial migration. Conversely, both CONV and ABX beetle feeding elicited a robust IAA increase (Fig. 3C). This proves that beetle-specific factors (chewing damage/oral secretions) drive the IAA response. To test for a generalized stress response, we expanded our analysis to include Jasmonic Acid (JA) and Chlorogenic Acid (CGA). While beetle feeding activates multiple defense pathways, *P. putida* recruitment is exclusively coupled with IAA dynamics, and the strain shows no chemotactic response to JA or CGA (Fig. 3C, S4).

5. Insect Health and Feeding Intensity: To address the concern that ABX treatment might make insects "unwell," we quantified the total feeding area as a proxy for physiological vigor. We found no significant difference in feeding intensity between CONV and ABX beetles (Revised Fig. S1).

Revised Figure 3



Revised Fig. S5



Comment 2. Leaf and insect bacterial load and diversity

A clearer characterization of bacterial diversity and load across insect and plant would improve the manuscript. It would have been useful (and expected in such study) to report a bar plots of bacterial community composition (16S rRNA sequencing) and absolute bacterial quantification (qPCR) for gut and leaf tissues to clarify whether *P. putida* behaves as a transient environmental bacterium or a persistent symbiont.

Additionally, it is unclear how *P. putida* was distinguished from other potentially present *Pseudomonas* taxa in the gut. Information on the number of operational taxonomic units (OTUs or ASVs) per genus, their geographic consistency, and the diversity of *Pseudomonas* strains isolated would help determine if *P. putida* is a dominant, recurrent associate or one of many closely related taxa.

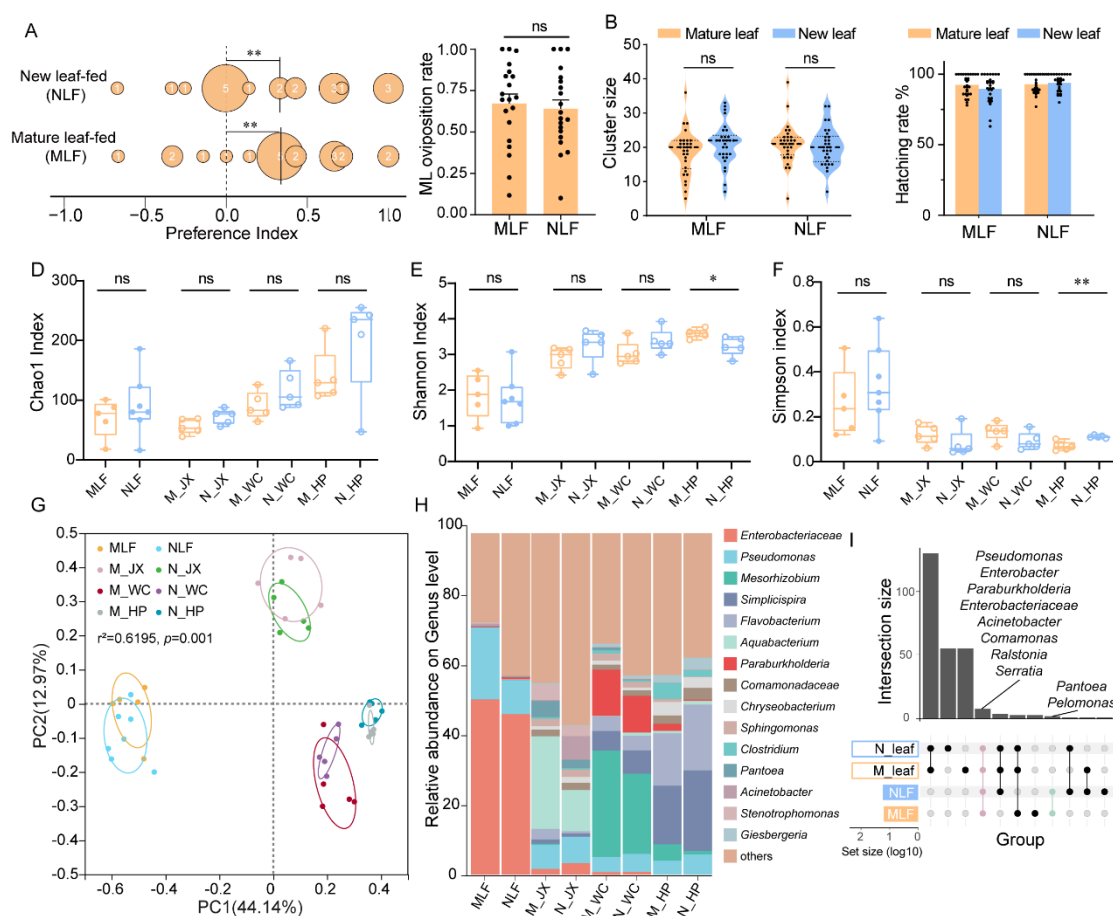
Response: We entirely agree that a high-resolution characterization of the microbial landscape is essential to define the nature of the *P. putida*–beetle association. In the revised manuscript, we have included extensive new data (Revised Fig. 2D-I, Fig. 3A, and Fig. S3G-I) to systematically address

the composition, absolute abundance, and geographic consistency of the microbiota.

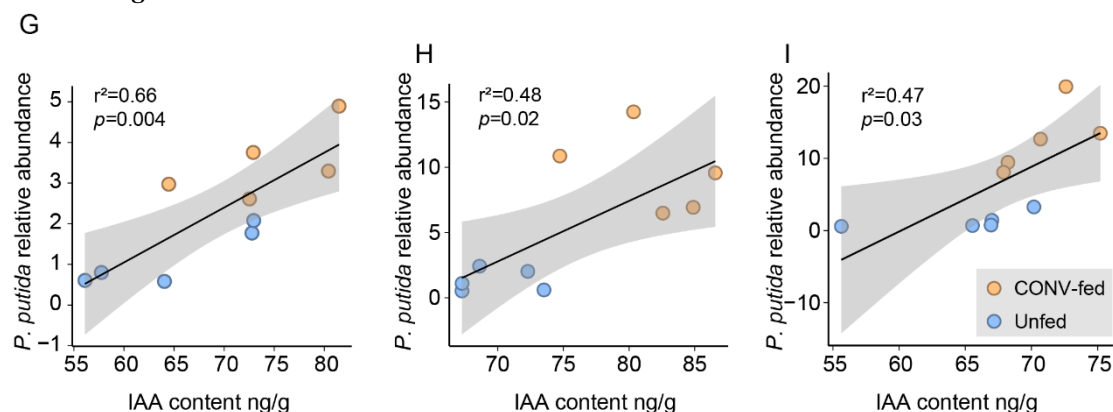
1. Systematic Characterization of Community Composition and Core Taxa: We now provide genus-level bar plots (Revised Fig. 2H) characterizing the bacterial community structures across the insect' guts (NLF, MLF) and the phyllosphere microbiota of leaves from multiple geographic sites (JX, WC, and HP in Wuhan). While PCoA analysis (Revised Fig. 2G) indicates that the phyllosphere and gut microbiota are distinct, the Upset plot (Revised Fig. 2I) identifies *Pseudomonas* as a core, dominant genus consistently shared across all biological and environmental compartments. Furthermore, absolute quantification via qPCR from samples collected across broad geographic regions—Qinhuangdao, Wuhan, and Guiyang (Fig. 1A)—confirmed the ubiquitous presence of *P. putida* in the willow phyllosphere (Revised Fig. S3G-I). Notably, its absolute abundance exhibits a significant positive correlation with IAA concentrations across all these sites (Revised Fig. S3G-I), reinforcing its status as a widespread and functionally relevant associate.

2. Cultivation-Based Species Identification: As noted by the reviewer, 16S rRNA sequencing (V3-V4) lacks the resolution to distinguish species. Therefore, we complemented our sequencing data with a cultivation-dependent approach. Using *Pseudomonas*-selective media, we isolated 92 strains from female guts. Taxonomic identification confirmed that *P. putida* is the most prevalent species within the *Pseudomonas* community (representing 45.7% of isolates, Fig. 3A). This dominance, coupled with its stable transgenerational transmission (as shown in Fig. 3I-L), identifies *P. putida* as a persistent symbiont within this system.

Revised Fig. 2A-I



Revised Fig S3G-I



Comment 3. Horizontal versus vertical transmission

The mode of *P. putida* transmission is still unclear. Fig. 3F shows that it is unclear whether transmission from beetles to leaves occurs via saliva during feeding, frass deposition, or another route. Quantifying the eGFP-tagged *P. putida* strain in the mouthparts, gut, frass, and leaf tissue over time would clarify this. Such experiment is necessary and should be easy to set up.

More broadly, the relative contributions of vertical and horizontal transmission are ambiguous. If *P. putida* is transmitted to eggs, this must be demonstrated directly. Conversely, if pupation eliminates gut bacteria, as is typical in holometabolous insects, then adult reacquisition of *P. putida* must occur via the environment. In that case, horizontal transmission would be obligatory, and the proposed ‘feedback loop’ would operate differently than implied. Therefore, clarifying when and where *P. putida* is present across life stages (eggs, larvae, and newly emerged adults) is essential. The difference between horizontal and vertical transmission needs to be clarified.

Response: We thank the reviewer for these insightful suggestions. Clarifying the transmission dynamics is indeed essential for understanding the stability of the *P. versicolora*-*P. putida* association. As suggested, we have performed a systematic quantification of eGFP-tagged *P. putida* across all life stages and relevant adult compartments to define the transmission modes.

- 1. To determine the persistence and contribution of different transmission routes**, we colonized female adults with eGFP-tagged *P. putida* and tracked the marker in the F1 generation without further supplementation. Results showed that eGFP-tagged *P. putida* was consistently detected on the egg surface (Revised Fig. 3L). Crucially, the eGFP copy numbers showed an increase from the 1st to the 3rd larval instars, indicating active microbial proliferation and enrichment within the developing host, and eGFP-tagged *P. putida* persists through the pupal stage and successfully colonizes newly emerged adults (Revised Fig. 3L). Besides, we observed that newly-emerged larvae engage in eggshell consumption upon hatching (RL Video 1), which serves as the primary behavioral mechanism for vertical transfer from the egg surface to the larval gut.
- 2. To clarify the beetle-to-leaf transmission route**, we quantified the bacterial load in specific adult compartments. Results showed that high titers of eGFP-tagged *P. putida* were detected in the mouthparts, gut, and frass of adult females (Revised Fig. 3L). These findings suggest that horizontal transmission occurs through a combination of mechanical deposition during feeding (via mouthparts) and fecal contamination (via frass). Our comparative assays using ABX and CONV beetles further clarify this dynamic. While ABX beetle damage can trigger a minor increase in indigenous *P. putida* (likely from pre-existing endophytic populations), the magnitude

of enrichment is significantly lower in CONV and CONV+*P. putida* groups (Revised Fig. 3C, F). This confirms that the beetle-associated *P. putida* population is the primary driver of the high-density bacterial pulses observed in the willows.

While we cannot assign a fixed percentage to each route, we can conclude that *P. versicolora* employs a mixed transmission strategy: Vertical transmission ensures the stable persistence of the bacterium across generations; Horizontal transmission facilitates its rapid enrichment in the local environment (the "feedback loop" in the phyllosphere). The stability of this loop is reinforced by the fact that even without environmental re-acquisition, the vertical route is sufficient to maintain the symbiont population within the host lineage.

Comment 4. Role of *Pseudomonas*

Quantifying *P. putida* in the eggs is necessary because it may provide protection against entomopathogenic fungi, a hypothesis that I suggest to the authors. There are many examples of such defensive symbionts in insect-microbe symbiosis, whether they are obligate or not. The presence of *P. putida* on the eggs could provide support for this hypothesis. Additionally, the author could inoculate germ-free eggs with *P. putida*, followed by an entomopathogen, to demonstrate antifungal protection. Ultimately, *P. putida* whole genome mining with antiSMASH software could provide a list of antimicrobial metabolite candidates. Bioassay and genome mining are suggestions, whereas DNA barcoding of the eggs is necessary to reveal *P. putida*'s presence and confirm vertical transmission.

Response: We appreciate the reviewer for the insightful hypothesis regarding the anti-fungal role of *P. putida*. We have confirmed the stable presence of *P. putida* on the egg surface through absolute quantification and eGFP-tracing (Revised Fig. 3L). This confirms that the bacteria are positioned to potentially influence offspring fitness from the earliest life stage. Regarding the functional significance of these egg-associated bacteria, recent research on *P. versicolora* (Li et al., 2025) indicates that egg deposition induces a significant increase in chlorogenic acid (CGA) in host leaves, which is detrimental to neonate survival. *P. putida* has been demonstrated to possess the capability to degrade CGA and contribute to larval tolerance against this induced plant defense (Li et al., 2025). This verified metabolic function provides a plausible explanation for the fitness benefits of maintaining *P. putida* through vertical transmission. We agree that investigating anti-fungal protection via antiSMASH mining or bioassays would be a valuable addition. However, since the primary focus of our study is the mediation of oviposition behavior and the resulting plant-insect-microbe feedback loops, we believe that exploring defensive symbiosis against entomopathogens falls beyond the current scope.

Following your suggestion, we have updated our Discussion (Lines 337-341) to acknowledge these potential multi-functional roles—including both the verified metabolic benefits and the hypothesized defensive roles—to provide a more balanced ecological context.

Revised Discussion:

Additionally, a previous study demonstrated that egg-surface P. putida can degrade chlorogenic acid (CHA)41, suggesting a potential protective role for neonates. How these diverse, stage-specific functions of P. putida—ranging from potentially pathogenic in new leaves to protective on egg surfaces—are spatio-temporally synchronized with the host's life-history stages warrants further investigation. (Lines 337-341).

Reference: Li B, Liu F, He X, et al. Leaf Beetle Symbiotic Bacteria Degrade Chlorogenic Acid of Poplar Induced by Egg Deposition to Enhance Larval Survival. *Plant, Cell & Environment*, 2025,

Comment 5. Positive feedback loop and adaptive interpretation

Although the title and discussion repeatedly invoke a 'positive feedback loop', no evidence is presented for generational reinforcement, increasing *Pseudomonas* prevalence across generations, or population-level amplification. The data support directional interactions, but they do not demonstrate a self-reinforcing feedback loop in an ecological or evolutionary sense.

Similarly, statements such as those in L277–279, which frame mature-leaf oviposition as an adaptive strategy to protect offspring, are speculative. Reduced larval performance on upper leaves alone does not demonstrate adaptive maternal behavior unless oviposition choice is linked to net reproductive success.

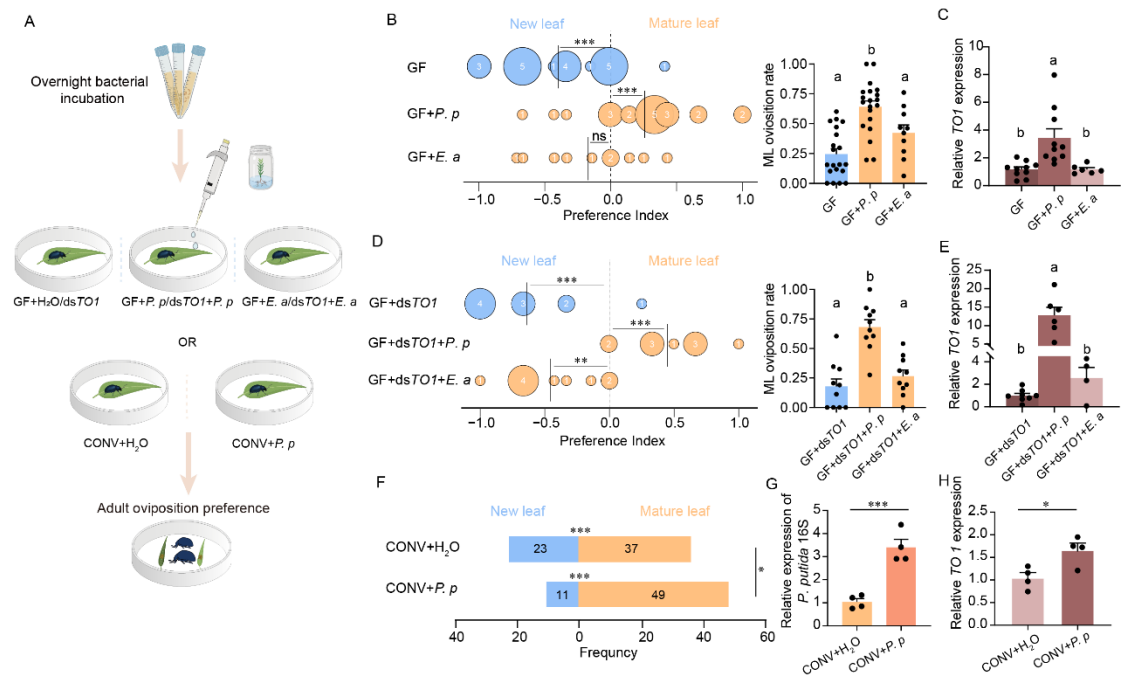
Response: We sincerely thank the reviewer for this critical evaluation of our conceptual framework. We agree that the term "positive feedback loop" in its strict ecological and evolutionary sense requires evidence of population-level amplification and generational reinforcement that exceeds our current datasets. Accordingly, we have toned down our claims and revised the manuscript to characterize these interactions as a "a plant-mediated feedback loop "

To provide more robust support for the stability and adaptive significance of this association, we have added the following data and clarifications:

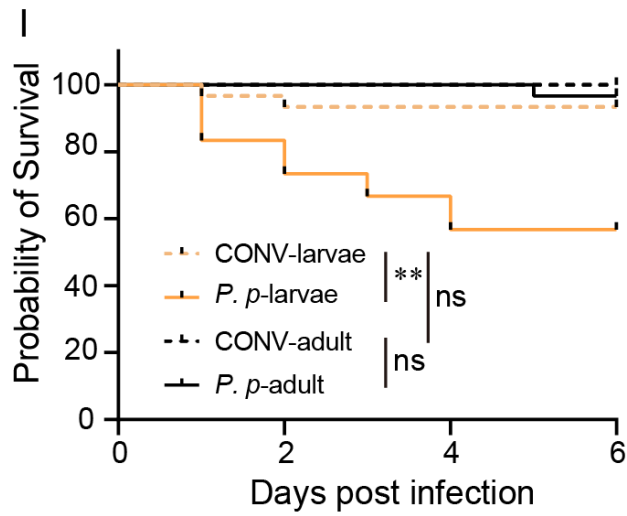
1. **To address the requirement for generational reinforcement**, we quantified eGFP-tagged *P. putida* across the F1 generation (Revised Fig. 3J & 3L). Our results demonstrate the stable transgenerational inheritance of the symbiont from mother to offspring, confirming that the association is not a transient environmental artifact but a persistent biological feature.
2. **To move beyond mere correlation**, we performed an experimental gain-of-function assay (Revised Fig. 6F–H). By artificially supplementing the *P. putida* load in CONV females, we observed a significant enhancement in their preference for mature-leaf oviposition. This causal evidence demonstrates that the microbiota directly modulates the intensity of maternal choice, providing a mechanistic basis for how microbial accumulation reinforces the behavioral phenotype.
3. **To provide a clear proximal fitness basis for this behavior**, we confirmed that while *P. putida* is pathogenic to larvae, the same bacterial load has no discernible impact on adult survival or egg viability (Revised Fig. 4I and Fig. S6). This disparity suggests a plausible adaptive trade-off: by ovipositing on mature leaves, females spatially segregate their susceptible offspring from the bacterial hotspots in new leaves, while still ensuring the vertical transfer of *P. putida* necessary for later life stages (e.g., for degrading plant toxins like chlorogenic acid).

In response to your suggestion, we have replaced "positive feedback loop" with more precise terms such as "a plant-mediated feedback loop" throughout the Title and Discussion.

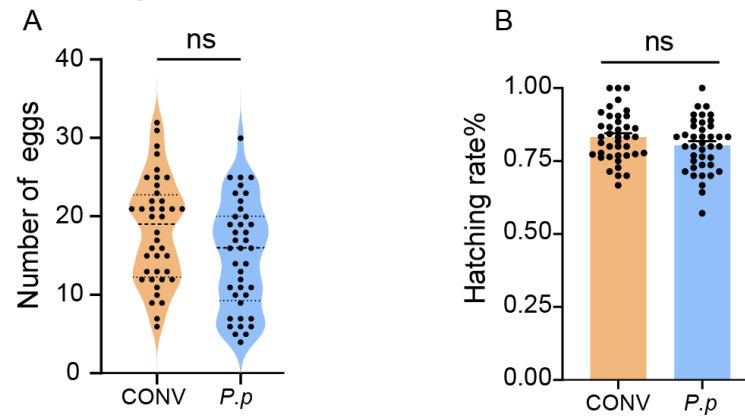
Revised Fig. 6



Revised Fig. 4I



Revised Fig. S6



Comment 6. Use of ‘Dietary Specialization’

The phrase ‘dietary specialization’ is used multiple times throughout the paper in contradictory ways. ‘Dietary specialization’ is invoked in the introduction and discussion (L69, L220) to signal the different tissue feeding habits between adult and larval *P. versicolora* on the same host plant, which is at odds with the usual meaning of the term to describe general host species monophagy in an herbivore species without reference to life stages or plant tissues. However, in the term's first instance (L64), the words following the comma ‘which reduces both inter- and intraspecific competition’ seems to indicate both the usual meaning of ‘dietary specialization’, which reduces interspecific competition, as well as this unique meaning that distinguishes adult and larval habits, which reduces intraspecific competition. I recommend the addition of clarifying language to indicate this dual meaning of ‘dietary specialization’ in this first instance (L64), because all later instances of the phrase in this paper refer exclusively to the unique, adult-versus-larval-feeding-habits meaning.

Response: We appreciate the reviewer’s suggestion to clarify the definition of "dietary specialization." As noted, this term describes a dual ecological strategy: (1) host specificity, which reduces interspecific competition with other herbivores, and (2) ontogenetic niche partitioning, which minimizes intraspecific competition by separating the leaf tissue preferences of adults and larvae. Following your advice, we have revised the introduction (L64) to explicitly define these two dimensions, providing clear context for all subsequent mentions of the term in the manuscript.

Revised manuscript: *To better exploit the limited leaf resources, the beetle has evolved an ecological strategy of dietary specialization. In this context, the term encompasses both host-plant specificity—which reduces interspecific competition—and ontogenetic niche partitioning, characterized by the distinct leaf tissue preferences of adults and larvae, which minimizes intraspecific competition. (Lines 73-77).*

Accordingly, we have also updated the discussion to use the term 'ontogenetic niche partitioning'. This ensures consistency with the dual-dimension definition of dietary specialization provided in the introduction, clarifying that this behavior serves to minimize intraspecific competition between life stages.

Revised manuscript: *In *P. versicolora*, this linkage is reflected in dietary specialization and ontogenetic niche partitioning: adults preferentially feed on new leaves but lay eggs on mature leaves (Lines 270-272).*

Comment 7. Inconsistency in the proposed *TO1*-mediated mechanism

The author proposes that *Pseudomonas putida* modulates oviposition preference via regulation of the takeout gene *TO1*. However, this model is inconsistent since while *TO1* knockdown abolishes the mature-leaf oviposition preference (Fig. 5H), *P. putida* reintroduction nevertheless restores this preference even under *TO1* knockdown (Fig. 6D). This result contradicts the proposed causal role of *TO1* as a required downstream mediator of *P. putida*’s effect on behavior. At minimum, these data suggest that *TO1* is not strictly necessary, or that parallel pathways exist. What was the level of *TO1* expression after the KO? Probably not 100% which means that it could interfere with the experiment’s results. As currently presented, the mechanistic model is therefore not supported by the data and clarification are required to carefully address the limitations of the results for understand the mechanism.

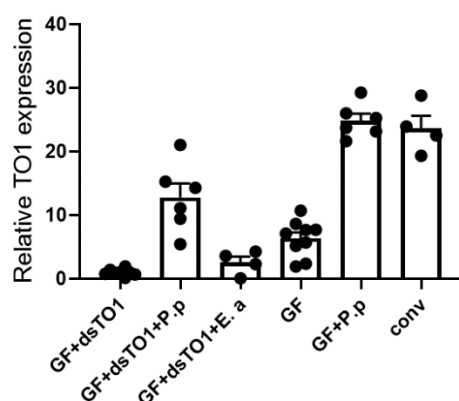
Response: We sincerely thank the reviewer for identifying this inconsistency in our mechanistic

model. The observation that *P. putida* reintroduction can restore mature-leaf preference even when *TO1* expression is significantly suppressed is a critical point that requires a more nuanced interpretation.

1. **To address the concern about the degree of *TO1* suppression**, we have synthesized the representative expression trends across our independent experimental batches for this response (RL Fig. 1). As the reviewer correctly noted, our RNAi approach (*dsTO1*) achieves a robust knockdown but not a 100% knockout. In the GF+*dsTO1*+*P. p* group, *TO1* expression was significantly lower (approx. 50%) than the induced levels in the GF + *P. p* and CONV groups. Crucially, even with this partial transcript suppression, the mature-leaf preference was restored. This suggests that the behavioral output is not strictly linear with *TO1* transcript abundance. **Please note** that these measurements were obtained from independent experimental sets to ensure biological reproducibility. While we present them together here to facilitate a direct comparison of the trends, they remain separated in the manuscript to maintain experimental rigor.
2. The reviewer's comment that "*TO1* is not strictly necessary, or that parallel pathways exist" is highly insightful and aligns with our data. We have moved away from a strictly linear model to a more nuanced "multifaceted regulatory" framework. We explicitly acknowledge that while *TO1* plays a significant role, the presence of parallel pathways means it is not the sole, indispensable determinant. These limitations have been carefully addressed in the revised Discussion.

Revised manuscript: Intriguingly, however, *P. putida* reintroduction remained capable of rescuing the mature-leaf preference even when *TO1* was targeted by RNAi (Figure 6D), suggesting that the behavioral response is robust to transcript reduction and potentially triggered by a low functional threshold of *TO1* expression (Figure 6D). Specifically, the capacity of *P. putida* to significantly elevate *TO1* levels relative to the *dsTO1* control—effectively partially overriding the RNAi-mediated suppression—was sufficient to drive the behavioral transition (Figure 6E). Such partial decoupling between the magnitude of *TO1* expression and the behavioral output suggests that *TO1* may operate within a threshold-dependent framework. The ability of *P. putida* to override *TO1* knockdown further points to the existence of parallel regulatory pathways—potentially involving other bacterial metabolites or neuromodulatory genes—that can compensate for reduced *TO1* availability. (Lines 377-381).

RL Fig. 1



Miscellaneous

Comment 8. L277-279 ‘This suggests that the adult’s preference for laying eggs on mature leaves, away from the *P. putida*-enriched new leaves, serves as a strategy to avoid the negative impact of

the bacterium on their offspring.' This is purely speculative.

Response: Thank you for your reminder. In the revised manuscript, we have added data demonstrating a significant increase in larval mortality when exposed to supplemental *P. putida* (Revised Fig. 4I). In contrast, the same bacterial load has no discernible impact on adult survival, fecundity, or egg hatchability (Revised Fig. 4I, S6). This ontogenetic shift in susceptibility provides a proximal fitness rationale for maternal avoidance of bacterial hotspots. To avoid over-interpretation, we have removed the definitive "strategy" phrasing and reframed the conclusion as a mechanistically-grounded hypothesis as follows.

Revised manuscript:

*Overall, this tripartite interaction between *P. versicolora*, *P. putida*, and the willow host suggests a potential trade-off in which maternal behavior appears to balance the requirements of gut bacteria transmission with the necessity of offspring survival. (Lines 342-344).*

Comment 9. L280 'While *P. putida* negatively affects larval fitness, we observed no significant impact on adult survival or clutch size of eggs.' None of the experiments in this work test and demonstrate this hypothesis. The negative effect could come from the chemical defense induced by IAA production for example. How would the *P. putida* have a negative effect on larvae but not on adult?

Response: We appreciate the reviewer's concern regarding the evidence for stage-specific impacts of *P. putida*. To address the concern, we conducted additional independent bioassays where equal concentrations of *P. putida* were directly applied to the diet, thereby decoupling the bacterial effect from plant-mediated defenses. Results showed that *P. putida* significantly increases larval mortality but has no statistically significant impact on adult survival, clutch size (fecundity), or egg hatching rates (Revised Fig. 4I, S6). The disparity in susceptibility likely stems from an ontogenetic shift in immune competence or gut physiology. Studies in *Tribolium castaneum* have also shown that immune responses differ markedly between larval and adult stages, suggesting that these stages may exhibit distinct levels of tolerance to the same bacterial challenge (Khan et al., 2016; Tate et al., 2021). Accordingly, we have incorporated these new data and the corresponding analysis into the revised manuscript.

Reference :

Khan I, Prakash A, Agashe D. Divergent immune priming responses across flour beetle life stages and populations. *Ecology and Evolution*, 2016, 6(21): 7847-7855.

Tate A T, Perry A, Jent D. Larvae and adults exhibit contrasting patterns of immune gene expression and infection resistance in wild flour beetle populations. *Ecological Entomology*, 2021, 46(5): 1230-1235.

Comment 10. Minor Comments: Introduction: **L64: L70-73:** Omit final sentence. Repetition from the end of the previous paragraph (L59-61)

Response: Thank you for your reminder. As suggested, we have removed the repetitive final sentence (originally L70-73).

Comment 11. L84: Replace "P putida" with "one *Pseudomonas* species, *P. putida*,". Since both *Plagioderia* and *Pseudomonas* abbreviate to "P", further clarification would better signal to readers that *P. putida* is a microbial symbiont, rather than a related beetle species.

Response: Thank you for your reminder. The sentence has been revised as follows: *we revealed that one Pseudomonas species, P. putida, influences this preference by modulating the expression of the takeout gene in P. versicolora* (Line 94).

Comment 12. Results: L139-141: Edit to clarify that the "significant increase in IAA concentration" was specifically observed in the upper leaves.

Response: Thank you for your suggestion. As mentioned above, in addition to IAA, we have now included measurements of JA and CGA, and incorporated spatiotemporal dynamics into the analysis to improve the overall coherence of the study. In this context, we focused specifically on the upper leaves. Accordingly, in the revised manuscript, we have clearly specified that both the quantified metabolites and the *Pseudomonas* abundance were measured in upper leaves (see revised manuscript).

Comment 13. Discussion: L250-251: Elaboration needed. In what sense is leveraging the plant environment more effective at transmitting *Pseudomonas* relative to leaf surface acquisition? This notion seems to depend on an unwritten premise that *P. versicolora* adults prefer compensatory growth-produced leaves relative to adult beetle-damaged leaves, which is not suggested by the data, and different from a preference for new over mature leaves indicated in other sections. It's perfectly reasonable to hypothesize such a preference for compensatory growth leaves, but it should be stated explicitly here.

Response: We appreciate the reviewer's insightful critique. We agree that our current data do not directly compare adult preference between compensatory growth-produced leaves and other damaged leaves. To ensure scientific rigor, we have revised this section as follows:

Revised manuscript: *Theoretically, leveraging the plant environment to enrich and transmit these bacteria may represent a more effective strategy than simple passive acquisition from leaf surfaces, particularly if beetles preferentially feed on plant tissues (e.g., compensatory growth leaves) where these bacteria are enriched* (Lines 297-300).

References: Nakamura M, Utsumi S, Miki T, et al. Flood initiates bottom-up cascades in a tri-trophic system: host plant regrowth increases densities of a leaf beetle and its predators. *Journal of Animal Ecology*, 2005, 74(4): 683-691.

Comment 14. L279: Soften language. The data presented does not demonstrate that *P. putida* specifically reduces the fitness of larvae fed upper leaves from CONV-damaged plants, as opposed to any other potential chemical or microbial factors that distinguish those leaves from those of the other treatments.

Response: We agree with the reviewer that the reduced larval fitness observed on upper leaves likely involves a complex interplay of multiple factors, including plant defensive chemicals and shifts in the broader microbial community. As noted in **Response to Comment 9**, we have added new experiments demonstrating that *P. putida* exhibits differential virulence toward larvae and adults. To avoid over-interpretation, we have removed the definitive "strategy" phrasing and reframed the conclusion as a mechanistically-grounded hypothesis (Lines 327-337).

Revised Manuscript:

This observed fitness cost is consistent with the hypothesis that the female's preference for ovipositing on mature leaves, effectively distancing neonates from P. putida-enriched tissues, may

*serve as an adaptive life-history interpretation to mitigate the detrimental effects of the bacterium on the F1 generation while still ensuring necessary maternal colonization. Furthermore, the observation that *P. putida* increases larval mortality without affecting adult survival, fecundity, or egg viability (Figure 4I, S6) reveals a pronounced ontogenetic shift in immune competence or physiological tolerance. This stage-dependent susceptibility suggests that adults may function as stable, resilient vectors for the bacterium, whereas larvae remain vulnerable. Whether this shift is driven by a more robust adult immune system or distinct physiochemical constraints within the adult gut—which may suppress the bacterium's pathogenic potential—remains to be elucidated (Lines 327-337).*

Comment 14. Materials and Methods: L342: Replace "*S. babylonica*" with "*Salix babylonica*".

Response: Done

Comment 15. Figures and Tables Figure 1C, L740: Since the pie charts contain the same information as the bar chart, the pie charts should be omitted for simplicity.

Response: Thank you for your reminder. The pie charts have been removed.

Comment 16. Figure 3, L781-784: Mislabelled caption. Should be "(C) Relative abundance of *P. putida* ..." and "(D, E) Chemotactic response of *P. putida* (D) and *E. aerogenes* (E)..." or the labels in the figure should be switched.

Response: Thank you for your reminder. We have corrected the caption for Figure 3.

Comment 17. Figure 4D, E L802-803: Clarify that the different shades in the bar graphs denote the larval instars

Response: In accordance with the reviewer's suggestion, we have clarified the legend for Figure 4D and E. We now explicitly state that the progressively darker shades in the bar graphs denote the developmental progression of larval instars (from the 1st to the 3rd instar), ensuring a clearer interpretation of the temporal growth data.

Revised Figure legend: *The color gradient from light to dark within each bar represents the developmental duration of the 1st, 2nd, and 3rd larval instars, respectively.*

Reviewer #3 (Remarks to the Author):

General Comment: The manuscript by Li et al combines field studies of the leaf beetle *Plagioderma versicolora* with oviposition bioassays and the molecular characterization (RNAi knock-down) of a candidate gene. This is an interesting manuscript and the suggested link between specific gut bacteria and behavioral adaptation sounds fascinating. One can appreciate many aspects of this work, and the general system of the beetle herbivore-gut microbiome association can be considered to be of potential broad interest. The overall impression of this manuscript is that it has lots of experiments and data analysis and is mostly written well. However, the interpretation of the data goes beyond what is justified, with quite some level of speculation based on rather scarce or unclear evidence. Unfortunately, it is therefore not a major advance in research on interactions between gut microbial partners and symbiont-driven behavioral adaptation (i.e. oviposition). More specifically, the data presented is not sufficient to support the proposed role of the takeout gene *TO1* as a link between *Pseudomonas putida* and the suggested manipulation/induction of *P. versicolora* female oviposition preference.

In general, one does get the impression that the authors seem to approach the whole system in a somewhat biased way. **(1)** For example, they seem to neglect several issues related to the methods and approaches used, e.g. the problems associated with disrupting the normal gut microbiota-host insect interface and the possible subsequent alterations in response to pathogen encounters. **(2)** In addition, they overlook the link between the potential pathogenicity of *P. putida* in the larvae and the adult immune response observed in the transcriptomic (RNAseq) data. Originally, avoidance of intraspecific competition was suggested as the main argument for female oviposition preference for old leaves. This now clashes with the new argument of female avoidance of elevated *P. putida* levels found in new leaves. Several of the main questions remaining are: where does *P. putida* come from, who transfers what amounts to the plant and who picks up *P. putida* from the ingested plant material again? This is important to know, since *P. putida* is an environmental bacterium, frequently found in the rhizosphere as well as on/in plants, but obviously also in adult (and larval?) *Plagioderma versicolora* beetles. If eggs are laid on old leaves, eggs and newly hatched larvae will potentially be exposed to lower (?) *P. putida* levels before later larval instars will eventually have to move to newer leaves. Since adult beetles seem to even prefer young (upper) leaves, one could ask why those adults seem to not be negatively impacted by the elevated levels of the opportunistic entomopathogen *P. putida*. **(3)** Instead of proposing a direct link between the differences in relative abundance of *P. putida* in mature and young leaves and larval performance, one could also argue for other differences between the leaves (i.e. unrelated to *P. putida* loads), including plant defense metabolites. Differential induction of plant metabolites as a result of tissue damage exerted by beetles with and without gut microbiomes could be the reason for the variable performance observed in subsequent larval feeding assays. In general, it needs to be noted that young and mature leaves can respond very differently to the same wounding/feeding/microbe stimulus.

In general, after looking at the many different experiments performed by the authors in more detail, the reader is left with more questions in mind, instead of the conclusion of the authors that they show "...gut bacteria ... ultimately sustaining a symbiont-driven behavioral adaptation".

Response: We sincerely appreciate your recognition of the fascinating link between gut microbiota and host behavior, as well as your constructive comments. We have systematically addressed your general concerns, which we have categorized into three key areas for clarity. Detailed, point-by-point responses to the remaining comments are provided below, following the response to the

general comment.

(1) On Methodological Rigor and the Microbiota-Host Interface.

We appreciate the reviewer's concern regarding the potential physiological artifacts of disrupting the gut microbiota. To address this, we have included additional data on feeding preference and feeding area. Both CONV and ABX females preferentially fed on new leaves (RL Fig. 1), and no significant difference in feeding area was observed between the two groups (Fig. S1E), indicating no detectable effect on feeding behavior. In the revised Materials and Methods, we emphasize that gut microbiota depletion (ABX and GF groups) specifically shifts the oviposition preference index without compromising the oviposition motivation (total egg count, clutch size, and more importantly ~100% of the females in the ABX and GF groups successfully deposited eggs). This distinction indicates that the behavioral change is a deliberate recalibration of site selection rather than a secondary symptom of impaired nutrient sensing or reduced health.

To move beyond the limitations of GF/ABX models, we introduced a dietary supplementation of *P. putida* experiment in CONV females (Revised Fig. 6F-H). By increasing the *P. putida* load within the native gut environment, we reinforced the preference of oviposition for mature leaves. This indicates that bacterial abundance itself can modulate this behavior.

Contrary to the hypothesis that preference is a stress response to pathogen encounters, our transcriptome data revealed that the majority of immune-related DEGs were actually upregulated in GF females (which lost the preference, Table S2). Furthermore, RNAi knockdown of an immune gene *defensin* failed to alter oviposition preference (Fig. S7), suggesting that the behavior is not a byproduct of immune activation.

(2) On Ecological Synergy and Transmission Dynamics.

We appreciate the reviewer's thoughtful critique of our ecological model. The avoidance of intraspecific competition and the avoidance of opportunistic pathogens (*P. putida*) are not mutually exclusive; rather, they represent synergistic selection pressures that reinforce the observed oviposition preference. In the revised manuscript, our eGFP-tagged *P. putida* assays (Revised Fig. 3I-L, Fig. S5) demonstrate that a gut-colonized strain translocates to upper leaves during feeding and is subsequently acquired by other females, who then transmit the bacteria to the next generation via egg-surface contamination. Notably, eGFP copy numbers progressively increased from the 1st to the 3rd larval instars, indicating active microbial proliferation within the host and suggesting an ontogenetic increase in microbial tolerance or immune competence as the larvae mature. This hypothesis is further supported by our infection assays: while exposure to *P. putida* at 10^4 CFU/cm² leads to significant mortality in neonates (1st instars), the same bacterial load has no discernible impact on adult survival or fecundity (Revised Fig. 4I, Fig. S6). This disparity in life-stage susceptibility explains why adults can exploit enriched new leaves as high-quality feeding sites without incurring fitness costs, while selectively shielding their more vulnerable offspring through precise oviposition site selection. Furthermore, new data (Revised Fig. 3B-F) comparing *P. putida* levels across various treatments clarify the beetle's role in the environment. While ABX-treated (microbiota-free) beetle damage can trigger a transient mobilization of indigenous endophytic *Pseudomonas*, this population returned to baseline by 48h. In contrast, feeding by CONV and CONV+*P. putida* beetles resulted in significant and sustained colonization at 48h. This divergence confirms that while *P. putida* exists in the environment, the beetle serves as the essential biological reservoir for establishing the high-density symbiont pulses required for behavioral modulation.

(3) Distinguishing Bacterial Effects from Plant Defensive Metabolites.

We agree with the reviewer that plant-derived chemical changes following herbivory could potentially confound the results. To decouple the direct impact of *P. putida* from potential confounding effects of plant defensive chemistry, we firstly quantified *P. putida* levels in both leaf tissues and the larvae that fed upon them (Revised Fig. 4F-G), showing a direct correlation between larval acquisition and stunted growth. To further isolate the microbial effect, our supplemental experiments show that *P. putida* alone is sufficient to impair larval fitness, confirming the bacterium as a direct selective agent. In contrast, the same concentration of *P. putida* had no impact on adult survival or fecundity (Revised Fig. 4I)

In response to the specific comments, we have also incorporated several new experiments and revised the manuscript accordingly, as detailed in the point-to-point response below.

Below several of the abovementioned and other issues are listed in more detail.

Comment 1. Gut microbiota and oviposition preference:

The authors have shown that germ-free females shifted their oviposition preference towards new leaves, whereas the re-introduction of gut microbiota restored their preference for mature leaves. This could have multiple reasons, e.g. it could be the result of a shift in gut nutrient sensing, changing the female oviposition choice to more nutrient-rich plant material. Alternatively, depleting the gut microbial community could also result in the avoidance of mature leaves with potentially altered microbial (not only *Pseudomonas*) loads. It is noteworthy that the oviposition rate on mature leaves dropped considerably in germ-free females, to about one third of the rate observed in both conventional and females where bacteria were re-introduced (Figure 1E, right panel). However, information on overall oviposition differences (i.e. how many of the germ-free females actually laid any eggs) is missing.

Response: We sincerely thank the reviewer for this critical observation. We have addressed these concerns by clarifying our metrics and providing additional physiological data.

- 1. Clarification of "ML Oviposition Rate":** We would first like to clarify that the "ML oviposition rate" in Fig. 1E (right panel) represents the proportion of eggs deposited on mature leaves; the remaining eggs were deposited on new leaves. We apologize if this presentation caused any confusion regarding the total fecundity. We have added a detailed description in the revised M&M. In our study, gut microbiota depletion (both GF and ABX) did not impair the fundamental capacity or motivation to oviposit. Nearly 100% of the surviving females in all treatment groups (CONV, GF, and ABX) successfully deposited eggs within the 24-hour assay period. We clarified this in the revised M&M section as follows.
- 2. Decoupling Gut Nutrient Sensing from Oviposition Choice:** To address the concern regarding a "shift in gut nutrient sensing," we quantified the feeding preferences of CONV and ABX females. Our results show that both groups exhibited a robust and significant preference for feeding on new leaves (RL Fig. 1). Although feeding and oviposition are distinct behaviors, this consistent feeding preference across all groups indicates that the fundamental nutrient-sensing apparatus remains intact regardless of microbiota status. Furthermore, to address the concern that ABX treatment might make insects "unwell," we quantified the total feeding area as a proxy for physiological vigor. We found no significant difference in feeding intensity between CONV and ABX beetles (Revised Fig. S1E)

Revised manuscript:

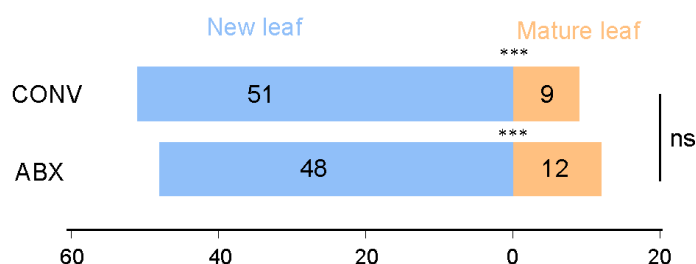
The mature leaf (ML) oviposition rate was determined by dividing the number of eggs deposited on

mature leaves by the total egg count, calculated according to the formula: $ML \text{ oviposition rate} = (\text{number of eggs on mature leaves}) / (\text{number of eggs on new leaves} + \text{number of eggs on mature leaves})$. (Lines 464-467)

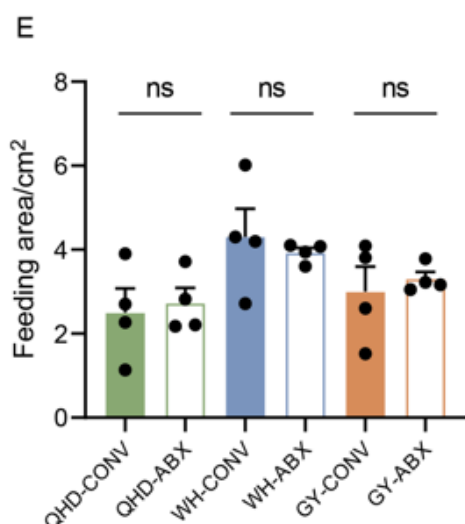
Throughout the experiments, nearly 100% of the 35 pairs per group successfully laid eggs, from which 30 samples were randomly selected for statistical analysis ($n=30$). (Lines 442-444).

Notably, as nearly 100% of both GF and CONV females successfully initiated oviposition within the 24-hour period, the oviposition rate was not quantified as a separate variable. (Lines 459-461).

RL Fig. 1 Feeding preference for new versus mature leaves in CONV and ABX adults.



Revised Fig. S1E



Comment 2. *Pseudomonas* abundance in mature leaves:

There seems to be decent evidence supporting a higher abundance of *Pseudomonas*-like gut microbes in beetles feeding on mature leaves. However, when having a closer look at Figure 2G, the absolute abundance of *Pseudomonas* does not seem to differ much between beetles feeding on young or mature leaves. Furthermore, the significant results in Figure 2G (top panel) seem to be driven by two samples showing very high relative abundance levels of *Pseudomonas*. Considering the above, evidence that *Pseudomonas* is really highly correlated with “mature-leaf oviposition preference” of the females is not really convincing. It would have been important to also perform 16S rRNA sequencing and bacterial community assessments of the mature and young leaves – without beetle feeding. Multiple dietary factors can alter the gut microbial composition of herbivorous insects, including plant defense metabolites and leaf nutritional content differences. In this specific context, one of the major questions is whether there is a shift in relative abundances of permanent or transient (i.e. obtained from the diet) bacteria in the beetles’ gut.

Furthermore, what remains to be shown is the identity of the *Pseudomonas* species grouped as

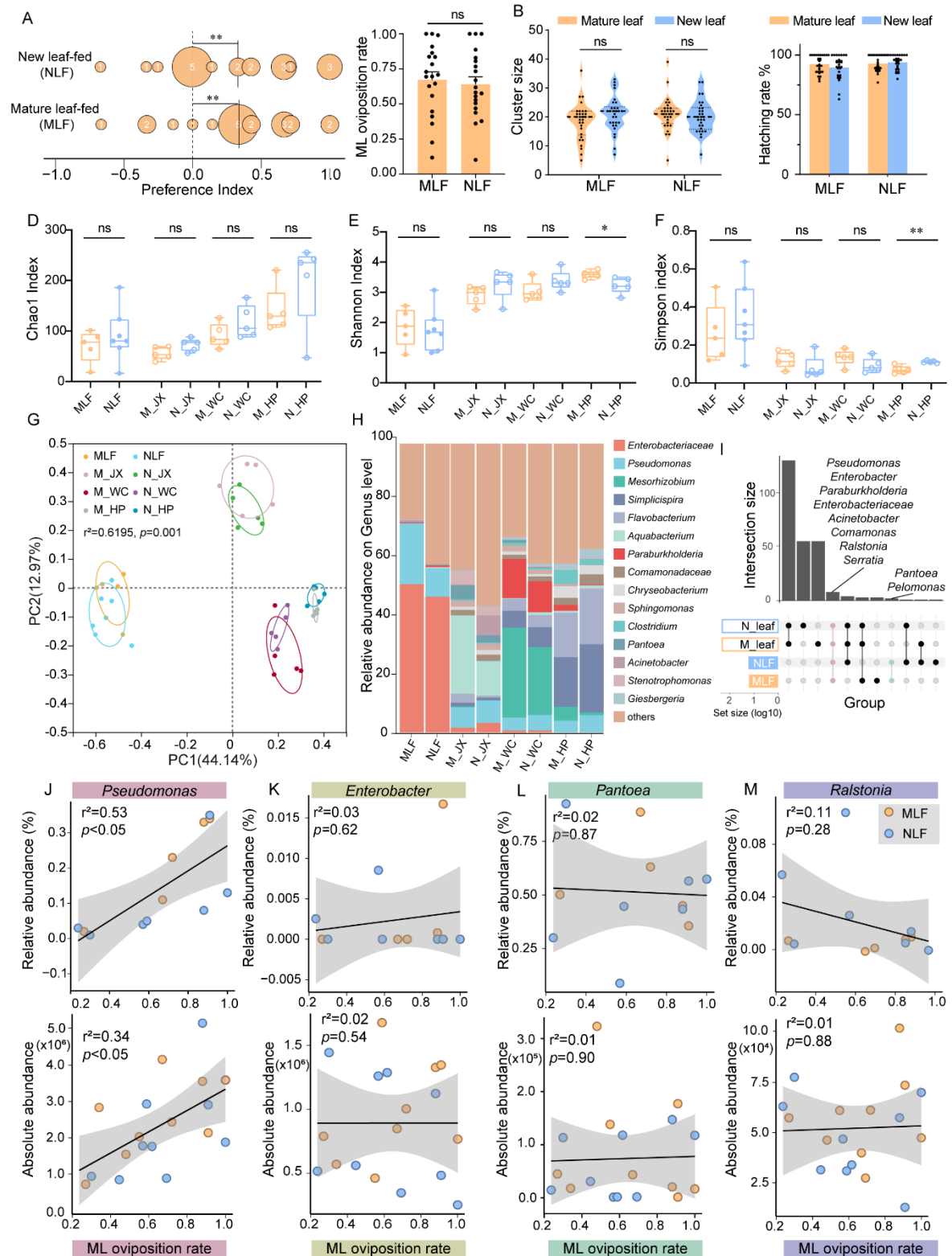
Pseudomonas in all the microbiome analysis. It is unclear why the authors subsequently only focus on *Pseudomonas putida* for all of the other assays. Important questions remain unanswered:

- What is the evidence supporting the notion that only a single *Pseudomonas* species is found among the gut bacteria?
- What is the prevalence of *P. putida* in the leaves of host plants and/or beetles from the field?
- Have *P. putida* levels increased in larvae fed on the new leaves damaged by conventional beetles?
- How much variation in *Pseudomonas* levels can be found among individual females and how would this translate to differences in oviposition preferences?

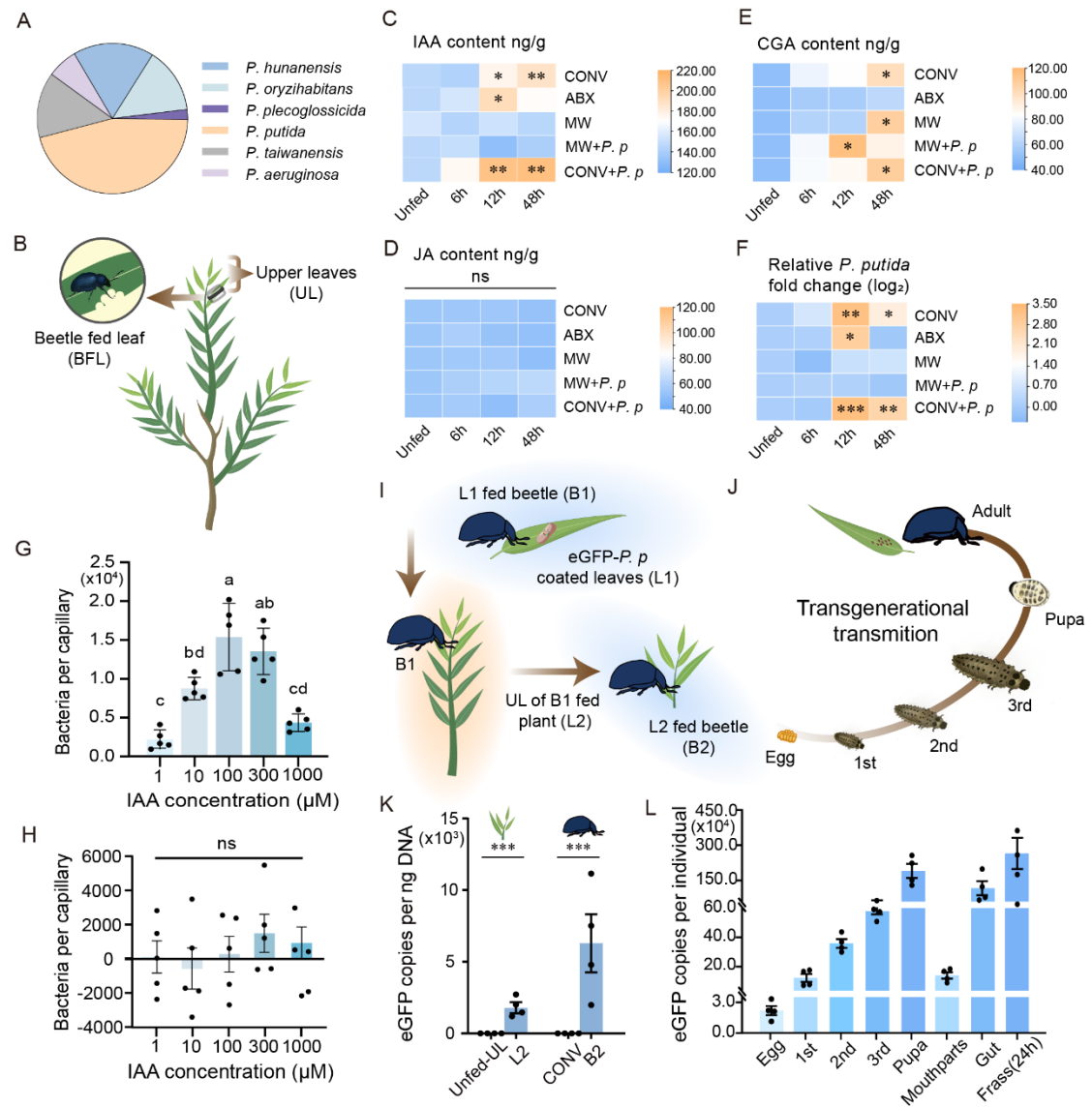
Response: We thank the reviewer for their critical eye regarding the potential outliers in Fig. 2G. We have addressed these concerns with both re-analysis and extensive new datasets:

- 1. Statistical robustness of the *Pseudomonas* correlation:** To address the influence of potential outliers in Fig. 2G, we re-analyzed the data. The correlation remains significant even after removing the two influential data points (revised Fig. 2J). To move beyond correlation and mono-association in germ-free (GF) females, we added a new selective enrichment experiment (revised Fig. 6F-H). By feeding CONV females *P. putida*-coated leaves (at a concentration that does not impair adult performance, Revised Fig. 4I), we selectively increased the titer of this specific bacterium within an intact, conventional microbiota. This resulted in a reinforced mature-leaf preference, directly linking *P. putida* abundance to behavioral changes (revised Fig. 6F-H).
- 2. Gut microbiota independence and environmental prevalence:** Following your suggestion, we sampled healthy (undamaged) new and mature willow leaves across three sites in Wuhan for 16S rRNA sequencing. The results showed that while the microbiomes of healthy leaves from different locations clustered separately, they were all significantly distinct from the gut microbiomes of the beetles (Revised Fig. 2D-I). This indicates that the gut community is not a mere reflection of dietary intake. Additionally, we used qPCR to assess the environmental prevalence of *P. putida* across Qinhuangdao, Guiyang, and Wuhan (revised Fig. S3). While qPCR confirmed *P. putida* is a common environmental resident, our eGFP-tracking demonstrates that it can be transmitted transgenerationally via egg-surface contamination (revised Fig. 3L).
- 3. Justification for focusing on *P. putida*:** We appreciate that the reviewer correctly asked about the diversity of *Pseudomonas* species. To justify our focus on *P. putida*, we isolated 92 strains from female guts using *Pseudomonas*-selective media (Wang et al., 2024, *Microbiome*). These isolates belonged to six species, with *P. putida* accounting for 45.7% (42 strains) (Revised Fig. 3A). Coupled with our eGFP-tracking data across different life stages, this confirms *P. putida* as the dominant and representative strain in the adult gut (Revised Fig. 3L).
- 4. Larval gut loading of *P. putida* and fitness:** We have now quantified *P. putida* levels across treatments (unfed control, ABX-fed, and CONV-fed plants) in both leaves and larval guts following feeding. Our results show that larvae feeding on upper leaves (UL) from CONV-fed plants exhibit significantly higher gut *P. putida* levels compared to unfed and ABX-fed groups (Revised Fig. 4F-G), revealing an association between elevated bacterial load and impaired larval development. In addition, we conducted *in vitro* feeding assays with *P. putida*, demonstrating its ability to infect larvae (Revised Fig. 4I).

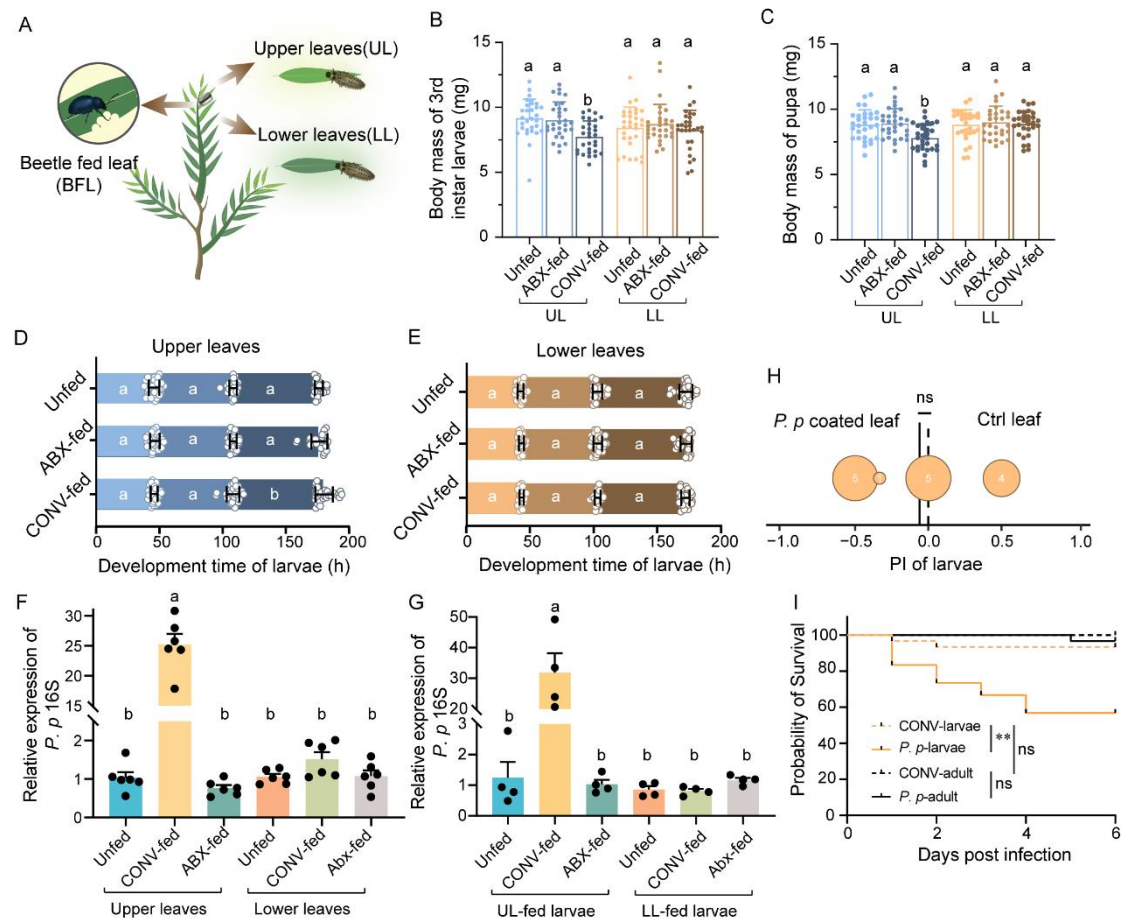
Revised Fig. 2



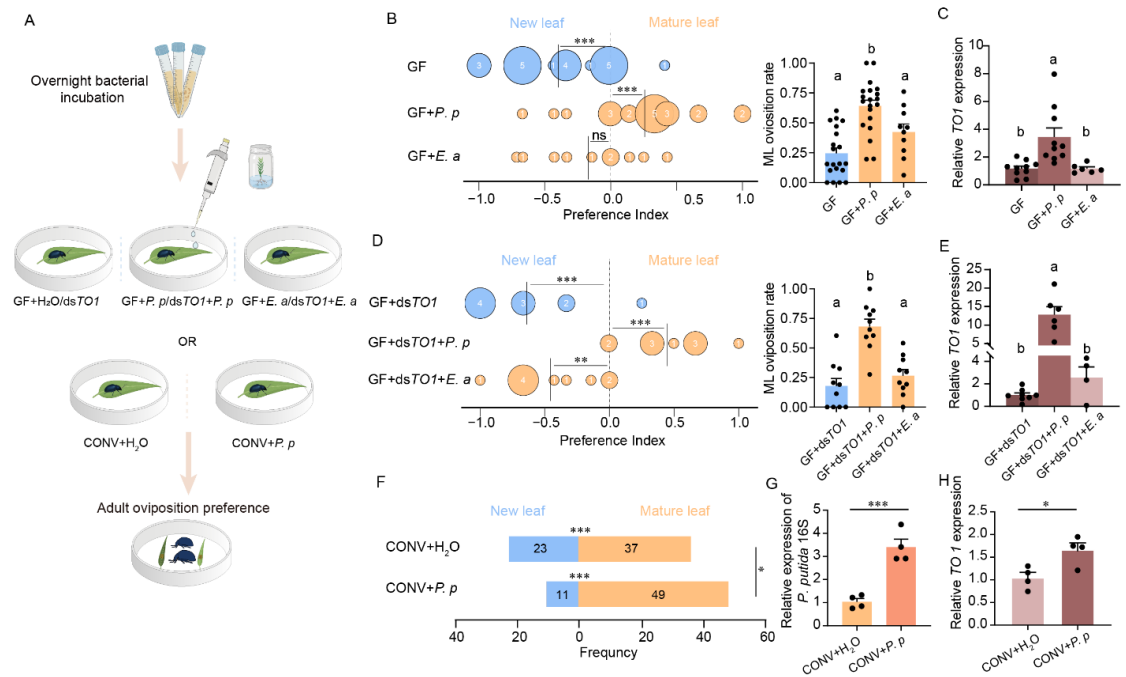
Revised Fig. 3



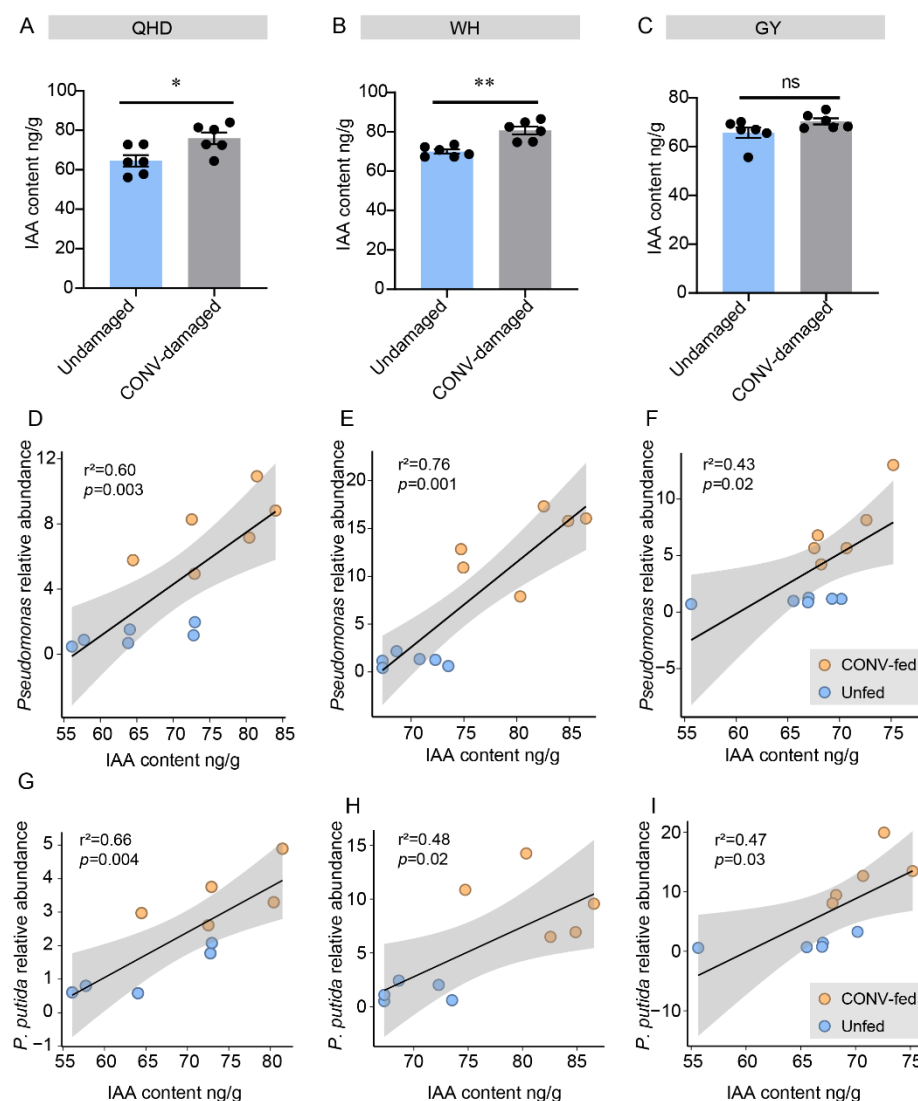
Revised Fig. 4



Revised Fig. 6



Revised Fig. S3



Comment 3. Chemotactic response of *P. putida*:

Whether *P. putida* is actively migrating towards younger leaves or whether bacterial cells are transported by the plant remains unclear. Although the authors state that “These results (Figure 3D) demonstrated that *P. putida* exhibited positive, concentration-dependent chemotaxis towards IAA ..”, the supposed “migration upwards from the feeding site to new leaves by sensing IAA ..” could also be due to other compounds (i.e. defense metabolites) produced by the plant during beetle feeding. In other words, whether IAA itself mobilizes *P. putida* to migrate away from the feeding site is not well supported, even if demonstrated with IAA in isolation. Leaf damage by e.g. feeding herbivores induces a multitude of plant responses, including defense responses mainly orchestrated by jasmonic acid and its derivatives. Plant wounding and additional herbivore-derived factors usually result in local responses, but also systemic spreading of the response to other parts of the plant (e.g. other leaves). Considering the multitude of changes in damaged versus undamaged leaves, it remains unclear why the authors specifically focus on IAA only.

Response: We sincerely thank the reviewer for this insightful concern. Our initial focus on IAA was primarily based on established literature identifying chemotaxis toward IAA as a typical trait of *Pseudomonas* (Rico-Jimenez et al., 2022). To clarify the role of IAA and exclude passive transport

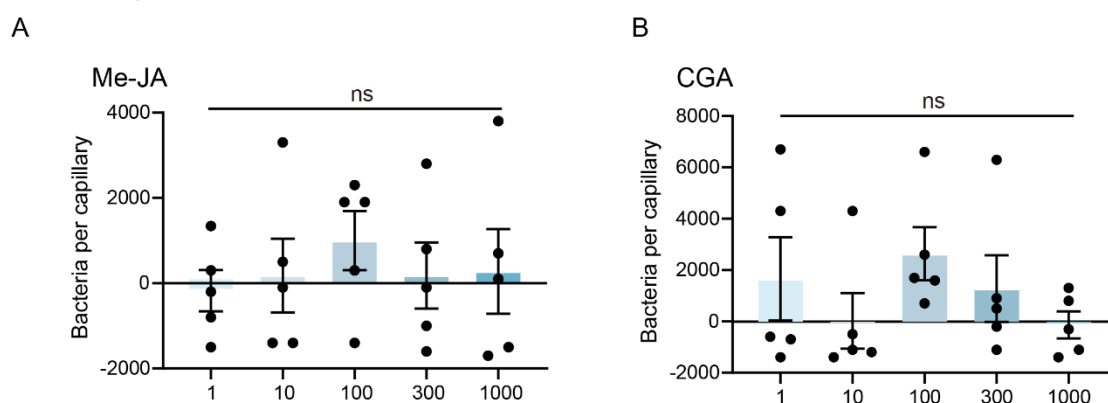
or interference from defense metabolites, we performed a spatiotemporal analysis (6h, 12h, 48h) of IAA, JA, and Chlorogenic Acid (CGA, a primary defensive chemical in willow upon *P. versicolora* feeding, Zhu et al., 2026) in upper leaves located above beetle-fed leaves (ABX, CONV, and CONV + *P. putida*) or mechanical wounding (MW, MW + *P. putida*) (Revised Fig. 3).

1. Excluding Defense Metabolites: While herbivory induces JA and CGA, their spatiotemporal patterns showed no correlation with the arrival of *P. putida* in upper leaves. In contrast, bacterial concentration exhibited a distinct correlation exclusively with the IAA gradient (Revised Fig. 3C-F). Furthermore, *in vitro* assays confirmed that *P. putida* exhibits no chemotactic response to JA or CGA (Revised Fig. S4).

2. Herbivory vs. Mechanical Wounding: We found that mechanical wounding (even with topical *P. putida* application) failed to trigger either IAA accumulation or bacterial enrichment in upper leaves, despite inducing CGA changes. Conversely, both CONV and ABX herbivory triggered an IAA rise followed by *P. putida* enrichment (potentially from residual or endophytic populations in ABX group). These results indicate that specific herbivory-induced IAA signaling, rather than generic wounding or passive plant transport, is the primary driver of bacterial migration.

3. Interpretation revision: We have revised the Discussion to acknowledge that while our evidence identifies IAA as a key driver, the potential influence of other feeding-induced phytochemicals cannot be entirely excluded (Lines 307-308).

Revised Fig. S4



Revised Manuscript:

...the potential influence of other feeding-induced phytochemicals cannot be entirely excluded... (Lines 307-308)

Reference:

Zhu P, Zhang Y, Zhang Y, et al. Endophytic entomopathogenic fungi promote growth and prime phenylpropanoid defenses for enhanced insect resistance in willows. *Industrial Crops and Products*, 2026, 240: 122631.

Rico-Jiménez M, Roca A, Krell T, et al. A bacterial chemoreceptor that mediates chemotaxis to two different plant hormones. *Environmental Microbiology*, 2022, 24(8): 3580-3597.

Comment 4. RNAseq analysis:

In the RNAseq analysis, 63 differentially expressed genes were identified, predominantly associated with structural proteins, but also immune response factors. However, the authors solely focus on two genes supposedly related to neural signaling, the takeout homologs TO1 and TO2.

Among the differentially expressed genes, there are several genes potentially related to immune signaling, pattern recognition and/or regulation, i.e. a lectin, an amidase and a proteinase inhibitor.

Furthermore and even more importantly, three different attacins, a defensin and a coleopteracin are differentially expressed, all of which are immune effectors (or more precisely antimicrobial peptides) utilized by insects to counter pathogen attacks and to sometimes control the uncontrolled spread of mutualists.

Given that the two takeout genes identified in the RNAseq analysis are only moderately differentially expressed, one does wonder why exactly those two genes have been selected for further analysis. In general, the Supplemental Material does not provide any information on fold-change levels and the direction of change (increased or decreased transcript levels) for the genes listed in Table S3.

Pseudomonas is known to have insecticidal toxins similar to nematode-vectorized insect pathogens. Considering the role of *P. putida* as an opportunistic pathogen (which was e.g. shown in another beetle species, *Leptinotarsa*) one does wonder about the differential expression of such a high number of AMPs. Are these AMPs more abundant in the presence of *P. putida* specifically - or are AMP expression levels linked to the presence/absence of a complex gut microbiome in general?

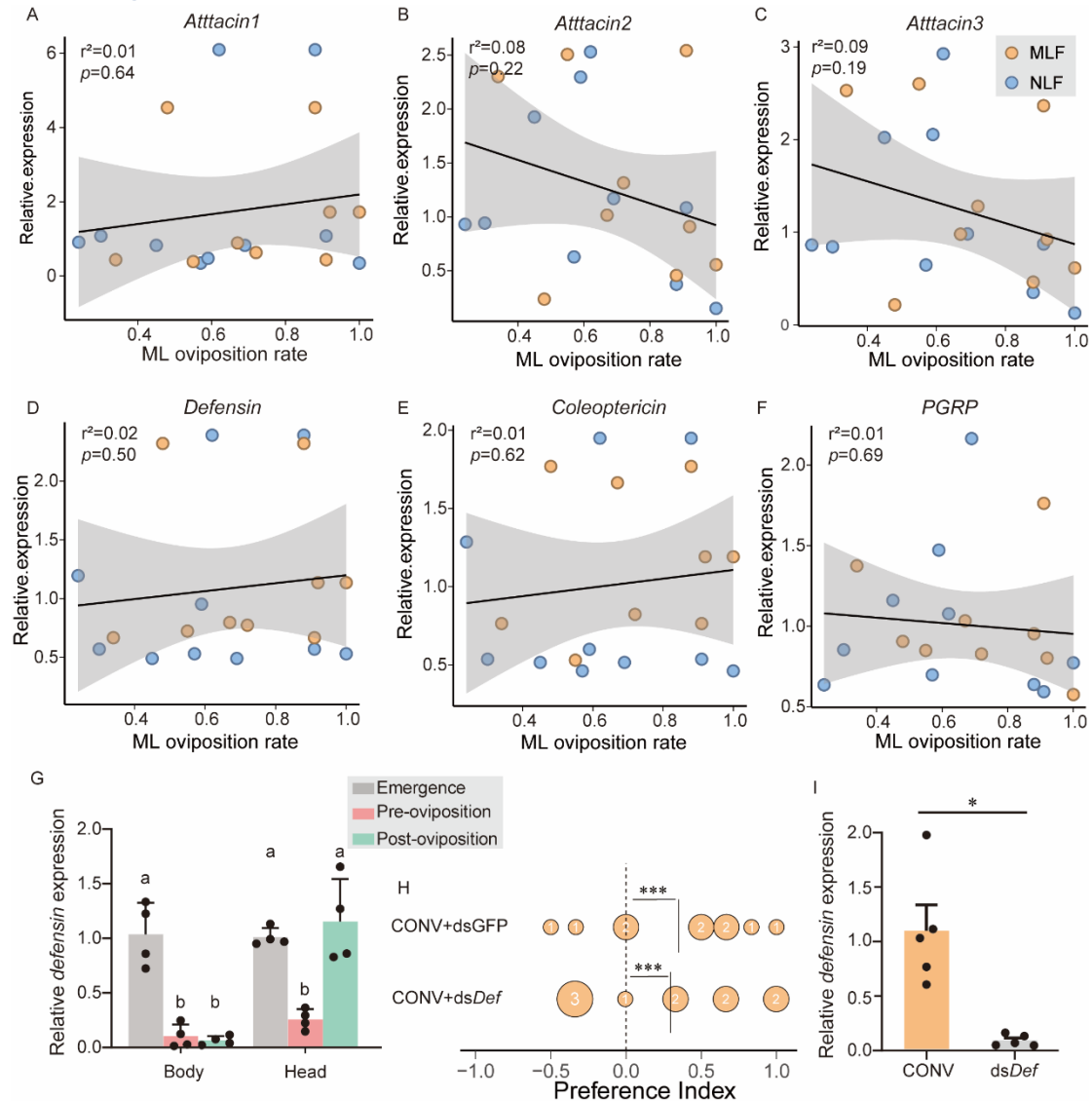
Response: We appreciate the reviewer's detailed scrutiny of the RNA-seq data and the potential role of immune signaling. This feedback allowed us to more clearly articulate our selection criteria and empirically address the potential contribution of immune signaling to the observed behavior.

1. Data Transparency and Table S2 Update: As requested, we have updated and included TPM values for all 61 DEGs.

2. Empirical Validation via Immune Gene Knockdown: While the reviewer correctly identifies several antimicrobial peptides (AMPs)—including *attacins*, a *defensin*, and a *coleopteracin*—as DEGs, our data reveal a directional conflict with the behavioral phenotype. Most immune-related DEGs (AMPs) are aberrantly upregulated in GF females compared to CONV controls, likely reflecting immune dysregulation in the absence of a normal microbiome, as reported in other systems (Belkaid et al., 2014; Wu and Wu, 2012). If elevated immune signaling were the primary driver of the mature-leaf preference, the GF group (which exhibits higher AMP levels) should logically display a reinforced or at least maintained preference. Instead, GF females lose this preference entirely. This inverse relationship suggests that the host's baseline immune activation is not the causal mediator of the behavioral shift toward mature leaves. To further test this, we performed a targeted RNAi knockdown of the *defensin* gene, which was the most highly expressed immune effector in the GF group. As shown in Revised Fig. S7H-I, suppressing *defensin* in the beetles did not alter their oviposition preference. This result allows us to rule out these specific immune pathways as direct behavioral mediators.

3. Rationale for Selecting *Takeout* (TO1): Our selection of *TO1* was based on its established role as a regulatory hub rather than a mere effector (As discussed in the revised manuscript lines 354-360). Unlike AMPs, which are physiological responses to stress, *Takeout* proteins are known to integrate nutritional and hormonal cues (JHBP domain) to modulate complex behaviors. Given its significant upregulation in CONV females and its positive correlation with preference strength (Revised Fig. 5C), *TO1* provided the most promising candidate for a symbiont-driven behavioral adaptation.

Revised Fig. S7



Revised Manuscript:

As a member of the Takeout/JHBP family—a group known for integrating nutritional status, circadian rhythms, and endocrine signaling—TO1 aligns with the typical profile of TO proteins, being approximately 250 amino acids long, containing a signal peptide, and possessing a conserved JHBP domain 42-44. Given that TO proteins often function as circulating carriers for lipophilic factors like juvenile hormone, TO1 likely serves as a physiological bridge between the host's internal state and its external behavior. (Lines 354-360)

Reference:

Belkaid Y, Hand T W. Role of the microbiota in immunity and inflammation. *Cell*, 2014, 157(1): 121-141.

Wu H J, Wu E. The role of gut microbiota in immune homeostasis and autoimmunity. *Gut microbes*, 2012, 3(1): 4-14.

Comment 5. Takeout genes and RNAi:

Takeout proteins, part of the Takeout/juvenile hormone-binding protein (JHBP) family, are implicated in regulating feeding behavior, circadian rhythms, and potentially social interactions in insects. In general, takeout gene(s) encode a secreted protein related to circulating carrier proteins of lipophilic factors, such as juvenile hormone. It would be important to know whether the candidate takeout gene is regulated by JH signaling, similar to other takeout genes. Expression of takeout

genes is not limited to neuronal tissues, since members of this gene family are also expressed in e.g. gut or fat body tissue. In addition, it has been shown that the expressions of takeout genes is regulated by age and nutritional status, and in some Lepidoptera even by the application of insecticides. In *Drosophila*, the takeout mutant dies rapidly in response to starvation, indicating a link between survival and food status sensing. Since the above suggests multiple roles of takeout genes in insects, the authors should refrain from linking the role of TO1 and TO2 to neural signaling only. It is interesting to note that both TO1 and TO2 (for which overall expression profiles look almost identical) are expressed also in the body, not only in the head. Any knock-down approach would thus also impact TO gene expression/function in the body.

Although the authors only mention 2 takeout-like genes, in insects the takeout gene family can be quite large, with usually 10-20 and in some cases even more (e.g. 25 in termites) genes. With these numbers in mind and given the sometimes very high level of sequence conservation among takeout genes, controlling for off-target effects of gene knock-down experiments is crucial. This could even be done using a transcriptome only. However, since a reference genome exists for this species, the authors need to clarify this shortcoming by applying an off-target search across the complete genome. For the RNAi experiment, only a single dsRNA fragment was generated for the TO1 candidate gene, but at least two different regions of the gene should be considered for dsRNA generation. There is also no information about the length of the dsRNA fragment. Screening for off-targets using all possible short interfering RNAs deduced from the long dsRNA fragment, allowing for mismatches would have been important to exclude the untargeted knock-down of other takeout-like (or completely unrelated) genes.

Response: We sincerely appreciate the reviewer's comprehensive and constructive overview of the *takeout* gene family. These insights have been instrumental in refining our molecular analysis and ensuring the technical rigor of our RNAi experiments.

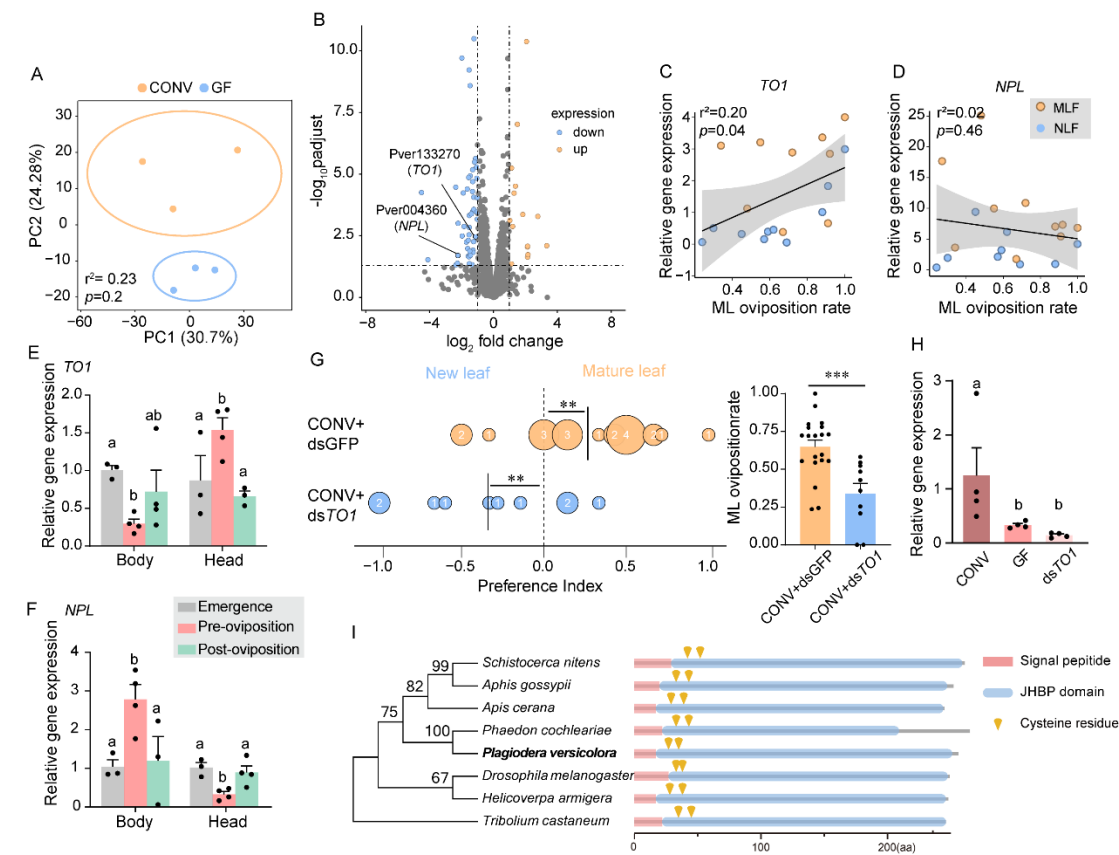
- 1. Broadening the Functional Context of *TO1*:** We agree that our previous emphasis on neural signaling was too narrow. As suggested, we have revised the manuscript to reflect the broader roles of the *TO/JHBP* family in integrating nutritional status, circadian rhythms, and endocrine signaling (Lines 354-360). Specifically, we confirmed that *TO1* possesses a conserved Juvenile Hormone Binding Protein (JHBP) domain, suggesting its potential role as a carrier for lipophilic factors such as JH. We now characterize *TO1* as a physiological bridge between the host's internal state and its external behavior.
- 2. Resolving the *TO1/TO2* Haplotype Mis-assembly:** Regarding the nearly identical profiles and 99.3% sequence identity between *TO1* and *TO2*, our read-depth analysis revealed that both loci exhibited significantly lower coverage (160× and 131×, respectively) compared to the chromosomal average (204×). This "depth-splitting" pattern strongly indicated that these two loci represent misassembled haplotypes of a single gene rather than true segmental duplications. This interpretation was further supported by genomic qPCR (gqPCR), which showed that the total genomic abundance of the *TO1/TO2* sequence was significantly lower than the expected abundance for a *bona fide* two-copy gene (RL Fig. 2). Consequently, we corrected the gene model and re-analyzed our transcriptomic data based on this single-gene locus (Revised Fig. 5).
- 3. Characterization of the *TO* Gene Family:** By searching the *P. versicolora* genome, we identified a total of 17 *takeout-like* genes. Interestingly, while these genes are distributed across the genome, none of them (except *TO1*) were DEGs in our pre-oviposition transcriptome. We have provided the full list of these 17 *TO* genes, including their genomic IDs and expression levels (TPM), in the revised manuscript (Table S2).
- 4. Validation of RNAi Specificity and Off-target Control:** To address the concerns regarding RNAi rigor, we performed a genome-wide *in silico* off-target search using TBtools-II. The 546

bp *dsTO1* sequence (length specified in Table S1) was screened against all predicted coding sequences (CDS). Using the "blastn-short" algorithm to simulate Dicer-processed siRNAs, we found no continuous perfect matches of ≥ 19 nt with any other *TO* family members or unrelated genes. For the most closely related *TO* family members (those with TPM > 10), we performed qPCR following *dsTO1* treatment. The results showed that their expression levels have no significant differences between *dsTO1* treatment versus *dsGFP* (Revised Fig. S8), confirming the high specificity of our chosen *dsRNA* fragment. We also acknowledge that the specific physiological consequences of *TO1* knockdown outside the neuronal tissues also warrant further investigation (Lines 391-393).

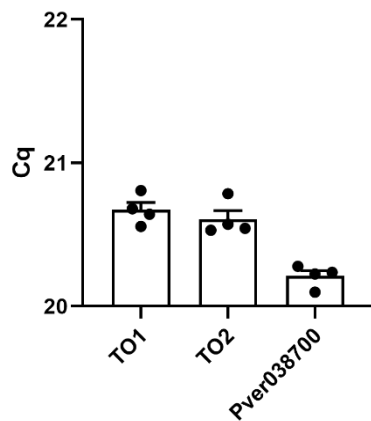
Revised Manuscript:

Furthermore, as *TO1* is expressed in both the head and body, our systemic RNAi approach targets both compartments globally; thus, the specific physiological consequences of *TO1* knockdown outside the neuronal tissues also warrant further investigation (Lines 391-393).

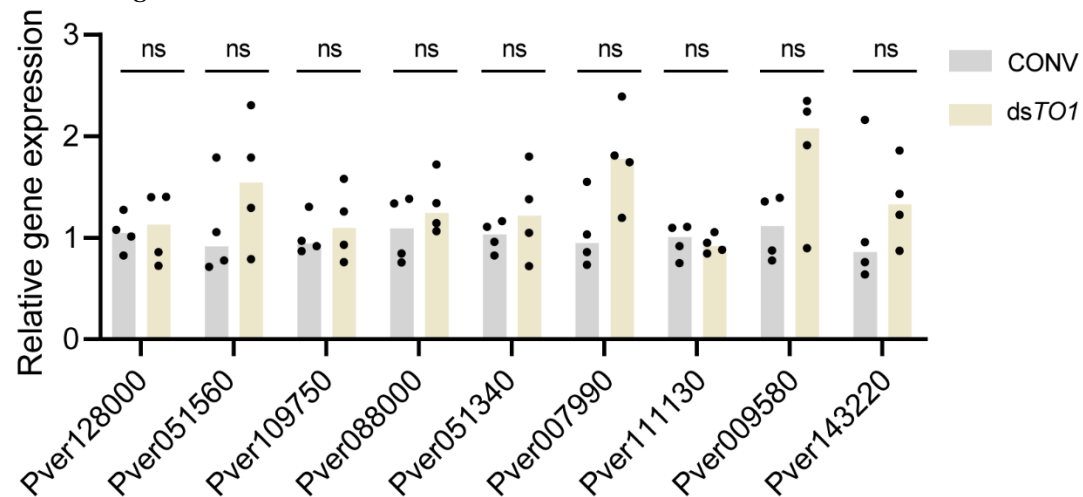
Revised Fig. 5



RL Fig. 2



Revised Fig. S8



Comment 6. Tissue material used for RNAseq:

In their RNAseq experiment, the authors used complete heads, but mentioned that “the chitinous cuticle was removed from the heads prior to sample preparation”. I am not really sure why the authors have performed this step, since this approach can certainly introduce a bias in some or even all of the samples. Removing the cuticle without affecting any other, sometimes rather brittle, easy-to-fragment tissues such as fat body can be quite tricky. Gene expression levels could be affected by the length of the dissection procedure as such and by how much of other tissue material remains on the cuticle which was removed after beetle decapitation. Furthermore, it remains unclear whether - together with the cuticle – the complete antennae were also removed. The authors need to address the issues mentioned above, and should also provide information about biological variability of the RNAseq samples, by e.g. providing a Multidimensional Scaling plot displaying all biological replicates of the treatments.

Response: We thank the reviewer for these important points regarding the dissection procedure. The removal of the chitinous cuticle is to improve RNA extraction efficiency by removing the presence of acellular chitinous material, which contributes significantly to mass but not to the RNA pool.

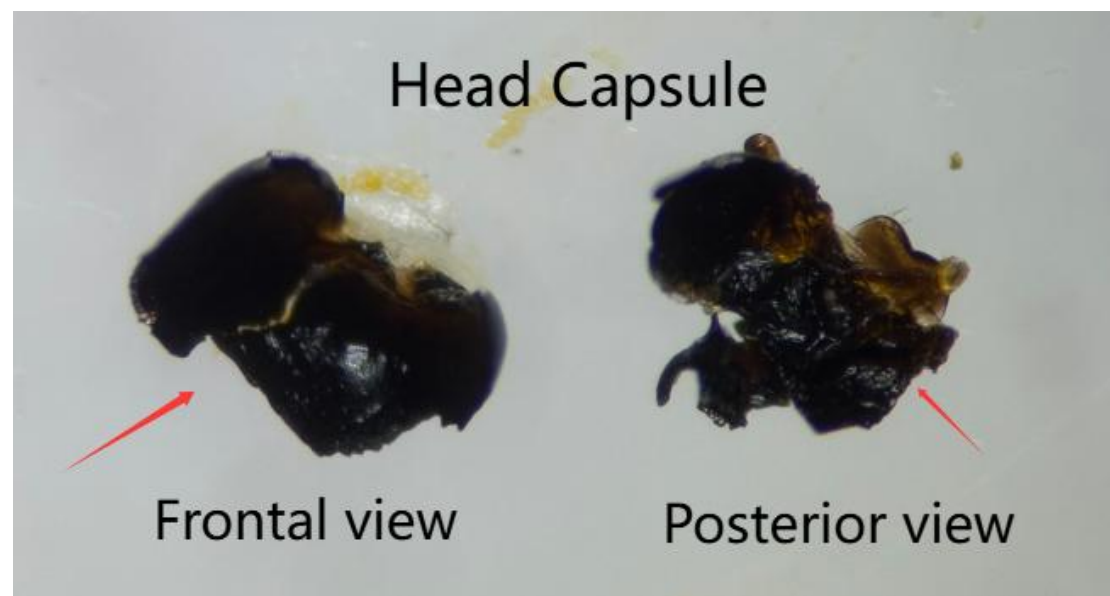
1. To address the concern regarding potential bias and tissue loss: All dissections were performed by the same researcher under a stereomicroscope using a rapid, standardized protocol

to minimize RNA degradation and technical variability. As shown in our representative dissection photographs (RL Fig. 3), the internal surfaces of the removed cuticles show negligible tissue adherence, confirming that the soft tissues remained intact within the sample pool. The antennae were indeed removed along with the cuticle. Given that our study focuses on central gut–brain signaling axes rather than peripheral olfactory or sensory perception, the exclusion of antennal transcripts does not affect the interpretation of the *TOI*-mediated behavioral mechanism.

2. Biological Reproducibility and Variability: Following your suggestion, we have included a Principal Component Analysis (PCA) plot in the revised manuscript (Revised Fig. 5). The PCA, based on the top 2,000 most variable genes, shows that biological replicates within each treatment group (CONV vs. GF) cluster together. More importantly, the distinct separation between groups along PC2 indicates that the transcriptomic shifts driven by the microbiota are far greater than any minor variation introduced by the dissection process.

These clarifications and the PCA data have been integrated into the revised Methods and Results sections.

Response Fig. 3: representative dissection photograph



Minor comments:

Comment 7. - The references are formatted inconsistently

Response: Thank you for your reminder. We have reorganized and standardized the format of the references.

Comment 8 - Line 37ff: In the introduction, the authors state that “Among these decisions, ...” – but fail to explain which decisions they actually mean.

Response: We thank the reviewer for pointing out the ambiguity in our phrasing. We have revised the text to explicitly define the "decisions" referred to in this context. The sentence now specifies that these refer to fundamental life-history choices, such as foraging, mate selection, and habitat colonization, which result from the integration of sensory signals and neural pathways. This provides a clearer logical transition to the subsequent discussion on the criticality of oviposition site selection.

Revised Manuscript: *Ultimately, these processes manifest as a series of life-history decisions—such*

as foraging strategies, mate selection, and habitat colonization—that aim to maximize the individual's or their offspring's fitness (Lines 40-42).

Comment 9 - Line 44: what is “fundamental insect ecology” supposed to mean?

Response: We thank the reviewer for the suggestion to use more precise terminology. We have replaced “fundamental insect ecology” with “advancing our understanding of insect-plant interaction and life-history strategies.” (Lines 48-49).

Comment 10 - In general, the authors list almost exclusively mutualistic interactions between insects and microbes in the introduction. They seem to completely ignore the multitude of antagonistic and pathogenic interactions.

Response: We thank the reviewer for this insightful comment. We agree that focusing solely on mutualism overlooks the significant role of antagonistic interactions. In the revised Introduction, we have included a statement acknowledging that gut-resident communities can include antagonistic or pathogenic species.

Revised Manuscript:

- 1) *Recent studies have revealed that microorganisms—ranging from beneficial symbionts to opportunistic pathogens—can influence nearly every aspect of insect reproductive behavior...* (Lines 52-53).
- 2) *These resident communities encompass a diverse spectrum of interactions, ranging from beneficial mutualists to antagonistic or opportunistic pathogens...* (Lines 63-65).

Comment 11 - Lines 55ff: Only in very few cases (if at all) is there clear evidence for real co-evolution between insects and microbes in general. The authors should therefore refrain from stating co-evolution in the context of gut-resident microbes and their hosts.

Response: We appreciate the reviewer’s rigorous attention to evolutionary terminology. We agree that the term “co-evolution” carries a specific requirement for reciprocal genetic changes that is difficult to prove definitively in most host-microbe systems. Following your suggestion, we have replaced the term using “...often stable association with their hosts” (Line 63).

Comment 12 - Lines 59-61 and 71-73 are the exact same sentence. Consider rephrasing one of them.

Response: We apologize for this redundancy and have removed the duplicated sentence on lines 71-73 to ensure the clarity and flow of the text.

Comment 13 - Line 150: were the bacteria used in this assay also isolated from the beetles as stated in line 158? If so, please state it here.

Response: Yes, as detailed in our response to Comment 2, we have now included comprehensive data on the systematic isolation and taxonomic identification of the gut microbiota. Our results confirm that *P. putida* is the most abundant culturable symbiont within the *P. versicolora* gut (Revised Fig. 3A).

Comment 14 -Line 364: this looks like a header that wasn't properly formatted.

Response: Thank you for your reminder. The redundant “Laboratory oviposition preference experiments” has been deleted.

Comment 15 - The change in nomenclature of the leaves halfway through from new and mature to upper and lower seems unnecessary and adds confusion.

Response: We apologize for the confusion caused by the inconsistent nomenclature. We have standardized the terminology to “new leaves” and “mature leaves” throughout the manuscript. The spatial definitions (e.g., upper vs. lower leaves) have been explicitly clarified in the revised Materials and Methods section (Lines 567-570) to ensure consistency.

Comment 16 - Throughout the results, the mentioning of some data regarding the observed effects for main results such as the differences in oviposition preference, larval and pupal weight is missing.

Response: We thank the reviewer for this suggestion. We have updated the Results section to include key numerical data, such as Preference Indices (PI) and representative larval/pupal weights. To maintain the narrative flow, especially for experiments with multiple treatment groups, we have prioritized the most critical values in the text, while the complete raw datasets are provided in the Source Data files as required by *Nature Communications*.

Comment 17 - Gene expression patterns of TO1 and TO2 seem to be almost identical. However, except for briefly mentioning some differences between TO1 and TO2, no direct comparison of TO1 and TO2 sequences and domains are shown (neither in Figure 5 nor as a supplement). If TO1 plays the suggested central role in the interaction between *P. putida* and the host, what role might TO2 have, given its highly similar expression pattern?

Response: As we have detailed in our response to **Comment 5**, we have confirmed through read-depth analysis and genomic qPCR that *TO1* and *TO2* are actually misassembled haplotypes of the same gene, rather than two distinct paralogs. In the revised manuscript, we have corrected the gene model, re-analyzed the transcriptomic data based on this unified locus, and updated Fig. 5 and Table S2 accordingly.

Comment 18 - In the discussion a figure is cited in the first paragraph and then never again. I think having figure citations in the discussion helps tie it to the results.

Response: We sincerely thank the reviewer for this constructive suggestion. In the revised version, we have integrated figure citations throughout the Discussion section to ensure that each major interpretation is directly supported by our experimental evidence.

Comment 19 - For the cultivation of the bacteria from beetle guts, did the authors know beforehand that the kind of cultivation medium they used for the experiments would select for the most representative members of the microbiota? If so, it would be nice to state that somewhere. Otherwise, it could be nice to show that the post-incubation microbes match the natural composition.

Response: We appreciate the reviewer's concern regarding potential cultivation bias. To ensure we captured the most representative members of this genus, we employed *Pseudomonas*-selective media for isolation. As now detailed in the revised Materials and Methods and Results (lines 537-555; lines 157-159), we recovered 92 isolates from female guts. Taxonomic identification revealed that these belonged to six species, with *P. putida* being the most prevalent (42 strains, 45.7%) (Revised Fig. 3D). This high isolation frequency, coupled with our 16S sequencing results, confirms that *P. putida* is a dominant and representative member of the natural microbiota. We have added this species-level distribution data to Fig. 3D to provide a more transparent view of the gut community's cultivable composition.

Reviewer #1 (Remarks to the Author):

General Comment: I deeply appreciate all the edits, adjustments, responses, and additional experiments that significantly improve the manuscript, clarify the mechanisms, and broaden the impact of this remarkable work.

I am very happy with all the changes, and in my humble opinion, the manuscript is now ready for publication in *Nature Communications*.

Responds: We sincerely thank the reviewer for these incredibly encouraging and supportive comments. It is highly rewarding to receive such a positive assessment of our efforts. We are grateful for the rigorous and thoughtful peer-review process, which significantly improved the clarity and impact of our manuscript. Thank you again for recognizing the value of our work and for supporting its publication in *Nature Communications*.

Reviewer #3 (Remarks to the Author):

General Comment: The authors have done a thorough job of responding to the reviewer comments and have addressed most major concerns. The manuscript was greatly improved as the authors went to great lengths to address the comments not only by changing phrasing throughout the manuscript and providing a better contextual framework, but also by reanalyzing existing data and performing additional experiments. However, some issues remain or were added in this process.

Responds: We are grateful for your positive assessment of our revisions and for the recognition of our efforts to strengthen the manuscript's framework. We also appreciate you pointing out the remaining and newly identified issues. Your ongoing critical feedback is essential to the quality of this work. Accordingly, we have carefully addressed each of these points in the point-by-point responses below to further enhance the study's clarity and rigor.

Comment 1: Takeout genes

The authors' re-analysis of the genomic and transcriptomic data is appreciated and very valuable. The process of streamlining the 2 takeout genes into a single gene model which was explained in the rebuttal should be described in the methods and mentioned in the results section. This would prevent future efforts to characterize this gene coming to the same initial conclusion of the existence of 2 highly similar takeout genes.

Responds: We sincerely thank the reviewer for this constructive comment. As suggested, we have incorporated the description of the bioinformatic and molecular validation process into the Materials and Methods section. Besides, to maintain the narrative flow and ensure the Results section remains focused on the primary biological observations, the specific findings of this validation (including sequencing depth and qPCR data) were integrated directly into the Methods section.

Revised manuscript:

Initial transcriptomic profiling identified two DEGs encoding putative takeout proteins (TO1 and TO2). Subsequent analysis revealed that these two sequences share exceptionally high sequence identity (~99.3%). To determine whether they represent assembly-derived redundancies, locus-specific read depth was assessed using raw genomic data (BioProject: PRJNA1045849). Clean genomic short reads (second-generation sequencing) were re-mapped to the reference genome using BWA-MEM (v.0.7.19-r1273), and the per-base sequencing depth was extracted

using SAMtools (v.1.22.1) depth. To establish a $1\times$ genomic coverage baseline, we utilized the median depth of 2,785 “Complete” single-copy orthologs identified by BUSCO (v.5.4.3) against the *insecta_odb10* database. Both TO1 and TO2 ($131\times$ and $160\times$, respectively) exhibited significantly reduced coverage compared to the genomic median coverage of $261.7\times$. To further validate this at the molecular level, we conducted genomic qPCR using a bona fide two-copy gene, *Pver038700*, as a positive control (Table S1). The relative genomic dosages of TO1 and TO2 were 0.73- and 0.76-fold, respectively, compared with *Pver038700*. Collectively, these bioinformatic and molecular lines of evidence confirmed that TO2 was a misassembled redundancy; it was therefore streamlined into a single gene model (TO1) and excluded from all subsequent functional analyses (Lines 683-698).

Comment 2: Defensin knockdown

It is appreciated that the authors placed more relevance into the differences in immune regulation between conditions and tried to address the comments.

However, since immune responses are usually triggered by signalling cascades leading to transcriptional regulation, the knockdown of a single immune effector, even if this is the most highly differentially expressed one, cannot provide information on whether or not differences in immune regulation lead to differences in behavior. Especially in insects there is a large amount of overlap between activities of individual antimicrobial peptides. Furthermore, we did not intend to imply that differential expression of a single AMP would result in behavioral differences. I am afraid to say that the defensin knock-down experiment is essentially useless and would be misleading to the readers.

The recommendation would be that, rather than including this experiment in the final manuscript, the authors try to discuss a bit better what the immune upregulation in GF conditions may mean in this context.

Responses: We sincerely thank the reviewer for this insightful suggestion. We agree that the knockdown of a single immune effector is insufficient to rule out the behavioral influence of broader immune signaling cascades, especially given the functional redundancy and overlap among antimicrobial peptides (AMPs).

To prevent any potential misinterpretation, we have followed your recommendation and removed the *defensin* knockdown results from the revised manuscript. As suggested, we have expanded our discussion to address the biological implications of immune gene upregulation in GF females. We now frame these observations within the context of microbiota-mediated immune homeostasis and “calibration”, providing a more nuanced ecological interpretation of our transcriptomic data.

Revised Manuscript:

Interestingly, most immune-related DEGs (AMPs) are aberrantly upregulated in GF females compared to CONV controls (Table S2). In insects, the gut microbiota plays a key role in maintaining immune homeostasis by modulating basal immune signaling pathways (Tafesh-Edwards, 2023). Evidence from diverse systems suggests that the depletion of commensal cues can destabilize the homeostatic set point of innate immunity, potentially impairing regulatory mechanisms and leading to dysregulated immune activation (Belkaid et al., 2014; Wu and Wu, 2012). We therefore hypothesize that a similar microbiota-mediated immune calibration occurs in *P. versicolora*. While this systemic immune activation in GF individuals coincides with the loss of mature-leaf oviposition preference, the precise regulatory mechanisms linking immune status to

behavioral output warrant further investigation (Lines 346-355).

Reference:

Tafesh-Edwards G, Eleftherianos I. The role of *Drosophila* microbiota in gut homeostasis and immunity. *Gut Microbes*. 2023

Belkaid Y, Hand T W. Role of the microbiota in immunity and inflammation. *Cell*, 2014, 157(1): 121-141.

Wu H J, Wu E. The role of gut microbiota in immune homeostasis and autoimmunity. *Gut microbes*, 2012, 3(1): 4-14.

Comment 3: *P. putida*'s ecological relevance in larvae compared to adults

The authors argue in line 219 that the fact that newly emerged larvae are more susceptible to *P. putida* compared to newly eclosed adults represents an "ontogenic shift in susceptibility, indicating that *P. putida* may function as a stage-specific conditional pathogen".

This sentence should be moved to the discussion where further context should be added on whether this is known for other insect-pathogen interactions.

Responds: We thank the reviewer for this helpful suggestion. In accordance with this advice, we have moved the statement regarding the ontogenetic shift in susceptibility from the Results to the Discussion section (Line 325). We have also expanded this section by incorporating a broader biological context, comparing our findings with established patterns of stage-specific immune remodeling and susceptibility in other insect-pathogen systems.

Revised Manuscript:

Such ontogenetic variation mirrors broader patterns observed in other insect-pathogen systems. For instance, developmental transcriptomics in *Spodoptera frugiperda* has uncovered dynamic immune remodeling across life stages (Wang 2025), while studies in *Tribolium castaneum* confirm that life stage strongly influences both immune gene expression and infection resistance (Tate 2021). More broadly, immune priming studies suggest that insect immune responsiveness is shaped by the interplay between developmental context and prior microbial exposure, contributing to stage-dependent variation in host defence (Sheehan G 2020) (Lines 327-333).

Reference:

Wang, G., Ran, J., Jia, C. et al. Developmental transcriptomics reveals stage-specific immune gene expression profiles in *Spodoptera frugiperda*. *Sci Rep* **15**, 25044 (2025).

Tate A T, Perry A, Jent D. Larvae and adults exhibit contrasting patterns of immune gene expression and infection resistance in wild flour beetle populations. *Ecological Entomology*, 2021, 46(5): 1230-1235.

Sheehan G, Farrell G, Kavanagh K. Immune priming: the secret weapon of the insect world. *Virulence*. 2020 Dec;11(1):238-246.

Minor comment:

- Please add the mean development times and weight of the control group as well in line 210.

Responds: Thank you for your reminder. We have added the mean development times and weight of control group in Lines 209-211.