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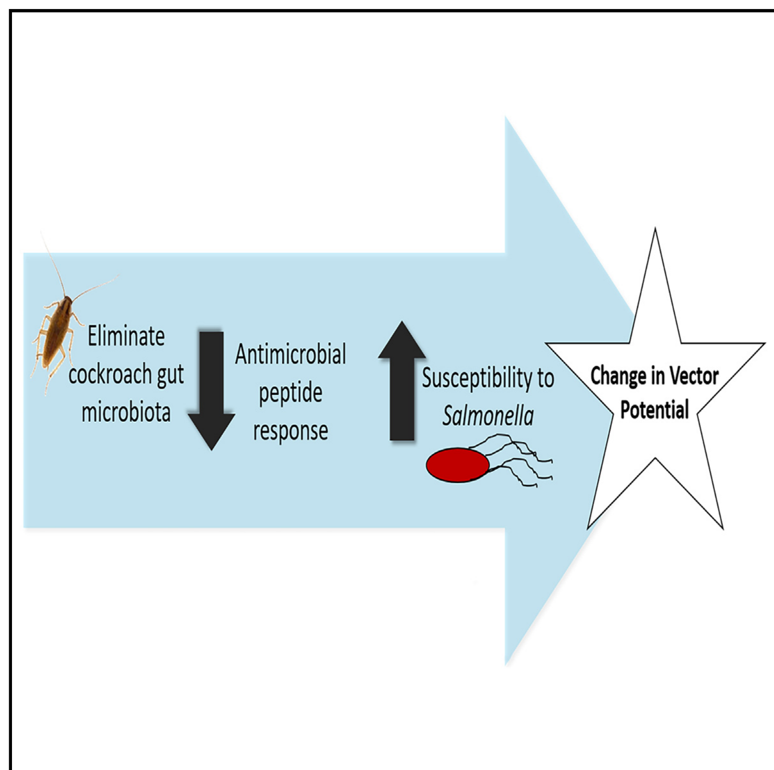
# The gut microbiota confers resistance against *Salmonella* Typhimurium in cockroaches by modulating innate immunity

## Graphical abstract




## Highlights

- The gut microbiota of cockroaches confers resistance to *Salmonella* infection
- The gut microbiota of cockroaches primes antimicrobial peptide expression
- Several minority taxa were identified as indicators of *Salmonella* resistance
- Cockroaches can be a useful model for host-microbiota-pathogen interactions

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## In brief

Biological sciences; Microbiology; Microbiome

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# The gut microbiota confers resistance against *Salmonella* Typhimurium in cockroaches by modulating innate immunity

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## SUMMARY

Cockroaches exhibit unexplained intra- and interpopulation variation in susceptibility to *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium) infection. Here, we show that the gut microbiota has a protective effect against colonization by ingested *S.* Typhimurium in cockroaches. We further examine two potential mechanisms for this effect, showing that commensal bacteria present in the gut do not compete with *S.* Typhimurium during growth in cockroach feces, but rather prime expression of host antimicrobial peptide genes that suppress *S.* Typhimurium infection. Lastly, we determine that neither absolute abundance of the microbiota nor its overall diversity is linked to infection susceptibility. Instead, we identify several minority bacterial taxa that exhibit interindividual variation in abundance as key indicators of infection susceptibility among genetically similar individuals. These findings illuminate the potential of cockroaches as an invertebrate model for interspecies microbial interactions and provide insight into vector-borne *Salmonella* transmission, suggesting that the microbiota of cockroaches could be targeted to reduce pathogen transmission.

## INTRODUCTION

The field of microbiology is currently amid a massive bloom in understanding the structure and functions of microbial communities (microbiota) associated with a wide range of host organisms. Studies of these relationships have elucidated critical roles for the microbiota in numerous fundamental physiological processes, including infection and immunity.[1–4] Studies have also identified variation in the microbiota as a key driver of phenotypic variation between closely genetically related individuals during infection. Of note, following reports suggesting that gut microbiota changes result in variably penetrant phenotypes in Toll-like receptor (TLR) knockout mice during *Salmonella* infection,[5] landmark work by Velazquez et al. revealed that variation in low abundance keystone enterobacteria in the guts of genetically identical mice from different vendors underlies significant differences in susceptibility to infection by *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium).[6] Velazquez et al. also identified a mechanism for this phenomenon, which was dependent on aerobic competition for resources. Additional work in this field has further unraveled multiple mechanisms by which the gut microbiota confers colonization resistance against *Salmonella* spp. and other gut pathogens in mice, underscoring the intricacy of this phenomenon.[7,8]

*S*. Typhimurium is an important human pathogen that has long been used as a model for investigating the regulation of bacterial virulence strategies.[9] It has a broad host range and can be transmitted, among several routes, by insect vectors such as the German cockroach (*Blattella germanica*).[10–15] Recently, due to their low cost, ease of manipulation and diverse microbiota, cockroaches have also been emerging as invertebrate models for investigating the impact of a complex microbiota on host gut health and development.[16,17] Due to their promiscuous diet, German cockroaches can naturally acquire *S*. Typhimurium by ingesting a variety of infectious materials, including human or animal feces. Subsequently, the bacteria colonize and replicate in the gut, where they can persist for weeks without causing mortality.[14,15,18] Infectious bacteria are shed in the feces but not oral regurgitations.[14,15] Despite recent advances in resolving the spatiotemporal dynamics of *S.* Typhimurium infection of cockroaches, a significant knowledge gap remains in understanding the biological challenges faced by this and other enteric pathogens in the cockroach vector, and how these are overcome. For instance, cockroaches harbor communities of gut bacteria that are highly diverse among insects and may compete with ingested bacteria.[19,20] They are also capable of mounting specific and robust antibacterial immune responses, including the production of an array of >30 antimicrobial peptides (AMPs), some of which (e.g., blattellicins) are unique to cockroaches.[18,21,22]

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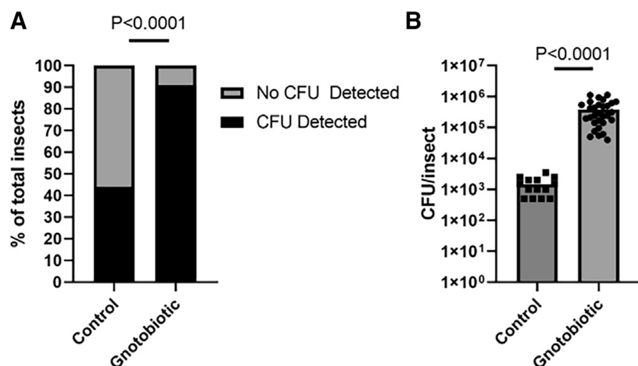


**Figure 1. An intact gut microbiota reduces *S.* Typhimurium load during infection of the cockroach gut**

Control cockroach nymphs with an intact gut microbiota and gnotobiotic cockroach nymphs lacking a gut microbiota were orally infected with *S.* Typhimurium. Three days later, internal loads of *S.* Typhimurium were determined by selective plating of insect homogenates. Both (A) the prevalence of infection (proportion of insects harboring detectable colony forming units; CFU) and (B) the intensity of infection (CFU/insect for those with detectable infection) were quantified. Data were collected from a total of 35–36 individual insects per group derived from three independent biological replicates. Bars represent the mean. Prevalence data were analyzed by chi-square test and intensity data were analyzed by t test performed on log transformed CFU values.

The complexity of the gut environment of the German cockroach sets the foundation for a myriad of host-polymicrobial interactions, providing opportunities to probe the conservation and divergence of the biology of pathogens that colonize taxonomically diverse hosts. That is, a variety of pathogens, including *S.* Typhimurium, have evolved a broad host range and can colonize hosts from distantly related taxa. While much is known about how *S.* Typhimurium colonizes the mammalian gut, less is known about how it colonizes other hosts. By investigating how *S.* Typhimurium (and other pathogens of interest with similarly diverse host ranges) interacts with a model invertebrate such as a cockroach, important knowledge can be gained about the strategies that such pathogens use to colonize distantly related hosts, including which aspects are conserved across hosts, and which diverge.

Notably, we previously reported that, like mice, adult male cockroaches exhibit significant but unexplained intra- and interpopulation variation in susceptibility to infection with *S.* Typhimurium,[15,23] leading to the hypothesis that variation in the microbiota exerts significant influence on infection outcome. Accordingly, here we investigated the role that the microbiota plays in influencing *S.* Typhimurium infection of the cockroach gut, leveraging both a gnotobiotic cockroach nymph model and naturally occurring variation in infection susceptibility among adult cockroaches with an intact microbiota to examine several possible mechanisms.

## RESULTS

### An intact gut microbiota reduces *S.* Typhimurium load during infection of the cockroach gut

German cockroaches acquire their diverse gut microbiota horizontally from the environment via their diet and conspecific coprophagy during development.[24–26] Therefore, to test the impact of the gut microbiota on *S.* Typhimurium infection, we first generated gnotobiotic cockroach nymphs lacking a gut microbiota using an axenic rearing protocol. We orally infected these cockroaches along with matched controls harboring an intact gut microbiota, then compared the load of *S.* Typhimurium colony forming units (CFUs) by plating three days post-infection, a critical point in colonization of the cockroach gut at which post-bottleneck replication has occurred[15] (Figure 1). The gnotobiotic cockroaches had a significantly higher prevalence of detectable infection (91%) than controls (44%) ($p < 0.0001$, chi-square test) (Figure 1A), as well as more than 100-fold higher average loads of *S.* Typhimurium ($3.85 \times 10^5$ CFU/infected insect) than controls ($1.46 \times 10^3$ CFU/infected insect) ($p < 0.0001$, t test) (Figure 1B), demonstrating that the gut microbiota provides resistance against *S.* Typhimurium infection in cockroaches.

### Lack of competition between gut microbiota constituents and *S.* Typhimurium during growth in cockroach feces

To determine whether the effect of the gut microbiota on *S.* Typhimurium infection could be due to direct competition, we examined the impact of gut microbiota constituents on *S.* Typhimurium growth in cockroach feces *in vitro* (Figure 2). There is major overlap between the gut and fecal microbiomes of *B. germanica*,[19] and a similar assay has been used to identify competition between *S.* Typhimurium and constituents of the mouse gut microbiota,[6] supporting this approach. When *S.* Typhimurium was inoculated into homogenized cockroach feces (in PBS) and incubated overnight at room temperature, replication was observed under both aerobic conditions (Figure 2A) and anaerobic conditions (Figure 2B) as indicated by significant increases in CFUs relative to inoculation into sterile PBS, and similar CFU counts relative to inoculation into LB medium. Further, *S.* Typhimurium remained viable in cockroach feces under aerobic conditions at room temperature for a 24-day period of investigation without a significant decline in viability until 17 days post-inoculation (Figure 2C). However, when feces were filter sterilized to remove potentially competing bacteria prior to inoculation with *S.* Typhimurium, this had no significant effect on growth relative to unfiltered feces under either aerobic or anaerobic conditions (Figures 2A and 2B), indicating a lack of significant competition between *S.* Typhimurium and other bacteria from the cockroach gut. In fact, under aerobic conditions, *S.* Typhimurium grew slightly more on average in the presence of commensal bacteria than without them, even though this effect was not statistically significant.

### An intact gut microbiota primes AMP gene expression in the cockroach gut during *S.* Typhimurium infection, enhancing resistance

We next investigated whether the gut microbiota indirectly affects *S.* Typhimurium by modulating the host innate immune response. To do so, we examined the expression of three AMP genes known to be specifically upregulated in the cockroach gut within 1 h of ingestion of *S.* Typhimurium but not *Escherichia coli*[18] (Figure 3). We compared expression of attacin 1 (Figure 3A), attacin 2 (Figure 3B), and blattellicin 1 (Figure 3C) in

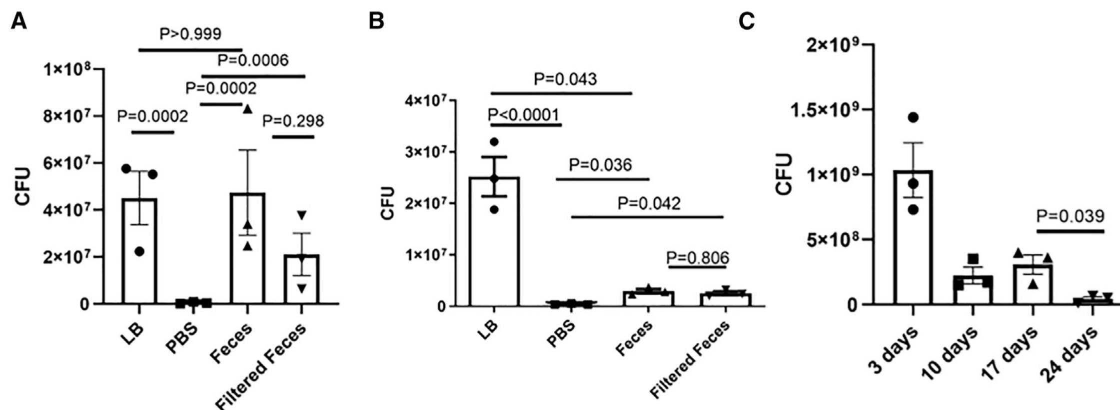


**Figure 2. Lack of competition between gut microbiota constituents and *S.* Typhimurium during growth in cockroach feces**

(A and B) Equal doses of *S.* Typhimurium were inoculated into equal volumes of sterile LB medium, sterile PBS, homogenized cockroach feces in PBS, or homogenized cockroach feces in PBS that were filter sterilized. The samples were incubated overnight under aerobic (A) or facultative anaerobic (B) conditions at room temperature to mimic cockroach body temperature, then the load of *S.* Typhimurium was quantified by selective plating.

(C) *S.* Typhimurium was inoculated into samples of homogenized cockroach feces in PBS and incubated under aerobic conditions at room temperature. The load of *S.* Typhimurium was quantified by selective plating at various periods to determine the duration of viability.

Each of the experiments was independently replicated three times, and individual data points, the mean, and SEM are shown. The data were analyzed by ANOVA with Tukey's post-hoc test on log transformed CFU values.

gnotobiotic cockroach nymphs lacking a gut microbiota and matched controls harboring an intact microbiota that were both orally infected with *S.* Typhimurium. On average, expression of all three genes was lower in the gnotobiotic cockroaches than in controls, but only the reduction in attacin 2 expression was statistically significant ($p$ = 0.045, t test) (Figure 3B). Nevertheless, together these results suggested that the presence of a gut microbiota shapes the ability of the innate immune system of cockroaches to appropriately respond to and resist ingested *S.* Typhimurium. RNA interference (RNAi)-mediated knockdown of attacin 2 expression in cockroaches harboring an intact microbiota further supported this premise (Figures 3D and 3E). Systemic injection of dsRNA targeting the attacin 2 gene 24-h prior to oral infection with *S*. Typhimurium resulted in a 66.5% reduction in attacin 2 expression 1-h post-infection relative to infected cockroaches injected with a control dsRNA (Figure 3D). Subsequently, cockroaches in which attacin 2 expression was knocked down had a significantly higher prevalence of detectable *S*. Typhimurium infection (40.5%) than matched controls (23.1%) three-days post-infection ($p$ = 0.046, chi-square test).

## Neither absolute abundance of the microbiota nor its diversity is associated with *S.* Typhimurium susceptibility

To further understand the characteristics and components of the cockroach gut microbiota associated with the observed effect on *S.* Typhimurium infection, we orally infected adult male cockroaches harboring an intact gut microbiota then compared the microbial communities of individuals that, through natural variation, either became colonized or cleared the infection after three days, as determined by plating (Figure 4). First, DNA was isolated from individual insects and we used quantitative PCR (qPCR) with primers targeting a conserved region of the bacterial 16S rRNA gene to assess absolute abundance of the microbiota[27–29] (Figure 4A). The mean cycle threshold (CT) values of insects that became colonized or that naturally cleared *S.* Typhimurium were not significantly different, indicating a lack of difference in total concentration of the bacterial microbiota between these groups. We next conducted high throughput 16S rRNA amplicon sequencing to profile gut microbiota diversity.[30] Similar to the trend observed in the qPCR assay, analyses of alpha and beta diversity metrics, including number of amplicon sequence variants (ASVs), Shannon index, and inverse Simpson index (Figure 4B**)**, as well as unweighted and weighted Bray-Curtis distances (Figure 4C), found no significant differences between cockroaches that became colonized and those that cleared infection. These results suggest that protection against *S.* Typhimurium is not driven simply by the non-specific load of the gut microbiota or by the balance of its major constituents.

## Low abundance gut microbiota constituents are indicators of *S.* Typhimurium susceptibility

Given that we did not identify links between absolute gut microbiota abundance or diversity and *S.* Typhimurium susceptibility, we also conducted indicator species analysis on 16S sequencing data from adult male cockroaches that became colonized or naturally cleared *S.* Typhimurium to determine if specific bacterial taxa of low abundance and with low impact on overall diversity are associated with infection susceptibility (Figure 4D). In this analysis, bacterial taxa were assigned an indicator value (ind val) ranging from 0 to 1 based on their frequency of occurrence and relative abundance in each experimental group, with a value of 1 signaling a taxon that is exclusively found in all samples of one group at a high relative abundance (i.e., a perfect indicator), and a value of 0 signaling a taxon that exhibits uniform presence and abundance across experimental groups (i.e., not an indicator).[31] We identified eight taxa with indicator values above 0.5. Of these, *Parabacteroides* (ind val = 0.63,

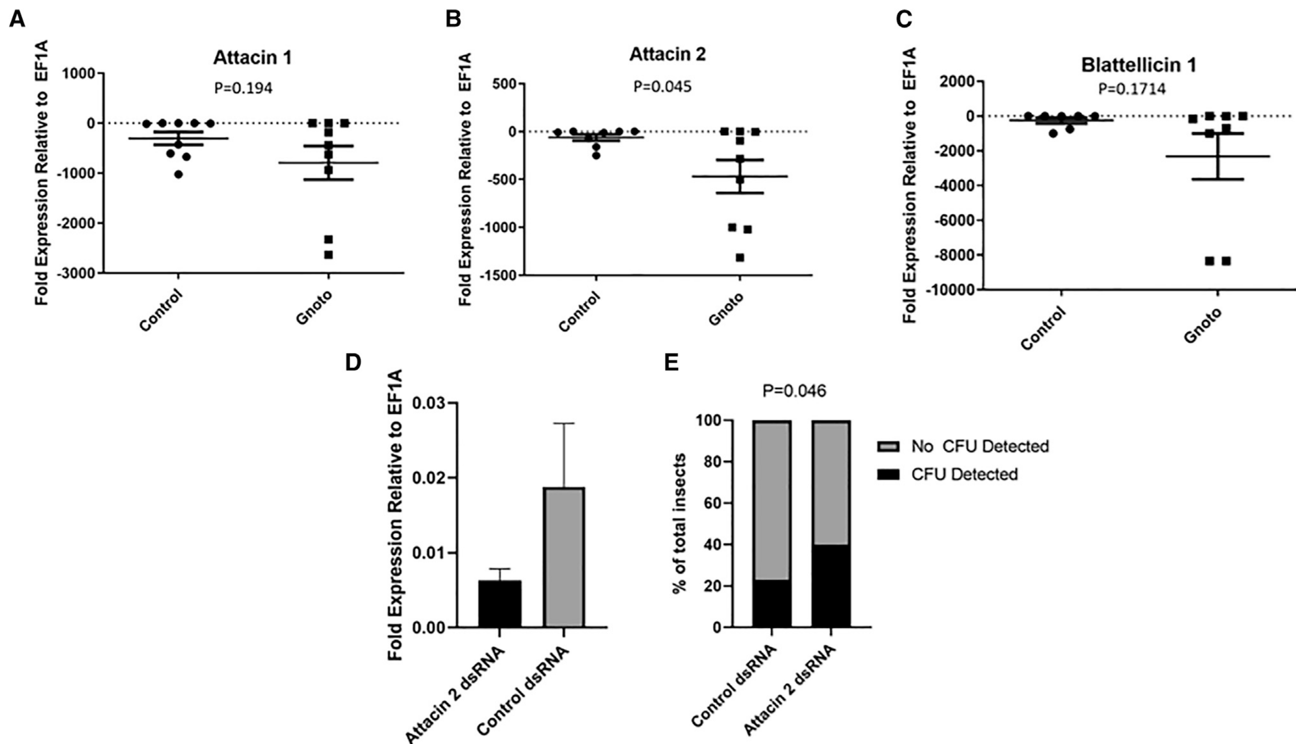


**Figure 3. An intact gut microbiota primes antimicrobial peptide gene expression in the cockroach gut during *S.* Typhimurium infection, enhancing resistance**

(A–C) Control cockroach nymphs with an intact gut microbiota and gnotobiotic cockroach nymphs lacking a gut microbiota were orally infected with *S.* Typhimurium. One hour later, insects were harvested for RNA extraction and qRT-PCR analysis of expression of three antimicrobial peptide genes previously shown to be upregulated in response to *S.* Typhimurium infection: (A) attacin 1, (B) attacin 2, (C) blattellicin 1. Expression is shown relative to the housekeeping gene elongation factor 1 alpha (EF1A). Points represent individual insects derived from two independent biological replicates and bars represent the mean and SEM. The data were analyzed by t test.

(D) Systemic injection of dsRNA was used to knock down attacin 2 gene expression by RNAi in cockroaches with an intact gut microbiota 24-h prior to oral infection with *S.* Typhimurium, and attacin 2 expression relative to EF1A was measured 1-h-post infection to determine knockdown efficiency relative to insects injected with control dsRNA. The data consist of 14 insects in each group. Mean and SEM are shown.

(E) The prevalence of detectable *S.* Typhimurium three-days post-infection was determined by selective plating of cockroaches with an intact gut microbiota injected with dsRNA targeting attacin 2 or control dsRNA. The data consist of a total of 39–42 individual insects per group derived from four independent biological replicates and were analyzed by chi-square test.

$p = 0.03$), Victivallaceae (ind val = 0.52, $p = 0.03$), *Paludibacter* (ind val = 0.67, $p = 0.057$), and *Mucispirillum* (ind val = 0.64, $p = 0.11$), which are present in the feces of German cockroaches as well as the gut,[19] were indicators of cockroaches that naturally cleared *S.* Typhimurium, exhibiting higher relative abundances in this group. Importantly, these are all anaerobic minority taxa, with relative abundances generally under 2%. Additionally, four taxa (*Elizabethkingia*, Tannerellaceae, Enterobacterales, and *Serratia*), had higher relative abundances in cockroaches that became colonized by *S.* Typhimurium and were indicators of this group, possibly providing some benefit to *S.* Typhimurium. These results indicate that the effects of the gut microbiota on *S.* Typhimurium susceptibility are driven by minority taxa.

## DISCUSSION

Our results demonstrate the importance of the gut microbiota in conferring resistance to *S.* Typhimurium infection through priming of the innate immune response in cockroaches and identify several minority taxa that are potentially involved in this phenomenon. These findings have implications for understanding and mitigating vector-borne *Salmonella* transmission as well as for more broadly understanding fundamental host-polymicrobial interactions and developing cockroaches as a model for these interactions.

In mice, the gut microbiota can confer resistance to enteric pathogens via a number of mechanisms. Minority keystone enterobacteria have been specifically found to act through competition for nutrients using aerobic metabolism to hinder *S.* Typhimurium growth,[6] while commensal *Bacteroides* can limit *S.* Typhimurium by producing the toxic metabolite propionate.[32] On the other hand, perturbing the microbiota with antibiotic treatment downregulates expression of Reg3g, a secreted host AMP that protects against pathogenic *Enteroccocus* infection, by reducing TLR4 signaling, exemplifying a form of innate immune priming.[33] Similarly, in a murine model of cytokine storm, antibiotic treatment to disrupt the microbiota dampens

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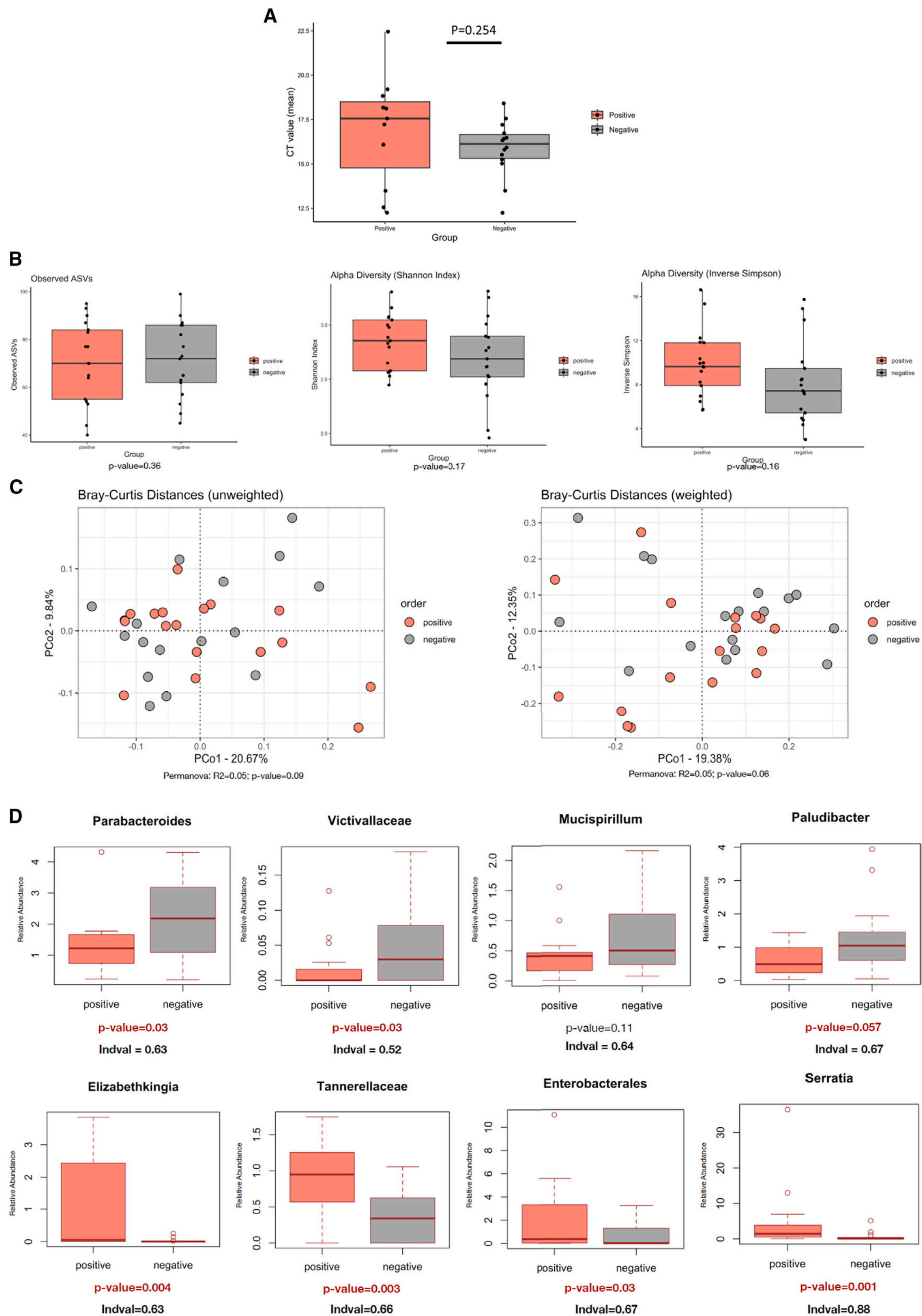


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TLR-induced proinflammatory cytokine production in a JAK signaling dependent manner.[34] The phenomenon of microbiota-mediated protection has also been demonstrated against several insect pathogens in their respective hosts.[35–40] Though mechanistic information from insects remains limited, microbiota-mediated protection against enteropathogenic virus (DCV) infection in *Drosophila* has been attributed to priming of NF-kB signaling by peptidoglycan specifically from the Gram-negative commensal *Acetobacter ponorum*, which subsequently triggers antiviral ERK signaling via a secreted host factor, Pvf2.[40] In the context of the current state of knowledge, our findings are a novel example of "microbial education" of the host immune system by commensal bacteria to confer resistance to pathogen infection.[41,42] This example is particularly compelling because it comes from an insect-pathogen system that is ecologically relevant and also mirrors aspects of the effect of the microbiota on the same pathogen in its mammalian hosts.[6] That is, both cockroaches and mice are hosts for *S.* Typhimurium, and in both the microbiota has a protective effect against infection. However, while in mice competition is a prevailing mechanism underlying this effect, priming of the host AMP response and not competition appears to be the driver in cockroaches.

Along with the many logistical advantages of experimenting on cockroaches, our results further support the utility of these insects as models for studies of not only the effects of a complex microbiota on host gut health and development, but also relationships between the host, its microbiota, and ingested pathogens. In particular, cockroaches can shed light on the conservation and divergence of the colonization mechanisms used by pathogens that colonize taxonomically diverse hosts, including *S.* Typhimurium and potentially other enteric pathogens that have been found to associate with cockroaches, such as *Shigella* spp. and *Campylobacter* spp. Indeed, not only do our results highlight how important mechanisms of microbiota-pathogen relationships can change in the context of different hosts, but our previous work has demonstrated that *S.* Typhimurium type III secretion systems 1/2 are necessary for efficient transmission in cockroach feces,[15] as they are for pathogenesis in mice. Since cockroaches are highly adaptable and omnivorous, this model will facilitate studies considering additional complex variables, such as environmental changes and dietary shifts.[20,43]

Knowledge of the relationship between *S.* Typhimurium and cockroaches may also specifically inform interventions to prevent cockroach-borne transmission. Long-standing dogma has stipulated that cockroaches are passive mechanical vectors of *S.* Typhimurium, rather than active biological vectors that become colonized, limiting infection prevention efforts that target cockroaches to unrefined approaches such as sanitation and insecticidal treatments. However, this concept has been challenged by several recent studies,[15,23] as well as the results presented here, underscoring the potential for more sophisticated approaches, such as manipulation of the gut microbiota to increase resistance to infection. This approach could be applied using existing ingestible bait technologies to deliver compounds that alter the microbiota, or even to deliver certain protective bacteria directly. Moreover, our results provide an explanation for previously unexplained variation in susceptibility to *S*. Typhimurium within and between adult cockroach populations.[15,23]

### Limitations of the study

Despite its important implications, our work has several limitations. Although our experiments found no evidence of competition between *S.* Typhimurium and commensal bacteria during aerobic or anaerobic growth in cockroach feces *in vitro*, these conditions do not completely account for the complex environment and host metabolism in the cockroach gut *in vivo*. Thus, it is possible that a secondary mechanism involving competition *in vivo* may also contribute to microbiota-mediated resistance. In addition, the specific molecular interactions between microbiota constituents and the cockroach immune system that prime AMP expression are unresolved. Among insects, cockroaches encode a highly expanded arsenal of both bacterial recognition proteins (e.g., PGRPs and GBBPs) and AMPs.[21,22] It is possible that canonical peptidoglycan recognition pathways (e.g., Toll/IMD) are involved in sensing *S.* Typhimurium and are upregulated by some gut microbiota constituents. However, alternative pathways may be at play, as expression of attacin 2 during *S.* Typhimurium appears to be highly specific, induced only in response to live *S.* Typhimurium but not heat-killed *S.* Typhimurium nor live *E. coli*.[18] Furthermore, although our studies demonstrate the involvement of a single AMP (attacin 2), likely other AMPs are also primed and involved in microbiota-mediated resistance to *S.* Typhimurium. Insect attacins have been shown to have broad spectrum activity against Gram-negative bacteria, including *S.* Typhimurium.[44,45] Therefore, attacins produced during infection may both directly inhibit *S.*

**Figure 4. Low abundance gut microbiota constituents, but not absolute bacterial abundance nor diversity of the microbiota, are indicators of *S.* Typhimurium susceptibility**

Adult male cockroaches with an intact gut microbiota were orally infected with *S.* Typhimurium. Three days later, *S.* Typhimurium was detected by selective plating of insect homogenates. Samples were grouped into those that harbored detectable *S.* Typhimurium colony forming units (positive, pink color) and those that naturally cleared the infection (negative, gray color). DNA was then extracted from the homogenates to analyze the microbiome by qPCR and 16S rRNA amplicon sequencing.

(A) The abundance of total bacteria in each sample was measured by qPCR targeting a conserved region of the bacterial 16S rRNA gene and cycle threshold (CT) values were compared by t test.

(B) Alpha diversity indices for each sample were compared by Wilcoxon rank-sum test or t test.

(C) PCoA based on unweighted and weighted Bray-Curtis distances were analyzed by PERMANOVA to examine beta diversity.

(D) Indicator species analysis was carried out to identify taxa associated with positive or negative *S*. Typhimurium infection status. Taxa with indicator values greater than 0.5 and their respective relative abundances are shown. The data were analyzed by Wilcoxon rank-sum test or t test.

All data points in (A–D) represent individual insects and the mean is indicated by black (A and B) or red (D) bars.

Typhimurium and also act indirectly by affecting other constituents of the gut microbiota.

The minority bacterial taxa that we identified as indicators of resistance to *S.* Typhimurium have not yet been isolated in culture, which is necessary to directly test their effects. *Parabacteroides*, Victivallaceae, *Paludibacter*, and *Mucispirillum* have all been previously reported in German cockroaches and other cockroach species at variable abundances.[19,30,46–49] *Parabacteroides* in particular appears to be a core member of the microbiota of *B. germanica* that is prevalent across populations.[19] Intriguingly, there is evidence of the protective abilities of some of these taxa against *Salmonella* in vertebrate hosts. For example, *Mucispirillum schaedleri* antagonizes *S.* Typhimurium and alters its gene expression in mice by competing for anaerobic electron acceptors.[50] In chickens, an increase in the relative abundance of *Parabacteroides* following fecal transplant is correlated with an increased immune response and reduced *Salmonella* colonization.[51] Given the implication of these minority taxa as potential causal agents of resistance against *S.* Typhimurium, the lack of correlation between *S.* Typhimurium resistance and overall microbiome abundance or diversity that we observed is unsurprising.

Lastly, generating gnotibiotic cockroaches, and the total lack of microbiota itself, involves a variety of artificial manipulations and conditions that could possibly influence infection and immunity in an unnatural manner.

Filling the aforementioned knowledge gaps is currently complicated by a dearth of genetic tools in *B. germanica* that is slowly improving, as well as by the difficulty of culturing many cockroach gut commensals, which is ongoing. Future studies should continue to mechanistically dissect the *Salmonella*-microbiota relationship in *B. germanica*, building upon new resources in order to develop an integrative understanding of the effects of the gut microbiota on pathogen infection under natural contexts in this vector and emerging model. These effects are ultimately likely to be pleotropic, driven by complex interplay between multiple mechanisms involving agonists and antagonists,[27] and understanding them holds unique potential to broaden fundamental knowledge of interspecies microbial interactions across the animal kingdom.

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests should be directed to and will be fulfilled by the lead contact, Jose E. Pietri (jpietric@purdue.edu).

### Materials availability

This study did not generate any new unique reagents or materials. Insect strains from the study will be made available upon request to the lead contact.

### Data and code availability

- This study did not generate any new unique code.
- Raw 16S sequencing data were deposited into the NIH NCBI SRA database. The accession number is provided in the key resources table.
- Any additional information regarding the data reported in this publication will be made available by the lead contact upon request.

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## AUTHOR CONTRIBUTIONS

J.E.P., M.T., and L.V.H. conceptualized and designed experiments. M.T. and L.V.H. conducted experiments. J.E.P., M.T., L.V.H., K.G., and D.A. analyzed the data. J.E.P., M.T., L.V.H., and K.G. wrote and edited the manuscript.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

## STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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# STAR★METHODS

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---|---|---|
| Bacterial and virus strains | | |
| *Salmonella enterica* serovar Typhimurium strain 14028s GFP | This study | N/A |
| Deposited data | | |
| 16S rRNA gene amplicon sequences from cockroaches | This study | NCBI SRA BioProject PRJNA1136928 |
| Experimental models: Organisms/strains | | |
| *Blattella germanica* American Cyanamid Orlando strain | This study | N/A |
| Oligonucleotides | | |
| *B. germanica Attacin 2* forward primer for qPCR | Sigma Aldrich | GCACTGTCTGGGAGAGCAAA |
| *B. germanica Attacin 2* reverse primer for qPCR | Sigma Aldrich | ATCGAAACTGACGCCTCCTC |
| dsRNA targeting *B. germanica Attacin 2* | Integrated DNA Technologies | 5′-GGUCAAAGGUCAUCGAUG GACCCCA-3’ \| -: 5′- UGGGGU CCAUCGAUGACCUUUGACCAG-3′ |
| dsRNA negative control | Integrated DNA Technologies | catalog #51-01-14-04 |
| Bacterial *16S rRNA* 331F forward primer for qPCR | Sigma Aldrich | TCCTACGGGAGGCAGCAGT |
| Bacterial *16S rRNA* 797R reverse primer for qPCR | Sigma Aldrich | GGACTACCAGGGTATCTAATCCTGTT |

## EXPERIMENTAL MODEL AND STUDY PARTICIPANTS

### Cockroaches

The American Cyanamid Orlando laboratory strain of German cockroach (*Blattella germanica*) was used for all experiments. Cockroach colonies were maintained in plastic enclosures at room temperature (25 ± 1°C) and 40%–45% relative humidity on a 12:12 (L:D) hour photoperiod. The colonies were provided dog chow (Purina, St. Louis, MO, USA) and tap water freely, and were given egg carton harborages for shelter.

## METHOD DETAILS

### Generation of gnotobiotic cockroaches

German cockroaches lacking an environmentally acquired gut microbiota were generated using an axenic rearing process as previously described by our lab and others.[25,52,53] Mature oothecae were gently removed from gravid females using forceps, then their surface was sanitized by rinsing with 10% bleach and 70% ethanol. Sanitized oothecae were allowed to hatch inside sterile petri dishes within a sterilized, air-tight plastic chamber ventilated through a 0.2 μm filter. Newly hatched nymphs were maintained on autoclaved water and dog chow (Purina) and handled strictly inside a laminar flow hood with sterilized tools. Prior work confirmed that autoclaving dog chow has no effect on its nutritional quality based on cockroach fitness.[54] To verify the success of the axenic rearing protocol, multiple individual insects derived from each ootheca were sampled over a period of several weeks. Whole insect homogenates were plated on LB agar without antibiotics and a lack of bacterial growth was confirmed. Once the gnotobiotic cockroaches molted into middle instars, these were infected with *Salmonella*, as described below. The gnotobiotic cockroaches exhibit delayed development, so nymphs rather than adults were used for infection experiments. Controls were matched based on size given the general similarity of microbiome composition and abundance in 2$^{nd}$ and 3$^{rd}$ instar nymphs.[46] No other abnormalities or excess mortality was noted in gnotobiotic nymphs.

### Infection of cockroaches with Salmonella

*S*. Typhimurium was provisioned orally as described in our previous work.[14,15,27] Groups of cockroach nymphs were separated into experimental enclosures and starved of food and water for three days to promote consistent experimental feeding. Following the starvation period, a shallow Petri dish containing a stationary-phase culture of kanamycin-resistant *S*. Typhimurium (strain 14028) grown overnight in LB medium at 37°C was provided to the cockroaches as a sole food source for 30 min. The culture was diluted to $OD_{600} = 1$ prior to feeding. This concentration results in an average ingested bacterial load of ~3.5 × $10^6$ CFU per insect,[15] which is naturally feasible for cockroaches to acquire by consuming infectious feces. Non-toxic blue food dye (blue #1/Brilliant Blue FCF) was added to the bacterial culture to enable visual tracking of feeding[55] and insects that did not feed during the 30-min period were

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excluded. The cockroaches were maintained under the colony rearing conditions described above. Three days after infection, *S.* Typhimurium loads were examined by selective plating. Insects were washed successively with 10% bleach, 70% ethanol, and water, to remove surface contaminants, then mechanically homogenized in sterile PBS using a hand-held tissue homogenizer. The samples were serially diluted and plated on LB agar with kanamycin, which prevents growth of commensal bacteria from the cockroach gut, as determined by plating of uninfected controls. CFUs of *S.* Typhimurium were counted after overnight incubation of plates at 37°C. Both the prevalence of infection (proportion of insects with CFU counts above the limit of detection of 500) and the intensity of infection (CFU/insect for those with detectable infection) were quantified. Data were collected from a total of 35–36 insects per group derived from three independent biological replicates. Infection prevalence data were analyzed using a chi-square test. For analysis of infection intensity data, one outlier data point was identified and excluded by the ROUT method, then CFU values were log transformed and ANOVA with Tukey's post-hoc test was conducted. Cleaned data (after outlier removal) are shown in (Figure 1B).

### Growth of Salmonella in cockroach feces

There is major overlap between the gut and fecal microbiomes of *B. germanica*.[19] Fecal pellets were manually collected from a mixed stage cockroach colony. A mass of 0.026 g of feces was homogenized in 1 mL of sterile PBS three times for 30 s using a BeadBug microtube homogenizer (Benchmark Scientific, Sayreville, NJ) and lysing matrix D tubes (MP Biomedicals, Santa Ana, CA). 1 μL of an overnight culture of kanamycin-resistant *S*. Typhimurium (strain 14028s) diluted to $OD_{600}$ = 1 was inoculated into either 40 μL of the homogenized feces in PBS, or 40 μL of sterile LB medium, sterile PBS, or homogenized feces in PBS that was first passed through a 0.2 μm syringe filter to remove potentially competing bacteria. The effectiveness of the filtration was confirmed by plating both unfiltered and filtered fecal homogenates on LB agar without antibiotics. Samples were incubated overnight on a shaker at room temperature (25 ± 1°C) to mimic cockroach body temperature, either under aerobic conditions or under facultative anaerobic conditions generated using a BBL GasPak Plus Anaerobic system (BD, Franklin Lakes, NJ). Serial dilutions were then plated on LB agar with kanamycin and CFUs of *S.* Typhimurium were counted after overnight incubation of plates at 37°C. In a separate set of experiments, samples were incubated in homogenized feces (in PBS) for longer periods (3–24 days) to determine the duration of *S.* Typhimurium viability. The data were analyzed by matched ANOVA with Tukey's post-hoc test on log transformed CFU values.

### Antimicrobial peptide (AMP) gene expression

Expression of three AMP genes was evaluated in gnotobiotic cockroach nymphs lacking a gut microbiota and control cockroaches with an intact gut microbiota in the context of *S.* Typhimurium infection. Groups of cockroaches were orally infected as described above. One-hour post-infection, RNA was isolated from individual insects using TRIzol reagent (ThermoFisher, Waltham, MA) according to the manufacturer's protocol. RNA samples were treated with DNase (ThermoFisher) to remove contaminating traces of genomic DNA. Subsequently, the RNA concentration in each sample was determined using a Qubit fluorometer (ThermoFisher) and cDNA was synthesized using the high-capacity cDNA reverse transcription kit (Applied Biosystems, Waltham, MA). qRT-PCR was performed on a QuantStudio 3 instrument (Applied Biosystems) using the PowerUp SYBR Green Master Mix (Applied Biosystems) with previously validated primers targeting the attacin 1, attacin 2, and blattellicin 1 genes at a concentration of 500 nM[18]. The amplification conditions were set to the instrument default for a fast run as follows: 95 C for 20 s, 40 cycles of 1 s at 95 C and 20 s at 60 C. Triplicate reactions were run for each sample and gene target and CT values were averaged. From CT values, expression of each AMP gene was calculated relative to a common housekeeping gene, elongation factor 1 alpha (EF1A),[18,25] using the delta-CT method.[56] Data were collected from nine individual insects derived from two independent biological replicates. Outlier values were identified and excluded by the ROUT method. One outlier data point was removed from the attacin 2 experiment and 3 outlier data points were removed from the blattellicin 1 experiment. The data were compared by t-test. Cleaned data (after outlier removal) are shown in (Figures 3A–3C).

### RNAi knockdown of attacin 2

The effect of the AMP attacin 2 on *S*. Typhimurium infection was further evaluated via RNAi using injection of dsRNA for systemic knockdown, an established method for silencing gene expression in cockroaches.[57] Groups of adult male cockroaches were starved of food and water for three days before infection. On the second day of starvation, 2 μL of 1 μg/μL dsRNA targeting the attacin 2 gene (Integrated DNA Technologies, Coralville, IA, 5′- GGUCAAAGGUCAUCGAUGGACCCCA-3' | -: 5′- UGGGGUCCAUCGAUGACC UUUGACCAG-3′) was injected into the abdomen of individual male cockroaches in one group using an aspirator and pulled glass capillary needle. A second group was injected with 2 μL of 1 μg/μL dsRNA negative control (Integrated DNA Technologies, catalog #51-01-14-04). The groups of cockroaches were then infected after the third day of starvation as described above. The efficiency of RNAi knockdown was determined by measuring attacin 2 expression in whole insects via qRT-PCR 1-h post-infection, and the prevalence of *S*. Typhimurium infection (proportion of insects with detectable CFUs) was determined by selective plating three-days post-infection. Three independent biological replicates including a total of 14 cockroaches per group were conducted to assess RNAi efficiency, and four independent biological replicates including a total of 39–42 cockroaches per group were conducted to measure infection prevalence. Infection prevalence data were analyzed by chi-square test.

### Microbiota abundance qPCR

A semi-quantitative real-time PCR assay (qPCR) was carried out as described in our previous work[27,28] to determine if cockroaches that naturally clear *S*. Typhimurium harbor a more abundant microbiota than those that become colonized. Groups of adult male cockroaches were orally infected with *S.* Typhimurium and their infection status was determined by plating three days post-infection using the methods above. DNA was isolated from the same insect homogenates using the DNeasy blood and tissue kit (Qiagen, Germantown, MD) according to the manufacturer's protocol. DNA concentration was measured using a Qubit fluorometer (ThermoFisher). qPCR was performed on a QuantStudio 3 instrument (Applied Biosystems) using the PowerUp SYBR Green Master Mix (Applied Biosystems), 50 ng of DNA as template, and previously published primers targeting a conserved region of the bacterial 16S rRNA gene (331F/797R, F: 5′-TCCTACGGGAGGCAGCAGT-3′, R: 5′-GGACTACCAGGGTATCTAATCCTGTT-3′).[29] These primers cover 83.1% of bacterial taxa based on estimation using the SILVA TestPrime tool,[29] allowing for simple simultaneous quantitation of diverse microbiota constituents, which would not be possible via culture based methods. Amplification conditions were as follows: 95 C for 5 min, followed by 40 cycles of denaturation at 95 C for 15 s, annealing at 50 C for 20 s, and extension at 72 C for 30 s. Mean CT values were obtained for individual samples as a proxy for microbiota abundance. The Dixon test was used to identify outliers. One data point was excluded, and the data were analyzed by t-test. Cleaned data (after outlier removal) are shown in (Figure 4A).

### Microbiota diversity and indicator species analysis (16S sequencing)

DNA isolated from the same whole adult male cockroaches used for qPCR analysis of microbiota abundance was also used for 16S sequencing. Primers targeting the V4 hypervariable region of the bacterial 16S rRNA gene (515/806) were used for PCR with HotStarTaq Plus Master Mix (Qiagen). 95 C, 53 C, and 72 C were used as denaturation, annealing, and extension temperatures, respectively. PCR products underwent electrophoresis on 2% agarose gel to verify successful amplification. Samples were multiplexed using unique dual indices and pooled together at equal concentrations before being purified using Ampure XP beads (Beckman Coulter, Brea, CA). Pooled and purified PCR products were used as input for Illumina DNA library preparation. Sequencing was performed on an Illumina MiSeq instrument (Illumina, San Diego, CA). 16S rRNA sequences were processed using the Qiime2 pipeline (QIIME 2–2024.2; qiime2.org) as previously described (30). In brief, raw sequencing data were processed to remove primers and low-quality reads (phred quality score <25). High-quality reads were considered for denoising, merging and chimera removal, and to generate unique ASVs using the Dada2 plugin within Qiime2.[58] Representative sequences of each ASV were aligned using MAFFT and phylogenetic trees both rooted and unrooted were constructed with FastTree.[59] Taxonomic assignments were based on reference sequences (clustered at 99% sequence identity) from the pre-trained Naive Bayes Classifier Silva 138.[60,61] ASVs which were not present in at least three samples were omitted from the dataset. Sequencing depth ranged from 226,357 to 556,060 sequences per sample. Downstream analyses were performed after removal of reads derived from endosymbionts that reside outside the gut (*Blattabacterium* and *Rickettsiella*). All analyses were performed in the R statistical platform version 4.3.2 (2023-10-31). Briefly, for alpha diversity, beta diversity and PERMANOVA, vegan and ape packages were used.[62,63] Significantly discriminating bacterial taxa (indicator species) were identified using the species indicator analysis in the labdsv package, which calculates the indicator value using the fidelity and relative abundance of species.[31,64] The Shapiro test was used to determine normality, and the appropriate statistical tests (Wilcoxon rank-sum test or t-test) were used to assess significant differences in alpha diversity values and relative abundances of indicator.

## QUANTIFICATION AND STATISTICAL ANALYSIS

GraphPad Prism and RStudio were used for quantification and statistical analyses. The specific statistical tests used for each experiment are described in the corresponding method details section. Specific *p*-values for comparisons made are reported in the figures. Information on replication for each experiment is provided in the corresponding method details section and in the figure legends.