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RESEARCH ARTICLE

Opposing Growth Responses of Lepidopteran Larvae to the Establishment of Gut Microbiota

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ABSTRACT Gut microbiota can have diverse impacts on hosts, the nature of which often depend on the circumstances. For insect gut microbes, the quality and nature of host diets can be a significant force in swinging the pendulum from inconsequential to functionally important. In our study, we addressed whether beneficial microbes in one species impart similar functions to related species under identical conditions. Using fall armyworm (*Spodoptera frugiperda*), beet armyworm (*Spodoptera exigua*), and other noctuid hosts, we implemented an axenic rearing strategy and manipulated gut bacterial populations and dietary conditions. Our results revealed that some gut *Enterococcus* and *Enterobacter* isolates can facilitate utilization of a poor diet substrate by fall armyworm, but this was not the case for other more optimized diets. While *Enterococcus* provided benefits to fall armyworm, it was decidedly antagonistic to beet armyworm (*Spodoptera exigua*) under identical conditions. Unique isolates and bacterial introductions at early growth stages were critical to how both larval hosts performed. Our results provide robust evidence of the roles in which bacteria support lepidopteran larval growth, but also indicate that the directionality of these relationships can differ among congener hosts.

IMPORTANCE Insects have intimate relationships with gut microbiota, where bacteria can contribute important functions to their invertebrate hosts. Lepidopterans are important insect pests, but how they engage with their gut bacteria and how that translates to impacts on the host are lacking. Here we demonstrate the facultative nature of gut microbiota in lepidopteran larvae and the importance of diet in driving mutualistic or antagonistic relationships. Using multiple lepidopteran species, we uncover that the same bacteria that can facilitate exploitation of a challenging diet in one host severely diminishes larval performance of another larval species. Additionally, we demonstrate the beneficial functions of gut microbiota on the hosts are not limited to one lineage, but rather multiple isolates can facilitate the exploitation of a suboptimal diet. Our results illuminate the context-dependent nature of the gut microbiomes in invertebrates, and how host-specific microbial engagement can produce dramatically different interactions.

KEYWORDS bacteria, dysbiosis, *Enterococcus*, microbiome, *Spodoptera*, symbiosis

There is a widespread recognition that gut microbiota can contribute substantially (for better or worse) to vertebrate and invertebrate phenotypes (1–3). For insects, gut microbes can facilitate digestive and nutrient acquisition processes (4–7), potentially allowing some host species to expand their diets (8–11). However, these budding partnerships require host recognition and regulation of the microbiota in order to achieve realized mutualisms. In some circumstances, destabilization of the interaction may occur, and cause disengagement of the relationship or degenerative metabolic syndromes (12–14).

Although there have been incredible advances in microbiome research over the past decade, we have poor reconciliation of host gut microbiome modulation between closely related species and their resulting phenotypes. Host evolution and life history undoubtedly contribute to the host's ability to filter undesired microbes and mediate community

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assembly (15), but the strength of the relationships between host and microbial assemblage and, ultimately, impacts on host fitness, are not well-resolved. For instance, while microbial assemblages can pair with host phylogeny (16, 17), some do not exhibit strong patterns (15, 18). Although similar microbial taxa can confer different metabolic potentials and phenotypes to the host (19, 20), there are other instances where unrelated bacteria perform similar functions in their hosts (21, 22). Further complicating matters, the host's environment (i.e., population source, diet) may shape both the composition and phenotypic potential of the intestinal microbiota of some insects. So, while insects can serve as excellent models for microbiome research due to their simplicity and reproducibility (23), ascribing broad functions across insect taxa or host conditions may be tenuous.

Despite being economically important pests and experimental systems in plant-herbivore interactions, lepidopterans lag other groups regarding defined roles of gut microbes (24). There are several possible explanations for this trend, such as the wide variation observed in microbiome composition between individuals (18, 25–27). This is compounded by the fact that microbial effects on their lepidopteran hosts are not ubiquitous mutualists, with documented functions mostly occurring under facultative circumstances with nutritionally limiting or toxin-laden diets (28–30). Thus, the development of experimental systems has been hampered (31), making comparisons between different bacterial isolates and host species under common conditions challenging.

Noctuid caterpillars are among the most important agricultural pests. Individuals from this family have received much attention related to the composition and membership of their gut microbiota (25, 32, 33), but broad knowledge is lacking about how microbial consortia translate to beneficial or antagonistic interactions (31, 34). Recently, we determined that the fall armyworm, *Spodoptera frugiperda* (Lepidoptera: Noctuidae), receives considerable growth benefits from *Enterococcus* when fed on a bean-based diet (35) that has been used since the 1960s for lepidopteran larval rearing (36). This diet was developed to support multiple lepidopteran genera and species, and these bean-based diets are still utilized in laboratory rearing and comparative experiments (37–40).

Here, we implemented an axenic and defined population rearing strategy developed for fall armyworm larvae (28, 38) to determine if beneficial insect-microbe interactions can be applied broadly to other species of the Noctuidae. We used bacterial isolates commonly detected in Lepidoptera, *Enterococcus* and *Enterobacter*, and orally administered them to multiple hosts. While our initial hypothesis was that comparable patterns would occur among the closely related insect species, our results indicate that the same bacteria may be beneficial or antagonistic to different hosts and on different diets.

RESULTS

***Enterococcus* colonization mediates fall armyworm diet utilization when fed on a concentrated diet but is antagonistic on a diluted diet.** We first determined how fall armyworm performed with and without gut bacteria on an array of diets of different nutritional quality. We used pinto bean diet and made serial dilutions by adding cellulose to the diets to make three dietary concentrations: normal (no cellulose added), 35% dilute (0.35 g g^{-1} added cellulose), and 70% dilute (0.7 g g^{-1} added cellulose). Diet concentration ($F_{2,79} = 9.32$; $P < 0.001$), axenic status ($F_{1,79} = 9.32$; $P = 0.002$), and their interaction ($F_{2,79} = 38.0$; $P < 0.001$) all influenced fall armyworm performance. Axenic fall armyworm larvae performed poorly on normal and 35% diluted diets compared to the 70% diluted diet, having 3× less biomass after 10 days post hatch (dph) (Fig. 1). Fall armyworm orally inoculated with *Enterococcus* FAW2-1 exhibited inverted trends compared to axenic caterpillars on the same pinto bean diet diets.

Different armyworm species exhibit disparate patterns of pinto bean diet usage and microbial engagement. After observing significant differences between axenic and mono-associated fall armyworm on pinto bean diet (no cellulose), we sought to address how other armyworm (*Spodoptera*) species would perform under the same dietary conditions. Beet armyworm (*Spodoptera exigua*) eggs were subjected to the same sterilization and bacteria inoculation procedures as for fall armyworm. Like our first experiment, *Enterococcus* FAW2-1 improved fall armyworm growth (Fig. 2A), increasing body mass by 4.5× ($F_{2,26} =$

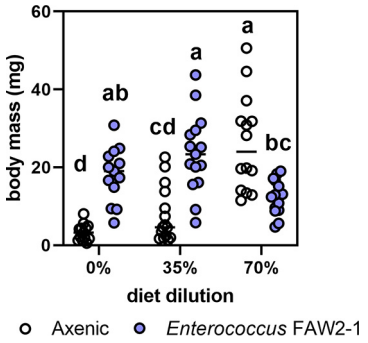

FIG 1 Influence of *Enterococcus* FAW2-1 on fall armyworm body mass under different pinto bean diet formulations. Neonates were inoculated with bacteria for 3 days before transferring to a fresh diet of the same concentration. Body masses were obtained 10 d after hatching. Different letters represent statistically significant differences.

17.7; $P < 0.001$). In complete contrast to fall armyworm, axenic beet armyworm performed very well on pinto bean diet (Fig. 2B), accumulating 10× the body mass of fall armyworm at 14 days postinoculation (dpi). *Enterococcus* FAW2-1 reversed beet armyworm growth entirely, where inoculation of this isolate reduced growth by 15× compared to the axenic larvae ($F_{2,26} = 12.4$; $P < 0.001$). Autoclaving the isolate yielded larval growth patterns indistinguishable from their axenic status for both species (Fig. 2). A separate experiment indicated that the 3-day bacterial introduction period after hatching imparted long-term consequences on both species. Axenic fall armyworm larvae had ~2× lower pupation success (Fig. S1A; $Z = 5.62$; $P < 0.001$), pupae that were 13% smaller (Fig. S1B; $T_{df,54} = 3.024$;

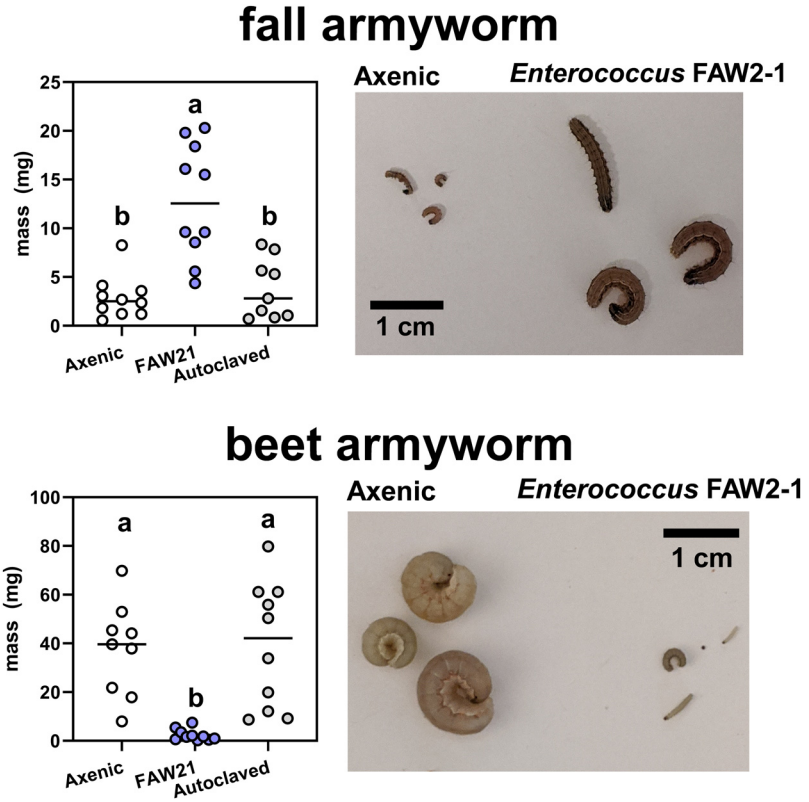

FIG 2 Body mass of fall armyworm (A) and beet armyworm (B) orally inoculated with *Enterococcus* FAW2-1. Neonate larvae were inoculated for the first 3 days after hatch before individuals were transferred to fresh diet. Larvae were weighed 10 days after hatch and photos were taken 14 days after hatch. Different letters and asterisks represent statistically significant differences ($P < 0.05$). Viable *Enterococcus* FAW2-1 elicited negative effects on both insect species fed on nutrient rich wheat germ diet under identical experimental conditions (Supplemental material).

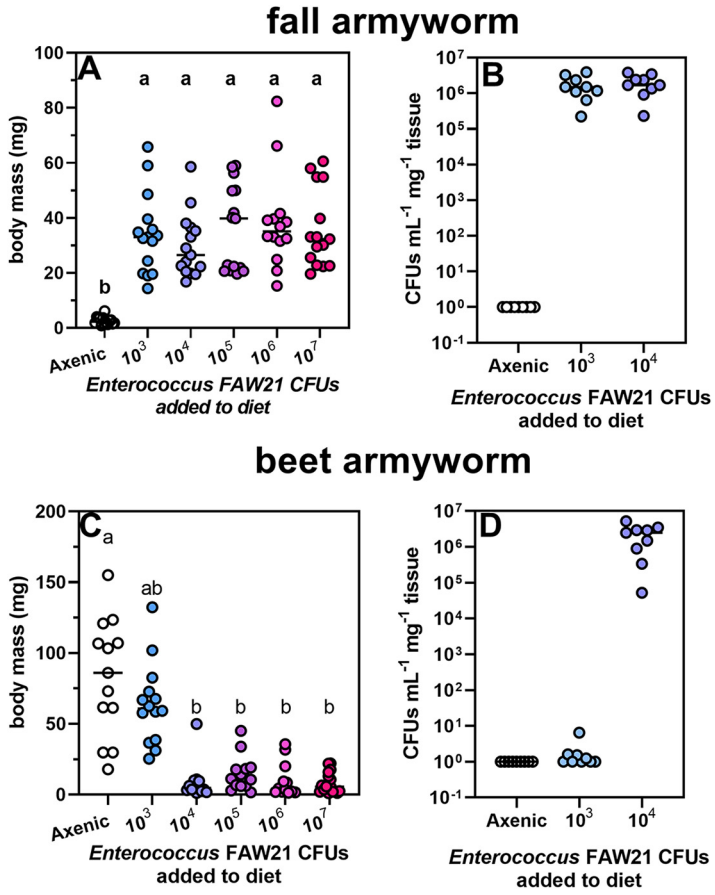

FIG 3 Dose-response of *Enterococcus* FAW2-1 on mass (left) and respective gut bacterial titers (right) of fall armyworm (A & B) and beet armyworm (C & D) to initial inoculation 14 d postinoculation. Different letters represent statistically significant differences ($P < 0.05$).

$P = 0.004$), and delayed development by 27% (Fig. S1C; $T_{df,54} = 9.31$; $P < 0.001$) compared to those that were inoculated with *Enterococcus* FAW2-1. Inoculated beet armyworm had lower pupation success (Fig. S1D; $Z = 2.09$; $P = 0.036$), pupal mass reduction by 16% (Fig. S1E; $T_{df,55} = 4.92$; $P < 0.001$) and slowed development by 30% (Fig. S1F; $T_{df,55} = 9.51$; $P < 0.001$).

Given that a portion of fall armyworm and beet armyworm larvae successfully pupated under challenging circumstances suggests that both insect species could overcome their respective axenic and bacteria-derived limitations. Daily monitoring of caterpillar masses indicated that *Enterococcus* slowed beet armyworm mass accumulation immediately (Fig. S2), but after achieving a body mass of >5 mg, growth rates increased rapidly. However, axenic fall armyworm body mass accumulation was less rapid throughout larval development. We hypothesized that larval ontogeny may dictate both the positive and negative interactions for the respective species. To address this question, we conducted a bioassay where we administered bacteria later in larval development by inoculating larvae in the second instar with *Enterococcus* FAW2-1 before moving to a fresh, sterile diet. The isolate still improved fall armyworm growth by 2.5× (Fig. S3A; $T_{df,18} = 5.82$, $P < 0.001$), while delayed inoculations did not have any apparent impacts on beet armyworm growth (Fig. S3B; $T_{df,18} = 1.12$, $P = 0.276$).

***Enterococcus* established in fall armyworm at low titers but failed to establish in beet armyworm fed on pinto bean diet.** Since there were sustained effects of early instar inoculations on both *Spodoptera* species, we evaluated how initial inoculum density affects larval performance (Fig. 3). For most doses, *Enterococcus* FAW2-1 was an effective colonizer of both insects achieving high densities throughout both species' development. We found that inoculating with 103–107 CFU of *Enterococcus* FAW2-1 led to increased (4–5×) larval body mass accumulation compared to axenic fall armyworm ($F_{5,82} = 14.0$; $P < 0.001$), with no differences occurring between doses (Fig. 3A). Resulting titers did not differ in fall

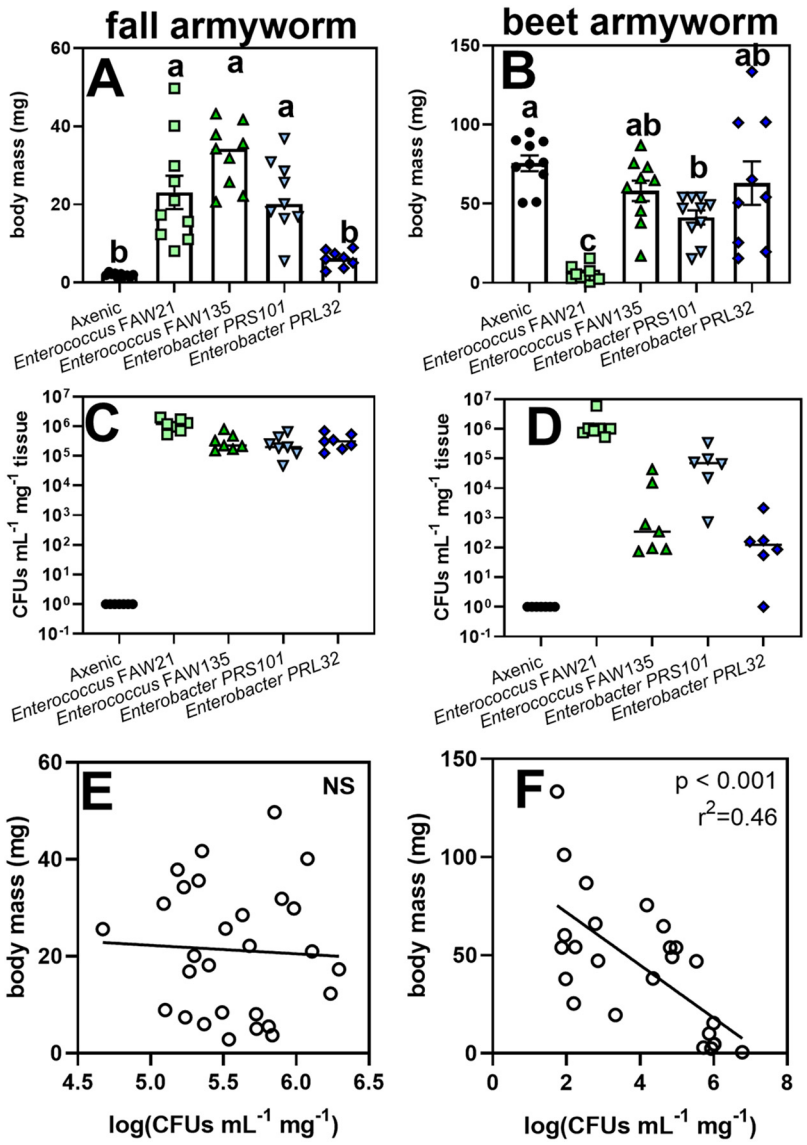

FIG 4 Growth (A & C) and associated gut microbial titers (B & D) of fall armyworm (left) and beet armyworm (right) 14 d postinoculation with 4 different isolates of bacteria. Different letters represent statistically significant differences ($P < 0.05$). Relationships between larval growth and associated bacterial titer indicate fall armyworm exhibited less of a relationship between which bacteria established and influence on larval performance (E). Beet armyworm had a negative relationship between gut microbial titer and body mass (F).

armyworm inoculated with 10^3 and 10^4 CFU ($P = 0.494$; both having $\sim 10^6$ CFU mL^{-1} mg^{-1}) while no culturable bacteria were detected in axenic individuals (Fig. 3B). Beet armyworm inoculated with *Enterococcus* FAW2-1 at 10^3 CFU did not differ in bacteria titer from axenic larvae (Fig. 3C), but at all other doses, body mass was reduced by $>8\times$ compared to the axenic larvae ($F_{5,75} = 29.1$; $P < 0.001$). The bacterial gut titers in beet armyworm were related to differences in larval growth. Beet armyworm receiving low *Enterococcus* doses (10^3 CFU/mL) had 0–320 CFU mL^{-1} mg^{-1} (Fig. 3D) while the higher dose (104) resulted in colonization densities comparable to fall armyworm ($\sim 10^6$ CFU mL^{-1} mg^{-1}).

***Enterococcus* and *Enterobacter* isolates behave differently in armyworm hosts.** In prior experiments, we observed that fall armyworm had different growth responses on pinto bean diet to *Enterococcus* and *Enterobacter* isolates (Fig. S4) (36). Here, we evaluated how different isolates would establish and perform differently in fall and beet armyworm. We selected two *Enterococcus* isolates and one *Enterobacter* isolate, which improved the growth of fall armyworm compared to axenic larvae (Fig. 4A). Despite different effects on larval

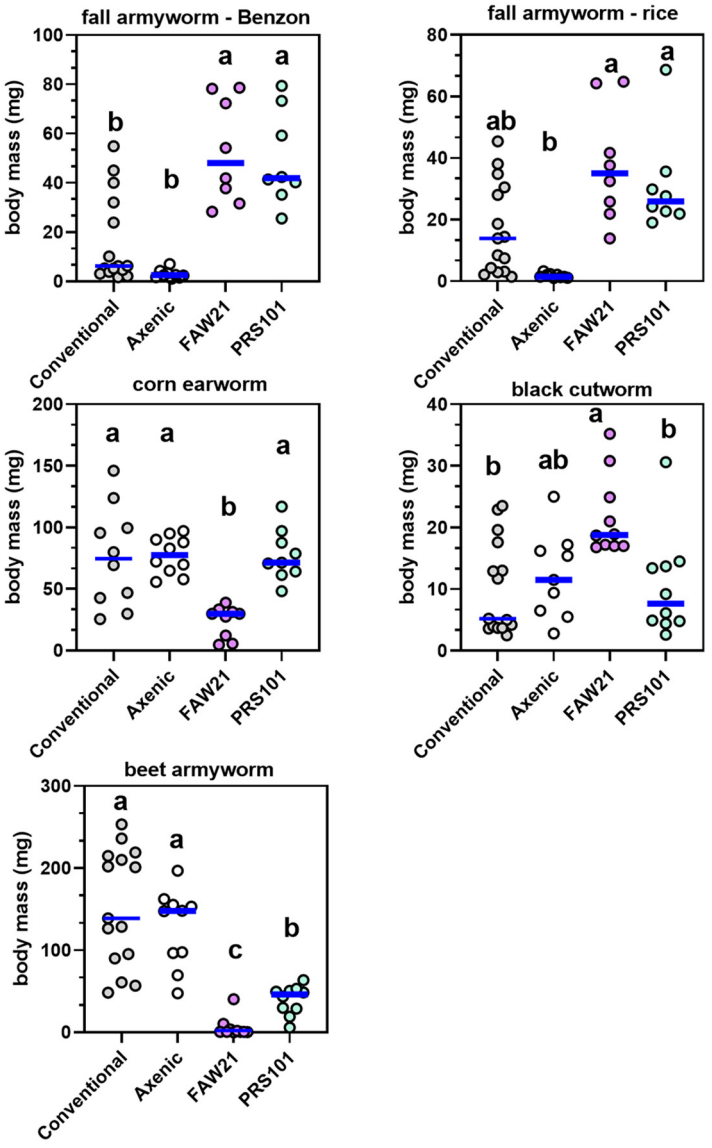

FIG 5 Impacts of *Enterococcus* FAW2-1 and *Enterobacter* PRS101 on larval performance for several noctuid species and fall armyworm strains 16 d postinoculation compared to conventional (unsterilized eggs) and axenic larvae. One egg source of fall armyworm was derived from a colony fed on rice, while the other was purchased from Benzon. Different letters represent statistically significant differences.

body mass accumulation ($F_{4,41} = 21.2$; $P < 0.001$), all four bacterial isolates established at relatively high titers ($\sim 10^6$ CFU mL^{-1} mg^{-1}; Fig. 4B), indicating establishment and persistence regardless of whether the outcome was beneficial to the host (Fig. 4E). As observed previously, bacterial inoculation reduced beet armyworm body mass ($F_{4,41} = 11.0$; $P < 0.001$), but the magnitude of this response differed between isolates (Fig. 4C). Isolates also contrasted in their ability to persist in the beet armyworm gut (Fig. 4D), with gut CFU titers exhibiting a negative relationship with body mass (Fig. 4F; $P < 0.001$; $r^2 = -0.46$).

Bacterial-mediated effects contrast between Noctuidae hosts. To determine if broader patterns emerge among other lepidopterans, we evaluated how the bacterial isolates *Enterococcus* FAW2-1 and *Enterobacter* PRS10-1 performed in fall armyworm, rice-adapted fall armyworm, beet armyworm, corn earworm (*Helicoverpa zea*), and black cutworm (*Agrotis ipsilon*) (Fig. 5). Both fall armyworm strains followed identical patterns. Both beet armyworm and corn earworm body mass accumulation exhibited similar trends, where they were negatively affected by both bacterial strains with *Enterococcus* having a larger effect. Black cutworm had mixed responses to inoculation, with *Enterococcus* improving growth and

Enterobacter not differing from the axenic larvae (Fig. 5). Results of rearing of conventional (nonsterilized eggs) on pinto bean illustrate trends that support a hypothesis that larvae are selecting for microbial populations to maximize fitness benefits. Growth of conventionally reared beet armyworm, corn armyworm, and black cutworm did not differ from axenic larvae. Conventionally reared fall armyworm did not exhibit significant differences from axenic either, but some individuals performed at a substantially higher rate than the others (Fig. 5).

Beneficial effects by *Enterococcus* on fall armyworm are not universal. For our final bioassay, we evaluated how *Enterococcus* FAW2-1 influences fall and beet armyworm on a different artificial diet formulation (SI Fig. 5). When fed a wheat germ diet, fall armyworm larvae inoculated with *Enterococcus* FAW2-1 had a 75% reduction in growth compared to axenic larvae at 10 dpi ($T_{df,18} = 9.26$, $P < 0.001$). Beet armyworm had growth responses comparable to fall armyworm, with inoculated individuals having a 52% reduction in weight gain compared to axenic larvae ($T_{df,18} = 5.94$, $P < 0.001$). Notably, the larvae generally performed much better on this diet under all conditions compared to pinto bean diet.

DISCUSSION

Facultative host-microbial partnerships involve the alignment of ecological and biochemical processes to realize potential mutualisms. Here, we demonstrate that gut bacteria can promote the success of caterpillars consuming a suboptimal diet. These findings are important unto themselves as it expands our understanding of direct performance benefits of microbes to lepidopterans, especially as clear microbial mutualisms are lacking in this order (2, 24, 41). However, contrary to our initial expectations, the beneficial effects were absent in other noctuid caterpillars fed the same diet. Instead, the microbial interactions that emerged were dysbiosis and parasitism. Across several experiments, our results revealed that host species, microbial isolate, larval ontogeny, and dietary conditions help dictate the directionality of host-microbe interactions. Furthermore, we observed that the initial encounter between the caterpillar hosts and bacteria in the first days after hatch persisted for weeks across multiple diet replacements. While our results shed new light on the context-dependent benefits bacteria provide for lepidopterans, they suggest that extrapolation beyond the system in question could lead to dubious interpretations.

Diet is a pivotal factor affecting gut microbiomes and is the main driver of the patterns we document here. For fall armyworm, *Enterococcus* provided massive benefits when its host encountered challenging diet conditions (pinto bean diet). As the dietary challenge was relaxed with either a diluted diet or a different, more optimized formulation, *Enterococcus* instigated costs (Fig. 1; Fig. S1), similar to observations in other lepidopteran systems (42–44). Maintaining partnerships require metabolic currency and, clearly, the benefits of possessing these associates outweigh the costs on the pinto bean diet. We presume the benefits administered by *Enterococcus* are due to some intractable component of pinto bean diet adversely impacting fall armyworm digestive processes. We previously reported that fall armyworm consuming pinto bean diet has elevated oxidative stress in the midgut (35), and here we observed that diluting the diet with cellulose led to better performances (Fig. 1). We posit that the underlying mechanism of this interaction is likely a toxin, but something nutritional, hormonal, or immunological should not be discounted. Destabilizing the gut microbiome of fall armyworm leads to changes in metabolic homeostasis (45), so the mechanisms underlying our observations may be complex.

Like what has been observed in other instances (21, 22, 46), we identified distantly related symbionts that can confer comparable benefits to the host. Some of the *Enterococcus* and *Enterobacter* isolates promoted fall armyworm growth on a pinto bean diet, while others did not. Irrespective of benefits, different isolates colonized fall armyworm at identical densities, suggesting that the gut system is a hospitable environment to bacteria under these dietary conditions. Also, *Enterococcus* FAW2-1 was able to efficiently colonize and propagate in fall armyworm larvae even at lower introductory titers. The same isolates had negative or no effect on beet armyworm growth, but the responses were more strongly tied to the establishment and propagation of the bacteria. However, *Enterococcus* FAW2-1 appears to be adept at

evading beet armyworm gut microbial suppression, as it colonizes at higher densities than the other isolates we tested, but at low initial titers, establishment can be abated by the host.

Enterococcus has been documented as common taxa that populate larval guts in noctuids in the field and laboratory, with *Enterobacter* more often being associated with larvae consuming plants (25, 45, 47, 48). While these genera are common features in the gut microbiomes of lepidopteran larvae, their functions are not widely resolved (31). Specifically, evidence of direct beneficial impacts of these bacteria on their hosts is limited. *Enterobacter* in diamondback moth metagenomes has been shown to encode multiple genes involved with detoxification (49) and *Enterococcus* from velvetbeen caterpillar can provide proteases to circumvent potential inhibitors (50, 51). Both genera can have negative effects when the host is engaged with pathogen and plant-derived toxins (28, 52, 53). Like all bacteria, there is probably strain-level variation that may impact their interactions.

Some of the most formative lepidopteran-microbe relationships appear to engage immediately upon larval hatch. For instance, beet armyworm was not impacted by the introduction of *Enterococcus* later in development (Fig. S3). Similarly, the late instar fall armyworm was more resilient when fed on a pinto bean diet than earlier in its development (36). Of course, other bacteria and fungi may invade and establish in the larval gut microbiome, but perhaps they are not as integral to the host from a diet exploitation perspective since older larvae can more readily overcome negative dietary components. By evaluating the role of the gut microbiome in older lepidopteran larvae, studies may be inadvertently overlooking or diminishing crucial interactions. Simultaneously, much of the microbial variation observed in older larvae may not be important to the larvae at that point in development. However, these hypotheses are speculative and require further experimental attention.

A major question that emerges from our study is: why do the same isolates facilitate fall armyworm digestion but exhibit antagonistic behaviors under other circumstances? Based on our results here, the host's resources may be shunted to the microbiota or redirected to host immune responses (43, 54). Either way (or a combination of both), host utilization of diet drives these dynamics and ultimate costs and benefits. How gut bacteria enable fall armyworm to exploit a suboptimal pinto bean diet is also currently unknown. Our findings do not appear to be an artifact of extended laboratory rearing. All isolates used in this study originated from field-collected larvae that never experienced larval diet rearing before, indicating strains occupying the fall armyworm gut possess this feature even in natural larval populations. Perhaps this is not surprising, as there is a large amount of strain variation even among taxonomically simple insect gut microbiomes (20). Overall, it may be more important that an isolate contributes a metabolic function than its originating source.

Despite the level of interest lepidopteran microbiomes have received with metabarcoding sequencing approaches, details on microbial functions are very limited and not well-documented (31, 55). Our study helps address this deficiency, but there are some limitations to our experiments and how it relates to some concepts in host-microbe interactions, particularly phylosymbiosis (56). For instance, we did not explicitly perform reciprocal microbial transplants between larval species (57–59) or with novel non-host isolates, which would help address whether there are adaptions between the gut microbiota and their respective hosts. 16S-rRNA sequencing indicates that the beet armyworm harbors *Enterococcus* as part of its gut microbiome (32), but we do not know how different isolates would compare to those used in our study. Further isolations, whole genome sequencing, meta-transcriptome approaches, and reintroduction of *Enterococcus* strains would better elucidate the mechanisms underlying the pervasiveness and functions of these bacterial genera in lepidopteran larvae.

Exploiting a sterile semi-artificial diet, our results detail that strong positive and antagonistic gut bacterial interactions persist among lepidopteran larvae. Our study supports the concept that bacteria can expand insect dietary breadth, but there are caveats. Pertinent to the magnitude and directionality of these partnerships is the hosts' own ability to utilize diets. Not only can responses vary between isolates, but the timing in larval development is also critical. We only have a nascent understanding of lepidopteran-gut microbiome interactions, and *Spodoptera* may provide an accessible, tractable experimental system to tease apart governing principles in comparative contexts. Determining mechanisms that facilitate establishment or

enable larvae to manage detrimental populations are important steps forward. For instance, a major question to be addressed is how different life histories resulted in the discrepancy between fall- and beet armyworm's associations with gut microbiota? However, as illustrated by our study, how broadly those conclusions can be applied may unfortunately be narrower than in other instances.

MATERIALS AND METHODS

Insect sources, bacterial isolates, and diet formulations. Fall armyworm, beet armyworm, corn earworm, and black cutworm eggs were obtained from colonies maintained by Benzon Research Laboratories (Carlisle, PA, USA). Rice-adapted fall armyworm colonies initially were collected from rice and eggs were kindly provided by Dr. Michael Stout at Louisiana State University. All bacterial strains were isolated from fall armyworms and recovered from storage at −80°C by inoculation of 2× yeast tryptone (YT) agar plates prior to experiments (25). Bacterial strains were initially identified using two methods: Bruker Biotyper MALDI-TOF MS (Bruker Daltonics, Billerica, MA, USA) and near-full length 16S rRNA gene sequencing (25). *Enterococcus* isolates were selected due to previously reported fall armyworm-pinto bean diet interactions (35), and *Enterobacter* isolates through preliminary screens of fall armyworm performance (Fig. S4). Pinto bean diet was formulated as previously described (36, 38) (Table S1), with dilution modifications done using non-nutritive cellulose filler on a percent weight basis (35). Wheat germ diet was also formulated as described previously with no modifications (39) (Table S2). Strong antibiotics (i.e., streptomycin) that are normally included in such artificial diets were excluded. All diets were autoclaved before feeding to larvae and a portion was plated to ensure sterility.

Sterilization procedures, growing conditions, inoculation protocols. Sterilization of eggs to generate initially axenic larvae followed procedures previously described (28). First, eggs were immersed in 4% bleach with light agitation followed by sterile water rinses. Eggs were air dried in a biosafety cabinet, then transferred to an autoclaved 250 mL polypropylene container without any food. Larvae were maintained in a 28°C growth chamber under a 16:8 h Light:Dark regime for all experiments. Bacterial inocula were generated from glycerol stocks propagated on solid media and bacteria were grown in 2× yeast-tryptone broth overnight. Cells were pelleted, resuspended in phosphate-buffered saline (PBS) pH 7.0, and diluted at previously determined concentrations related to OD_{600} measurements. For all but one experiment, neonate larvae were inoculated with bacteria on the specific diet treatment.

Rearing cups (22.5 mL) were surface sterilized with 70% ethanol and by placing under UV light for 30 min. Bacteria was introduced by applying 20 μL of cells onto an ~0.5 cm diet cube to achieve an inoculation dose of 10^7 CFU as determined by previously determined OD_{600} concentrations for the isolates. Newly hatched larvae from the sterilization procedure described above were transferred to inoculation cups within 24 h of hatch. Larval groups (~5–10 individuals per cup) were placed onto each treatment and allowed to feed for 72 h. For each experiment, 10 inoculation cups were designed in an array to provide a potential pool of >50 individuals to select from. For each experiment, single larvae were randomly selected and transferred to a new container where they were allowed to feed in isolation. New cups contained a fresh diet and no bacterial inoculum. One to two larvae were selected from each rearing cup, except when noted below. The replicate for the experiments we describe below are therefore individual larvae. If any larvae were visibly damaged or killed in the transfer process, they were removed from the experiment. Diets were replaced every 5 days for 2 weeks and larvae were fed *ad libitum*.

Bioassays. Larvae were obtained at different intervals over the course of a year, so each experiment was performed as separate comparisons. We initially established a level of consistency of the axenic treatments, and therefore performed permutations to evaluate different components of the host-microbe-diet interactions. All diets were made fresh for each set of experiments and portioned among larval species equally to eliminate any technical biases in the diet making process. While all the insects we used are in the same family, they have different growth rates and allometry patterns that make it challenging to make explicit comparisons. As such, each bioassay that compared different insect species and bacterial isolates was conducted simultaneously and is not explicitly (statistically) compared with each other across time.

All neonate experiments followed the same procedure, albeit with different diet and bacterial permutations. To determine the effect of diet diluted with cellulose on larval growth and pupal development, axenic fall armyworm larvae were inoculated with *Enterococcus* FAW2-1. Larvae (n = 13–15) were weighed at 10 days post-hatch (dph).

Initially, the effects of living and heat-killed *Enterococcus* on fall and beet armyworm performance were determined by autoclaving bacteria prior to inoculating insects. Larvae were weighed (n = 9–10) 10 dph. A separate experiment was performed to determine impacts of *Enterococcus* FAW2-1 on pupation, where 4–5 larvae were selected from each rearing cup. Axenic fall armyworm (n = 40), inoculated fall armyworm (n = 49), axenic beet armyworm (n = 28), and inoculated beet armyworm (n = 38) were reared until pupation after which larval duration (days to pupation), pupal mass, and pupal success were measured. Growth of fall and beet armyworm over time was determined by weighing individuals (n = 5–7) on an ultramicrobalance. For the first 10 days, individual larvae were weighed daily to minimize the risk of environmental contamination and accidental sample loss. Subsequently, the same samples were weighed daily until the onset of axenic beet armyworm pupation. Body masses of axenic and *Enterococcus* FAW2-1-inoculated fall and beet armyworm (n = 9–10) on wheat germ diet was determined 10 dph.

Determination of initial bacterial dose on fall (n = 14–15) and beet armyworm (n = 12–15) body mass accumulation was accomplished by applying serial dilutions of *Enterococcus* FAW2-1 onto a pinto bean diet (10^3–10^7 CFU) for neonate larvae to consume. At 14 dph, insects were weighed and microbial titers of axenic larvae and those receiving 10^3 and 10^4 CFU doses were determined from a randomly selected subset (n = 9). Briefly, larvae

were surface sterilized in 70% ethanol for 15 s, dried on paper towels, and had their guts dissected and homogenized. Due to their small size, axenic fall armyworm larvae were not dissected but instead, the whole body was crushed. Homogenates were serially diluted in PBS, plated on 2×YT medium, and incubated until CFUs were enumerated.

The effects of unique *Enterococcus* and *Enterobacter* isolates on fall and beet armyworm performance were determined 14 dph ($n = 8$–10). CFU titers were determined as described above for fall armyworm ($n = 8$) and beet armyworm ($n = 6$–8). Larvae that weighed <10 mg were subjected to whole body homogenization while larger larvae had their guts dissected.

Effects of *Enterococcus* FAW2-1 and *Enterobacter* PRS10-1 (for each isolate $n = 8$–9) isolates on fall armyworm (Benzon and rice strain), beet armyworm, corn earworm, and black cutworm were measured 10 dph. Host responses to the isolates were compared for each larval species to those with axenic status ($n = 9$–10) and larvae from eggs which were not sterilized (conventional; $n = 15$). Masses were determined 16 dph.

Statistical analyses. Statistical analyses were performed with GraphPad Prism (v. 9.3.1). Assumptions of normality and heteroscedasticity were evaluated and data were transformed as necessary. Growth and developmental data between treatments and bacteria within a larval species were analyzed using Welch's t-tests and analysis of variance (ANOVA) with *post hoc* comparisons performed with a Tukey HSD (honestly significant difference) adjustment. Pupation success rate was analyzed using χ^2 tests. Regressions between insect microbial titer and insect growth were evaluated using $\log_{10}(y + 1)$ transformed CFU values.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

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C.J.M., K.H., and G.W.F. determined the overall objectives; C.J.M. and B.C. designed the experiments; C.J.M. and M.P. performed the experiments and data collection; C.J.M. analyzed the data; C.J.M. wrote the first manuscript draft; M.P., B.C., K.H., and G.W.F. provided editorial guidance.

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