An Indigenous Gut Bacterium, Enterococcus faecalis (Lactobacillales: Enterococcaceae), Increases Seed Consumption By Harpalus pensylvanicus (Coleoptera: Carabidae)

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AN INDIGENOUS GUT BACTERIUM, *ENTEROCOCCUS FAECALIS* (LACTOBACILLALES: ENTEROCOCCACEAE), INCREASES SEED CONSUMPTION BY *HARPALUS PENSYLVANICUS* (COLEOPTERA: CARABIDAE)

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ABSTRACT

Microbial symbioses likely drive the evolution of diet within animals, yet these symbiotic relationships remain poorly understood for many organisms. The bacterial endosymbiont *Enterococcus faecalis* is found in the intestinal tract of the beetle *Harpalus pensylvanicus* (DeGeer) (Coleoptera: Carabidae) and is thought to contribute to the digestion of the insect's seed diet. We tested whether *E. faecalis* increases seed consumption by *H. pensylvanicus*. The feeding assay consisted of 4 dietary treatments fed: 1) antibiotics and *E. faecalis*; 2) antibiotics and no *E. faecalis*; 3) no antibiotics and *E. faecalis*; and 4) no antibiotics and no *E. faecalis*, in which seed consumption of the beetles was measured. Beetles administered antibiotics and then *E. faecalis* consumed greater weights of seeds and had both decreased efficiency of conversion of ingested material to biomass (E.C.I.) per beetle and decreased efficiency of conversion of digested material (E.C.D.) to biomass per beetle. These data provide further evidence that a gut microbiota dominated by *E. faecalis* facilitate seed consumption by *H. pensylvanicus*, possibly by contributing digestive enzymes to their host. Further research is needed on the evolutionary relationship between *E. faecalis* and granivorous insects, and on how these facultative symbioses could influence the trophic placement of animals within complex food webs.

Key Words: bacteria, Carabidae, *Chenopodium album,* granivore, seed predation, symbiont

RESUMEN

La simbiosis microbial probablemente impulsa la evolución de la dieta dentro de los animales, sin embargo, estas relaciones simbióticas siguen siendo poco conocidas para muchos organismos. Se encuentra la bacteria endosymbionte *Enterococcus faecalis* en el tracto intestinal del escarabajo *Harpalus pensylvanicus* (DeGeer) (Coleoptera: Carabidae) y se cree que contribuyen a la digestión de la dieta de semillas de este insecto. Probamos si *E. faecalis* aumenta el consumo de semillas de *H. pensylvanicus*. Esto fue probado a través de un ensayo de alimentación de laboratorio que consistió de un tratamiento con antibióticos, para eliminar la flora intestinal natural, y un tratamiento con *E. faecalis*. El ensayo de alimentación consistió en 4 tratamientos dietéticos alimentados con 1) antibióticos y *E. faecalis* 2) antibióticos y no *E. faecalis*, 3) no antibióticos y no *E. faecalis* y 4) antibióticos y no *E. faecalis*, en el que se midió el consumo de semillas de los escarabajos. Los escarabajos que recibieron los antibióticos y luego *E. faecalis* consumieron un peso mayor de semillas y tenían una eficacia disminuida de la conversión de los alimentos ingeridos a la sustancia corporal (ECI) y una eficiencia de conversión del alimento digerido a la sustancia corporal (ECD). Estos datos proveen más evidencia de que una microbiota intestinal dominada por *E. faecalis* facilita el consumo de semillas por *H. pensylvanicus,* posiblemente contribuyendo enzimas digestivas para su hospedero. Se necesita más investigación sobre la relación evolutiva entre *E. faecalis* y los insectos granívoras y cómo estas simbiosis facultativas podrían influir en la colocación trófica de los animales dentro de las redes alimentarias complejas.

Palabras Clave: bacterias, Carabidae, *Chenopodium album*, granívoro, depredación de semillas, simbionte

Trophic placement of an organism is important to understanding its evolutionary history, phylogenetic relatedness to other organisms, and how it coexists with other members of its community.

One factor that influences the trophic behavior of an organism is its symbiotic relationships with beneficial microbes. Certain endosymbionts contribute a range of nutritional benefits to their host. In insects, these contributions include synthesis of essential nutrients, production of vitamins and sterols, processing of foods such as cellulose, food detoxification, and determination of food utilization (Douglas 2009). Often when an insect's diet lacks essential nutrients, endosymbionts play pivotal roles in providing the host with the limited nutrients. Blood-feeding insects are a good example of insects living on a nutrient-limited diet in which endosymbionts supplement essential vitamins for the host's survival (Pais et al. 2008; Hosokawa et al. 2010). This phenomenon of nutritional upgrading is not exclusive to blood-feeding insects. In the ominivorous carpenter ant *Camponotus floridanus* (Buckley) (Hymenoptera: Formicidae) the gut endosymbiont *Blochmannia floridanus* provides essential amino acids thought to contribute to the ant's nutrition when those amino acids cannot be found in the environment (Feldhaar et al. 2007). A major problem that omnivorous insects face is breaking down and digesting tough plant material. In the cricket *Acheta domesticus* (L.) (Orthoptera: Gryllidae), the gut microbial community helps to digest carbohydrate oligomers and polymers, leading to faster growth rates, quicker maturation, larger adult body size, and greater egg production (Kaufman & Klug 1991).

Seeds are a common part of many insects' diet. Insects have developed a number of adaptations to consume and digest seeds. Some key adaptations that determine rate of granivory could be categorized as morphological, physiological, or enzymatic (Lundgren 2009). Mandibular structure and size is one key morphological adaptation which determines seed consumption. For example granivorous/herbivorous carabids have evolved a large retinacular ridge on their mandible, which is used to grind seeds (Acorn & Ball 1991). Also, the mandibles of granivorous carabids tend to be more quadrate in shape than those found in predaceous species, and they are often asymmetric (Acorn & Ball 1991; Arndt & Kirmse 2002). Also, the psamnophore in harvester ants is a morphological adaptation used to carry seeds (Brown et al. 1979). Size of the insect also affects seed selection; all else being equal, insects prefer to eat the largest seeds they can physically consume to maximize their foraging efficiency (Lundgren 2009). Omnivorous insects have also adapted features within their intestinal tract to destroy the hard portions of the seed that could damage the midgut; the adaptations these insects have developed include sharp raduli covering the anterior portion of the proventriculus, thought to better destroy the hard seeds and protect the mid- and hindgut (Forsythe 1982). Enzymes play a key role for the digestion of seeds by many insects. Some insects regurgitate fluids which contain enzymes that break down the seed, allowing the insect to consume the emulsified liquid (Woodring et al. 2007). Many cellulose-feeding Coleoptera digest much of the material in the midgut by using specialized enzymes and elevated pH (Terra 1990). Symbiotic bacteria and fungi are often a source of the digestive enzymes found in the intestinal tract of insects.

Harpalus pensylvanicus (DeGeer) (Coleoptera: Carabidae) is an omnivorous carabid commonly found in cropland throughout North America, benefitting agricultural producers by consuming insect pests and weed seeds (Eskelson et al. 2011; Ward et al. 2011). This beneficial insect harbors a relatively simple gut community of bacterial populations (Lundgren et al. 2007; Lehman et al. 2009; Lundgren & Lehman 2010). The bacterial populations residing within the gut of *H. pensylvanicus* are considered facultative or secondary endosymbionts, as opposed to obligate or primary symbionts. Within that endosymbiont community, the bacterium *Enterococcus faecalis* (Lactobacillales: Enterococcaceae) has been isolated (Lundgren & Lehman 2010). *Enterococcus faecalis* is a widely distributed bacterium found in a variety of environments including animals, humans, and food (Klein 2003; Getachew et al. 2013). *Enterococcus faecalis* is commonly associated with the gastrointestinal tract of their host organism, and has been observed as being both a pathogenic and beneficial symbiont (Sava et al. 2010). Within *H. pensylvanicus*, *E. faecalis* is hypothesized to be responsible for increased seed consumption by the beneficial beetle (Lundgren & Lehman 2010). We used a combination of antibiotic treatment and inoculation with *E. faecalis* to directly test if the endosymbiont *E. faecalis* is responsible for the increased seed consumption by *H. pensylvanicus*.

Materials and Methods

Beetles

Harpalus pensylvanicus used in the experiment were collected from crop land and surrounding areas in Brookings, South Dakota. The collected beetles were kept in a colony at 27 °C and 16:8 h L:D. The beetles were raised in clear plastic containers, n ≤ 25 per container, in damp soil which consisted of a 4:2:1 mixture of field soil from the local site, peat moss (Spagnum Peat Moss, Waupaca Northwoods LLC, Waupaca, Wisconsin), and vermiculite (Vermiculite, Therm-O-Rock West Inc., Chandler, Arizona). The beetles were fed cat food (Iams Original with Chicken Proactive Health, The Iams Company, Cincinnati, Ohio). Active *H. pensylvanicus* from the colony that visually lacked external, saprophytic fungal infections were advanced for the assay.

Enterococcus faecalis

Intestinal tracts of 10 field-collected *H. pensylvanicus* were aseptically dissected, homogenized with a sterile mortar and pestle, suspended in phosphate buffer, and spread onto m-Enterococcus selective growth medium (Difco m-Entercoccus Agar, Becton, Dickinson and Company, Sparks, Maryland). Agar plates were incubated at 35 °C for 72 h. Morphologically-distinct colonies were selected and restreaked until purified. DNA was extracted from isolated colonies using DNeasy Blood and Tissue kit (DNeasy Blood and Tissue Kit, Catalog No. 69506, Qiagen Sciences, Germantown, Maryland) per manufacturer's instructions for gram positive bacteria. DNA extractions were screened on 0.7% agarose gel (100V, 25 min). The 16S rRNA genes from these isolates were PCR-amplified using the eubacterial primers 8F (5' – AGAGTTTGATCCTGGCTCAG – 3') and 1492R (5' – GGTTACCTTGTTACGACYT – 3') with the reaction mixture and PCR conditions described in Lundgren and Lehman (2010). PCR products were screened on 1.2% agarose gel (75 V, 45 min) with positive (*E. coli* DNA) and negative (reagents only) controls and then purified using Wizard PCR Preps DNA Purification System (Promega). The PCR products were sequenced at the Iowa State DNA Sequencing Facility using the eubacterial primers 8F, 530F (5' – GTGCCAGCMGCCGCGG – 3'), and 1100F (5' – GCAACGAGCGCAACCC – 3'). Edited, assembled, aligned, and chimera-checked 16S rRNA sequences were compared to entries in GenBank database using BLASTn to determine the closest match. Two distinct phylotypes resulted from these isolations, a strain (Egut10) that closely matched (>99.5% similarity) *Lactococcus garvieae*, and a strain (Egut6) that closely matched (>99.5% similarity) members of the *Enterococcus faecalis* group as described by Byappanahalli et al. (2012). The *E. faecalis* isolate (Egut6, GeneBank Accession # KF178438) was used for inoculations of *H. pensylvanicus* in the feeding assay.

Feeding Assay

As seen in Fig. 1, *H. pensylvanicus*, n = 160, were placed individually into clean Petri dishes (100 × 20 mm, Falcon BD Optilux No. 351005, Fisher Scientific, Pittsburgh, Pennsylvania) containing only a water-saturated cotton wick. The beetles were starved for 24 h at 27 °C, after which they were weighed to the nearest 0.01 mg and placed into seed dishes containing lambsquarter (*Chenopodium album* L.; Caryophyllales: Amaranthaceae) seeds for a pre-treatment assessment of seed feeding. The seeds were presented on double sided tape stuck to the bottom of the Petri dish; non-sterile silicon sand covered the exposed areas of the tape to prevent the insects from becoming stuck. Two sets of seed dishes were created to examine the number and weights of seeds consumed. Seed dishes (n = 10 per treatment) each contained 150 undamaged lambsquarter seeds; these seeds were later inspected for damage after the assay (see below). The remaining 120 seed dishes (n = 30 per treatment) contained 0.06 g of uninspected lambsquarter seeds. *Harpalus pensylvanicus* were exposed to the seeds for 24 h, after which they were weighed again and placed into Petri dishes containing a water-saturated cotton wick and diet (Appendix 1) (Lundgren et al. 2005).

To eliminate the gut microbiota, 2 cohorts (n = 40 each; each beetle was housed individually) received diet containing the antibiotics erythromycin, tetracycline, and rifampicin. The 2 other cohorts received the same diet but without the antibiotics. Fresh diet was given daily over 10 days. After this period *H. pensylvanicus* were placed into Petri dishes containing a cotton wick saturated in 1 of 2 aqueous 20% sucrose-solutions (w/v). Beetles in 2 treatments (one fed antibiotic diet, and one not) received cotton wicks saturated in 20% sucrose-solution containing *E. faecalis*. The cotton wicks were placed in 0.5 mL microcentrifuge tubes (Seal Rite Natural Microcentrifuge

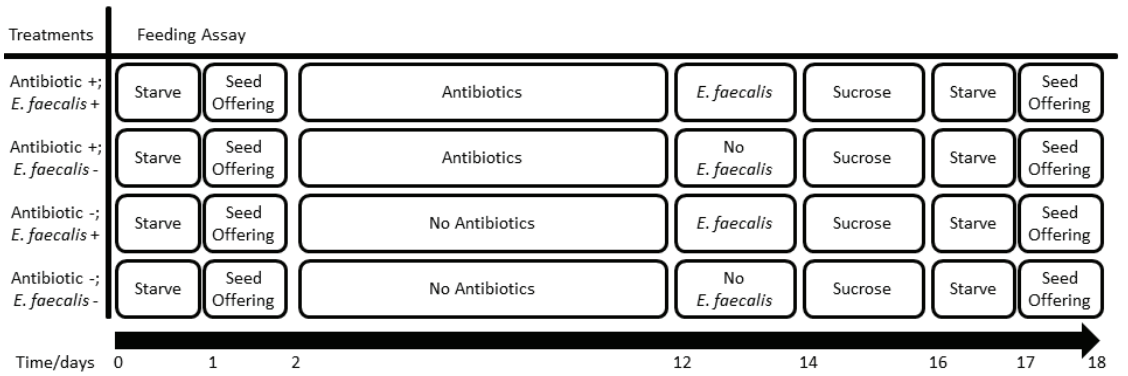

Fig. 1. Diagram outlining the treatment structure of the feeding assay.

tubes Catalog No. 1615-0000, USA Scientific, Ocala, Florida) containing sugar water and *E. faecalis* mixture with the end of the wick sticking out of the microcentrifuge tube. The sucrose-solution contained 2×10^8 *E. faecalis* cells mL^{-1}. The other 2 treatments (one fed antibiotic diet, and one not) were fed 20% sucrose-solution without *E. faecalis*. The cotton wicks containing *E. faecalis* were offered for 2 days. After this period, cotton wicks saturated in 20% sucrose-solution were offered to all treatments for an additional 2 days. Beetles were then starved for 24 h, and weighed. Then for a second time beetles were placed individually into seed dishes (as above) for 24 h, and reweighed. Thus, 4 treatment cohorts were created hereafter referred to as antibiotic +, *E. faecalis* +; antibiotic +, *E. faecalis* -; antibiotic -, *E. faecalis* +; and antibiotic -, *E. faecalis* -. At the end of the experiment, the intestinal tracts of randomly selected *H. pensylvanicus* (n = 31 Antibiotic +, *E. faecalis* + and Antibiotic -, *E. faecalis* - treatments; n = 30 Antibiotic +, *E. faecalis* - and Antibiotic +, *E. faecalis* + treatments) were dissected under sterile conditions and placed into 1 mL of 1 × PBS. The parts of gut that were dissected were crop, proventriculus, midgut, and hindgut; these gut samples were examined for the presence of *E. faecalis* as described below.

The weight of seeds consumed during each seed dish feeding, and weight of feces produced during the 24 h period by each *H. pensylvanicus* was recorded. Additionally, the number of inspected lambsquarter seeds eaten by each beetle was recorded in those administered the dishes with 150 seeds each. Using these measurements, efficiency of conversion of ingested food to body substance (E.C.I.), approximate digestibility (A.D.), and efficiency with which digested food is converted to body substance (E.C.D.) were calculated using the formulas of Waldbauer (1968).

Several metrics were used to ensure that all randomly assigned treatment groups were equivalent prior to experimentation. All 4 treatments consumed similar numbers of seeds ($F_{3, 155} = 1.89$, $P = 0.13$), and had similar ECI ($F_{3, 155} = 1.13$, $P = 0.34$), AD ($F_{3, 155} = 1.21$, $P = 0.31$), and ECD ($F_{3, 155} = 0.86$, $P = 0.46$). Sex ratios (proportion male) were similar in the 4 treatments ($\chi^2 = 1.61$; $P = 0.66$); treatments had 0.43, 0.33, 0.30, and 0.38 proportion male in the antibiotic +, *E. faecalis* +; antibiotic +, *E. faecalis* -; antibiotic -, *E. faecalis* +; and antibiotic -, *E. faecalis* -.

Examination of *H. pensylvanicus* for the Presence of *E. faecalis*

Within 24 h of dissection, individual guts were processed and spread on m-Enterococcus growth media as described above. The m-Enterococcus growth media was chosen due to its selectivity for *E. faecalis*, which made it highly useful for the purposes of examining *H. pensylvanicus* for the presence of *E. faecalis*. Using a less selective media or next generation sequencing may have revealed additional microbiota members. For each gut, morphologically-distinct colonies were identified. Three to 6 replicates of each morphologically distinct colony type were streaked for isolation.

DNA was extracted and 16S rRNA genes were amplified and sequenced from isolated colonies as previously described. 16S rRNA sequences were compared to entries in GenBank database using BLASTn to determine the closest match (> 99.5% similarity). The closest matching phylotypes were recorded to determine the bacterial community in each cohort and to determine the effect of treatment on *E. faecalis* presence in the gut.

Data Analysis

The number of seeds consumed, weight of seeds consumed (adjusted for body mass of the beetle), E.C.I., A.D., and E.C.D. (feeding metrics were all log-transformed) were compared among treatments using two-way ANOVAs (with treatments receiving antibiotics and *E. faecalis* as factors in the analysis). Data from beetles that died during the seed dish feeding were excluded, which left 151 beetles at the end of the experiment. A similar number of beetles died from each treatment, ranging from 2 to 3 beetles per treatment. Statistical significance for P-value was set at $\alpha = 0.05$, and a marginally significant P-value was set at $\alpha = 0.10$.

Results

Effects of *E. faecalis* Treatment on Seed Consumption

The difference in the seed weight consumed was marginally significant between beetles fed *E. faecalis* or not (antibiotic: $F_{1, 147} = 7.72$, $P = 0.01$; *E. faecalis*: $F_{1, 147} = 3.18$, $P = 0.08$; antibiotic × *E. faecalis*: $F_{1, 147} = 3.04$, $P = 0.08$) (Fig. 2). The marginally significant interaction term was caused by the different responses observed in the treatments that received antibiotics versus no-antibiotics. Within the antibiotic-fed treatments, the sub-treatment fed *E. faecalis* consumed a greater weight of seeds (0.41 ± 0.05 g) than the treatment that received no *E. faecalis* (0.26 ± 0.04 g). There was no difference between the treatments receiving *E. faecalis* or not when antibiotics were not administered to the beetles. The *E. faecalis* fed treatments had a lower E.C.I. (2.38 ± 0.15) than the treatments that received no *E. faecalis* (2.82 ± 0.15) (antibiotic: $F_{1, 147} = 2.87$, $P = 0.09$; *E. faecalis*: $F_{1, 147} = 4.41$, $P = 0.04$; antibiotic × *E. faecalis*: $F_{1, 147} = 0.83$, $P = 0.36$) (Fig. 3A). The antibiotic treatments also had a lower E.C.I. (2.42 ± 0.15) than the treatments fed no antibiotics (2.77 ± 0.15), but this difference was only marginally signifi-

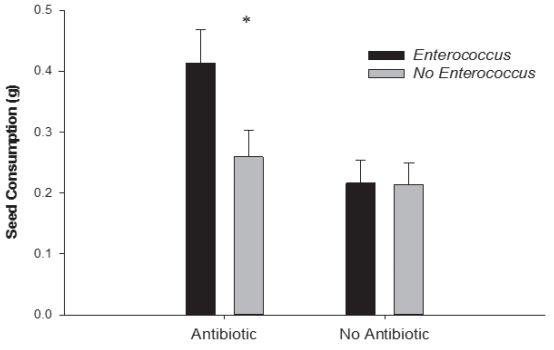

Fig. 2. The effects of *Enterococcus faecalis* on seed weight consumed per beetle by *Harpalus pensylvanicus*. The asterisk denotes significant differences within these antibiotic or no antibiotic treatments ($\alpha = 0.05$). Antibiotic +, *E. faecalis* + n = 58; Antibiotic +, *E. faecalis* - n = 58; Antibiotic -, *E. faecalis* + n = 58; and Antibiotic -, *E. faecalis* - n = 57.

cant (Fig. 3A). The *E. faecalis* fed treatments had a lower E.C.D. (2.39 ± 0.15) than the treatments that received no *E. faecalis* (2.85 ± 0.16) (antibiotic: $F_{1, 147} = 3.09$, $P = 0.08$; *E. faecalis*: $F_{1, 147} = 4.59$, $P = 0.03$; antibiotic × *E. faecalis*: $F_{1, 147} = 0.68$, $P = 0.41$) (Fig. 3B). Again, the antibiotic treatments had a lower E.C.D. (2.43 ± 0.15) than the treatments fed no antibiotics (2.81 ± 0.16), but this difference was only marginally significant (Fig. 3B). All treatments had similar A.D.s of seeds (antibiotic: $F_{1, 147} = 1.50$, $P = 0.22$; *E. faecalis*: $F_{1, 147} = 1.14$, $P = 0.29$; antibiotic × *E. faecalis*: $F_{1, 147} = 0.67$, $P = 0.41$) and consumed similar numbers of seeds (not weight of seeds) (antibiotic: $F_{1, 33} = 0.22$, $P = 0.65$; *E. faecalis*: $F_{1, 33} = 1.24$, $P = 0.27$; antibiotic × *E. faecalis*: $F_{1, 33} = 0.17$, $P = 0.68$). Beetle wet weights (mean ± SEM) were 141 ± 4, 146 ± 4, 143 ± 4 and 139 ± 5 mg for the antibiotic +, *E. faecalis* +; antibiotic +, *E. faecalis* -; antibiotic -, *E. faecalis* +; and antibiotic -, *E. faecalis* – treatments, respectively. Feces dry weights were 0.41 ± 0.07, 0.53 ± 0.08, 0.52 ± 0.07, and 0.64 ± 0.08 mg for the antibiotic +, *E. faecalis* +; antibiotic +, *E. faecalis* -; antibiotic -, *E. faecalis* +; and antibiotic -, *E. faecalis* - treatments, respectively.

Recovery of *E. faecalis* from Treated Beetles

The different treatments had an effect on *E. faecalis* recovered on the selective media. The 2 bacterial DNA sequences from the antibiotic +, *E. faecalis* + treatment were identified as *E. faecalis*. No DNA sequences were obtained from the antibiotic +, *E. faecalis* - treatment. The antibiotic -, *E. faecalis* + had 2 bacterial colonies identified as *E. faecalis*, 2 colonies most closely matching *Enterococcus* sp., and 1 colony most closely matching *Stenotrophomonas* sp. Lastly, the antibiotic -, *E. faecalis* - treatment contained 1 bacterial colony that

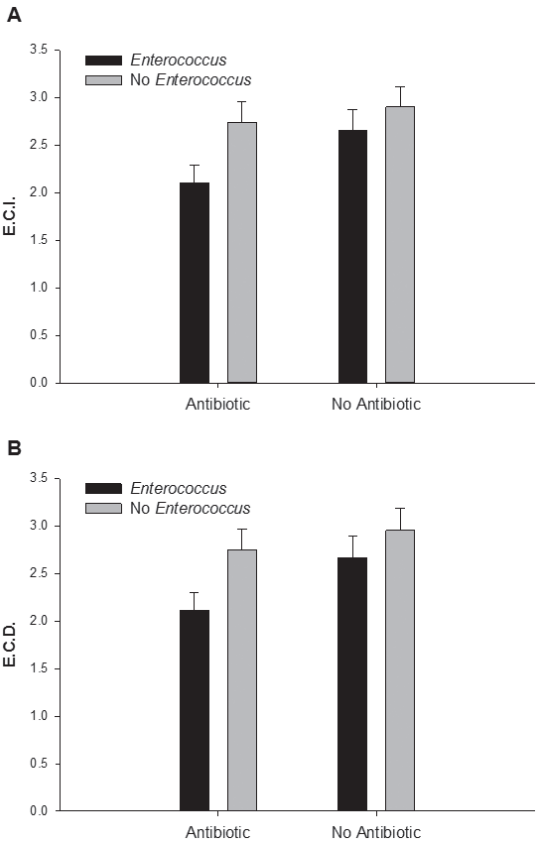

Fig. 3. The effects of *Enterococcus faecalis* and antibiotic treatment on A. efficiency of conversion of ingested material (E.C.I.) to biomass and B. efficiency of converted digested material (E.C.D.) to biomass per beetle of *Harpalus pensylvanicus*. Antibiotic +, *E. faecalis* + n = 58; Antibiotic +, *E. faecalis* - n = 58; Antibiotic -, *E. faecalis* + n = 58; and Antibiotic -, *E. faecalis* - n = 57.

matched *E. faecalis* and 1 bacterial colony that matched an *Enterococcus* sp. As can be seen from the DNA sequencing results, it appears as though our antibiotic and *E. faecalis* treatments produced the desired effects. The antibiotics reduced gut microbiota as the antibiotic +, *E. faecalis* - treatment produced no DNA sequences, and the antibiotic +, *E. faecalis* + treatment only produced *E. faecalis* which was expected. Also, the antibiotic -, *E. faecalis* + treatment contained several bacterial species along with *E. faecalis* which was the intended result. Finally the antibiotic -, *E. faecalis* - treatment provided an unaltered gut community to study.

Discussion

Our research supports the hypothesis that facultative symbioses between gut bacteria and insects can have important implications for insect diet consumption rates, but that these relation-

ships may be more complicated than was previously proposed. Treating beetles with antibiotics (i.e., antibiotic +) followed by the facultative symbiont *E. faecalis* increased the weight of seeds consumed and decreased the E.C.I. and E.C.D. In contrast, beetles that had their gut endosymbiont community intact (i.e., antibiotic -) when *E. faecalis* was administered did not consume additional seeds. Lack of increased seed consumption (by weight) by antibiotic -, *E. faecalis* + beetles could have been caused by several reasons, such as suppression of *E. faecalis* colony establishment by the existing gut microbiota, endosymbiont competition, or niche overlap within the gut bacteria. Another result, which was unexpected, was that administering solely antibiotics (antibiotic +, *E. faecalis* - treated beetles) did not reduce seed consumption relative to the treatments receiving no antibiotics (i.e., antibiotic -, *E. faecalis* -), a frequently observed result in this study system (Lundgren & Lehman 2010). One possible explanation for this discrepancy in the current work is that pathogens or parasites in the antibiotic-free treatment may have lowered the seed consumption levels of the host; antiobiotics may have cured the pathogens from the guts, but also eliminated the beneficial microbes that encourage seed consumption, thus leading to equivalent seed consumption rates in these 2 treatments. Additional research that explores the complexities of these interactions under different scenarios may help to shed light on why we did not observe reduced seed consumption in antibiotic-treated beetles as expected.

The *E. faecalis* treatment with suppressed microbiota (antibiotic +, *E. faecalis* +) in the guts was correlated with an increase in weight of seed consumption and decreased E.C.I. and E.C.D. by *H. pensylvanicus.* E.C.I is described as the ability of an insect to utilize ingested food for growth, and E.C.D. is the efficiency that digested food is converted to body substance (Waldbauer 1968). A decrease in E.C.D. means that a larger proportion of digested food is being metabolized for energy (Waldbauer 1968). So, the decrease in E.C.I. and E.C.D. of *E. faecalis* fed beetles is simply due to the fact that the beetles are devoting less energy to support growth and more energy to support activity and maintaining physiological function (Waldbauer 1968). With E.C.I. and E.C.D. being tightly correlated with each other, the question then becomes what caused the weight of seed consumption to increase? The specific mechanism underlying increased weight of seed consumption is unclear at this point, but a potential answer is that *E. faecalis* complemented the digestive capacity of *H. pensylvanicus.* Microbial symbionts provide a variety of functions for the digestion of their host's diet. These functions include the provision of many essential nutrients such as nitrogen, amino acids, vitamins, and sterols (Douglas 1998; Nasir & Noda 2003; Douglas 2009; Snyder et al. 2010). Symbionts also provide protection from pathogens, provision of antibiotics, and digestive enzymes (Currie et al. 1999; Lundgren 2009; Vorburger et al. 2010). Perhaps *E. faecalis* complemented the digestive capacity of *H. pensylvanicus* by contributing enzymes that break down the unique nutritional components of seeds. Cellulose is a common component of seeds, and there are many gut endosymbionts which produce digestive enzymes that break down cellulose (Kukor & Martin 1983; Sinsabaugh et al. 1985; Kukor et al. 1988). The endosymbionts contributing to cellulose breakdown within the intestinal tract often originate within certain phylogenetic clades. The common cellulase-producing endosymbionts found in these organisms often fall in the classes of Clostridia and Bacilli (Hongoh 2010; Huang et al. 2010; Zhu et al. 2011). *Enterococcus faecalis* belongs to the class Bacilli, and it is feasible and testable that this symbiont could contribute to seed digestion by producing cellulolytic enzymes. *Enterococcus faecalis* has been found within the gut microbial community of insects such as gypsy moth larvae, green stink bugs, termites, and silkworms (Lu et al. 1994; Bauer et al. 2000; Hirose et al. 2006; Allen et al. 2009). These insects primarily consume leaves or other plant material which are high in cellulose. According to Broderick et al. (2004), *E. faecalis* is an essential endosymbiont to gypsy moths (*Lymantria dispar* [L.]; Lepidoptera: Erebidae) which primarily consume tree leaves. This is not to say that cellulolytic enzymes are the only explanation for the increased weight of seed consumption by *H. pensylvanicus*, as there are numerous other services that symbionts provide which may contribute to increased seed consumption such as improving food utilization or food detoxification (Douglas 2009). However, based on the results of previous studies where *E. faecalis* was closely associated with herbivorous insects and the fact that *E. faecalis* is a member of a taxa known for providing enzymes makes the hypothesis of cellulolytic enzymes production by *E. faecalis* one potential theory worth further investigation.

Interactions with the existing microbiota may affect the ability of *E. faecalis* to increase seed consumption by *H. pensylvanicus*. In treatments where *E. faecalis* was present along with the endemic gut bacteria (antibiotic -, *E. faecalis* +), weight of seed consumption did not increase over the control treatment that did not receive *E. faecalis* (antibiotic -, *E. faecalis* -) (Fig. 2); this is in contrast to the treatment with their endemic gut bacteria reduced that received *E. faecalis* (antibiotic +, *E. faecalis* + vs antibiotic +, *E. faecalis* -) (Fig. 2). In the antibiotic -, *E. faecalis* + treatment, *E. faecalis* may have not been able to establish or compete well with the existing microbiota, as microbes deploy various mechanisms that allow

them to overcome competitors in their environment (Refardt 2011; Rendueles & Ghigo 2012). Sometimes a simple mechanism such as large colony size gives a microbe a competitive edge (Li & Li 2012), and other times a more sophisticated mechanism such as bacteriocins is employed (Majeed et al. 2011). The naturally occurring gut microbiota in *H. pensylvanicus* is relatively simple ranging from 2-5 different symbiont species per beetle (Lundgren et al. 2007). The endosymbionts found in *H. pensylvanicus* were affiliated with Alphaproteobacteria, Gammaproteobacteria, Bacilli, and Mollicutes (Lundgren et al. 2007; Lundgren & Lehman 2010). Using the selective growth medium, we isolated a strain most similar to *Enterococcus* sp. in addition to *E. faecalis* from the digestive tracts of *H. pensylvanicus*. These species are both considered to be lactic acid bacteria that commonly ferment carbohydrates and are tolerant of an acidic microenvironment. Many *Enterococcus* and lactic acid producing bacteria produce bacteriocins or antimicrobials that are antagonistic against other bacteria including other *Enterococcus* species or lactic acid bacteria (Ott et al. 2001; Poeta et al. 2006; Theppangna et al. 2007; Brandao et al. 2010). For example *Enterococcus faecium* CE5-1 produces bacteriocins that have inhibitory activity against *E. faecalis* (Saelim et al. 2012). In fact many microbial species produce bacteriocins that inhibit closely related species as part of a survival mechanism to eliminate the competition (Cleveland et al. 2001). Thus, it is plausible to think that *E. faecalis* was not able to establish well in the treatments with an established bacterial community.

Before drawing sweeping conclusions on the importance of this symbiosis for the dietary breadth or niche separation among omnivorous insects, it is important to study the symbiotic interactions revealed here under more natural settings. Under natural conditions and in laboratory choice tests, *H. pensylvanicus* consumes numerous other foods, including pollen, fungi, and insect prey (Kirk 1973; Best & Beegle 1977; Larochelle 1990; Riddick & Mills 1994; Ahmad et al. 2006). Each of these foods is associated with unique defensive and nutritional characteristics. Also, the bacterial community within *H. pensylvanicus* and other insect guts is dynamic, and thus environmental changes could influence the strength of the *E. faecalis* - *H. pensylvanicus* symbiosis, and the predominance of bacterial taxa within insect guts. The results of this experiment indicate that the benefit of the symbiotic relationship between *E. faecalis* and *H. pensylvanicus* is dependent on the elimination of the natural occurring gut microbiota first. It is unpractical to administer antibiotics and then *E. faecalis* under field conditions, thus studying the factors that contribute to strengthening the symbiotic relationship between *H. pensylvanicus* and *E. faecalis* or other beneficial gut symbionts is an important next step. For example abiotic factors affect the bacterial diversity within an environment (Roesch et al. 2007; Fierer & Lennon 2011; Andrew et al. 2012; Wang et al. 2012). Bacterial-animal symbioses have important implications for the evolution of dietary specializations, and the facultative yet functional relationship described in our research may help better understand the continuum in this interaction between trophic behavior and the fidelity of a nutritional symbiosis.

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Appendix

Appendix 1. The diet, including antibiotics, fed to *Harpalus pensylvanicus* during the feeding assay.

Diet:

Cat food – 35 g – soaked in

Distilled (E Pur) H_20 – 70 mL
Chicken Liver – 25 g
Raw Chicken Egg – 1
Vitamin Solution (Made the day of) – 1.5 mL
Sorbic Acid – 1 g
Tetracycline – 0.5 g
Rifampicin – 0.1 g
Erythromycin – 0.1 g

Blended 3 minutes

Added agar solution = 3 g agar into 70 mL boiling H_20
Boiled 1 minute – (Add agar very slowly)

Blended 1 minute