# Gut Bacterial Symbiont Diversity Within Beneficial Insects Linked to Reductions in Local Biodiversity

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**ABSTRACT** Understanding the factors that constrain or promote symbiotic microbial communities gives a clearer picture of the niches that can be occupied by a host organism. Many insects harbor symbiotic microbes that can alter various aspects of insect behavior and biology including digestion, sex determination, and pathogen defense. Habitat diversity has a major influence on insect and microbial diversity within an environment. In the current study, we assessed how habitat biodiversity affects the bacterial species richness within the gastrointestinal tract of insects. We measured species abundance of plants and insects present in three replicated habitats (prairie, pasture, and maize fields) that inherently represent a continuum of biological diversities. Gut bacterial symbiont diversity of the crickets *Gryllus pennsylvanicus* Burmeister and *Allonemobius* sp. (Orthoptera: Gryllidae) were described using terminal restriction fragment length polymorphism analysis of rRNA genes. The resulting data show that gut bacterial diversity of both cricket species is positively correlated with biodiversity according to habitat type. This demonstrates that microbial diversity within insect gastrointestinal tracts, and possibly their functions within these insects, is tied to the biodiversity within the habitats where insects live. These results have important implications as to how reductions in habitat biodiversity may affect the ecological functions and services that the remaining species can perform.

**KEY WORDS** *Allonemobius*, cricket, symbiont, *Gryllus pennsylvanicus*, habitat

It is widely accepted that habitat is a major force that drives the biodiversity found in different landscapes (Tews et al. 2004, Rotholz and Mandelik 2013). Diversity of primary producers (namely plants) within a habitat often influences the overall biodiversity found within a habitat. Various metrics of plant diversity (phylogenetic diversity, vegetation structure, and landscape heterogeneity) correlate with the diversity of other groups of organisms within a habitat, including animals (Greenstone 1984, Thiollay 1990, Dennis et al. 1998, Atauri and de Lucio 2001, Dinnage et al. 2012) and soil microbes (Bakker et al. 2013, Saul-Tcherkas et al. 2013). Still, biodiversity of these different groups does not strictly scale uniformly; for example, animal community composition is also affected by both abiotic and biotic factors within a habitat such as temperature, foliage density height, and food availability (MacArthur and MacArthur 1961, Diehl et al. 2012, Seoane et al. 2013). One important group of organisms within a habitat that affects the range of niches occupied and services provided by higher organisms is symbiotic microorganisms. Characteristics of the external environment that drive or encourage the diversity of symbiotic microbes within their hosts remain poorly understood.

Microbial symbionts are typically an essential component of the biology and behavior of higher organisms. Pathogenic microbial symbionts are well studied (Vega and Kaya 2012), but symbionts can also be beneficial to host insects. Benefits provided by microbial symbionts to their hosts include food processing, detoxification, and utilization; production of enzymes; protection from predators, parasites, and pathogens; contribution of inter- and intraspecific communication; development; and synthesis of nutrients, vitamins, and sterols (Kukor and Martin 1983, Douglas 2009, Koch and Schmid-Hempel 2011, Engel and Moran 2013, Shi et al. 2013, Takasuka et al. 2013, Zindel et al. 2013). Symbiotic microbiota are so intrinsic to the biological function of many hosts that it has been suggested that the microbiota should be elevated to organ status (Backhed et al. 2005). There are numerous factors, both internal and external, which influence the composition of gut microbial communities. Internal factors affecting gut microbial community composition are often fueled by competition between microbes for space (Raaijmakers et al. 2002, Li and Li 2012, Rendueles and Ghigo 2012), while external factors could include a plethora of abiotic or biotic factors, such as diet (Schmid et al. 2014a,b). Landscape simplification

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associated with agricultural proliferation has led to a reduction in biodiversity of many organisms (Stoate et al. 2001, Purtauf et al. 2005); however, the effects of habitat biodiversity on gut microbial community diversity have yet to be examined.

In the current study, we measured the species abundance of plants and insects present in three habitats (prairie, pasture, and maize fields) that inherently represent a continuum of biological diversities. In each of these habitats, we used culture-independent techniques to examine the microbial diversity found within the gastrointestinal tracts of omnivorous crickets which provide ecosystem services important to their habitat. Crickets were selected because they were some of the only soil taxa common to all three habitats; their natural diet is extensive and includes a wide variety of insect prey, seeds, and other plant material (Lundgren and Fergen 2011, Lundgren and Harwood 2012). *Gryllus pennsylvanicus* Burmeister, one of the selected crickets for the study, has been demonstrated to be a beneficial insect by feeding on grasshopper eggs, apple maggot pupae, alfalfa weevil adults, and other insect pests (Criddle 1925, Monteith 1971, Barney et al. 1979), as well as weed seeds such as common ragweed, redroot pigweed, and other agriculturally related weed seeds (Brust and House 1988, Carmona et al. 1999). *Allonemobius* sp., the second cricket species selected for the study, has been documented as omnivorous, consuming plant material and seed of many plant species (Fulton 1931, Tennis 1983). The bacterial symbionts within the guts of crickets can have an impact on cricket diet selection, quantity of diet consumed, and the nutrition obtained from the diet (Kaufman et al. 1989, Kaufman and Klug 1991, Schmid et al. 2014a). With the resulting dataset we tested the hypothesis that biodiversity within a habitat (combined insect and plant species richness) is positively correlated with the enteric gut bacterial species richness of the insects within those habitats.

## Materials and Methods

**Site Criteria.** Three habitat types (prairie, grassland pasture, and maize field) were selected to represent habitats of high, medium, and low levels of biodiversity (each replicated at three locations), and each habitat type was represented by three experimental sites. The total area of each site comprised between 10 and 29 ha. The sites were located within a 48-km radius of Brookings, SD, USA. Prairie sites I, II, and III were located at 44.512, −96.532 (latitude, longitude); 44.261, −96.707; and 44.254, −96.811. These were native prairies; no fertilizers or pesticides had been used within the past 10 yr. Prairies I and II were burned on a 2–5 yr rotation. Prairie I was last burned in 2010, and Prairie II was last burned in 2008. Prairie III was burned only once (in 1990) since 1982. Pastures I, II, and III were located at 44.211, −96.753; 44.414, −96.963; and 44.338, −96.983. All pastures had been established for at least 10 yr. No fertilizers or pesticides were used in Pastures II and III. Pasture I was spot sprayed with a herbicide containing aminopyralid and 2, 4 D (ForeFront, The Dow Chemical Company, Midland, MI), and a fertilizer mixture of nitrogen and phosphorous was applied at least 30 d before sample collection. No cattle grazed Pasture II during sample collection, 25 head of cattle grazed Pasture III, and 30 head of cattle grazed Pasture I during sample collection. Maize fields I, II, and III were located at 44.308, −96.666; and 44.300, −96.666; and 44.356, −96.838. No insecticides were sprayed in any of the maize fields. Maize fields I and II were fertilized with 179 kg of nitrogen, 56 kg of phosphorous, and 56 kg of potassium per ha. Maize field III was fertilized with 118 kg of nitrogen, 67 kg of potassium, and no phosphorous per ha. Maize sites I and II were preceded by soybeans in the previous year, and Maize field III was preceded by winter wheat. Maize seed planted in field III was treated with clothianidin (Poncho, Bayer CropScience, Research Triangle Park, NC).

**Arthropod and Plant Diversity Sampling.** Arthropod and plant diversity sampling was done twice, once in mid-July (12–17 July, 2012) and once in mid-August (14–16 August, 2012). The arthropod community was collected from the plant foliage, on the surface of the soil, and below the soil surface. Only adult arthropods were considered. Arthropod samples were randomly collected from four locations at each site during each sample period. Arthropods within the soil column were sampled using soil cores measuring 10 cm (diam) × 10 cm (deep). Specimens were extracted from the soil cores using Berlese funnels over a 7-d period, and communities found within the cores at each site were pooled. Quadrats were used to focus the sampling on surface dwelling arthropods. Specimens found in the top 1 cm of soil within each quadrat (0.5 × 0.5 m and 15 cm tall) were aspirated. Foliar-dwelling arthropods were sampled from the vegetation using the vacuum setting of a leafblower (PoulanPRO 200 MPH Super, Electrolux Home Product, Inc., Augusta, GA), with a mesh screen placed inside the vacuum tube to collect the arthropods. Samples were collected from plants that intersected a 10-m transect. The arthropods collected from the soil cores, soil surface, and vegetation samples were preserved in 70% ethanol at room temperature until they could be processed.

The plant community was sampled using four transects measuring 100 m each. Every 20 m, beginning at 0 m, a 0.25- by 0.25-m quadrat was laid down adjacent to the transect line. Vegetation within each quadrat was clipped at ground level, placed in plastic bags, and stored at 10°C until the plants could be recorded. Insect and plant specimens collected across sample periods and across sample locations within a site were pooled to create a composite measure of biodiversity for each experimental unit (i.e., site).

To assess insect diversity, each insect collected was identified to as low a taxonomic level as possible, hereafter referred to as operational taxonomic units, OTUs (i.e., morpho-species). Immature insects did not count toward insect diversity. The number of each OTU was recorded for each site, creating a database of morpho-species. A similar method was followed for assessing plant diversity. Voucher specimens of each insect OTU were kept.

**Characterization of Symbiont Communities.** The two cricket species *G. pennsylvanicus* and *Allonemobius* nr. *fasciatus* (Orthoptera: Gryllidae) were chosen due to their abundance at every site in each habitat type. *G. pennsylvanicus* and *Allonemobius* sp. were collected using pitfall traps; 20–30 traps were placed no closer than 30 m from the habitat edge in any direction at each field site. Each trap consisted of two plastic cups, 10 cm in diameter and 15 cm deep, one stacked inside the other, and each trap was loosely covered with a plywood board 0.3 by 0.3 m. The traps were checked every 24 h for the two crickets species. The crickets collected were preserved in 70% ethanol and stored at −20°C until their intestinal tract could be dissected. The number of crickets collected from each site varied. In Prairies we collected 6, 18, and 20 *G. pennsylvanicus* and 16, 19, and 20 *Allonemobius*; in Pastures we collected 4, 10, and 20 *G. pennsylvanicus* and 20, 20, and 23 *Allonemobius*; in Maize fields we collected 12, 17, and 20 *G. pennsylvanicus* and 7, 9, and 18 *Allonemobius*.

Gut dissections were performed under sterile conditions, and resulting whole digestive tracts (crop, proventriculus, midgut, and hindgut) were placed into 1 ml of 1 × PBS, and stored at −20°C. Only adult cricket guts were used to characterize the symbiont communities. The bacterial community in each gut was analyzed separately. Guts were homogenized with a sterile mortar and pestle. DNA was extracted using DNeasy Blood and Tissue kit (DNeasy Blood and Tissue Kit, Catalog No. 69506, Qiagen Sciences, Germantown, MD) per manufacturer's instructions for purification of total DNA from animal tissues. DNA was concentrated using Amicon Ultra—0.5 ml Centrifugal Filters Ultracel—100K per manufacturer's instructions (EMD Millipore Corporation, Billerica, MA). DNA extractions were screened on 0.7% agarose gel (100 V, 25 min). For terminal restriction fragment length polymorphism (tRFLP) analysis, the 16S rRNA genes from DNA extractions were PCR-amplified along with positive (*E. coli* DNA) and negative (reagents only) controls, using the reaction mixture and conditions described in Lundgren and Lehman (2010) with the eubacterial primers 8F (5′–AGAGTTTGATCCTGGCTCAG–3′) labeled at the 5′ end with 6-carboxyfluorescein (FAM) and 1492R (5′–GGTTACCTTGTTACGACYT–3′). PCR products were screened on 1.2% agarose gel (75 V, 45 min). PCR product was purified with a 96-well PCR clean-up kit (Wizard SV 96 PCR Clean-Up System, Promega, Madison, WI). Purified PCR product was quantified using a spectrophotometer. Each sample was digested for 3 h at 37°C and terminated at 75°C with 10 U of RsaI and 1X CutSmart buffer (New England Biolabs, Beverly, MA). The DNA fragments were analyzed by capillary electrophoresis using filter D and Mapmarker 1000 size standards on an ABI Prism 3100 (Applied Biosystems Inc., Foster City, CA) per ABI's recommended parameters. The electropherograms were analyzed with GelQuest 3.1.7 (SequentiX-Digital DNA Processing, Warnow, Germany) using the Local Southern size calling method, a minimum peak height of 50, a fragment range of 80–980 bases, and a peak window of two base pairs. The number of individual tRFLP peaks approximates the number of bacterial species, and the number of bacterial species is equivalent to the bacterial species richness.

tRFLP is an excellent tool for analyzing microbial community dynamics and the effect of external factors on community structure and function (Schutte et al. 2008, Aiken 2011, Prakash et al. 2014); however, it has a few limitations that need to be taken into account when interpreting tRFLP results. The limitations include multiple restriction enzyme cutting sites for some bacterial species, which can cause an over estimation of the bacterial diversity. Another limitation is that certain bacterial species can have tRFLP fragments that are near equal in length, which can be interpreted as the same species, depending on the methodology and data analysis used, resulting in an under estimation of bacterial diversity (Marsh et al. 2000). Precautions were taken to ensure optimal tRFLP results, such as standardized DNA extraction and PCR amplification, purification of amplified product and digested DNA, standardization of DNA quantity used, and use of a standard and accepted data analysis methods, and these steps helped to mitigate any limitations or bias of our tRFLP results (Schutte et al. 2008, Prakash et al. 2014). We also expect that any bias or limitations of tRFLP were equally represented in each of the replicated plots. Although tRFLP may be considered less modern relative to the recent availability of next-generation sequencing (which have their own biases for interpretation), if the limitations of tRFLP are considered and the proper methodological precautions are taken, tRFLP remains a valid method to describe bacterial community diversity.

**Data Analysis.** We determined whether there were significant differences in the number of bacteria per cricket gastrointestinal tract among the three habitat types by first summarizing the average bacterial count per insect species of each individual site. For *G. pennsylvanicus*, the number of bacteria per insect fit a nonnormal distribution, and so the median number of bacteria per insect was calculated for each field. Bacterial number per *Allonemobius* followed a normal distribution, and so the mean number of bacteria per insect per plot was determined. The average (median or mean) number of bacteria per insect per plot was compared among the three habitat types using a Kruskal–Wallis nonparametric ANOVA. We also compared the number of bacterial species recovered in all of the crickets per location using a Kruskal–Wallis nonparametric ANOVA. Pairwise tests (Kruskal–Wallis nonparametric ANOVA) among the three treatments were conducted when the overall comparisons yielded a $P$-value $< 0.1$.

A correlation between biodiversity within a habitat and gut bacterial species richness was assessed through a regression model. The curve was fitted to the data using an exponential rise to maximum equation. Logarithmic transformation was performed on bacterial species richness in *G. pennsylvanicus* and *Allonemobius* sp. for regression model analysis. All statistics were

conducted using Systat 13 (Systat Software, Inc., Chicago, IL).

## Results

**Biodiversity Within the Habitats.** The richness of plant and arthropod OTUs varied among the three habitat types (prairies, pastures, and cornfields; $F_{2,6} = 33.00$, $P = 0.001$; Fig. 1). The mean (± SEM) species richness was highest in the prairies (148.67 ± 16.70), intermediate in the pastures (103.33 ± 2.84), and lowest in the cornfields (35.67 ± 2.67). Fourteen different orders of arthropods were collected across the habitats, representing 344 OTUs (Table 1). The majority of OTUs (87%) were found in the orders of Hymenoptera (84 OTUs), Coleoptera (75 OTUs), Hemiptera (90 OTUs), and Diptera (51 OTUs). The animal communities collected from the prairies contained 12 arthropod orders representing 226 OTUs, the pastures contained 10 arthropod orders representing 190 OTUs, and the maize fields contained 10 arthropod orders representing 67 OTUs (a supplementary table of the 344 arthropod taxa is included). Plant samples were not identified beyond being assigned an OTU number for the purposes of characterizing the plant diversity at each site. The total number of plant OTUs found across all of the sites was 75. The prairie plant communities contained 68 OTUs, pastures contained 34 OTUs, and the maize fields contained 2 OTUs (Table 1).

**Effect of Habitat Diversity on Gut Bacterial Species Richness**

*Bacterial Species Richness From All Crickets Collected at a Particular Habitat.* Gut bacterial species richness varied across the habitats (Fig. 2A). The total number of gut bacterial OTUs found in *Allonemobius* sp. across all of the sites was 58. Total gut bacterial species richness present in *Allonemobius* sp. was significantly affected by habitat ($\chi^2_2 = 6.83$; $P = 0.03$). In *G. pennsylvanicus* the total number of gut bacterial OTUs found across all of the sites was 41. However, the total gut bacterial species richness in *G. pennsylvanicus* was not significantly affected by habitat ($\chi^2_2 = 1.17$; $P = 0.56$).

*Gut Bacterial Species Richness per Cricket.* Bacterial species richness (median) per *G. pennsylvanicus* varied among the habitats (prairies, pastures, and maize fields; $\chi^2_2 = 5.64$; $P = 0.06$; Fig. 2B). Pairwise comparisons of the symbiont communities per cricket found in these three habitats revealed that *G. pennsylvanicus* from the prairies and pastures contained significantly more bacteria species per cricket than those from the maize fields (Prairie × Maize field: $\chi^2_1 = 4.36$; $P = 0.04$, Pasture × Maize field: $\chi^2_1 = 4.36$; $P = 0.04$). Symbiont species richness per *G. pennsylvanicus* was reduced by at least 70% in maize fields compared to the other habitats. However, no significant difference of gut bacterial species richness per cricket was seen between *G. pennsylvanicus* collected from the prairies and pastures

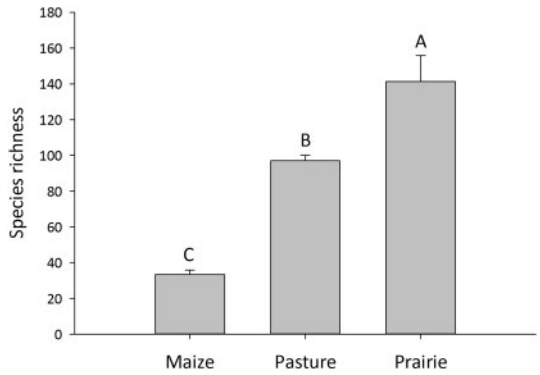


**Fig. 1.** The mean total species richness (plant and arthropod diversity) in each habitat ($n = 3$). Bars topped with different letters are significantly different from one another ($\alpha = 0.05$).

**Table 1. Arthropod and plant species richness in each of three habitat types**

| Habitat type | Arthropod species richness (OTUs) | Plant species richness (OTUs) |
|---|---|---|
| Prairie | 226 | 68 |
| Pasture | 190 | 34 |
| Maize field | 67 | 2 |

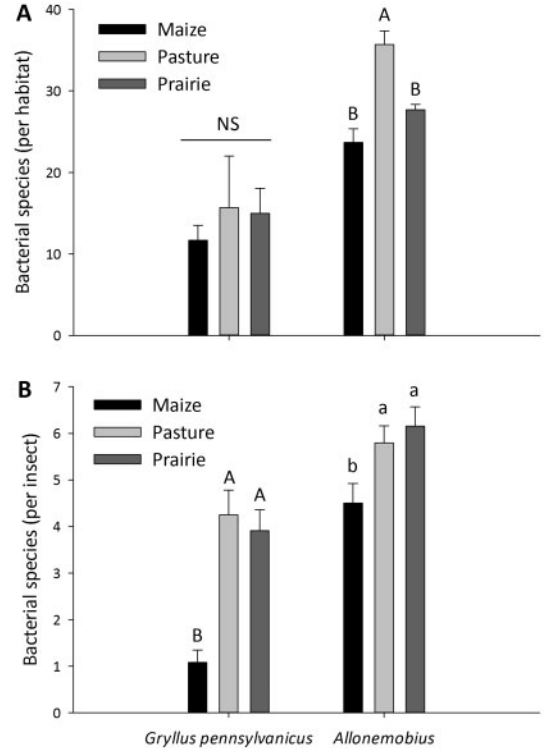


**Fig. 2.** The mean bacterial richness found in the guts of *G. pennsylvanicus* and *Allonemobius* sp. collected from three habitats. Bacterial species (A) per site (all crickets pooled) and (B) per cricket, are presented. Bacterial species richness in each cricket species were analyzed separately, and bars capped with different letters are significantly different from one another ($\alpha = 0.05$).

($\chi^2_1 = 0.00$; $P = 1.00$). When compared across habitat types, gut bacterial species richness of individual *G. pennsylvanicus* was positively correlated with habitat biodiversity at a significant level ($F_{1,8} = 12.55$, $P = 0.01$; Fig. 3).

Like the observations for *G. pennsylvanicus*, bacterial species richness (mean) in individual *Allonemobius* guts was also different (marginally significant) among the three habitat types ($\chi^2_2 = 5.6$; $P = 0.06$; Fig. 2B). Again, the differences were the result of significantly more symbiont richness per cricket in the prairies and pastures relative to that found in the *Allonemobius* guts from the maize fields (Prairie × Maize field: $\chi^2_1 = 3.86$; $P = 0.05$, Pasture × Maize field: $\chi^2_1 = 3.86$; $P = 0.05$). Symbiont species richness per *Allonemobius* was reduced by at least 20% in maize fields compared to the other habitats. Gut bacterial species richness per *Allonemobius* collected from the prairies and pastures were statistically similar ($\chi^2_1 = 0.43$; $P = 0.51$). A significant positive correlation was observed between habitat biodiversity and gut bacterial species richness per *Allonemobius* ($F_{1,8} = 7.18$, $P = 0.03$; Fig. 3).

## Discussion

A significant difference in bacterial species richness for both cricket species was observed between maize and the other two habitats (prairie and pasture; Fig. 2B), and this pattern is similar to the pattern of mean total species richness (plant and arthropod) observed across the habitats (Fig. 1). The patterns indicated a positive correlation between gut bacterial species richness and habitat species richness, which was substantiated through regression modeling (Fig. 3). An important caveat to this relationship is that crickets with the highest average bacterial species richness per individual did not come from habitats with the highest summed number of bacterial OTUs from all cricket guts (Fig. 2). Our results may also indicate a significant difference of gut bacterial species richness of both cricket species between crickets collected from the heavily managed maize field and the less managed prairie and pastures. This study demonstrates a correlation between reduced biodiversity in agricultural systems and decreased species richness of gut bacteria in *G. pennsylvanicus* and *Allonemobius* sp. This reduction of potential symbioses between bacteria and higher organisms can reduce the services provided by these species in agroecosystems.

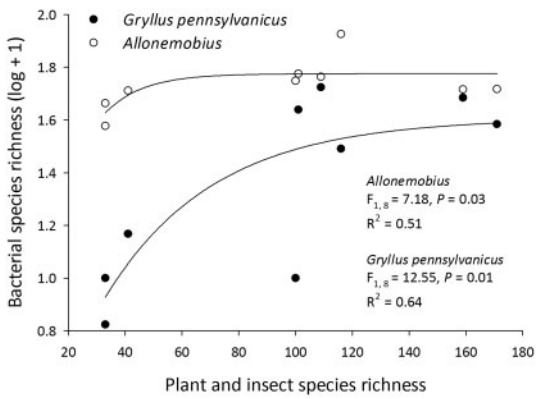


**Fig. 3.** Biodiversity in a habitat and the gut bacterial species richness in two cricket species. Habitats were prairies, pastures, and maize fields that captured a range of biodiversity (insect and plant species richness per habitat). Each data point represents a distinct experimental site. Plots are fitted with exponential rise to maximum models $y = a(1 - e^{-bx})$, where $a = 1.78$ and $b = 0.08$ for *Gryllus pennsylvanicus*, and $a = 1.61$ and $b = 0.03$ for *Allonemobius*.

The relationship between symbiont species richness and biodiversity within a habitat may be driven by a number of mechanisms. A potential explanation for these correlations between biodiversity and symbiont species richness may be simply explained by the increasing number of bacterial species present in a habitat. Biodiversity affects the bacterial diversity within a habitat, particularly in the soil. Many insects obtain their gut bacteria from the environment (Dillon and Charnley 2002, Kikuchi et al. 2007, Oliver et al. 2010, Woodbury et al. 2013), and it seems reasonable to expect that the observed increased gut symbiont species richness resulted from greater bacterial species richness encountered in biodiverse habitats. If this were the case we would expect the crickets collected from the prairies to have the highest number of total symbiont OTUs. This was not the case; the total species richness in the bacterial community collected from prairie crickets was not greatest among the habitats, even though the number of bacterial OTUs per cricket were highest in prairie-collected crickets (Fig. 2). Due to the high number of OTUs per cricket in the prairie-collected crickets and the total bacterial species richness being similar to other habitats we hypothesize that the total richness in the gut bacteria community in the prairie crickets is reduced but the remaining species have a more obligated or robust relationship with the host, resulting in the highest number of bacterial OTUs per cricket collected from the prairies.

It is important to point out that bacterial species richness did NOT plateau in the crickets collected from the maize fields, especially *G. pennsylvanicus*. Symbiont species richness reached its maximum under realistic settings (prairies and pastures) which are perennial or receive less management than cropland. The fact that diversity was lowest in the maize fields and quickly rose to maximum for crickets collected from the prairies is a similar pattern seen in organisms with a long evolutionary history between their habitat and gut bacterial diversity. Thus, the potential for similar evolutionary patterns exists for insects (Sullam et al. 2012), which may indicate a link between habitat stability and maximum gut bacterial diversity. The symbiont species richness in crickets collected in maize fields was significantly reduced below the levels observed in crickets from the prairies and pastures, which could reduce the number and diversity of services that these symbionts could provide to their hosts (Stier et al. 2012).

Our research may be the first to demonstrate a correlation between biodiversity within a habitat and gut bacterial diversity of insects living in that habitat. The level of biodiversity, not only gut bacteria per cricket but plant and insect species as well, in maize was consistently lower than the biodiversity found in the pastures and prairies. Increased habitat biodiversity can lead to a suite of ecosystem benefits, which include increased methane consumption, increased predation of pest eggs, and decreased pest pressure (Werling et al. 2014, Lundgren and Fausti 2015). Increasing gut bacterial species richness within insects is important because gut symbionts are key to digestion of many foods, and may be important in regulating pathogenicity (either positively or negatively) for a host. Thus expanding insect gut bacterial species richness can potentially expand the dietary breadth and services that insects can perform in a habitat (Lundgren 2009, Lundgren and Lehman 2010, Schmid et al. 2014b), and add another ecosystem benefit to the suite of benefits already known to be caused by increased habitat biodiversity (Werling et al. 2014). If symbiont communities are reduced alongside the diversity of other organisms within a habitat such as agroecosystems, then not only is the number of species reduced in these systems, but the potential ecosystem functions of the remaining species may also be diminished in less diverse systems.

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