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HOST MICROBE INTERACTIONS

Gut bacterial communities and their contribution to performance of specialist *Altica* flea beetles

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Abstract

Host plant shifts are a common mode of speciation in herbivorous insects. Although insects can evolve adaptations to successfully incorporate a new host plant, it is becoming increasingly recognized that the gut bacterial community may play a significant role in allowing insects to detoxify novel plant chemical defenses. Here, we examined differences in gut bacterial communities between *Altica* flea beetle species that feed on phylogenetically unrelated host plants in sympatry. We surveyed the gut bacterial communities of three closely related flea beetles from multiple locations using 16S rRNA amplicon sequencing. The results showed that the beetle species shared a high proportion (80.7%) of operational taxonomic units. *Alpha*-diversity indicators suggested that gut bacterial diversity did not differ among host species, whereas geography had a significant effect on bacterial diversity. In contrast, analyses of *beta*-diversity showed significant differences in gut bacterial composition among beetle species when we used species composition and relative abundance metrics, but there was no difference in composition when species presence/absence and phylogenetic distance indices were used. Within host beetle species, gut bacterial composition varied significantly among sites. A metagenomic functionality analysis predicted that the gut microbes had functions involved in xenobiotic biodegradation and metabolism as well as metabolism of terpenoids and polyketides. These predictions, however, did not differ among beetle host species. Antibiotic curing experiments showed that development time was significantly prolonged, and there was a significant decline in body weight of newly emerged adults in beetles lacking gut bacteria, suggesting the beetles may receive a potential benefit from the gut microbe-insect interaction. On the whole, our results suggest that although the gut bacterial community did not show clear host-specific patterns among *Altica* species, spatiotemporal variability is an important determinant of gut bacterial communities. Furthermore, the similarity of communities among these beetle species suggests that microbial facilitation may not be a determinant of host plant shifts in *Altica*.

Keywords Adaptation · Antibiotic treatment · Body weight · Development time · Speciation

Introduction

The high rate of diversification among phytophagous insects is thought to be a consequence of shifts to new host plant species and subsequent specialization [1–4]. Switching to a new host plant or extending the host plant range may require a number of adaptations, including physiological and behavioral adaptations that allow insects to effectively use a novel host. Particularly for host plants that are defended by secondary chemicals, there is likely to be strong selection on insects to adapt to the larval substrate [5–7]. Indeed, herbivorous insects have evolved adaptations that allow them to cope with the defenses of novel host plants, such as toxin avoidance, target-site alteration, and detoxification [8–10].

Studies of the mechanisms used in host plant shifts have primarily focused on evolutionary change within the insect

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lineage, yet adaptation to novel hosts could occur through alternative mechanisms. For example, there is an increasing realization that gut symbionts can influence insect development, physiology, ecological interactions, and evolutionary diversification [10–22], and these changes in turn, could assist insects in dealing with novel host plants. For instance, the guts of insects are characterized by a semi-open, dynamic habitat for microorganisms that can be impacted by the material consumed by the insect host. Especially in the case of phytophagous insects, the gut microbial communities are directly exposed to host plant tissues along with the secondary defense metabolites released during feeding [23, 24]. Consequently, microbial communities associated with herbivores have likely evolved to favor species with the ability to detoxify or resist the toxins liberated by digestion of host tissue. This has been proposed as the "gut microbial facilitation hypothesis" [20] that provides an alternative explanation for how insects shift onto plant species with novel suites of secondary defenses. As a result, gut microbes may be an important driver of insect species diversification [20].

Although microbial facilitation is one mechanism that could explain host plant shifts in phytophagous insects, the question remains as to how often microbes assist in this process. We have mounting evidence that demonstrates a direct benefit of microbes in the detoxification of plant-derived compounds [13, 18, 19], but recent work suggests that at least in some insect groups, gut microbes may not facilitate herbivore host shifts. Many lepidopterans, for instance, do not seem to benefit from their gut microbial communities [25–27]. Additionally, other insects such as dragonflies, fruit flies, and stick insects have also been shown to be less dependent on gut microbes for digestion [28–30]. These mixed results suggest that there is a strong need to examine the role of the gut microbial community in facilitating host plant shifts among a phylogenetically diverse assortment of phytophagous insects.

From the perspective of the gut microbial community, there are a number of elements that could contribute to diversity within the insect host, including the host habitat, diet, developmental stage, and even the phylogenetic position of the host [21, 31–33]. Within a single insect species, for example, host plant use can account for significant shifts in gut bacterial diversity as shown in the polyphagous lepidopteran pest, *Helicoverpa armigera* [34]. Similarly, in the fall armyworm (*Spodoptera frugiperda*) and corn earworm (*Helicoverpa zea*), host plant use had a greater impact on gut communities, although differences in insect physiology, gut region, and local factors also contributed to variation in the microbiomes [35]. Host plant use, however, is not always the key driver of gut bacterial communities [36, 37]. For instance, in the *Cryptocephalus marginellus* species complex, neither geographic distance nor host plant species correlated with bacterial composition, yet there was a significant effect due to altitude [38]. In addition, several large-scale studies spanning different insect families [39, 40] and orders [31, 32] show that a majority of the variation in gut microbiota between species was correlated with insect host phylogeny, potentially due to physiological constraints. Moreover, we also know that the biodiversity of gut microbiota can change significantly between developmental stages [32, 33], seasons [41], and sexes [33]. Together, these results suggest that while host plant use may be an important factor controlling gut bacterial community structure in some species, there are multiple additional effects that may make predicting bacterial communities complex.

Examining the relationship between host plant shifts and gut microbial communities is best conducted using a system in which there are closely related insect species using phylogenetically unrelated host plants in both sympatry and allopatry. For this reason, we focused our study on *Altica* Geoffroy (Coleoptera: Chrysomelidae) flea beetles because this is a species-rich genus [42] that is well-known as a model of ecological speciation triggered by host plant switching [43–45]. Within this group, there are three mostly sympatric species, *Altica cirsicola* Ohno, *A. fragariae* Nakane, and *A. viridicyanea* (Baly), which are closely related. These species are each specialized to different host plants that occur in different plant families [43]. For example, *A. cirsicola* feeds only on *Cirsium* species in the Asteraceae, *A. viridicyanea* occurs exclusively on *Geranium nepalense* Sweet (Geraniaceae), and the primary host of *A. fragariae* is *Duchesnea indica* (Andrews) Focke (Rosaceae) although it is more oligophagous than the other two species with four additional host plants in the Rosaceae. Moreover, the beetles are easy to keep under laboratory conditions [43, 46], making experimental manipulation of the gut bacterial communities possible.

In the present study, we characterized the gut bacterial communities of these three *Altica* species sampled from different localities. First, we used Illumina MiSeq sequencing of 16S rRNA genes to test whether beetle species and collection location affect the diversity and composition of gut bacterial communities. These data were also used to predict the metagenomic functionality of the gut bacterial communities. Second, we conducted a gut bacteria manipulation experiment in *A. viridicyanea* by using antibiotic treatment of lab-reared larvae to test whether gut microbes contribute to insect performance. Using these approaches, we show that although the gut bacterial communities did not differ among *Altica* species, there was significant geographic variation in the composition of bacterial communities. An analysis of metagenomic functionality showed roles consistent with degradation of plant secondary chemistry, and beetles treated with antibiotics had reduced performance. Together, the results are consistent with a beneficial role of gut microbial communities in these host specialist insects.

Materials and Methods

Sample Collection and Processing

To investigate the gut bacterial communities of field populations of *Altica cirsicola*, *A. fragariae*, and *A. viridicyanea*, adult beetles were collected from several locations near Beijing from April to August of 2016 and 2017 (Table 1). Because we were concerned that maintaining the insects in the lab might alter the gut bacterial community [47], the beetles were not provided with host plant material after being brought into the lab. We starved the beetles for 12 h to allow final digestion of any residual plant tissue, and then we surface-sterilized them with 75% ethanol for 90 s before rinsing with sterile, deionized water. We created "samples" for each site and species by pooling three randomly selected individuals regardless of sex. Each collection location and species had three samples except in three cases where we only had sufficient beetles for two samples. The entire digestive tract was pulled out from the head using sterile forceps, then we removed the head, pooled the samples, flash froze the tissue, and held them at − 80 °C until DNA extraction. We obtained 36 samples in total.

Table 1 Sample collection information of three *Altica* species from Beijing, China

Beetle species	Site	Geographic coordinates	Sample ID	Collection date
A. cirsicola	Kuaile shanzhuang (KLSZ)	40.33 N 116.52 E	AC11	2016 Jun 10
			AC12	
	Xiangyangkou (XYK)	40.09 N 115.76 E	AC21	2016 Jul 1
			AC22	
			AC23	
	Shahe (SHH)	40.17 N 116.22 E	AC31	2016 Jul 4
			AC32	
			AC33	
	Lang'eryu (LEY)	40.11 N 116.00 E	AC41	2017 Apr 25
			AC42	
			AC43	
	Sihai (SH)	40.54 N 116.42 E	AC51	2017 Apr 29
			AC52	
			AC53	
A. fragariae	Sihai (SH)	40.54 N 116.42 E	AF11	2016 Jul 14
			AF12	
			AF13	
	Sijiashui (SJS)	40.09 N 115.95 E	AF21	2016 Aug 5
			AF22	
	Lang'eryu (LEY)	40.11 N 1156.00 E	AF31	2017 Apr 25
			AF32	
			AF33	
A. viridicyanea	Sihai (SH)	40.54 N 116.42 E	AV11	2016 Jul 14
			AV12	
	Sijiashui (SJS)	40.09 N 115.95 E	AV21	2016 Aug 24
			AV22	
			AV23	
	Beizhaotai (BZT)	40.11 N 116.01 E	AV31	2016 Aug 24
			AV32	
			AV33	
	Lang'eryu (LEY)	40.11 N 116.00 E	AV41	2017 Apr 25
			AV42	
			AV43	
	Beijing Botanical Garden (BJBG)	39.00 N 116.21 E	AV51	2017 May 11
			AV52	
			AV53	

DNA Extraction, Bacterial 16s rRNA Gene Amplification, and High-Throughput Sequencing

Total DNA from the beetle gut was extracted using the Power Soil DNA Isolation Kit (MO BIO Laboratories) following the manufacturer's protocol. DNA quality and quantity were assessed by the ratios of 260 nm/280 nm and 260 nm/230 nm. We then used PCR to amplify the hypervariable V3 + V4 region of the 16s rRNA gene using the universal primers 338F (5′-ACTCCTACGGGAGGCAGCA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′), which contained Illumina TruSeq DNA adapters and barcodes [48]. PCR was carried out in a total volume of 50 μl containing10-μl buffer, 0.2-μl Q5 High-Fidelity DNA Polymerase, 10-μl High GC Enhancer, 1-μl dNTP, 10 μM of each primer, and 60-ng genome DNA. The thermocycler profile started with denaturation at 95 °C for 5 min followed by 15 cycles of denaturation at 95 °C for 1 min, annealing at 50 °C for 1 min, and extension at 72 °C for 1 min, and ended with a final extension step at 72 °C for 7 min. The PCR products from the first step PCR were purified through VAHTSTM DNA Clean Beads. A second round of PCR was then performed in a 40-μl reaction which contained 20-μl 2× Phusion HF master mix, 8 μl ddH_2O, 10 μM of each primer, and 10-μl PCR products from the first step. Thermal cycling conditions were as follows: an initial denaturation at 98 °C for 30 s, followed by 10 cycles at 98 °C for 10 s, 65 °C for 30 s, and 72 °C for 30 s, with a final extension at 72 °C for 5 min. PCR products were purified, quantified by Quant-iT™ dsDNA HS Reagent, and pooled together to establish a sequencing library. Standard library preparation was used to prepare libraries for sequencing on the Illumina HiSeq 2500 platform. A blank PCR control (sterile water) was used to check for contamination of the samples before they were sequenced. The library construction and sequencing steps were performed by Beijing Biomarker Technologies Co. Ltd. (Beijing, China).

Bioinformatic Analysis

The sequence reads were saved as FASTQ files that included both the reads and corresponding quality information. Subsequently, the obtained paired-end reads were merged using FLASH v. 1.2.7 [49]. Then, the raw reads were filtered and clustered in the next steps. Merged reads with an average quality score < 20 in a 50-bp sliding window were truncated using Trimmomatic [50], and merged reads shorter than 300 bp were removed. Chimeras were identified and removed with UCHIME v. 4.2 [51].

The denoised sequences were clustered into operational taxonomic units (OTUs) using a threshold similarity ≥ 97% in USEARCH v. 10.0 [52]. Bacterial taxonomy was assigned following the Silva database (Release119, http://www.arb-silva.de) taxonomy library. We used RDP Classifier v. 2.2 (http://sourceforge.net/projects/rdpclassifier/) [53] to determine the classification of the OTUs. Raw paired-end reads obtained in this study were deposited in the NCBI Sequence Read Archive (SRA) under the accession number PRJNA601281.

Bacterial Community Composition of Three *Altica* Species

Sequences assigned to cytoplasmic organelles and known endosymbionts (*Wolbachia* spp.) were removed before the downstream analysis. Although *Spiroplasma* is not strictly endosymbiotic as some species can colonize different habitats within the insect body, we decided to include these sequences in our analyses because many species within this genus are gut endosymbionts [54, 55]. Consequently, our analyses will be conservative as they contain all possible OTUs that may contribute to the gut communities. To ensure even sequencing depth across the samples, the dataset was sub-sampled to a depth of 3300 reads per sample before *alpha* and *beta*-diversity calculations. From the sequence results, we calculated two non-parametric richness indices (abundance-based coverage estimator (ACE) and bias-corrected Chao1), and three *alpha*-diversity indices (Shannon, Simpson, and Pielou's evenness) using Mothur v. 1.30 (http://www.mothur.org/) [56] and "vegan" package in R. Phylogenetic diversity (Faith's PD) was measured as the total shared branch length of OTUs within each host species [57] and was done using the Qiime software package [58]. We used the Kruskal-Wallis tests to examine differences in these estimators among host species and collection sites.

We also compared *beta*-diversity using the Jaccard presence/absence metric, the Bray-Curtis relative abundance metric, and the unweighted UniFrac distance metric [59]. Permutation-based multivariate analysis of variance (PerMANOVA) and analysis of similarities (ANOSIM) were used to assess the differences among beetle host species and among collection sites within each beetle species. We also used two-way PerMANOVA (9,999 permutations) conducted using PAST version 3.14 [60] to test the role of geography and collection year in structuring gut bacterial communities. For this analysis, we used "collection site" and "collection time" as main effects. Because all three beetle species were sampled from two locations and two of these species were also sampled from one additional location, we also used two-way PerMANOVA to jointly test the role of host species and geography in structuring gut bacterial communities from these sites using "host species" and collection site as main effects. We also used NMDS ordination plots to visualize dissimilarity in bacteria community composition among beetle species and collection sites. The above analyses were conducted on the Biomarker Biocloud Platform (http://en.biocloud.net/private-cloud) unless otherwise specified.

We identified OTUs with differential abundance between host species by using DESeq2 version 1.28.1 [61] in MicrobiomeAnalyst (https://www.microbiomeanalyst.ca/) [62, 63]. Finally, the metagenomic functionality of the gut bacterial communities from the different beetle species was predicted from the 16S rRNA sequences using PICRUSt [64]. PICRUSt analysis was performed using the predict_metagenomes.py script run against the functional database of KEGG Orthology.

Effect of Symbiotic Bacteria on Beetle Development

The potential effect of symbiotic bacteria on beetle development was studied using antibiotic treatment experiments. Newly hatched larvae of *A. viridicyanea* were reared in petri dishes containing moist filter papers placed under controlled laboratory living conditions of 16:8 LD and 25 °C. The larvae were fed with *Geranium nepalens* leaf material collected from the field. Antibiotic treatments were applied beginning with the second larval instar, as we determined that there is high mortality if antibiotics were administered earlier (HJX, unpublished data). The leaves were completely submerged in antibiotic solution for 60 s and allowed to dry before feeding them to the beetles. Molting second instar larvae were assigned to one of two treatment groups. Larvae in the antibiotic treatment were fed *Geranium nepalens* leaves soaked in an antibiotic cocktail containing rifampicin, streptomycin, and tetracycline (0.4% *w/v* in a 1:2:4 ratio) which has been shown to effectively suppress bacterial symbionts in other insect species [19, 65]. We confirmed the effectiveness of the antibiotic treatment in a preliminary experiment where we cultured bacteria in LB (Luria-Bertani) medium. Samples collected from antibiotic treated beetles did not grow bacteria (JW, unpublished data). Larvae assigned to the control group were provided with leaves treated with sterile, deionized water. We assessed two key fitness parameters using different sets of beetles. First, we calculated development time (the time required for second instar larvae to pupate) by using video recording data. Once each third instar larva ceased feeding, we removed them to a new petri dish containing moist filter papers. These petri dishes were video surveilled using an iPad (A1458). We recorded pupation time from the video data, and this was used to calculate development time. Second, we assessed the body weight of the newly emerged adults using an electronic scale (Sartorius AG BP211D). Mann-Whitney *U* tests were used to assess whether there was a difference between the treatments and control groups in fitness parameters. These statistical tests were conducted using IBM® SPSS® v. 21.0.0.

Results

Sequences Cluster, OTU Identification, and Classification

Most rarefaction curves reached an asymptote, indicating adequate sequencing depth. We obtained a total of 3,700,890 raw sequence pairs from the 36 pooled samples, and after quality filtering, we had 1,703,133 effective reads with an average of 47,309 reads per sample. At this point, we removed the *Wolbachia* and other non-gut symbiont sequences, and the remaining sequences (16,112 reads per sample) were used in the subsequent analyses. The sequences were clustered into 343 unique OTUs with a 97% similarity threshold. All of the OTUs were identified and classified into 15 phyla that included Proteobacteria, Firmicutes, Actinobacteria, Acidobacteria, Bacteroidetes, Nitrospirae, Chloroflexi, Gemmatimonadetes, Cyanobacteria, Tenericutes, Nitrospinae, Deinococcus-Thermus, Chlorobi, Spirochaetae, and Fusobacteria. Thirteen genera with a relative abundance > 1% belonged to five phyla: Proteobacteria, Tenericutes, Actinobacteria, Firmicutes, and Bacteroidetes (Table 2; Fig. 1; Table S1).

Community Structure of the Gut Bacteria of Three *Altica* Species

Analyses examining the gut bacterial communities of the three *Altica* species showed similar numbers of OTUs in each beetle species. There were 330 OTUs in *A. cirsicola*, 302 in *A. fragariae*, and 312 OTUs in *A. viridicyanea* (Table 3). In *A. cirsicola*, the dominant phyla included Proteobacteria (70.53%), Actinobacteria (8.90%), Acidobacteria (5.11%), and Firmicutes (4.80%), together occupying 89.34% of the total sequences. Phyla Tenericutes (74.28%), Proteobacteria (18.65%), Actinobacteria (2.84%), and Firmicutes (2.28%) constituted 98.05% of the total sequences in *A. fragariae*, and Proteobacteria (68.37%), Tenericutes (18.64%), Firmicutes (4.54%), and Actinobacteria (3.95%) accounted for 95.5% in *A. viridicyanea* (Table 3). Nearly all bacterial phyla showed non-significant differences among the three beetle species based on relative abundance data with the exception of Proteobacteria and Tenericutes (Table 3). The samples from the three host species shared 80.17% OTUs (275 of 343), and there were 12, three and two species-specific OTUs found in *A. cirsicola*, *A. fragariae*, and *A. viridicyanea*, respectively (Fig. 1).

Comparisons of Gut Bacterial Communities Among Different Species and Sites

Alpha-diversity of the gut bacteria among *A. cirsicola*, *A. fragariae*, and *A. viridicyanea* was similar to one another as there were no significant differences across any of the

Table 2 Distribution of bacterial OTUs at the phylum (relative abundance > 0.1%) and genus levels (> 1%) found in three *Altica* species

Phylum	Proportion	No. of OTUs	Genus	Proportion	No. of OTUs
Proteobacteria	58.09%	151			
			Acinetobacter	3.30%	5
			Endozoicomonas	5.35%	1
			Ensifer	2.19%	1
			Methylobacterium	1.74%	3
			Pannonibacter	5.56%	1
			Pantoea	2.26%	1
			Pseudomonas	5.17%	7
			Sphingomonas	2.81%	6
			Uncultured bacterium belong to family Holosporaceae	2.57%	1
Tenericutes	16.26%	3			
			Spiroplasma	16.20%	2
Actinobacteria	11.19%	42			
			Propionibacterium	1.16%	1
Firmicutes	6.76%	53			
			Staphylococcus	1.26%	1
Bacteroidetes	3.34%	22			
			Bacteroides	1.82%	8
Acidobacteria	1.75%	28			
Nitrospirae	0.95%	16			
Chloroflexi	0.69%	10			
Gemmatimonadetes	0.51%	9			
Spirochaetae	0.13%	1			
Nitrospinae	0.12%	3			
Unclassified				19.57%	50
Others	0.21%	5		29.06%	255

indicators (i.e., ACE, Chao1, Simpson, Shannon and Pielou's evenness; Kruskal-Wallis, $p > 0.05$ in all cases) (Table S2, S3). Collection location, however, had a significant effect on the *alpha*-diversity (ACE, Chao1 and Shannon indicators) of the gut bacterial community in *A. cirsicola* (Table 4). There were also marginally significant differences in the ACE and Chao1 indices among populations of *A. fragariae*, but none of the indices differed among populations of *A. viridicyanea* (Table 4; S4). The results also show that Faith's phylogenetic diversity metric was not different among the three beetle species ($X^2 = 0.388$, $p = 0.824$; Fig. 2), whereas collection location consistently had a significant effect on phylogenetic diversity (*A. cirsicola*: $X^2 = 11.448$, $p = 0.022$; *A. fragariae*: $X^2 = 6.250$, $p = 0.044$; *A. viridicyanea*: $X^2 = 10.790$, $p = 0.029$).

In contrast with *alpha* diversity, we identified significant differences in gut bacteria species composition among the three beetle species as measured by the Bray-Curtis dissimilarity metric (Fig. 3; PerMANOVA, $R^2 = 0.115$, $p = 0.004$; pairwise comparisons: *A. cirsicola*-*A. fragariae*, $p = 0.002$; *A. cirsicola*-*A. viridicyanea*, $p = 0.044$; *A. fragariae*-*A. viridicyanea*, $p = 0.084$), whereas the result was non-significant for the Jaccard similarity index (Fig. 3; PerMANOVA, $R^2 = 0.067$, $p = 0.227$). The results of the two-way PerMANOVA suggested that both geography and collection year structured gut bacterial communities within each host species (Table 5; Fig. S1). Furthermore, given that all three beetle species were sampled at two locations (LEY and SH, see Table 1), we also examined the contribution of host species and geography in structuring gut bacterial communities using two-way PerMANOVA. We found that gut bacterial communities were significantly different both among beetle species (Jaccard: $F = 2.018$, $p = 0.002$; Bray-Curtis: $F = 1.430$, $p = 0.004$) and among sites (Jaccard: $F = 3.730$, $p < 0.001$; Bray-Curtis: $F = 2.533$, $p < 0.001$). In addition, we sampled *A. fragariae* and *A. viridicyanea* at three shared locations (LEY, SH, and SJS; see Table 1), and a two-way PerMANOVA similarly showed a significant difference among sites (Jaccard: $F = 2.945$, $p < 0.001$; Bray-Curtis: $F = 2.448$, $p = p < 0.001$); however, there were no differences between host species (Jaccard: $F = 0.864$, $p = 0.204$; Bray-

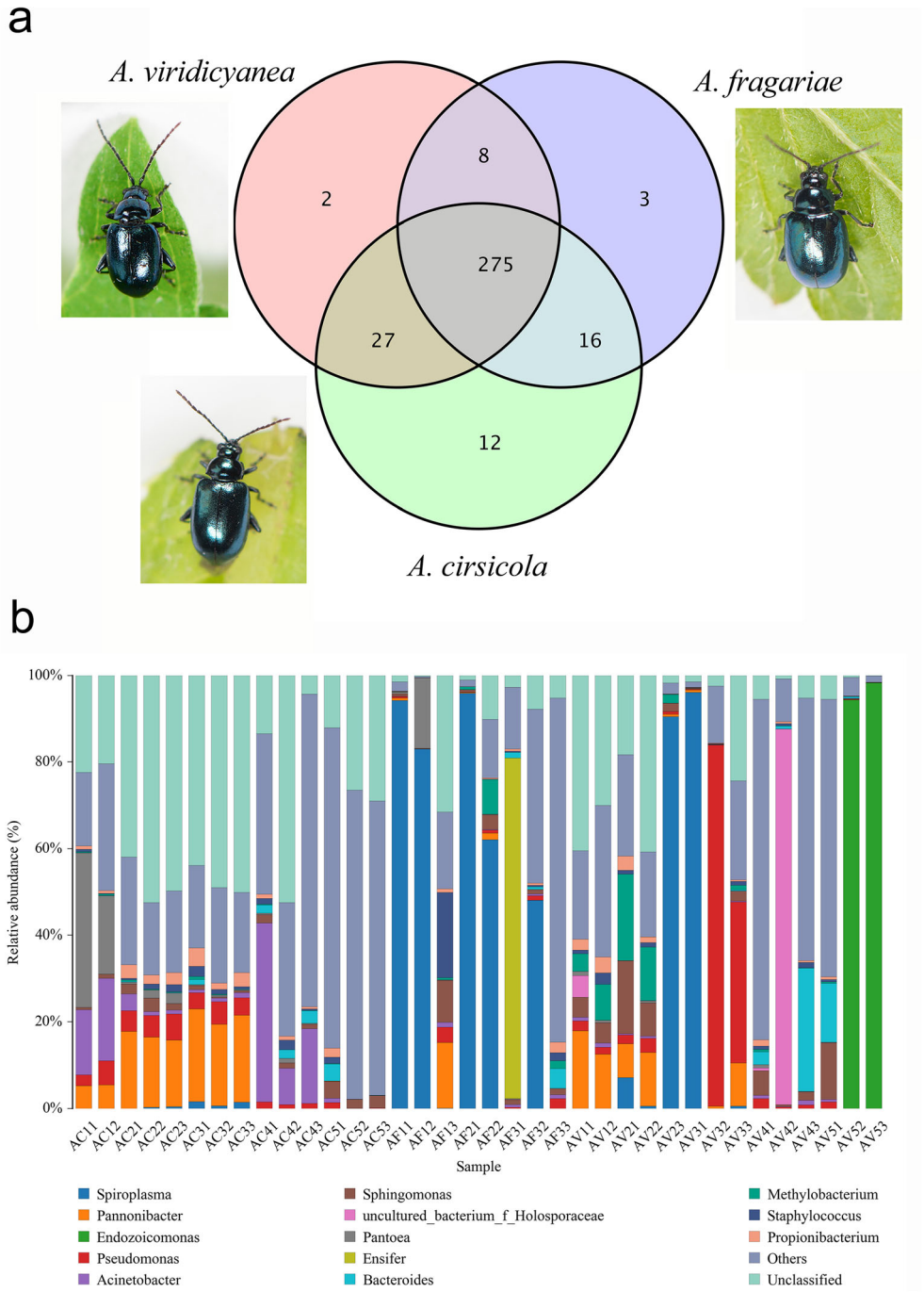

Fig. 1 Diversity of gut bacteria found in *Altica cirsicola*, *A. fragariae*, and *A. viridicyanea*. **a** Venn diagram represents the number of shared and species-specific OTUs. **b** Relative abundance of gut bacterial genera (> 1%) present in three *Altica* species

Curtis: $F = 0.955$, $p = 0.156$). Analyses based on unweighted UniFrac distances showed no significant differences in gut bacterial composition among beetle host species ($R^2 = 0.062$, $p = 0.316$), whereas significant differences were detected among locations within beetle species (*A. cirsicola*: $R^2 = 0.769$, $p = 0.001$; *A. fragariae*: $R^2 = 0.477$, $p = 0.001$; *A. viridicyanea*: $R^2 = 0.596$, $p = 0.006$) (Fig. S2).

Comparative abundance analysis between host species revealed differential abundance of six bacterial OTUs, including *Endozoicomonas* (OTU5), *Acinetobacter* (OTU27), *Kineococcus aurantiacus* (OTU327), *Nocardioides* (OTU208), *Comamonas testosteroni* (OTU62), and *Pseudomonas aeruginosa* (OTU6). Comparative functional analysis using PICRUSt showed similar patterns of metabolic functions among bacterial communities of the three beetle hosts. Although this analysis identified functions involved in xenobiotic biodegradation and metabolism as well as metabolism of terpenoids and polyketides (Fig. S3), there were no significant differences among beetle hosts ($p > 0.05$ for all pairwise comparisons).

Table 3 Number and relative abundance of OTUs per phylum found in three *Altica* species. For Proteobacteria and Tenericutes, the lowercase letters indicate significant differences between beetle species in relative abundance after Bonferroni correction for multiple testing

Phylum	No. and percentage of OTUs of AC	Relative abundance in AC (%)	No. and percentage of OTUs of AF	Relative abundance in AF (%)	No. and percentage of OTUs of AV	Relative abundance in AV (%)
Proteobacteria	147/44.55	70.53a	140/46.36	18.65b	139/44.55	68.37ab
Actinobacteria	39/11.82	8.90	41/13.57	2.84	42/13.46	3.95
Acidobacteria	28/8.48	5.11	22/7.28	0.81	22/7.05	0.21
Firmicutes	52/15.76	4.80	51/16.89	2.28	53/16.99	4.54
Nitrospirae	16/4.85	3.38	12/3.97	0.10	14/4.49	0.05
Bacteroidetes	21/6.36	2.40	19/6.29	0.57	21/6.73	3.88
Chloroflexi	9/2.73	1.91	8/2.65	0.27	6/1.92	0.11
Gemmatimonadetes	9/2.73	1.78	4/1.32	0.02	7/2.24	0.07
Nitrospinae	3/0.91	0.46	–	–	1/0.32	0.00
Cyanobacteria	1/0.30	0.36	–	–	–	–
Deinococcus-Thermus	1/0.30	0.17	–	–	2/0.64	0.09
Tenericutes	2/0.61	0.10c	3/0.99	74.28a	3/0.96	18.64b
Spirochaetae	1/0.30	0.06	1/0.3	0.08	1/0.32	0.04
Fusobacteria	1/0.30	0.06	–		1/0.32	0.04
Chlorobi	–	–	1/0.33	0.10	–	–
Total	330		302		312	

Effect of Symbiotic Bacteria on Insect Development

The mean development time of *A. viridicyanea* from second larval instar to pupation was increased in the group treated with antibiotics. In the control group, development time was 282.8 h, but the antibiotic-treated larvae required 325.8 h to pupate (Mann-Whitney *U* test, $U = 326.0$, $p < 0.001$, $n = 35$ for both antibiotic-treated and control group) (Fig. 4a). Similarly, we observed a significant decline in mean adult body weight of the antibiotic-treated beetles relative to untreated controls (Mann-Whitney *U* test, for males, $U = 364.0$, $p = 0.034$, 2.77 mg (treated, $n = 21$) versus 3.10 mg (control, $n = 51$); for females, $U = 740.5$, $p = 0.006$, 3.27 mg (treated, $n = 36$) versus 3.60 mg (control, $n = 62$)) (Fig. 4b).

Discussion

In the present study, we surveyed the gut bacterial communities of three closely related *Altica* flea beetles to determine whether host plant shifts are correlated with changes in gut communities. Although previous work suggests that host plant species is a major driver shaping gut microbiota in herbivorous insects [21, 34, 35, 66, 67], as a whole, we found little support for this idea, which is consistent with several studies on lepidopterans [36, 37]. Our results showed that *Altica* species using phylogenetically unrelated host plants shared more than 80% of their gut bacterial OTUs, and only a small number of species-specific OTUs were found. We found no significant difference in *alpha*-diversity indicators among hosts, and if only species presence/absence data were taken into consideration, there was no significant difference in the bacterial β-diversity among the three beetle species. Although it is possible that the handful of species-specific microbes are key mutualists that assist their beetle host with digestion of host plant tissues, we were surprised that there were no strong differences in community composition when comparing beetles feeding on different host plant species.

The sympatric habitats shared by the three *Altica* species in the present study are one possible reason why there were no

Table 4 Comparison of *alpha*-diversity indicators of gut bacterial communities among different collection sites in three *Altica* species using Kruskal Wallis tests

Host species	ACE		Chao1		Simpson		Shannon		Pielou's evenness	
	X^2	p	X^2	p	X^2	p	X^2	p	X^2	p
A. cirsicola	11.83	0.019	11.75	0.019	7.29	0.122	11.30	0.023	8.324	0.080
A. fragariae	5.14	0.077	5.14	0.077	2.00	0.368	2.00	0.368	2.000	0.368
A. viridicyanea	7.4	0.116	7.4	0.116	3.49	0.480	3.49	0.480	3.49	0.480

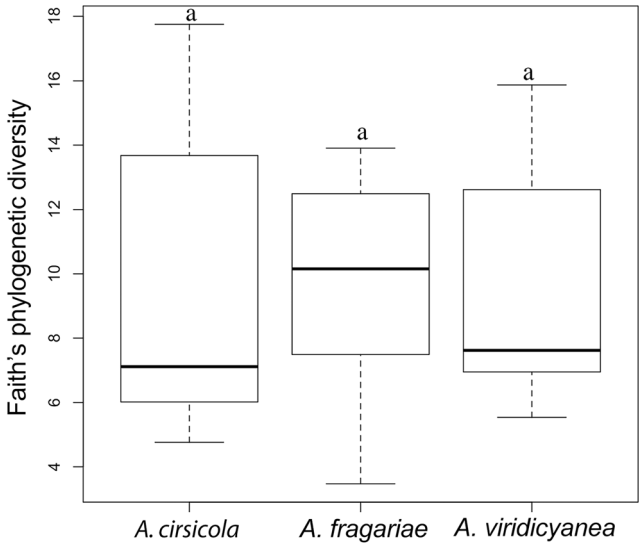

Fig. 2 Faith's phylogenetic diversity of gut bacterial community in different host species (*A. cirsicola*, *A. fragariae* and *A. viridicyanea*). Bars with different letters are significantly different ($p < 0.05$)

differences among host species based on presence/absence metrics. Since microbial associates are most likely obtained from the environment, *Altica* species living in close proximity may encounter the same gut bacteria OTUs. Although we expected that the microbiomes on leaves of different host plant species would differ due to the differences in the physical and chemical properties of leaves, a recent study suggests that leaf-feeding insects actually acquire their gut bacteria from the soil rather than the host plant [68]. As a result, beetles living in sympatry may be acquiring their gut bacteria from a common source. There are, however, some instances in which we would expect specialization of gut bacterial communities. Intimate associations with gut microorganisms, for instance, usually occur in social insects, such as termites, ants, bees, and dung beetles, because social interactions provide opportunities for vertical transfer of gut bacteria [16, 69–72]. In other species with unique behaviors such as the Japanese common plataspid stinkbug, *Megacopta punctatissima*, gut bacteria are transmitted from parents to offspring with the aid of a capsule [73]. Yet, in most cases, the lack of dependable transmission routes between host individuals means that most or all gut microorganisms are not transmitted between hosts. Instead, gut bacterial communities are transient and opportunistic associations that are dominated by widely distributed bacteria originating from changing environments [16, 22, 74, 75]. Indeed, in the present study, we also found that collection year affected gut bacterial communities. This spatiotemporal variability is perhaps additional evidence supporting the hypothesis of environmental acquisition of gut microbes in *Altica*.

The results of our study did, however, detect a significant difference in gut bacterial composition among host plant species when we examined relative abundance (i.e., Bray-Curtis metrics). This could be explained if host plant–based selection among bacterial species plays an important role in shaping gut community structure [76]. The physical conditions of insect host guts will vary in pH, redox potential, and the availability of particular substrates; therefore, the gut environment can filter out potential colonists [16, 40, 76]. In the closely related *Altica* species examined here, there are substantial differences in secondary plant chemistry that could feasibly act as a filter for bacterial communities. Although the presence/absence of specific bacterial species may not change among hosts, the significant shift in abundance among species could be adaptive if some species of bacteria are better equipped to handle the chemical composition of the host plant tissue being digested. Of course, there are potential drawbacks to interpreting metabarcoding data quantitatively [77], and the results presented here may be biased for a number of reasons. Consequently, a more clear understanding of the role of abundance shifts with respect to host plant use would benefit from closer examination of the bacterial species that are strongly affected by insect diet.

In contrast with beetle species identity, geographic location contributed substantially to gut bacterial composition. In most cases, there were significant differences among sites within species (Table 5), and the two-way PerMANOVA analysis also suggested that collection location contributed more to gut bacterial composition than host species. The lack of a pattern with respect to host species may be explained by relatively high variability in bacterial composition among populations that may have masked any host-specific patterns. That said, multiple factors often act synergistically to create the structure of gut bacterial communities. For example, a study of two serious lepidopteran pests showed that although host

Table 5 The role of collection site and collection year in structuring gut bacterial communities within *Altica* species. The analyses carried out using two-way PerMANOVAs and used "collection site" and "collection year" as main effects

Host species	Collection site				Collection year			
	Jaccard		Bray-Curtis		Jaccard		Bray-Curtis	
	F	*p*	*F*	*p*	*F*	*p*	*F*	*p*
A. cirsicola	0.860	< 0.001	1.247	< 0.001	2.385	< 0.001	2.619	< 0.001
A. fragariae	0.298	0.014	0.246	0.246	0.415	0.008	0.312	0.100
A. viridicyanea	0.434	< 0.001	0.305	0.018	1.261	< 0.001	0.361	0.026

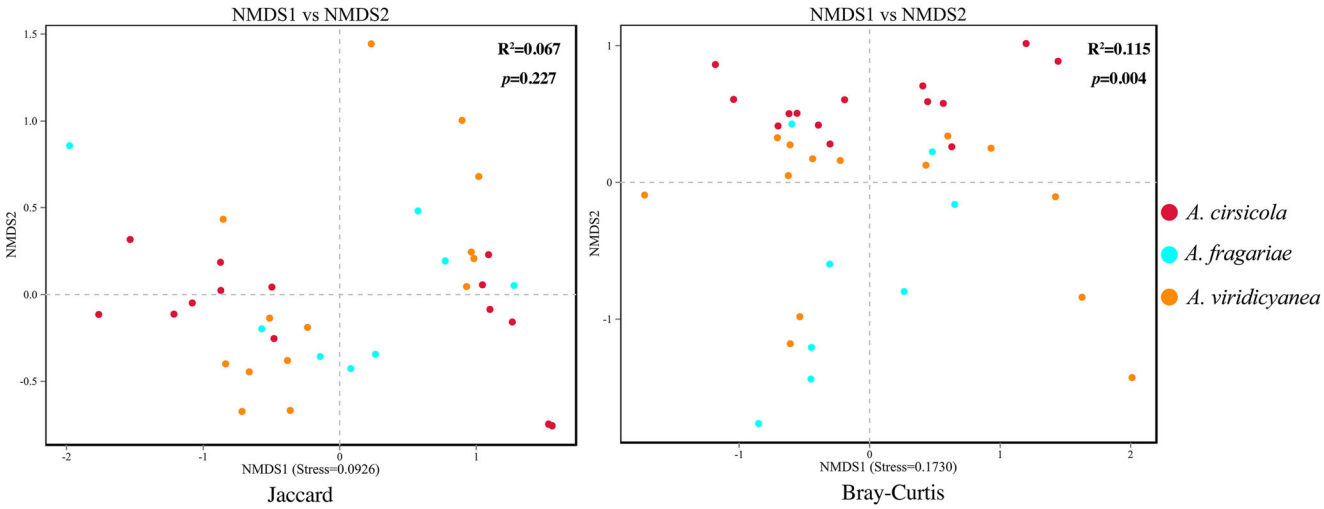

Fig. 3 Non-metric multidimensional scaling (NMDS) plots visualizing gut bacterial community dissimilarities of three *Altica* species. R^2 and p values are based on the similarity test of PerMANOVA

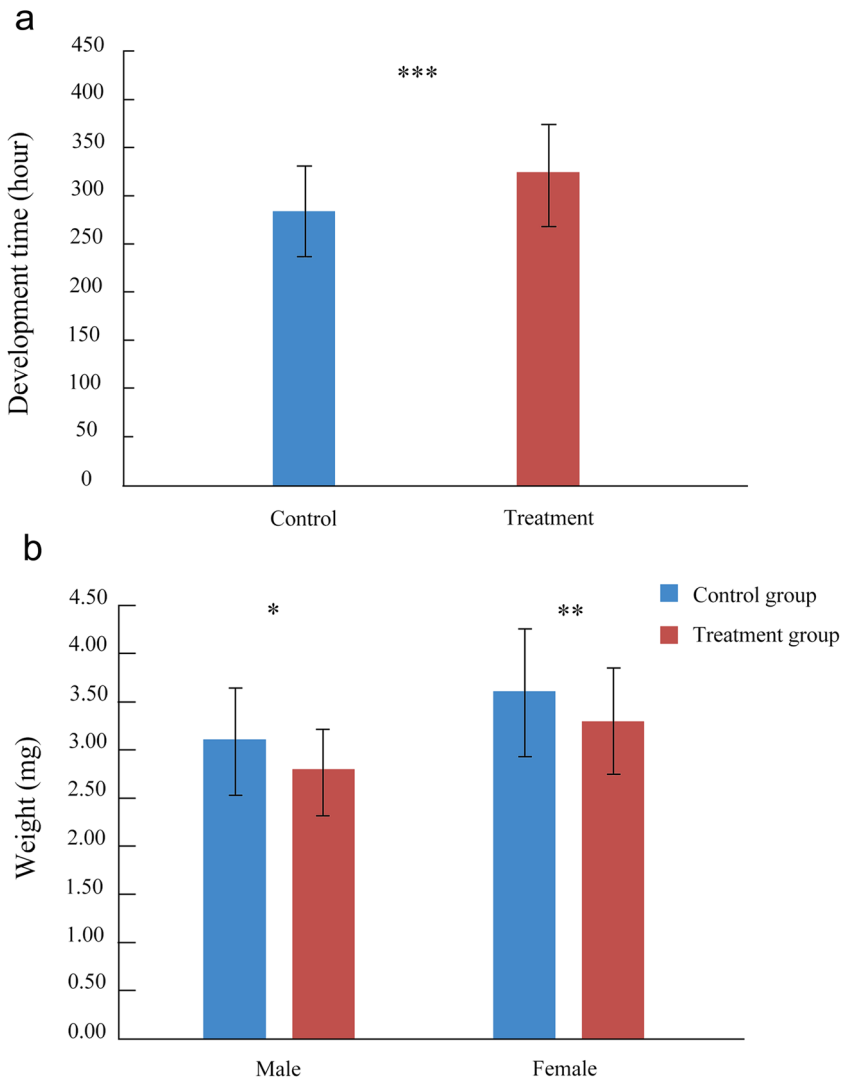

Fig. 4 Effect of gut bacteria on development and body mass of *A. viridicyanea*. **a** Mean development time from second larval instar to pupation of antibiotic treated and untreated beetles. **b** Adult body weight of the antibiotic treated and untreated beetles. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$

plant is a major driver shaping gut microbiota, insect physiology, gut region, and local factors can also contribute to variation in microbiomes [35]. Likewise, a meta-analysis indicated that insect gut bacterial diversity was determined by environmental habitat, diet, developmental stage, and phylogeny of the host [32].

Although the gut bacterial community did not show host-specific patterns among *Altica* species, pervasive gut bacteria might still contribute to performance of their *Altica* hosts. Specifically, we found that the development time of *A. viridicyanea* was prolonged, and adult body weight declined significantly in antibiotic-treated beetles. One possible explanation for this observation is that the gut bacterial communities provide benefits to the insects via a number of potential mechanisms. For instance, gut microbes may assist with digestion of toxic plant compounds, prevention of the invasion by pathogens, or production of other nutrients required for growth and development [78, 79]. Indeed, our functional analysis of gut bacterial genes identified gene functions associated with degradation of toxic compounds that could theoretically assist *Altica* in digestion and promote faster development and larger final body size. Furthermore, comparative abundance analysis detected six OTUs with differential abundance between host species. Among them, *Acinetobacter* was reported to degrade glycosides [18], and *Pseudomonas* can degrade tannins and terpenes [80, 81]. Such mechanisms have been shown for a number of species, including coleopterans. For example, gut bacteria facilitate adaptation to crop rotation in the western corn rootworm [13], and gut bacteria are proposed to degrade defense chemicals consumed by gypsy moths [18], coffee berry borers [19], and pine weevils [82]. In the present study, the results are only weakly supportive of the hypothesis of gut microbial detoxification as it remains unknown whether the microbial species present in *Altica* detoxify toxic compounds or whether the gene functions that we identified are exceptional among beetles. Chewing herbivorous insects, such as lepidopteran and sawfly larvae, beetles, and orthopterans, are predicted to be particularly reliant on microbes for detoxification [20]; however, several recent studies have shown different patterns: some caterpillars do not rely on specific bacterial symbionts and lack a resident gut microbiome [22, 26, 83]. Functional studies on the gut microbiota of foliar-feeding insects other than lepidopteran species are limited; thus, it remains unclear whether gut bacteria are universally required for herbivorous insects. Additional work in *Altica* and other species will thus increase our understanding of the linkages between gut bacteria communities and digestion of their host plants.

Although our results fit with the idea that gut bacterial communities benefit *Altica*, another possibility is that the antibiotic treatment itself negatively impacted insect performance. Due to the challenges of running experiments with controls in which beetles were treated with antibiotics and then subsequently re-inoculated with bacterial communities, we were unable to rule out whether the treatment itself caused the observed declines in performance. Changes in performance could have been a direct result of the antibiotic treatment if it was toxic to the beetle larvae, or alternatively, the treatment may have indirectly caused increased susceptibility to pathogen attack. Other work has shown that similar treatments had no effect on survival and fitness in some insects [84], but whether this is universally true remains to be tested.

Changes in performance may also be caused by the antibiotic treatment if removal of the non-gut symbionts had a negative impact on the beetle larvae. We notably discovered that a considerable proportion (59.5%) of the bacteria detected in the present study belonged to the endosymbiont *Wolbachia*. *Wolbachia* was detected in 94.4% (34/36) of samples (pooled by 2-3 beetles for each sample) in this study. A recent review showed that the typical infection rate of *Wolbachia* in beetles is about 38% [85]; however, a previous study showed that the infection rate can be as high as 100% at the species level and 97.0% at the population level across 11 *Altica* species [86]. These consistently high rates of infection in *Altica* suggest the opportunity for *Wolbachia* species to impact insect performance. Although the hypothesis of *Wobachia*-induced cytoplasmic incompatibility was rejected [87], other potential effects to *Altica* hosts remain to be explored, including iron metabolism [86], resistance to viruses [88], and protecting of the host by downregulation of defense genes in the host plants [89]. Thus, the possible negative effects on host performance due to depressing *Wolbachia* remain unclear.

Together, our results show that sympatric *Altica* species have largely overlapping bacterial communities that differ in relative abundance, suggesting that insect diet may act as an important ecological filter that shapes gut community structure. At the same time, temporal and geographic variation in gut communities within *Altica* species will create opportunities for insect hosts to partner with novel microbes that could have beneficial effects on insect performance.

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