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The Trichoplusia Ni Gut Microbiome and Its Derivation from the Phyllosphere of Its Food Plants

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THE *TRICHOPLUSIA NI* GUT MICROBIOME AND ITS DERIVATION FROM THE PHYLLOSPHERE OF ITS FOOD PLANTS

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ABSTRACT

Insects are the most abundant and diverse animals on planet Earth and rely on their diverse microbiomes to be so. The insect gut microbiome is vital in the growth and development of many insect species. *Trichoplusia ni*, the cabbage looper, is a generalist herbivore, but little is known about its microbiome. In this study, a metagenomic analysis of fecal samples was used to determine the effect of diet on the microbiome of *T. ni* larvae. Larvae were reared on six plant species, the microbiome was sampled from fecal material, and the phyllosphere was sampled from leaves the larvae ate. Bacteria were identified using 16S ribosomal RNA gene sequences, and diversity was quantified. Similar phyla, classes, and families were found in both types of samples, suggesting *T. ni* larvae primarily obtain their gut microbiome from their diet. However, the gut microbiome of *T. ni* larvae is not identical to the phyllosphere, particularly in relative abundances, suggesting that other factors in the insect gut environment may further modify the diversity of the microbiome. This study adds to the growing body of evidence about the implications of diet for the insect gut microbiome.

Keywords: 16S rRNA gene, Illumina sequencing, metagenomics, *Trichoplusia ni*

INTRODUCTION

Insects are members of the most abundant and diverse animal clade on planet Earth (Basset et al. 2012). The evolutionary success of insects depends partly on their symbiotic relationship with microorganisms (Geib et al. 2009). Similar to other complex animals, the alimentary canal of insects is the primary site of microbial colonization. The gut's collection of bacteria, fungi, and viruses form an insect's gut microbiome. The bacteria in the gut microbiome can be examined by directly analyzing the bacterial DNA from insect feces. This method is called metagenomics and involves sequencing the bacterial 16S rRNA gene. This gene codes for a ribosomal rRNA that is highly conserved among bacteria. However, within the gene there are hypervariable regions that are unique to each bacterial species. The conserved regions of the 16S rRNA are used for building universal primers while the hypervariable regions allow for bacterial classification (Ames et al. 2017).

Gut microorganisms aid in digestion of complex macromolecules and allow insects to feed on a myriad of diets. The insect microbiome also provides protection against other pathogens and predators, governs mating systems, and affects efficiency as disease

vectors (Engel and Moran 2013; Janzen et al. 2017; Ma and Leulier 2018; Yun et al. 2014). Therefore, the contribution of the microbiome to insect function is highly relevant to medicine, agriculture, and ecology.

Similar to mammals, some insect species acquire their microbiome via maternal transmission and social interactions with other insects (Engel and Moran 2013). However, studies have shown that many insect species acquire their gut microbiome primarily via their diet, specifically through the phyllosphere (Engel and Moran 2013). The phyllosphere is the microbiome that inhabits the surface of plants. While a few microbial species inhabit plant tissues, most colonize the surface (Lindow and Brandl 2003).

Lepidoptera is one of the most diverse insect orders, with 150,000 described species (Sree and Verma 2015). Most species are specialist herbivores as larvae (Robinson et al. 2002), feeding on a restricted range of host plants. Only a few species are generalists as larvae, able to consume members of several botanical families (Bernays and Minkenberg 1997). Some generalist lepidopteran larvae can be destructive agricultural pests and are well-studied as a result (Cunningham and Zalucki 2014; Franklin et al. 2011; Zhang et al. 2019). Bacterial symbionts are likely important in determining host plant use and establishment (Hammer and Bowers 2015), yet only a small number of species have been studied for bacterial symbionts (Mitter et al. 2017). Research by Hammer et al. (2017) suggests that many symbionts found in the lepidopteran gut are acquired from the diet. Analysis of the species and functional diversity of lepidopteran gut microbiomes may yield insights into their host plant use, which may have important implications in pest management.

In this study, a metagenomic analysis was carried out to determine the effect of diet on the gut microbiome of *Trichoplusia ni* Hübner (Lepidoptera: Noctuidae) larvae. *Trichoplusia ni* is a generalist herbivore with a host range that encompasses over 100 plant species (Robinson et al. 2002). Larvae are known as economic pests of plants in Brassicaceae, but are also considered pests of plants in Apiaceae, Amaranthaceae, Asteraceae, and other families (Capinera 1999; Hoo et al. 1984). Because this species is economically important and relatively easy to rear, its growth, metabolism, and behavior are well-studied (Capinera 1999; Shorey et al. 1962). However, much less is known about the gut microbiota of *T. ni* larvae. We hypothesized that the phyllosphere was the primary source of microorganisms found in the gut of *T. ni* larvae. This hypothesis led to the prediction that the same taxonomic groups of bacteria would be found in the phyllosphere of food plants of *T. ni* larvae and in their gut microbiomes.

METHODS

Samples were obtained from a course-based undergraduate research experience at the University of North Georgia (see Lampert and Morgan 2015 for background). The participants reared four sets of 12 *T. ni* larvae using four different plant species belonging to four different families (Table I) purchased from local supermarkets. Only organic plants were purchased to ensure no pesticide residues were present. Plants were removed unwrapped and intact from produce display bins except for *Coriandrum sativa*, which was purchased in bundles of stems. Plant materials were placed in water picks and refrigerated to remain fresh, but were not washed after purchase.

Table I. List of plant families used in this experiment

Plant species	Plant common name	Plant family
Beta vulgaris	chard	Amaranthaceae
Brassica oleracea	kale	Brassicaceae
Coriandrum sativa	cilantro	Apiaceae
Lactuca sativa	lettuce	Asteraceae

Sets of twelve, 7-day old *T.ni* larvae were transferred using sanitized forceps to leaves and reared under ambient conditions (22°C) in BugDorm tents (BioQuip Products, Inc., #1462W). Tents were sanitized in a dilute bleach solution before use. Leaves were replaced daily. The phyllosphere was sampled on the same day that *T. ni* larvae were placed on leaves. To sample the phyllosphere, 1 mL of 0.85% NaCl was pipetted onto both surfaces of each leaf, spread over the surface of the leaves with a sterile glass bar, and then collected in a 1.5 mL microcentrifuge tube. After a period of five days, ~50 fecal pellet samples (250–500 µL) from each rearing container were collected. Fecal pellets were transferred using ethanol-sanitized forceps into 1.5 mL microcentrifuge tubes containing 0.85% NaCl. Tubes were mixed using a vortex mixer to break up fecal pellets and suspend bacterial cells. Samples were frozen until DNA extractions were performed.

Metagenomic DNA was extracted from fecal samples and eluate of leaf samples using a ZymoBIOMICS DNA Microprep Kit (D4301/D4305) according to the manufacturer's directions. Briefly, each fecal sample was placed in a 2 mL collection tube with 750 µl of lysis solution, which contained 0.1 mm beads. Each sample was processed in a bead beater (Beadmill 24, Fisher Scientific) at maximum speed for 5 min, and then centrifuged at 10,000 × g for 5 min. After homogenization, the manufacturer's protocol was followed for eluting DNA from each sample. Quality and quantity of DNA was analyzed using a nanodrop spectrophotometer (Nanodrop Lite, Thermo Scientific). The extracted DNA was stored on ice and sent to GENEWIZ for 16S-genome sequencing with Illumina MiSeq and bioinformatic analysis. V3 and V4 hypervariable regions of the 16s rRNA gene were sequenced.

Reports generated by GENEWIZ for each of the sample runs included Shannon diversity, species richness, and number of reads from each of the eight most diverse bacterial taxa from phylum to order. Lower taxonomic ranks were not considered due to unknowns and high numbers of taxa with low relative abundance (<0.4–<2.0, depending on the run) and were grouped together as "other". The number of reads was used to calculate the relative abundance of each phylum, class, and order in each sample run. Relative abundances of the most abundant bacterial phyla, classes, and orders were compared between phyllosphere and gut microbiome samples using *Z*-tests for proportions. One *Z*-test was performed for each of the four most common bacterial taxonomic groups for each of the four food source species; thus, 48 *Z*-tests (4 × 4 × 3) were performed. *Z*-tests were performed using Microsoft Excel 2016.

The number of reads of each phylum, class, and order was used to perform three sets of multivariate analyses, one for each taxonomic level. Number of reads were standardized and square root transformed, and resemblances between each of eight samples (four food source species and two sources of bacteria [phyllosphere versus gut microbiome]) were measured with Bray-Curtis similarity. Transformed numbers of reads were used for analyses, and analyses included both cluster analysis and multidimensional

scaling. Multivariate analyses were performed using the *basic multivariate analysis* procedures in PRIMER 7 (PRIMER-e, Quest Research Limited).

RESULTS

Overall, the number of reads ranged from 348,357 (gut microbiome) to 568,963 (phyllosphere, both in the *B. vulgaris* system), and at least 93% of the reads could be identified to genus for each run. Although there was a high rate of reads that could not be identified beyond genus to species, the number of species identified ranged from 244 (gut microbiome, fed *Beta vulgaris*) to 695 (phyllosphere, *Brassica oleracea*). On average, the phyllosphere samples had greater Shannon diversity and species richness (Table II).

Table II. Summaries of Shannon index (*H'*) and species richness (*S*) of bacterial communities

Sample type	*n*	*H'*	*S*
Plant	4	2.76	540.75
Frass	4	1.89	350.25

Each of the *Z*-tests of proportions was found to be statistically significant, with $P < 0.0001$ except for the order *Enterobacteriaceae* in the *B. vulgaris* system ($Z = -2.27$, $P = 0.023$). The shifts in relative abundances of bacterial groups from phyllosphere to gut microbiome tended to differ in the *B. oleracea* system. For instance, the relative abundance of the most dominant phylum, *Proteobacteria*, was lower in the phyllosphere samples (Figure 1A) compared to the gut microbiome (Figure 1B) in the *B. oleracea* system, while the opposite was observed in the other systems (Figure 1). Within the *Proteobacteria*, samples from the *B. oleracea* system had lower relative abundances of *Gammaproteobacteria*, which was the dominant proteobacterium in the other systems (Figure 2). In the *B. oleracea* phyllosphere, *Actinobacteria* was the dominant class (Figure 2A), but its relative abundance decreased in the gut microbiome (Figure 2B). Relative abundance of bacteria in the class *Bacilli* also increased in the gut microbiome relative to the phyllosphere in the *B. vulgaris* and *Coriandrum sativa* systems (Figure 2). The order *Pseudomonales* was the dominant phyllosphere order in the *B. vulgaris*, *C. sativa*, and *Lactuca sativa* systems (Figure 3A), but its relative abundance decreased in the gut microbiome for every system except the *B. vulgaris* system (Figure 3B).

Our multivariate analysis revealed that bacterial communities clustered more by sample source (phyllosphere or gut microbiome) than by system. *Brassica oleracea* was the only system in which the phyllosphere and gut microbiome samples clustered together at the phylum, class, and order levels (Figure 4). At the phylum level, the two *B. oleracea* communities clustered with the gut microbiome from the *B. vulgaris* and *C. sativa* systems, while the gut microbiome of *L. sativa*-fed *T. ni* larvae was more like the phyllosphere of the other three systems but still fairly distinct (~80% similarity with their phyllospheres; Figure 4A). The communities could be separated into two major groups at the phylum level (Figure 4A). At the class level, the *B. oleracea* system bacterial communities were clearly different from any other communities (60% or less similarity; Figure 4B). The microbiomes from the *B. vulgaris* and *C. sativa* systems and the

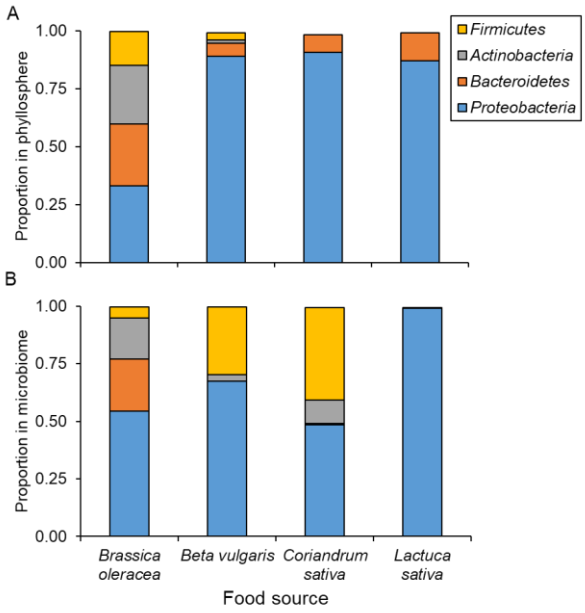

Figure 1. Proportions of four bacterial phyla found in the phyllosphere (A) and in the *Trichoplusia ni* gut microbiome (B).

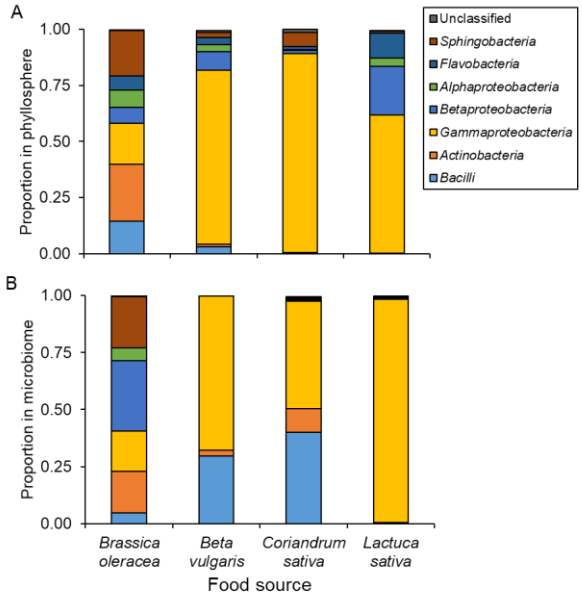

Figure 2. Proportions of seven bacterial classes and bacteria unclassified to class found in the phyllosphere (A) and in *Trichoplusia ni* gut microbiome (B).

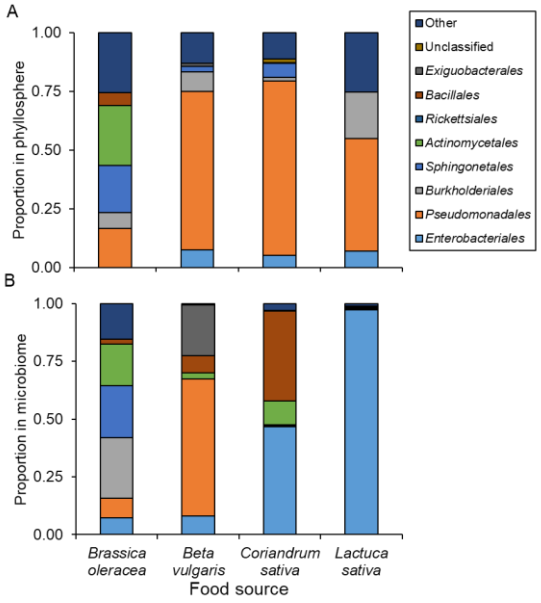

Figure 3. Proportions of nine bacterial orders, other minor orders, and bacteria unclassified to order found in the phyllosphere (A) and in *Trichoplusia ni* gut microbiome (B).

phyllospheres from those same two systems also clustered together at the class level (Figure 4B). At the order level, the gut microbiomes clustered together for each system except the *B. oleracea* system (Figure 4C). The unique bacterial communities at the order level were obtained from the *C. sativa* and *L. sativa* phyllospheres, which clustered together (Figure 4C).

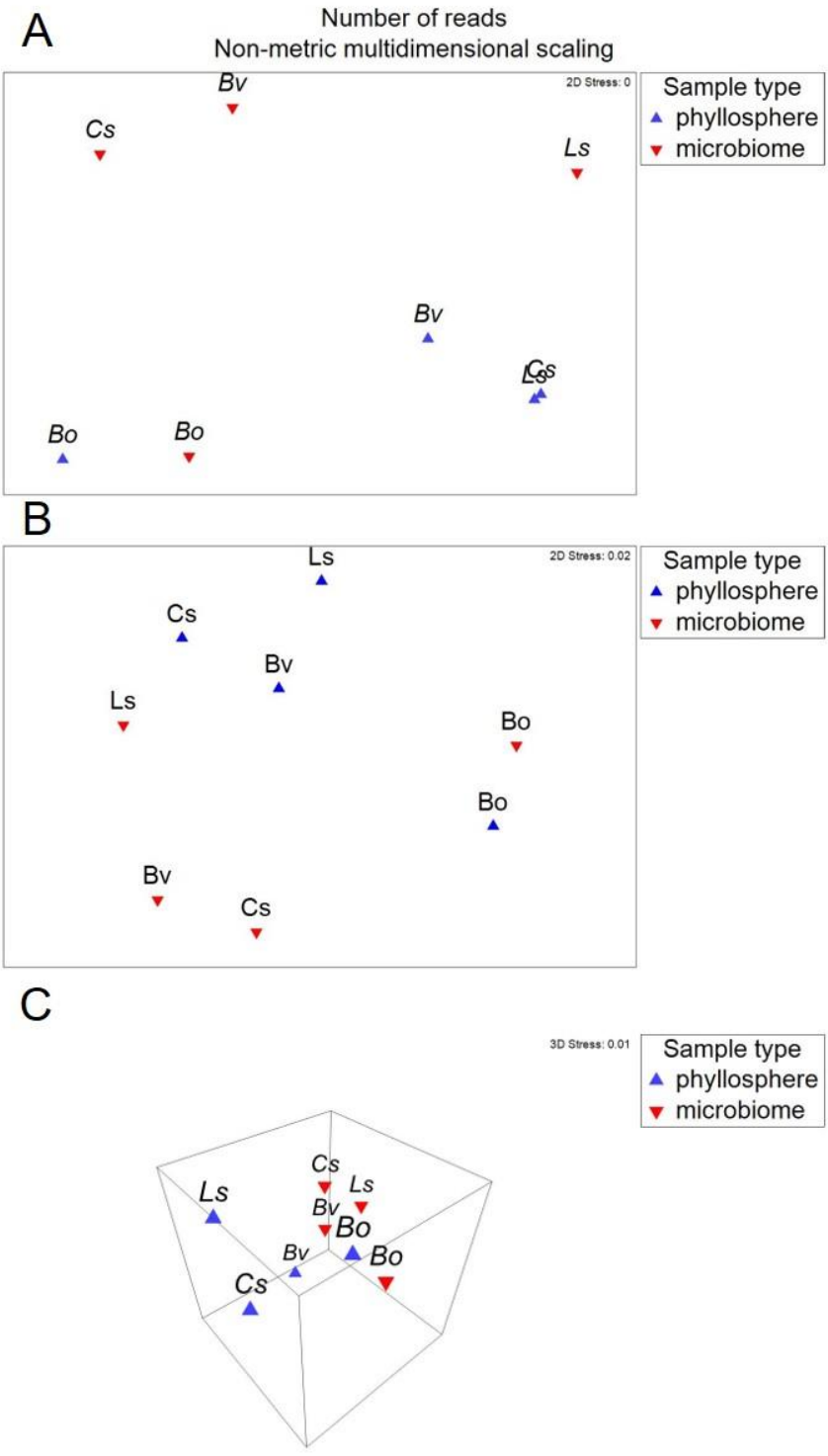

Figure 4. Scatterplots generated by multidimensional scaling analysis of the numbers of reads of different bacterial groups at the phylum (A), class (B), and order (C) levels identified from the phyllosphere or *Trichoplusia ni* larval gut microbiome. Two-dimensional plots represent phylum and class, while the three-dimensional plot due to reduced stress represents order. Labels represent names of plants upon which *T. ni* larvae were reared; Bo – *Brassica oleracea*; Bv – *Beta vulgaris*; Cs – *Coriandrum sativa*; *Ls – Lactuca sativa*.

DISCUSSION

Previous studies have shown that lepidopteran larvae are colonized by none or very few bacteria in comparison to other insect orders (Hammer et al. 2017). Therefore, it is thought that diet is the primary source of microbial communities in these insects. Our study provides some support for this hypothesis. Similar bacterial groups were found in both the *Trichoplusia ni* larval gut microbiome and the phyllosphere, suggesting that the diet is a source of the gut microbiome in *T. ni*. A small number of bacterial species were observed in the *T. ni* gut microbiome but were not observed in the plant phyllosphere. This suggests a second source for acquisition of gut microbes in *T. ni*. Similar to other lepidopterans, *T. ni* larvae bite through and ingest their eggshell while hatching (Hammer et al. 2017). Bacteria that may be present on the outer egg surface can potentially colonize these larvae.

In most cases, the phyllosphere and microbiome included the same bacterial taxa regardless of diet (*B. oleracea* was the only exception). At the phylum level, both phyllosphere (77%) and microbiome (67%) were dominated by *Proteobacteria*. Previous studies have shown that *Proteobacteria* predominate leaf surfaces while *Actinobacteria* and *Firmicutes* are abundantly found in root associated communities (Bodenhausen et al. 2013). Moreover, the majority of *Proteobacteria* in both the phyllosphere and microbiome were *Gammaproteobacteria*, a diverse and medically important group.

The gut microbiome of *T. ni* was not identical to the plant phyllosphere. First, some bacterial groups were found only in the phyllosphere or microbiome. For example, *B. oleracea* leaf surface showed no presence of *Enterobacteriales*, yet they were present in the gut microbiome of *T. ni* larvae fed on those leaves. Likewise, two sets of larvae, reared on *C. sativa* and *L. sativa*, contained no *Pseudomonales* in their microbiomes but *Pseudomonales* was a major constituent (52%) of the phyllosphere. *Betaproteobacteria*, a common component of the phyllosphere, was only found in the gut microbiome in the *B. oleracea* system. Second, the relative abundance of each bacterial species in individual groups of *T. ni* larvae was different from those found on leaf surfaces used for feeding. *Firmicutes*, a group of mostly Gram-positive bacteria that includes some pathogens, was found at a higher relative abundance in the gut microbiome compared to the phyllosphere.

The difference in bacterial abundance suggests that the insect gut environment selects and enriches for certain microbial species. This selection may be due to inherent alkaline conditions in the gut that may suppress the growth of certain bacteria. Some selection may be due to diet itself, which may promote growth of certain bacteria. For example, plants contain many phenolic compounds that can have a detrimental effect on digestive enzymes in the gut. These phenolic compounds can also promote production of reactive oxygen species under alkaline conditions. The *Enterobacteriales*, such as *Enterobacter* spp. in the lepidopteran gut, can provide reactive oxygen species-detoxifying enzymes like superoxide dismutase or catalase (Voirol et al. 2018; Xia et al. 2017).

The community composition of both the phyllosphere and microbiome differed substantially in the *B. oleracea* system compared to the other systems. Brassicaceae are characterized chemically by glucosinolate and myrosinase production. When insects feed on brassicaceous plants, the damaged leaf tissue releases myrosinase that hydrolyzes glucosinolate into isothiocyanates, which is toxic to most living organisms including insects and microbes. Some specialists such as *Pieris rapae* are capable of hydrolyzing glucosinolates into less toxic nitrile compounds (McKinnon 2016; Robinson et al. 2010).

However, diets high in glucosinolates significantly affect the gut microbiome of *P. rapae* (McKinnon 2016). Further research with the *B. oleracea* system, particularly controlled experiments in which the amount of dietary glucosinolates is manipulated, may explain the differences found in this study.

In conclusion, this study provides information about the microbiome of a generalist herbivore that is an economic pest. In further research, the addition of a group of larvae reared upon a sterilized diet would more precisely allow the determination of which microorganisms are acquired from the diet. Such a diet would contain no living microorganisms, and thus any microbiome established in those larvae would have been acquired by another method. Additional research into the phyllosphere of brassicaceous plants and how communities colonize the *T. ni* microbiome will be pursued. A clear understanding of the acquisition of the lepidopteran gut microbiome is valuable for answering questions in basic and applied ecology and may provide insights into controlling species that are herbivorous pests.

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