The Gut-Associated Microbiome of the Eastern Spruce Budworm Does Not Influence Larval Growth or Survival

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ABSTRACT

Microbial communities have been shown to play an important role for host health in mammals, especially humans. It is thought that microbes could play an equally important role in other animal hosts such as insects. A growing body of evidence seems to support this, however most of the research effort in understanding host-microbe interactions in insects has been focused on a few well-studied groups such as bees and termites. We studied the effects of the gut-associated microbial community on the growth of the eastern spruce budworm *Choristoneura fumiferana*, an economically important lepidopteran forest pest in eastern Canada and the northeastern United States. Contrary to our expectations, although antibiotics influenced spruce budworm microbial community structure, the gut microbial community of spruce budworm larvae did not influence host growth or survival. Our results agree with the hypothesis that lepidopteran larvae lack resident microbial communities and are not nutritionally dependent on bacterial symbionts. However, while most bacteria originating on foliage appear to be transient through the gut and could not be linked with host growth, some bacteria may thrive better in the *C. fumiferana* gut.

IMPORTANCE

The importance of bacterial symbiosis has been clearly shown in humans. This has led to a number of studies looking for similar levels of dependence on microbial communities for host health in other organisms. The eastern spruce budworm (*Choristoneura fumiferana*) is an economically important lepidopteran forest pest that feeds on the hard to digest needles of balsam fir and spruce trees making it an ideal candidate for studying host-microbe interactions. We found that disturbance of the gut microbiome with antibiotic treatment did not significantly affect the growth of *C. fumiferana* larvae and the majority of bacteria in the *C. fumiferana* gut were more abundant in diet samples. Our findings generally support the recent hypothesis that

lepidopteran larvae lack resident microbiota, however we also show that some rare taxa increase in relative abundance in the gut suggesting weak selective pressures are at play in the larval *C. fumiferana* gut.

INTRODUCTION

The eastern spruce budworm (*Choristoneura fumiferana*) is a forest pest native to the north eastern United States and eastern Canada that undergoes epidemic population outbreaks every 30-40 years. During these population outbreaks, lasting for approximately ten years, millions of hectares of balsam fir (*Abies balsamea*) and spruce (*Picea spp*.) trees are defoliated (1–4). Consequently spruce budworm has significant effects on forest productivity (5) is an economically important defoliator in coniferous forests (6) and. While the role of factors such as landscape context, parasitoids and other predators, and forest management practices have been hypothesized to play a role in governing spruce budworm health and population dynamics (7, 8), much less is known about the importance of the microbial life associated with spruce budworm for their growth, population dynamics, and outbreak status.

Insects are associated with diverse communities of microorganisms including bacteria and fungi. The collective set of microbial genomes associated with a host, the microbiome, has a much greater functional diversity than the eukaryotic host genome (9). One of the most important functions of the insect gut microbiome is its potential to aid in digestion by breaking down compounds the host cannot digest (10, 11). Thus the microbiome can act as an extension of the host gut. This is particularly important when considering the spruce budworm because it feeds on

conifer needles that are acidic, tough to digest and contain defensive compounds such as terpenes.

Drawing generalizations about the extent to which insect microbiota are important to their hosts is difficult due in large part to the morphological, physiological, and behavioral variation among insects. Different physiology, life histories, and feeding strategies can all influence how microbes interact with their host (12). There is evidence to support the assumption that gut microbial symbiosis is important in a number of insects (12–19). There is a body of evidence showing that termites depend on specialized bacteria or flagellates to allow them to digest cellulose (11, 12, 17), and various species of bees benefit greatly from their associated microbiota (13, 15 ,18, 19). Bee-associated microbes have been shown to contribute to immune function as well as to aid in nutrition through mediating the digestion of pectin (13, 19).

The lepidopteran microbiome has been described as a very simple microbial community compared to other insects (20–27). One reason that lepidopteran gut microbial communities tend to be simpler than other insects is that the lepidopteran larval gut is a simple tube without any specialized structure for microbial cultivation as is seen in termite guts (12). Another unique aspect of the lepidopteran larval gut is that unlike most insect midguts which are acidic and range in pH between 4-7, lepidopteran midguts are highly alkaline ranging from pH 8-12 (12, 20, 23, 25). The alkaline nature of the spruce budworm gut could provide some advantage in digesting acidic conifer needles.

The objectives of this study were to determine if the gut microbiota associated with spruce budworm larvae, resident or otherwise, influence larval growth rates and survival, and to determine if the eastern spruce budworm has a resident gut microbiome by comparing the community composition of foliage, spruce budworm guts, and frass community assemblages that

is distinct from the microbial assemblages associated with foliage. We also sought to quantify the effects that antibiotics would have on spruce budworm gut microbial diversity and community composition.

Given the challenges associated with a diet of conifer needles, we hypothesized that the eastern spruce budworm has a resident gut microbiome that contributes to larval growth and survival. Thus, we further hypothesized that the disturbance of microbial communities with antibiotics will negatively influence spruce budworm larval growth and survival. We also hypothesized that the use of antibiotics will both reduce diversity of the microbial communities associated with diet, guts, and frass, and would significantly alter the composition of the spruce budworm gut microbial community in a way that would negatively influence larval growth and survival.

RESULTS

Effects of diet and antibiotics on larval survival

Diet did not have a significant effect on eastern spruce budworm larval survival rates (logistic regression; z= -0.897, p=0.3695), however antibiotic treatment tended to favor survival (logistic regression; z= -1.810, p=0.0702) (Fig S1). Because the synthetic diet was designed to be optimal for spruce budworm growth and survival a second logistic regression was performed only on larvae that fed on foliage and we found that there was no longer a trend of antibiotic treatment on larval survival (logistic regression; z=0.110, p=0.913). The trend of antibiotic treatment favoring survival seems to be driven by the difference between larvae feeding on spruce (30% survival) that was not treated with antibiotics and larvae that fed on synthetic diet (60% survival) that contained antibiotics. Larvae feeding on spruce and fir treated with antibiotics both had 50% survival and larvae feeding on fir without antibiotics had 52.5% survival.

Effects of diet and antibiotics on larval growth rate

Antibiotic treatment and time significantly affected the weight of spruce budworm larvae (Table 1, Table S3). In the model, the estimates of time as a main effect represent larval growth rate. The overall differences in growth rate observed between spruce budworm larvae in different experimental groups were due to the antibiotic treatment, however this result must be interpreted carefully because it appears to be largely driven by an interaction between diet and antibiotic treatment rather than a consistent effect of antibiotics on growth rate. Individuals feeding on fir treated with antibiotics grew less than those feeding on antibiotic treated spruce foliage (-0.020 ± 0.005 (mean ± SE); $p<0.0001$), and larvae feeding on untreated fir foliage grew less than those feeding on untreated spruce (-0.017 ± .006; $p=0.032$, Fig. 1, Table 1,Table S4). Growth rates of larvae feeding on antibiotic treated and untreated foliage of the same type (i.e fir or spruce) did not differ. Therefore, differences in growth rate observed among different groups of larvae are due to differences in diet more so than any disturbance in the microbial community caused by antibiotic treatment.

The growth rate of spruce budworm larvae was not strongly correlated with the microbial community structure associated with foliage (Redundancy analysis (RDA); Fig. S2) or the community structure in guts (Fig. S3). The influence of microbial community structure on the growth rate of spruce budworm larvae was greater in foliage communities (19.01 % constrained variance explained) than in gut communities (9.85 %). These data suggest that although the gut microbial community is not well correlated with larval growth, there is some initial benefit from bacteria as they pass through the gut even if they do not colonize the gut tissue. Foliage samples collected at the first time point were used to determine the influence of foliage-associated

microbial communities because the community structure of foliage did not significantly differ between collection times (Procrustes test; sum of squares = 0.88, p=0.57).

Differences in the gut community of fast and slow growing larvae

We compared the gut-associated communities of the fastest and slowest growing larvae (upper and lower quartile of growth rates respectively) in each treatment group as an additional way to determine if gut community structure impacts spruce budworm larval growth. Gut communities of larvae did not differ between fast and slow growers regardless of diet, antibiotic treatment, or distance measure used (PERMANOVA on unweighted UniFrac; antibiotic treated fir F=1.89, R^2=0.32, p=0.10; untreated fir F=0.74, R^2=0.15, p=0.86; antibiotic spruce F=1.07, R^2=0.26, p=0.2; untreated spruce F=2.17, R^2=0.35, p=0.10).

Effects of antibiotic treatment on microbial communities

Foliage that was treated with antibiotics had slightly lower diversity (Shannon diversity = 1.8 ± 0.12) than untreated foliage (2.20 ± 0.12) (ANOVA; F=5.834 p=0.019) however the difference was not significant according to post hoc tests (Tukey's honestly significant difference test (TukeyHSD); p = 0.051, Fig. 2A). Larvae feeding on synthetic diet that contained antibiotics, had lower microbial diversity in their guts (0.52 ± 0.13) than larvae feeding on foliage treated with antibiotics (1.52 ± 0.13) or untreated foliage (1.47 ± 0.13) (ANOVA; F=20.5, p<0.001: TukeyHSD; p<0.001, Fig. 2B). The Shannon diversity of microbial communities sampled from spruce budworm frass did not differ among any of the treatments (Fig. 2C).

The composition of diet-associated microbial communities (fir foliage, spruce foliage, and synthetic diet) was different between antibiotic treatments (PERMANOVA on unweighted UniFrac; F=1.43, R^2=0.022, p=0.0027) regardless of the ecological distance measure used (Fig.

S4). When foliage samples (not including synthetic diet) were compared, antibiotic treatment only had an effect on foliage-associated community composition when UniFrac distances were weighted by relative abundance (Fig. S4D-C). Antibiotic treatment only affected gut microbial community structure when synthetic diet-fed larvae were excluded and the comparisons were made with weighted UniFrac distances (PERMANOVA on weighted UniFrac; $F=2.40$, $R^2=0.060$, $p=0.025$, Fig 3C-D). There was no effect of antibiotic treatment on microbial community composition associated with the frass of larvae from any treatment group.

Antibiotic treatment impacted the microbial community structure associated with the diets fed to larvae in each treatment. Spruce budworm gut-associated communities, however, were only affected by antibiotic treatment when distance measures were weighted by the relative abundance of OTUs. In addition, because the microbial diversity of spruce budworm gut-microbial communities was the same between larvae fed on treated and untreated foliage, this suggests that antibiotic treatment affected the relative abundance of OTUs in gut communities but not overall community membership.

Does spruce budworm have a resident microbiome?

To test if spruce budworm has a resident gut microbiota, we compared the associated microbial communities among diets (foliage and artificial diet), guts, and spruce budworm frass, both at a community-wide scale and at the scale of individual OTUs. Microbial diversity was significantly different among sample types (ANOVA; $F=23.68$, $p<0.001$, Fig. 2). Unsurprisingly microbial diversity was lower in guts (1.16 ± 0.09 (mean ± SE)) than in foliage samples (1.74 ± 0.1) (TukeyHSD; $p<0.001$). Interestingly microbial diversity was also lower in guts than in frass samples (1.76 ± 0.06) (TukeyHSD; $p<0.001$). There was no significant difference in microbial diversity between frass and foliage communities (TukeyHSD; $p=0.986$, Fig. 2).

The structure of microbial communities associated with diets (including artificial diet), guts, and frass were significantly different from each other based on the phylogenetic relatedness of OTUs among the communities weighted by their relative abundances (PERMANOVA on weighted UniFrac distance; $F=4.50$, $R^2=0.034$, $p<0.001$). Because these communities are different, we analyzed the effects of each sample type separately using both unweighted and weighted UniFrac. Overall diet-associated communities were different from each other regardless of distance measure used (PERMANOVA on unweighted UniFrac; $F=1.66$, $R^2= 0.052$, $p<0.001$, Fig. S4). The largest difference among diet-associated communities in terms of composition was between foliage communities and the communities in the synthetic diet (Fig. S4). Differences between spruce- and fir-associated communities were also evident (PERMANOVA on unweighted UniFrac; $F=1.27$, $R^2=0.0255$, $p=0.031$), however the difference between spruce- and fir- associated communities was not evident when OTUs were weighted by relative abundance which suggests that the abundant OTUs are shared between diet types and the difference between spruce and fir foliage is driven by rare taxa.

Gut-associated communities follow a similar trend but differences are less pronounced than observed in the foliage- and synthetic diet-associated communities. In most of the comparisons of diet-associated communities, diet type (tree species or artificial diet) was identified as being a significant factor driving community structure. Gut community structure on the other hand was only affected by diet type when larvae that fed on synthetic diet were included in the analysis (PERMANOVA on unweighted UniFrac; $F=12.97$, $R^2=0.31$, $p<0.001$, Fig. 3) regardless of distance measure. There was no difference in gut-associated community composition between spruce and fir foliage fed larvae (Fig. 3). This indicates that antibiotics affect the relative abundances of gut microbiota in the spruce budworm gut, but differences between host foliage

do not. Across all gut samples the five most abundant OTUs were identified as: *Enterococcus* sp. (39.7 % relative abundance), *Shewanella* sp. (13.6%), *Shewanella* sp. (8.8%), a member of the family *Halomonadaceae* (7.4%), and *Halomonas* sp. (7.4%) (Fig. 4).

Frass communities of spruce budworm larvae only differed in structure between diets when larvae fed on synthetic diet were included in the analysis (PERMANOVA on weighted UniFrac; $F=2.052$, $R^2=0.0493$ $p=0.0031$), there was no difference between frass-associated communities of foliage fed larvae. There was no difference among any of the treatments in frass based on unweighted UniFrac distances, a purely phylogenetic distance measure (Fig. S5). Frass-associated microbial communities in general were more similar among different diet types and treatments than either the guts or diet samples.

Generally, our results show that the type of diet (spruce foliage, fir foliage, or synthetic diet) and antibiotic treatment had a significant effect on the communities associated with the diets fed to larvae from each treatment. When we analyzed the gut-associated bacterial communities we were still able to detect effects on community structure attributed to diet type but that difference was driven entirely by the difference in community structure between synthetic diet and fresh foliage. When the guts of larvae that were fed synthetic diet were removed from the analysis, the only difference that we detected between communities was due to antibiotic treatment when weighted UniFrac was used as the distance measure. Because the only difference that we observed in community structure was calculated with a metric weighted by abundance, this suggests that antibiotic treatment applied to the different diets had some effect on spruce budworm microbiota relative abundances but not on overall community membership.

Are gut-associated bacteria more abundant in foliage or the gut?

We compared the relative abundances of individual OTUs between foliage, guts, and frass samples to identify OTUs that were differentially abundant between samples using ANCOM (Table S5). Identifying differentially abundant OTUs was done to further determine if the spruce budworm gut has a resident microbiome. Among all diets and treatments, ANCOM detected 9 differentially abundant OTUs between gut and foliage communities (Fig. 5). Each OTU identified as being differentially abundant was more abundant in foliage-associated communities. Within the group of larvae feeding on antibiotic treated fir however, there was one differentially abundant OTU between the gut (denovo8018: *Halomonas sp.*, relative abundance in gut: 0.0003 ± 0.0001) and foliage communities (relative abundance in foliage 0.0 ± 0.0) that was more abundant in the gut. *Halomonas sp.* accounts for less than 1% of the gut associated bacteria in larvae feeding on antibiotic treated fir however (Fig. S6). We also detected three differentially abundant OTUs between gut and frass communities two of which were more abundant in frass while the third, denovo4154: *Mollicutes*, was more abundant in guts (Fig S7). Finally, no OTUs were identified as being differentially abundant between fast and slow growing larvae.

DISCUSSION

Our results provide evidence to support the idea that microbial symbionts are not critical to lepidopteran nutrition\survival and that lepidopteran larvae lack a resident microbiota (28, 29). Although antibiotics influenced spruce budworm microbial community structure, the gut microbial community of spruce budworm larvae did not influence host growth. Contrary to other studies suggesting that microbial communities of lepidopteran larvae lack resident microbes (28, 29), we present evidence suggesting that a small number of bacterial taxa are able to persist and increase in relative abundance in the spruce budworm gut such as *Halomonas sp.* and a member

of *Molicutes*. These microbes do not appear to be necessary for the growth or survival of the spruce budworm, and their functions in the gut remain unclear.

Influence of the gut microbial community on larval health

One of our hypotheses was that the use of antibiotics would reduce the bacterial diversity and alter community composition in the gut of spruce budworm and that those differences in the microbial community would result in a reduction of spruce budworm larval growth. Although there are some differences in spruce budworm larval gut microbial communities due to diet choice and antibiotic treatment, none of the observed changes in gut microbiota associated with antibiotic treatment resulted in any significant change in larval survival or growth under laboratory conditions.

Taken together with similar results from a growth experiment where the microbiome of *Manduca sexta* was eliminated via antibiotic treatment and no change in growth was detected (28), our findings suggest that the eastern spruce budworm, and perhaps many other lepidopteran species, are not nutritionally dependent on a microbial symbiosis. One possible explanation for this could be the bulk feeding strategy utilized by spruce budworm and many other herbivorous lepidopteran species. It is possible that because spruce budworm larvae consume so much food during their development it is not as imperative to efficiently extract nutrients from their diet. Another possible explanation could be that the alkalinity of the spruce budworm guts allows them to extract nutrients or tolerate the secondary compounds associated with conifer foliage. Along with previous work showing that carnivorous and herbivorous larval microbiomes did not differ significantly in composition (29), this suggests that lepidopteran larvae may not select for gut bacteria based on their nutritional needs, providing further evidence lepidopteran larvae do not rely on microbial symbiosis to extract the necessary nutrients from food.

Is the spruce budworm gut microbiome resident or transient?

Although antibiotic treatment did not significantly affect the growth of spruce budworm, our results shed some light on an ongoing discussion in the literature about the nature of gut microbiota in lepidopteran larvae. Our second hypothesis was that as microbes are passed through the spruce budworm gut via feeding, the physio-chemical environment of the gut would select for bacteria that could thrive in that environment, but that the community would still reflect the source community originating from the foliage diet. This pattern would provide evidence that spruce budworm larvae possess a resident gut microbiome, however our results did not support this hypothesis, and our findings provide further evidence that lepidopteran larvae lack resident gut microbial communities. The main argument for this conclusion is that the larval midgut community is composed of diet- and environmentally-derived microbes, and further supported by evidence that lepidopteran larvae are not nutritionally dependent on microbial associations.

While we provide evidence that generally supports the hypothesis that lepidopteran larvae lack a resident microbiome, our data suggests that the microbial community associated with diet changes as it travels through the spruce budworm gut. As microbes pass through the spruce budworm gut, differences in the community structure attributed to diet and antibiotic treatment present in foliage diminish. Thus weak selective pressures seem to be at play in the spruce budworm gut, possibly due to the high pH in the lepidopteran larval gut. Looking at finer scales to examine the responses of individual taxa, we found that spruce budworm larvae host a few bacteria that increase in relative abundance while in the spruce budworm gut relative to the foliage diet such as *Halomonas sp.* and a member of the class *Mollicutes*. This supports the hypothesis that the dominant taxa in the spruce budworm gut are food-derived transient

microbes, but that rare foliage-associated microbes are able to persist in the spruce budworm gut. The functional role of these rare gut-associated taxa has yet to be fully elucidated. While it is clear that spruce budworm is not nutritionally dependent on its microbiota, it is possible that other aspects of spruce budworm health could be influenced by these rare taxa that are able to colonize the gut. More studies will be necessary to determine the influence of the microbiome on spruce budworm reproductive fitness, fecundity, and parasitism rates for example through field studies with wild populations of spruce budworm.

Although we observed weak selective pressures on the relative abundance of diet-derived bacteria as they passed through the spruce budworm gut, we were unable to determine whether these bacteria were dormant or active. If for example taxa that are active on foliage become dormant or are otherwise unable to colonize the gut but do not die, it is possible that their intact cells could survive the passage through the gut and still be detectable through DNA sequencing. Future studies that measure bacterial activity and function directly in the gut will be required to address this question.

Microbial communities in fecal material or frass are often presumed to provide a surrogate measure of the microbial communities present in the gut. In our study, microbial community structure in frass differed only between artificial diet versus all other treatments, in contrast with the results for gut and foliage associated communities. Due to logistical constraints, frass was allowed to accumulate over time before being collected, and it is possible that any differences in gut microbial community structure in the gut were obscured by microbial growth in frass after excretion. Because we observed weak selective pressures on the microbial community in the gut, it is likely that the observed phenomenon in the frass-associated microbial community is a combination of both selective pressures and methodological issues resulting in frass-associated

community structure being a less sensitive measure of variation in microbial community structure in the gut than direct sampling of gut tissues. For this reason we suggest that the use of frass to quantify insect gut microbiomes is not currently advised until a better method for collecting frass can be developed.

Conclusions

Overall we observed that spruce budworm does not maintain a resident gut microbiome but that there appears to be weak selective pressures on ingested bacteria in the spruce budworm gut. Our findings did not support our hypothesis that alteration of the spruce budworm gut-associated microbial community would result in a reduction of spruce budworm larval growth. We did however provide evidence in support of the hypothesis that, although some rare microbial taxa may be able to colonize the gut, the gut microbial community of lepidopteran larvae is largely transient and unimportant for larval growth.

MATERIALS AND METHODS

Insect rearing

We acquired approximately 1,000 spruce budworm second instar larvae that had completed diapause from the Insect Production Services at the Great Lakes Forestry Centre, (Sault Ste. Marie, ON, Canada). Larvae were packaged between a sheet of parafilm and a sheet of cheese cloth and stored at 4°C prior to the start of the experiment. Sections of the parafilm containing approximately 30-40 larvae were cut using scissors sterilized for 5 seconds with 70% ethanol and placed on cups of synthetic diet containing antibiotics in autoclaved magenta boxes. Larvae were allowed to emerge from their hibernacula and feed on the common diet for one week. The

purpose of rearing larvae on a common diet for the first week was twofold: to ensure larvae were large enough to successfully eat foliage, and so that all larvae started to feed on the same food to control for variation in the starting microbiota among second instar larvae. Throughout the experiment larvae were maintained at 24°C at 60% relative humidity under a 16h:8h light:dark cycle.

After 1 week of feeding on the common diet, 200 larvae were randomly selected and split equally among 5 treatments (n=40): artificial diet with antibiotics, black spruce (*Picea mariana*) foliage treated with antibiotics, untreated spruce foliage, balsam fir (*Abies balsamea*) foliage treated with antibiotics, and untreated balsam fir foliage. Each replicate consisted of an individual larva in an autoclaved magenta box. Spruce foliage was collected from saplings housed in the greenhouse at the Université du Québec à Montréal and stored at -20°C for approximately 4 weeks. Fir foliage was collected from trees near Baie Comeau, Québec and stored in sterile bags at -20°C for 4 weeks. In both cases we took care to use only foliage that had fresh growth. Foliage was placed in a 2ml microcentrifuge tube filled with sterile water, or 50 µg/ml streptomycin for antibiotic treatments, and sealed with parafilm to reduce desiccation of the cut foliage during the experiment. For antibiotic treatments, a 1500 ppm solution of methyl paraben and a 50 µg/ml solution of streptomycin were each sprayed on the foliage every other day. Untreated foliage were not manipulated other than placing the cut stem in a microcentrifuge tube containing sterile water.

Health assessment: measuring larval growth and survival

Larval health was assessed every other day by measuring larval weight and calculating growth rates. Overall survival was calculated as well. We chose larval weight as a measure of health because it is often used as a measure of fitness in pupae and therefore can also be used as a

representation of overall health (28). We removed each larva from its magenta box using a fine paintbrush, placed it on a sterile weigh boat, and recorded the mass. Re-application of antibiotics on foliage occurred at this time via spray bottle. All work was done in an ethanol sterilized fume hood. The paintbrush used to manipulate the larvae was sterilized for 5 seconds with 70% ethanol between each replicate. Larvae that were dead at the time of weighing were discarded.

Sample collection

We collected sixth-instar larvae just prior to pupation, placed them in microcentrifuge tubes, and left them at room temperature for 4 hours before freezing at -80°C. This was to allow for any remaining food to pass through their guts, providing us with a more accurate approximation of the true gut microbiota as opposed to microbes that simply pass through the gut along with the food. Larval midguts were extracted from surviving individuals, using forceps and scissors sterilized with 70% ethanol, by cutting the posterior and anterior ends of the individual off to separate the midgut from the hindgut and foregut; remaining midgut was extracted from the larva using the forceps. Extracted guts were placed directly in MoBio PowerSoil bead beating tubes (Qiagen) and stored at -20°C until nucleic acid extraction.

We sampled frass and foliage samples twice during the experiment, once 7 days after exposure to treatments and again after 14 days when larvae were also collected. Foliage was collected by taking 5 needles with ethanol sterilized forceps, placed in microcentrifuge tubes, and immediately frozen at -80°C. Frass was collected from the bottom of the magenta box, placed in microcentrifuge tubes, and immediately frozen at -80°C. Samples were then assessed for microbial community diversity and composition following DNA sequencing.

We sampled frass and foliage communities along with the gut microbiota. Using these three communities to determine how the relative abundance of microbes changes from the source (foliage) through an environmental filter (gut) and by comparing gut communities with frass communities (or foliage communities) makes it possible to determine which taxa are able to persist in the gut versus which taxa simply pass through the larval gut.

DNA extraction and processing

We extracted DNA from the midguts of all surviving larvae (n=96). In addition 10 individuals were selected randomly from each of the 5 treatment groups to extract DNA from foliage or synthetic diet (n=101) and frass (n=99), both collected at each of the two time points. All genomic DNA from the guts, foliage, and frass was extracted using the MoBio PowerSoil DNA extraction kit (Qiagen). We used a slightly altered protocol, as described below, in order to increase DNA yields. Guts were homogenized by vortexing for 10 minutes in the provided PowerSoil bead beating tubes and centrifuged at room temperature for 1 min at 10,000g. The supernatant was transferred to a sterile 2 ml microcentrifuge tube and sonicated with the Bioruptor UCD-200 sonicator (Diagenode) for 1 min on the low setting (160W at 20kHz) for 5 min. After sonication the DNA extraction proceeded as per the manufacturer's instructions.

Foliage and synthetic diet samples were placed in thick walled 2 ml tubes with three 2.3mm diameter stainless steel beads (BioSpec Products, Bartlesville, OK, USA) and 250 µl of the PowerSoil bead tube buffer. Diets were homogenized using a MiniBead Beadbeater-16 (BioSpec Products, Bartlesville.) for 1.5 minutes. The remaining buffer from the bead beating tube was added to the resulting homogenate, sonicated at the high setting (320W at 20kHz) for 2 minutes and re-introduced to the bead beating tubes. Frass samples were sonicated for 2 minutes at the high setting (320W at 20kHz) for 2 minutes with 250 µl of the bead beating buffer. Following

sonication, samples were transferred back to the bead beating tubes. For diet and frass samples, after sonication the DNA extraction was performed following the manufacturer's instructions.

Following DNA extractions all samples were cleaned using the Zymo OneStep-96 PCR inhibitor removal kit. Polymerase chain reaction (PCR) was used to amplify the 16S rRNA gene from the extracted DNA. We used the chloroplast excluding primers (799F and 1115R) (30) to target the V5-V6 region of the 16S rRNA gene. Each primer also contained 1 of 20 unique bar codes and an Illumina adaptor to allow sequences to bind to the flow cell of the MiSeq sequencer. PCR was performed using 25 µl reactions prepared with 1 µl genomic DNA diluted 1:10 in molecular-grade water, 5 µl 5x HF buffer (Thermo Scientific), 0.5 µl dNTP's (10 µM each), 0.5 µl forward and reverse primer (10 µM each), 0.75 µl DMSO, 0.25µl Phusion HotStart II polymerase (Thermo Scientific), and 16.5 µl molecular-grade water. Each reaction began with 30 seconds of denaturation at 98°C followed by 35 cycles of: 15s at 98°C, 30s at 64°C, 30s at 72°C, and a final elongation step at 72°C for 10 minutes. Each PCR included a positive control and a negative control that were verified using gel electrophoresis on an agarose gel prior to sequencing. Amplicons were cleaned and normalized to 0.55 ng/µl using the Invitrogen SequelPrep normalization plate kit. After normalization equal volumes of amplicon DNA per sample were pooled and sequenced. In addition to sequencing experimental samples, positive and negative controls from each PCR were sequenced.

Amplicon sequencing

We sequenced 16S rRNA gene amplicons using the Illumina MiSeq platform using V3 chemistry. After sequencing, we first trimmed Illumina adapters from our sequences using the program BBduk version 35.76 (https:// sourceforge.net/projects/bbmap) and created paired end sequences using PEAR version 0.9.5 (31). The resulting paired end sequences were

demultiplexed and passed through a quality control workflow using QIIME version 1.9.1 (32) where chimeric sequences, sequences with more than two errors in the primer sequence, and sequences with an average quality score lower than 25 were removed. Except where otherwise noted, default settings were used for all bioinformatics analyses. In total 3,568,621 sequences were obtained across all samples after the initial quality control steps and the removal of chimeric sequences.

Sequences passing quality control parameters were binned into 8,593 operational taxonomic units (OTU) based on a 97% sequence similarity using the uclust algorithm. The most abundant sequence for each OTU was used as a representative sequence that was taxonomically identified to the lowest possible level (50% consensus cutoff) using a BLAST search of the Greengenes 16S gene database (33). In addition an alignment of each OTU's representative sequence was used to create a phylogeny using the FastTree 2 software (34). Positive controls were identified as *E. coli* and were distinct from experimental samples as expected. Negative controls had few to zero sequences and did not pass quality control steps. Following quality control, we analyzed community composition, structure, and the effects of gut communities on spruce budworm growth and survival using the statistical software R (35).

Growth and survival analysis

The effects of antibiotic treatment and diet (spruce or fir) on spruce budworm growth and survival were tested using two separate models. A mixed-effects model implemented with the R package nlme (36) was used to test for differences in larval growth. Larval weights were log transformed and used as the response variable in the model. Time, antibiotic treatment, diet, and their interactions were used as fixed effects, where time as a fixed effect is an estimate of growth rate, and time nested within individual larvae was used as the random effect for the model.

Differences in larval survival were tested using a separate logistic regression with survival as a binary response variable and antibiotic treatment, diet, and their interaction as main effects.

Community analysis

We tested for differences in community composition and diversity both among and within sample types (foliage, guts, frass). When we made comparisons among sample types, data were analyzed as a single dataset so we could ensure that each sample type had equal sampling depth for comparisons. When comparing treatments within sample types we analyzed separate datasets for each sample type that were rarefied separately. Sample types were rarefied separately to ensure the maximum number of sequences could be used in our analysis, allowing for more statistical power when testing within sample type differences.

Prior to our analysis we removed extremely rare OTUs (< 10 sequences) and samples that had fewer than 500 total sequences. A total of 1,020 OTUs remained after removing rare OTUs. When all sample types were analyzed together samples were rarefied to 1,000 sequences. When analyzed separately, gut samples were rarefied to 2,500 sequences per sample while diet and frass samples were rarefied to 1,000 sequences each. We calculated Shannon diversity based on relative abundances of rarefied samples for each data set as a measure of diversity (37).

Community structure was explored using non-metric multidimensional scaling (NMDS) using two complementary distance measures; unweighted UniFrac, and weighted UniFrac (38). Permutational multivariate ANOVA (PERMANOVA) with 10,000 permutations was used to test for differences in community structure among diets and between antibiotic treatments using the unweighted UniFrac and weighted UniFrac distance measures. PERMANOVA was implemented using the R package Vegan (39)

Gut communities were further analyzed to test for correlations between growth rate (calculated from log transformed weights as the slope of a simple linear model for each larva) and gut community structure using redundancy analysis (RDA) with growth rate, diet, and treatment as environmental variables. Individual growth rates were used for redundancy analysis rather than the estimate derived from the mixed effects model in order to incorporate the communities of each sample separately rather than a single average community. Individual growth rates calculated in this manner were comparable to the estimate derived from the mixed effects model. Redundancy analysis was also performed on foliage communities collected at the first time point to test for correlations between plant-associated microbial communities and larval growth. A Procrustes analysis of NMDS ordination results calculated with weighted UniFrac was used to test for differences in foliage-associated community structure between collection times.

In addition to using redundancy analysis to examine how growth rate and gut community structure are related, the fastest and slowest growing larvae, defined as the larvae in the upper and lower quartile of growth rates respectively, were selected for each experimental group and we compared their gut community structure.

Finally to test for differences in relative abundance of individual taxa between groups we used a differential expression analyses using the R package ANCOM (40), which tests for differences in relative abundances of taxa between communities. Differentially abundant OTUs were identified using the Kruskal-Wallis test with the less stringent multiple testing correction option provided by ANCOM and an alpha of 0.05, based on samples rarefied to 1000 sequences per sample and a pseudocount of 1 added to each abundance. We chose to use this method for calculating differentially abundant OTUs because it has been shown to perform better than other methods

(41). A table summarizing all of the statistical tests used in this study and the hypotheses tested by each can be found in the supplementary material (Table S2)

Nucleotide sequence accession numbers

16S rRNA amplicon sequence reads, including positive and negative controls, were submitted to the NCBI sequence read archive under the SRA accession number SRP139053 which covers all samples collected for this study between the accession numbers SRX3908565 and SRX3908861 (https://www.ncbi.nlm.nih.gov/sra/SRP139053).

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REFERENCES

1. Boulanger Y, Arseneault D. 2004. Spruce budworm outbreaks in eastern Quebec over the last 450 years. Can J For Res 34:1035–1043.

2. Royama AT, Mackinnon WE, Kettela EG, Carter NE, Hartling LK, Ecology S, May N. 2005. Analysis of Spruce Bud Worm Outbreak Cycles in New Brunswick, Canada, Since 1952 86:1212–1224.

3. Sainte-Marie GB, Kneeshaw DD, MacLean D a., Hennigar CR. 2015. Estimating forest vulnerability to the next spruce budworm outbreak: will past silvicultural efforts pay dividends? Can J For Res 45:314–324.

4. Burton PJ, Svoboda M, Kneeshaw D, Gottschalk KW. 2015. Options for Promoting the Recovery and Rehabilitation of Forests Affected by Severe Insect Outbreaks. Restor Boreal Temp For 495–517.

5. MacLean DA. 1984. Effects of spruce budworm outbreaks on the productivity and stability of balsam fir forests. For Chron 60:273–279.

6. Fournier C, Bauce É, Dupontb A, Berthiaumea R. 2010. Wood losses and economical threshold of Btk aerial spray operation against spruce budworm. Pest Manag Sci 66:319–324.

7. Robert L, Kneeshaw D, Sturtevant BR. 2012. Effects of forest management legacies on spruce budworm (*Choristoneura fumiferana*) outbreaks. Can J Res 42:463–475.

8. Régnière J, Nealis VG. 2007. Ecological mechanisms of population change during outbreaks of the spruce budworm. Ecol Entomol 32:461–477.

9. Lapierre P, Gogarten JP. 2009. Estimating the size of the bacterial pan-genome. Trends Genet 25:107–110.

10. Feldhaar H. 2011. Bacterial symbionts as mediators of ecologically important traits of insect hosts. Ecol Entomol 36:533–543.

11. Douglas AE. 2009. The microbial dimension in insect nutritional ecology. Funct Ecol 23:38–47.

12. Engel P, Moran NA. 2013. The gut microbiota of insects - diversity in structure and function. FEMS Microbiol Rev 37:699–735.

13. Kwong WK, Mancenido AL, Moran NA. 2017. Immune system stimulation by the native gut microbiota of honey bees. R Soc Open Sci 4:170003.

14. De Souza DJ, Lenoir A, Kasuya MCM, Ribeiro MMR, Devers S, Couceiro JDC, Della Lucia TMC. 2013. Ectosymbionts and immunity in the leaf-cutting ant Brain Behav Immun 28:182-187.

15. Koch H, Schmid-Hempel P. 2011. Socially transmitted gut microbiota protect bumble bees against an intestinal parasite. Proc Natl Acad Sci USA 108:19288-19292.

16. Prado SS, Hung KY, Daugherty MP, Almeida RPP. 2010. Indirect effects of temperature on stink bug fitness, via maintenance of gut-associated symbionts. Appl Environ Microbiol 76:1261-1266. doi: 10.1128/AEM.02034-09

17. Rosengaus RB, Zecher CN, Schultheis KF, Brucker RM, Bordenstein SR. 2011. Disruption of the termite gut microbiota and its prolonged consequences for fitness. Appl Environ Microbiol 77:4303-4312. doi: 10.1128/AEM.01886-10.

18. Emery O, Schmidt K, Engel P. 2017. Immune system stimulation by the gut symbiont Frischella perrara in the honey bee (Apis mellifera). Mol Ecol 26:2576–2590. https://doi.org/10.1111/mec.14058.

19. Engel P, Martinson VG, Moran NA. 2012. Functional diversity within the simple gut microbiota of the honey bee. Proc Natl Acad Sci USA 109:11002-11007.

20. Broderick NA, Raffa KF, Goodman RM, Handelsman J. 2004. Census of the Bacterial Community of the Gypsy Moth Larval Midgut by Using Culturing and Culture-Independent Methods. Appl Environ Microbiol 70:293–300. doi: 10.1128/AEM.70.1.293-300.2004.

21. Mason CJ, Raffa KF. 2014. Acquisition and structuring of midgut bacterial communities in gypsy moth (*Lepidoptera: Erebidae*) larvae. Environ Entomol 43:595–604.

22. Xiang H, Wei G-F, Jia S, Huang J, Miao X-X, Zhou Z, Zhao L-P, Huang Y-P. 2006. Microbial communities in the larval midgut of laboratory and field populations of cotton bollworm (*Helicoverpa armigera*). Can J Microbiol 52:1085–1092.

23. Tang X, Freitak D, Vogel H, Ping L, Shao Y, Cordero EA, Andersen G, Westermann M, Heckel DG, Boland W. 2012. Complexity and variability of gut commensal microbiota in polyphagous lepidopteran larvae. PLoS One 7:1–9.

24. Brinkmann N, Martens R, Tebbe CC. 2008. Origin and diversity of metabolically active gut bacteria from laboratory-bred larvae of *Manduca sexta* (*Sphingidae, Lepidoptera, Insecta*). Appl Environ Microbiol 74:7189–7196. doi: 10.1128/AEM.01464-08

25. Belda E, Pedrola L, Peretó J, Martínez-Blanch JF, Montagud A, Navarro E, Urchueguía J, Ramón D, Moya A, Porcar M. 2011. Microbial diversity in the midguts of field and lab-reared populations of the European Corn Borer *Ostrinia nubilalis*. PLoS One 6: e21751. https://doi.org/10.1371/journal.pone.0021751.

26. Robinson CJ, Schloss P, Ramos Y, Raffa K, Handelsman J. 2010. Robustness of the bacterial community in the cabbage white butterfly larval midgut. Microb Ecol 59:199–211.

27. Landry M, Comeau AM, Derome N, Cusson M, Levesque RC. 2015. Composition of the Spruce Budworm (*Choristoneura fumiferana*) Midgut Microbiota as Affected by Rearing Conditions. PLoS One 10:e0144077. https://doi.org/10.1371/journal.pone.0144077

28. Hammer TJ, Janzen DH, Hallwachs W, Jaffe SL, Fierer N. 2017. Caterpillars lack a resident gut microbiome. PNAS 249:84–89.

29. Whitaker MRL, Salzman S, Sanders J, Kaltenpoth M, Pierce NE. 2016. Microbial communities of lycaenid butterflies do not correlate with larval diet. Front Microbiol 7:1920.

30. Chelius MK, Triplett EW. 2001. The Diversity of Archaea and Bacteria in Association with the Roots of *Zea mays* L. Microb Ecol 41:252–263.

31. Zhang J, Kobert K, Flouri T, Stamatakis A. 2014. PEAR: A fast and accurate Illumina Paired-End reAd mergeR. Bioinformatics 30:614–620.

32. Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, Fierer N, Peña AG, Goodrich JK, Gordon JI, Huttley G a, Kelley ST, Knights D, Koenig JE, Ley RE, Lozupone C a, Mcdonald D, Muegge BD, Pirrung M, Reeder J, Sevinsky JR, Turnbaugh PJ, Walters W a, Widmann J, Yatsunenko T, Zaneveld J, Knight R. 2010. Correspondence QIIME allows analysis of high- throughput community sequencing data Intensity normalization improves color calling in SOLiD sequencing. Nat Publ Gr 7:335–336.

33. DeSantis TZ, Hugenholtz P, Larsen N, Rojas M, Brodie EL, Keller K, Huber T, Dalevi D, Hu P, Andersen GL. 2006. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Appl Environ Microbiol 72:5069–5072. doi: 10.1128/AEM.03006-05

34. Price MN, Dehal PS, Arkin AP. 2010. FastTree 2 - Approximately maximum-likelihood trees for large alignments. PLoS One 5:e9490. https://doi.org/10.1371/journal.pone.0009490

35. R Core Team. 2017. R: A language and environment for statistical computing. R Foundation for Statistical Computing. Vienna, Austria. https://www.R-project.org/.

36. Pinheiro J, Bates D, DebRoy S, Sarkar D, R Core Team. 2017. nlme: Linear and Nonlinear Mixed Effects Models. R package version 3.1-131.

37. Haegeman B, Hamelin J, Moriarty J, Neal P, Dushoff J, Weitz JS. 2013. Robust estimation of microbial diversity in theory and in practice. ISME J 7:1092–1101.

38. Lozupone C, Knight R. 2005. UniFrac : a New Phylogenetic Method for Comparing Microbial Communities. Appl Environ Microbiol 71:8228–8235. doi: 10.1128/AEM.71.12.8228-8235.2005

39. Oksanen J, Blanchet FG, Friendly M, Kindt R, Legendre P, McGlinn D, Minchin PR, O'Hara RB, Simpson GL, Solymos P, Stevens MHH, Szoecs E, Wagner H. 2017. vegan: Community Ecology Package. R package version 2.4-3. https://CRAN.R-project.org/package=vegan

40. Mandal S, Treuren W Van, White RA, Eggesbø M, Peddada SD, Mandal S, Treuren W Van, White RA, Eggesbø M. 2015. Analysis of composition of microbiomes: a novel method for studying microbial composition. Microbial ecology in health and disease 26:2235.

41. Weiss S, Xu ZZ, Peddada S, Amir A, Bittinger K, Gonzalez A, Lozupone C, Zaneveld JR, Vázquez-Baeza Y, Birmingham A, Hyde ER, Knight R. 2017. Normalization and microbial differential abundance strategies depend upon data characteristics. Microbiome 5:27.

FIGURES AND TABLES

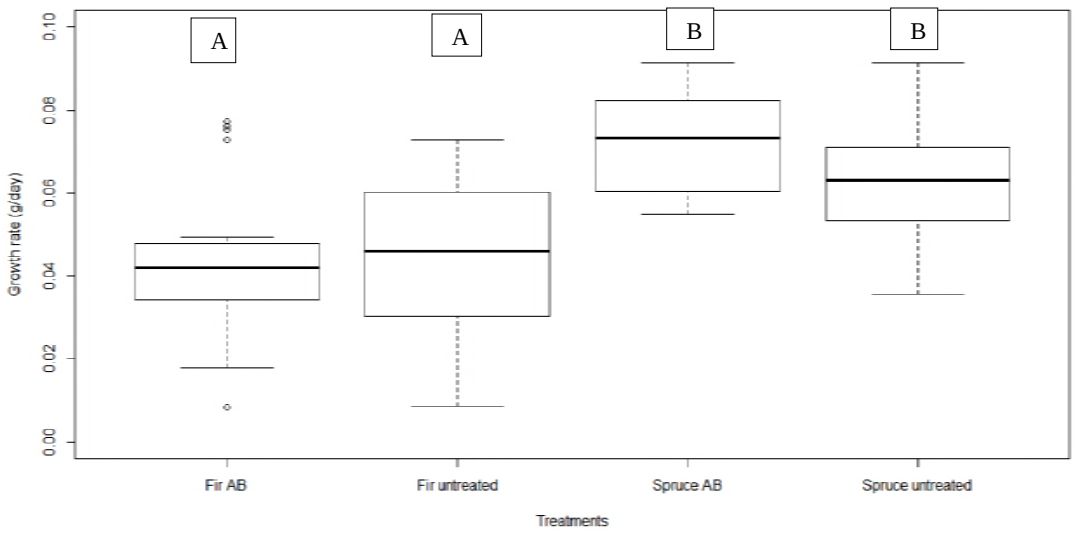
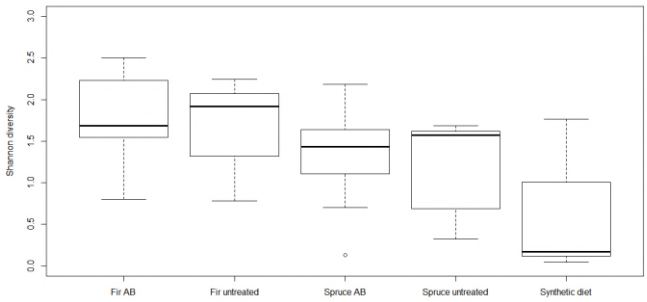


Figure 1. Growth rate (grams/day, ± S.E) of spruce budworm larvae among different diets (spruce versus fir foliage) and antibiotic treatments (AB = antibiotic treated). Letters indicate treatment combinations that differed significantly ($p < 0.05$) according to a Tukey's Honest Significant Difference post-hoc test, based on a mixed model (see Methods section for details).

A)



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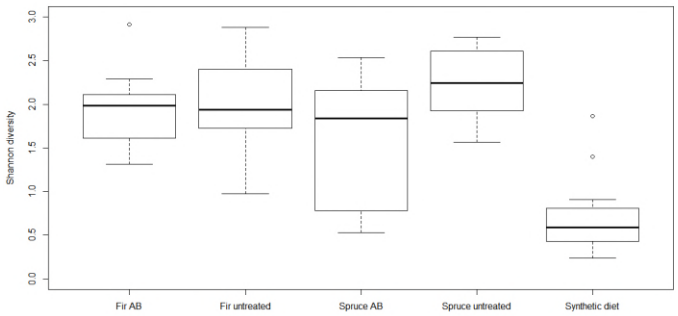

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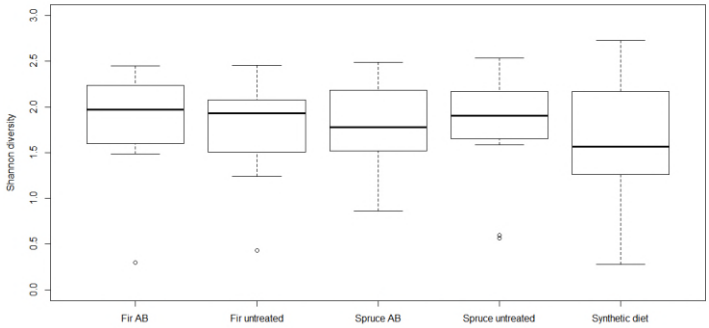
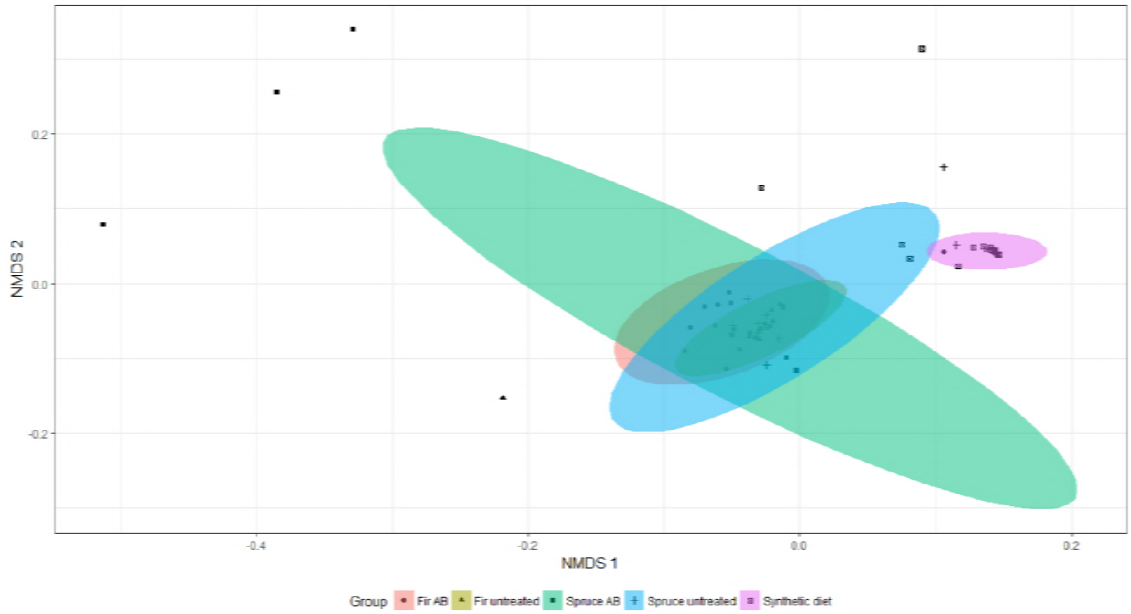
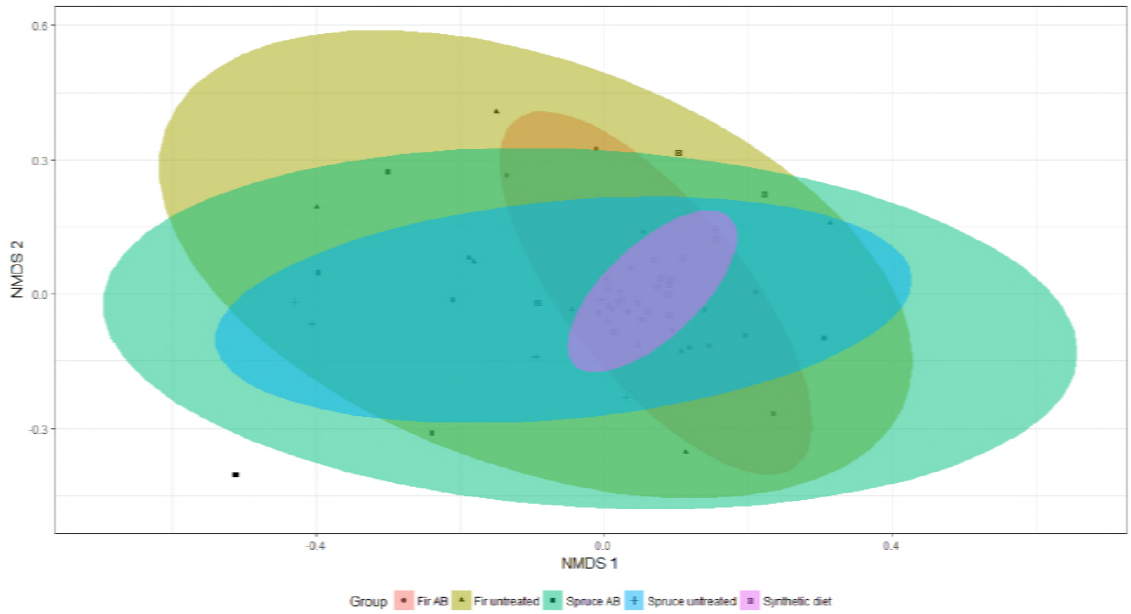


Figure 2. Mean (± SE) Shannon diversity of (A) spruce budworm diets (B) guts and (C) frass associated microbial communities among different diets (spruce versus fir foliage and synthetic diet) and antibiotic treatments (AB= antibiotic treated).

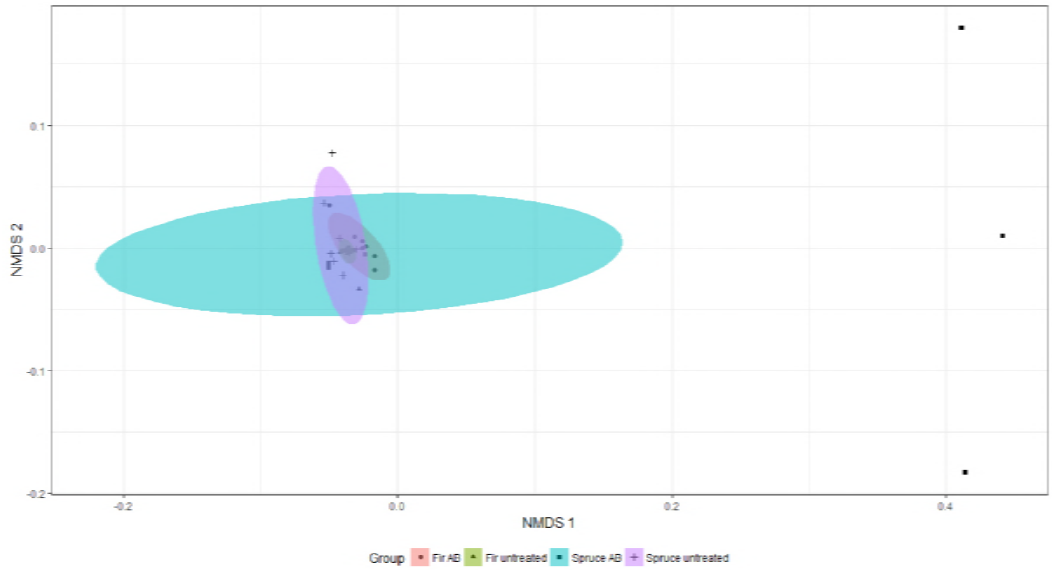
A)


0.2
0.0
-0.2
NMDS 2
-0.4
-0.2
0.0
0.2
NMDS 1
Group
Fir AB
Fir untreated
Spruce AB
Spruce untreated
Synthetic diet

B)


0.6
0.3
0.0
-0.3
NMDS 2
-0.4
0.0
0.4
NMDS 1
Group
Fir AB
Fir untreated
Spruce AB
Spruce untreated
Synthetic diet

C)



D)

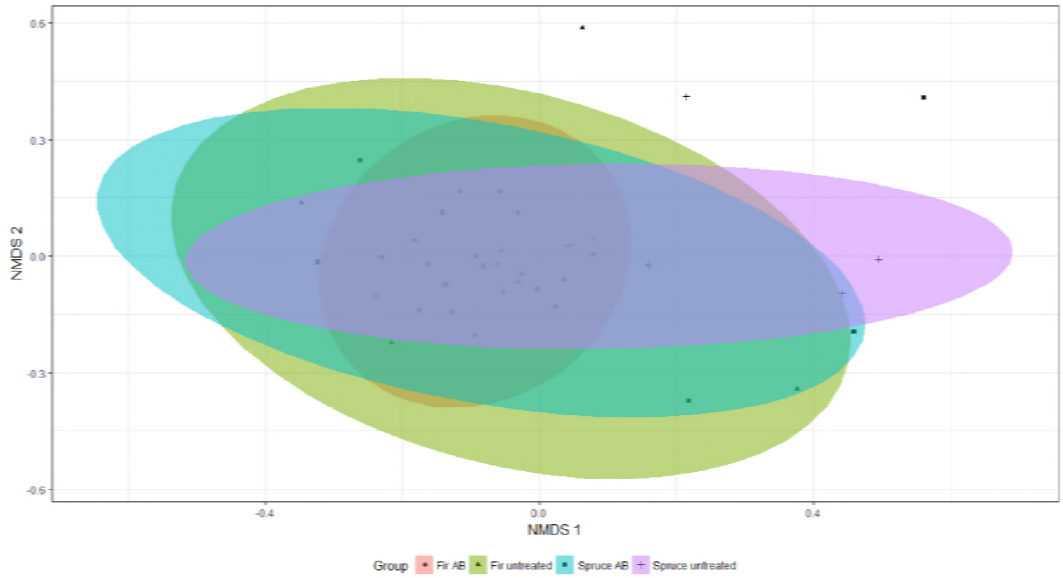


Figure 3. NMDS ordinations of gut-associated microbial communities based on weighted and unweighted UniFrac distances. (A: all guts weighted UniFrac stress =0.07, B: All guts UniFrac stress =0.21, C: guts of larvae feeding on foliage weighted UniFrac stress =0.07, D: guts of larvae feeding on foliage unweighted UniFrac stress =0.10). Ellipses represent 95% confidence

intervals around samples from different treatments (Fir.AB = antibiotic treated fir, Spruce.AB = antibiotic spruce, Fir.untreated = untreated fir, and Spruce.untreated = untreated spruce).

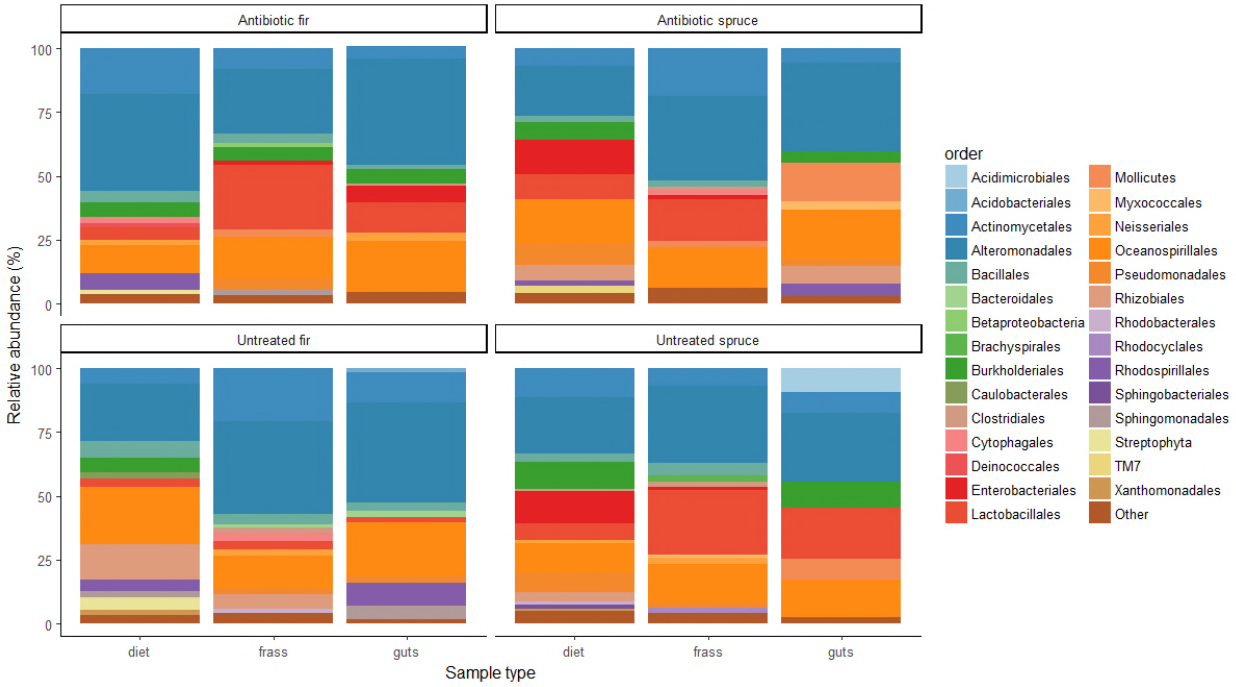


Figure 4. Mean relative abundance (%) of bacterial taxa across sample types, i.e diet (either spruce or fir foliage), budworm midguts, and budworm frass in each treatment group. Taxa were identified to the level of order, however if taxa remained unassigned at the order level they were labelled by taxonomic phylum or class.

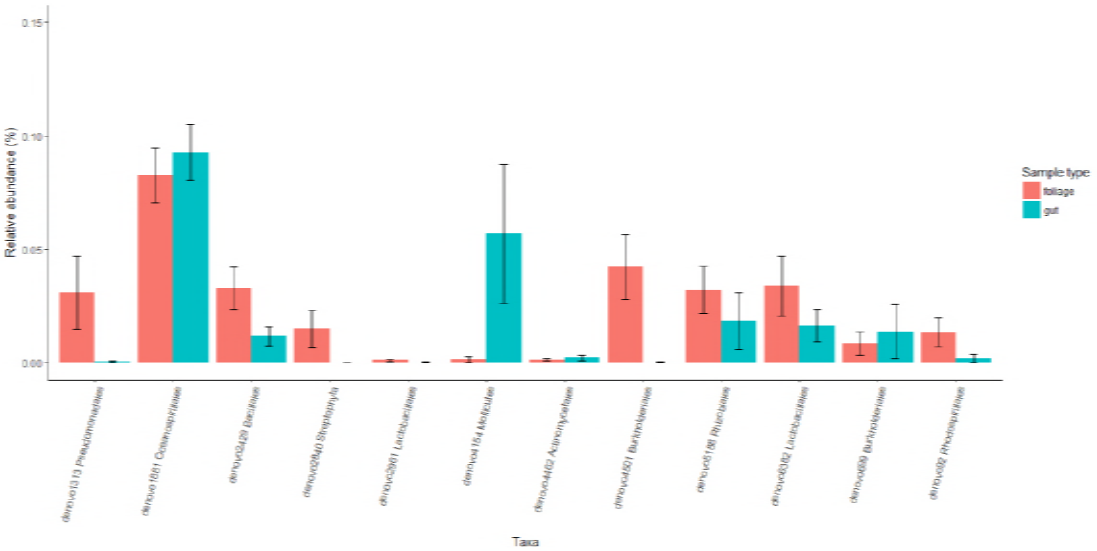


Figure 5. Mean relative abundance (± SE) of OTUs identified as being differentially abundant between foliage- and gut-associated communities based on an ANCOM test (ANCOM; adjusted $p < 0.05$). Blue bars represent gut samples and red bars represent foliage samples.

Table 1. Growth rates of spruce budworm larvae feeding on spruce and fir needles with and without antibiotics calculated as the estimate of time as fixed effect of a mixed effect model comparing larval weights with time, antibiotic treatment, and diet and their interactions as fixed factors and time nested within individual as random factors. In our model time as a fixed effect represents the growth rate of larvae

Diet	Treatment	Growth rate (g/day)	Standard error	Lower confidence limit	Upper confidence limit
Fir	AB	0.044	0.0039	0.036	0.052
Fir	None	0.045	0.0038	0.038	0.053
Spruce	AB	0.073	0.0038	0.065	0.081
Spruce	None	0.062	0.0049	0.053	0.072