UNIVERSITÉ DU QUÉBEC À MONTRÉAL

INVESTIGATING THE EFFECTS OF THE GUT-ASSOCIATED MICROBIAL COMMUNITY ON THE GROWTH OF THE EASTERN SPRUCE BUDWORM

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EFFETS DE LA COMMUNAUTÉ MICROBIENNE INTESTINALE DE LA TORDEUSE DES BOURGEONS DE L'ÉPINETTE SUR LA CROISSANCE DE L'HÔTE

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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 animals 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. The effects of host-associated microbial communities in *Lepidoptera* remains relatively unstudied. We studied the effects of the gut-associated microbial community in the eastern spruce budworm, *Choristoneura fumiferana*, an economically important forest pest in eastern Canada by studying the effects of an antibiotic treatment and diet on larval *C. fumiferana* growth. Studying host-microbe interactions in *C. fumiferana* not only provides us with answers to fundamental questions in lepidopteran biology, but has applications in terms of forest management practices. We hypothesized that antibiotic treatments would disturb or remove the resident *C. fumiferana* microbiome, resulting in decreased growth and survival. We show that the antibiotic treatment was sufficient to cause shifts in the microbial communities associated with balsam fir and black spruce foliage as well as in the guts of *C. fumiferana* larvae under laboratory conditions. Contrary to our expectations, we found that the observed antibiotic treatment did not significantly alter larval growth. We did find, however, that under laboratory conditions *C. fumiferana* larvae performed better when feeding on black spruce foliage compared to balsam fir which is widely understood to be the preferred food of *C. fumiferana* due to phenology. We show that although most bacteria originating on foliage appear to be transient through the gut, some bacteria may thrive better in the *C. fumiferana* gut. Therefore the *C. fumiferana* gut is only colonized by rare environmentally derived bacteria that are able to persist through the weak selective pressures exerted by the physiochemical properties found in the gut. Finally, our results suggest that *C. fumiferana*, and perhaps other lepidopteran species, are not nutritionally dependent on a resident microbial community.

Keywords : Spruce budworm, microbiome, host-microbe interactions, lepidoptera

RÉSUMÉ

Les communautés microbiennes jouent un rôle important pour la santé de leurs hôtes chez les mammifères, en particulier pour les humains. Les microorganismes pourraient jouer un rôle important pour les autres animaux, dont les insectes. Plusieurs études ont montré l'importance du microbiome pour les insectes, mais la majorité des recherches sur les interactions entre les insectes et la vie microbienne s'est focalisée sur certains groupes taxonomiques, dont les abeilles et les termites. Les effets des communautés microbiennes sur leurs hôtes chez les lépidoptères sont cependant encore mal compris. Dans le cadre de notre projet, nous avons étudié les effets du microbiote associés à l'intestin de la tordeuse des bourgeons de l'épinette (*Choristoneura fumiferana*), un insecte ravageur important au Canada. Nous avons quantifié en laboratoire les effets des traitements antibiotiques et les différentes diètes sur la croissance et la santé des larves de *C. fumeriferana*. Notre hypothèse était que le traitement antibiotique modifierait le microbiome de la tordeuse et ainsi diminuerait la croissance et la survie de la tordeuse des bourgeons de l'épinette. Nos résultats ont montré que les traitements antibiotiques n'avaient pas modifié la croissance des larves. La croissance des larves de *C. fumeriferana* était augmentée lorsque leur diète était composée d'épinette noire par rapport à lorsqu'elle était composée de sapin baumier, malgré le fait que le sapin baumier est supposé être la diète préférée de *C. fumeriferana* en raison de sa phénologie. Finalement, nous avons montré que la majorité des bactéries dans l'intestin de la tordeuse sont des bactéries transitoires originaires des feuilles, mais il y a également certains taxons bactériens avec une bonne performance dans l'intestin de la tordeuse. Le rôle fonctionnel de ces bactéries reste inconnu.

Mots clés : la tordeuse des bourgeons de l'épinette, microbiome, interactions hôtes-microorganismes, lépidoptères

INTRODUCTION

The eastern spruce budworm, *Choristoneura fumiferana*, is an economically important forest pest native to eastern Canada. Spruce budworm primarily feeds on balsam fir, *Abies balsamea*, and white and black spruce, *Picea spp.* Although *C. fumiferana* is polyphagous, they prefer to feed on balsam fir due to synchronized phenology between larval emergence and fir's budburst. Normally the impact of endemic spruce budworm populations on the boreal forest is insignificant, however every 30-40 years *C. fumiferana* undergoes a massive population outbreak which results in millions of hectares of defoliation (Boulanger and Arseneault, 2004; Sainte-Marie *et al.*, 2015; Burton *et al.*, 2015).

Because outbreaks can last for approximately 10 years, the amount of defoliation over such an extensive period causes massive growth reduction and tree mortality in balsam fir dominated, spruce dominated, or mixed stands. For example, during the last spruce budworm outbreak, between 1975 and 1992, an estimated 52 million ha of forest were defoliated and during the peak of that outbreak (1977-1981) it was estimated that 44 million m^3 of timber was lost annually. (Sainte-Marie *et al.*, 2015; Burton *et al.*, 2015)

What makes *C. fumiferana* so devastating during these population outbreaks is that *C. fumiferana* primarily feeds on recently emerged foliage. During the early stages of its life cycle *C. fumiferana* creates a silk hibernaculum in the new-year buds of balsam fir or spruce trees and after a 6-8 month period of diapause, spruce budworm larvae feed on recently emerged current year foliage. Over a period of 4-6 weeks larvae continue to increase in size and go through 4 more instars with the later instars

consuming the most foliar biomass. Larvae prefer to feed on current year growth because it is more nutritious but if the population size is high enough larvae will also eat older needles.

The Spruce budworm is a member of the order *Lepidoptera* (moths and butterflies) which is one of the largest insect orders and contains numerous, mostly herbivorous, economically important species including a number of agricultural pests, such as the gypsy moth *Lymantria dispar dispar*, the tobacco hornworm *Manduca sexta*, and the diamondback moth *Plutella xylostella* (Broderick *et al.*, 2004; Brinkmann *et al.*, 2008; Lin *et al.*, 2015). Like most lepidopteran pests, spruce budworm is most damaging during its larval stages or instars.

Adult spruce budworm mate and lay their eggs in mid-late summer. When first instar (L1) larvae hatch, they quickly locate an overwintering site where they, molt into the second instar (L2), build a silk hibernacula, and go into a state of diapause for approximately 6 months. After diapause they begin feeding on the newly emerged buds and over a period of 4-6 weeks will undergo four more instars with L6 larvae being the most voracious feeders. L6 larvae will pupate in late June to mid-July depending on the temperature and geographical location.

Generally, there are two approaches to reducing damage caused by spruce budworm: silvicultural (forest management) strategies aimed at reducing overall forest vulnerability (Sainte-Marie *et al.*, 2015; Burton *et al.*, 2015) and the use of biological pesticides such as *Bacillus thuringensis ssp kurstaki* (Btk), a soil microbe, at specific points in the budworm life cycle to protect foliage by locally reducing spruce budworm numbers (Fournier *et al.*, 2010). Although Btk toxin causes mortality on its own in the tobacco hornworm (*Manduca sexta*) it has been shown that mortality increased when the toxin was combined with the bacterium *Enterococcus faecalis*. The interaction between Btk toxin and *E. faecalis* is an example of how interspecific

interactions between the resident microbiota, a pathogenic invader, and the host can have drastic effects on host fitness. Btk toxins allow *E. faecalis* to move from the gut where it is benign into the hemoceol through openings in the gut lining where it becomes toxic (Graf, 2011; Mason *et al.*, 2011). There is still a debate about the exact mechanism of induced mortality, however, one recent study using RNA inhibition to silence the 102 Sl gene (a gene involved in immunosuppression) in *Spodoptera littoralis* larvae provides further evidence to suggest that septicemia is an important factor in Bt induced mortality (Caccia *et al.*, 2016).

Despite being one of the most studied forest pests in Canada (Régnière and Nealis, 2007), collectively our knowledge of spruce budworm's associations with bacteria is limited. Microbial assemblages are well studied in humans and other mammals and it is generally understood that microbes, especially those associated with the gut, are critical to the growth and development of their host. Our knowledge of gut associated microbial communities in insects is not nearly as expansive however. To date, only one study has been published surveying the gut-associated microbial community in spruce budworm (Landry *et al.*, 2015).

Because our understanding of spruce budworm microbial associations is still limited, my goal in this study was to better understand the role, if any, that gut bacterial assemblages play in maintaining spruce budworm growth. Studying bacterial associations with spruce budworm can provide valuable insights into what influences spruce budworm biology and ultimately fitness. From this new information we can potentially develop new, microbial based, control options for spruce budworm outbreaks. In addition, this study will provide additional insights into lepidopteran-microbe interactions in general. Because many lepidopteran species are considered to be pests, new information in controlling lepidopteran pests will prove useful.

Much of the research investigating host-microbe interactions in insects is focused on economically important species like pollinators, insect pests, vectors of human disease and some model species such as termites. Due to the vast diversity, both in terms of physiology and morphology, within insects it is difficult to draw general conclusions about the importance of insect microbiota for their hosts. Many factors influence insect-associated microbial community function and structure including diet, gut morphology, physiochemical properties of the gut, host-specificity to microbes through co-evolution, host life stage, and host environment (Engel and Moran, 2013; Colman *et al.*, 2012). In a recent study, Colman *et al.* performed a meta-analysis of 81 bacterial communities across 62 insect species representing multiple orders and 9 feeding types (herbivorous, xylophagous, etc....) from independent studies all using 16S rRNA gene clone libraries and found that both diet and host taxonomy play a role in shaping the insect gut community. However, diet and host taxonomy alone cannot explain all of the variability among insect bacterial communities. Colman and colleagues also suggest that, with a few exceptions, most insects do not maintain communities that are taxonomically distinct from the environment (Colman *et al.*, 2012).

Although insect gut microbial communities are typically taxonomically similar to the environment, the overall function, or gene expression, of the community could be drastically different (Franzosa *et al.*, 2014). The environmental conditions experienced by a group of bacteria on the surface of a leaf would be different from the environmental conditions experienced in the spruce budworm gut. Assuming the spruce budworm acquires its gut microbiota directly from the environment, through feeding, the gut microbial community would be taxonomically similar to or a subset of the microbial community associated with the foliage the spruce budworm is feeding on (Hammer *et al.*, 2017). Although the gut-associated community may be taxonomically similar to the diet-associated community, bacterial taxa being exposed to very different environmental conditions would likely express different genes in

order to stay competitive thus changing the overall functional profile of the community.

One can draw on previous studies to hypothesize what functions gut microbial communities may provide to different hosts. Gut microbiota have been previously shown to aid in the digestion of hard to process compounds. For example, bacteria in honey bee guts have been shown to aid in the degradation of the pectin found in pollen (Engel *et al.*, 2012) and bacteria have been shown to aid a number of xylophagous insects in the degradation of cellulose (Engel and Moran, 2013). In addition to microbially mediated digestion of food, gut microbes aid some herbivorous insects in processing secondary compounds which would otherwise be toxic (Hammer and Bowers, 2015). Spruce budworm must digest conifer needles which are high in lignin and terpenes and thus symbiosis with bacteria could be beneficial. Another common function that microbes may provide is providing their host with protection against pathogens either by buffering the host's immune response (Kwong *et al.*, 2017; Emery *et al.*, 2017; De Souza *et al.*, 2013) or by out-competing invading pathogens or parasites (Koch and Schmid-Hempel, 2011).

The midgut microbial community among *Lepidoptera* has been shown to be very simple, low species richness, in terms of community composition. For example a study on Gypsy moth larvae reported a community consisting of just 7 phylotypes (Broderick *et al.*, 2004). A more recent study of gypsy moth associated microbes (Mason and Raffa, 2014) reported an average of 52 operational taxonomic units (OTU) in the midgut, however most of those OTU's represented less than 1.0 % of the relative community structure. The increase in diversity shown in that study is likely because the sequencing technology used (Mason and Raffa, 2014) was more advanced than that of Broderick *et al.* (2004). Using 16S rRNA gene amplicon sequencing the cabbage white butterfly was reported to harbor only 6-15 species of bacteria (Robinson *et al.*, 2010). To date the most complex lepidopteran microbial

community to be studied is that of the European corn borer, where metagenomics sequencing revealed a community consisting of 240 genera (Belda *et al.*, 2011). Many of the genera, however, were considered rare (making up less than 1% of the community). These results reveal, when one considers differences in methodology and advances in sequencing technology, relatively simple communities comprised of a few dominant environmentally derived taxa and a number of rare taxa. Although one must be cautious when making generalizations about midgut communities of lepidopterans, there are some trends evident in the literature. Some common phyla in lepidopteran microbial communities include *Firmicutes*, *Proteobacteria*, and *Bacteroidetes*, all common gut microbes in animals (Belda *et al.*, 2011; Tang *et al.*, 2012; Broderick *et al.*, 2004; Xiang *et al.*, 2006).

Landry *et al.* (2015) is the first study of spruce budworm microbial communities using next generation sequencing. The study found a simple community dominated by *Proteobacteria*, with *Pseudomonas* being the most abundant genus within *Proteobacteria*. Other groups that were present in spruce budworm midguts include *Actiniobacteria*, *Firmicutes*, and *Bacteroidetes*. Interestingly Landry *et al.* (2015) found higher diversity within midguts of spruce budworm that were reared on artificial diet containing 0.56% of the antibiotic Aueromycin. A possible explanation for this could be that although the antibiotics did not eliminate the gut community it could have reduced the abundance of the more dominant taxa easing competition thus allowing rarer taxa to increase in abundance. Regardless of the mechanism causing increased diversity in communities of larvae reared on artificial diet containing antibiotics, the differences were driven by an increased presence of *Bacteroidetes,* the vast majority (97%) of which in artificial diet fed individuals belonged to the family *Chitinophagacaea*. The principal finding of this study is that diet does appear to significantly affect gut microbial community structure in spruce budworm. The fact that diet is a main determining factor in spruce budworm gut community assembly

because it suggests that one could alter the spruce budworm microbiome by altering the microbial community associated with the host diet.

Belda *et al.*, 2011, one of the only studies thus far to examine function of the microbial community in *Lepidoptera*, reported significant differences in the taxonomic abundances between field caught and lab-reared larvae, a finding which has been shown in other studies (Xiang *et al.*, 2006). Although microbial communities were taxonomically different between lab and field populations of the European corn borer, metabolic profiles were similar between the two populations (Belda *et al.*, 2011).

The overall function of the European corn borer (and possibly other lepidopteran species), as described by Belda *et al.* (2011), was heavily invested in the breakdown of food with 10-11% of the genes detected being associated with amino acid transport and metabolism and 8-9% being associated specifically with carbohydrate metabolism. Sixteen to twenty percent of genes were linked to general or unknown functions. One interesting difference in function between the field caught and lab reared populations was that the field metagenome had 1.3% of its genes associated with cell motility while no genes of that nature were detected in the lab metagenome (Belda *et al.*, 2011).

The importance of microbial communities for insects is not universal. Despite evidence to suggest that microbes perform important functions within the guts of their insect hosts, there is evidence to support the opposite. For example one study showed that antibiotic treatment of cows was sufficient to alter the microbial communities in cow dung and in the guts of dung beetles feeding on that dung, but that there was no observable effect on the health of the dung beetle i.e survival or growth (Hammer *et al.*, 2016).

A survey of 124 taxonomically diverse lepidopteran species representing 15 families revealed that the lepidopteran gut microbiome is not distinct from the microbial communities on the food they consume (Hammer *et al.*, 2017). Most lepidopteran species are herbivorous, however many Lycaenid butterflies are carnivorous. A survey of Lycaenid butterflies provides further evidence suggesting that regardless of dietary needs (herbivorous or carnivorous) the gut microbial community of lepidopteran larvae are all food-derived transient microbes (Whitaker *et al.*, 2016). This suggests that lepidopteran larvae may not draw any inherent benefit from their gut microbiota. The conclusion that lepidopteran microbes are only transient microbes presented in these two papers is based only on taxonomic identification of microbes. To fully elucidate the question of whether or not lepidopteran larvae maintain a distinct microbiome would require functional profiling of microbial communities.

One possible explanation for why gut-microbial communities in *Lepidoptera* appear to be transient is the morphology and physiochemical properties of the larval lepidopteran gut. While many insect guts employ a more complicated morphology with specialized structures, the larval lepidopteran gut is relatively simple morphologically in that it is a simple tube (Engel and Moran, 2013). Additionally, unlike most guts (insect or otherwise) the larval lepidopteran gut is an alkaline environment with the typical pH ranging from 8-12 compared with most other insect which have a gut pH ranging from 4-7 (Broderick *et al.*, 2004; Tang *et al.*, 2012; Engel and Moran, 2013; Belda *et al.*, 2011).

Another challenge to microbes trying to colonize the gut is that many lepidopteran species go through multiple larval instars meaning that part of the midgut, the peritrophic matrix, is shed multiple times during the lifecycle when the larvae undergo ecdysis (Engel and Moran, 2013). The shedding of the peritrophic matrix and the relatively hostile environment of the lepidopteran midgut, where most of the

nutrient extraction would be taking place makes it very difficult for microbes to successfully colonize the gut. It is likely that the physiochemical conditions in the lepidopteran midgut are an important factor in selecting the gut microbiota.

Conclusions about the relative importance of gut microbiota in Lepidoptera remain incomplete. The main argument suggesting that the lepidopteran larval gut is inhabited by only transient microbes is based on observations suggesting that the membership of the gut microbiome is largely food derived. Even though the membership of the gut community mirrors the membership of the microbial community associated with the food it is possible that the relative abundances of different taxa will change as they are consumed and passed through the gut. Considering the harsh conditions of the lepidopteran larval gut (high pH, disturbances via shedding of the peritrpophic matrix) it is not unreasonable to consider the larval lepidopteran gut as an environmental filter which would preferentially select a resident microbial community able to colonize the gut under those conditions. If a group of microbes, derived from foliage, increases in relative abundance in the gut compared to the foliage it could be assumed that those microbes are metabolically active in the gut and could be considered a resident microbiome. Sequencing of the metatranscriptome, the collective RNA of the microbial community showing gene expression, will be needed to confirm this.

I chose to investigate the gut microbial community of *Choristoneura fumiferana* to better understand host-microbe interactions in an organism capable of defoliating huge areas, particularly during outbreaks, of spruce and fir trees. The principal objectives of this study were to determine if *Choristoneura fumiferana* selects for a resident microbial community that is shared among. In this memoire I used an experiment designed to eliminate the gut-associated microbiome of spruce budworm larvae with antibiotics and track larval growth to test the hypothesis that spruce budworm has a resident microbiome which is required to maintain spruce budworm

growth and survival. Considering that the efficacy of the *Bacillus thuringensis* toxin on lepidopteran larvae is partially tied to the presence of commensal gut bacteria, gaining a better understanding of spruce budworm-microbe interactions could inform future research into spruce budworm management.

CHAPTER I

INVESTIGATING THE EFFECTS OF THE GUT-ASSOCIATED MICROBIAL COMMUNITY ON THE GROWTH OF THE EASTERN SPRUCE BUDWORM

1.1 Introduction

The eastern spruce budworm (*Choristoneura fumiferana*) is a forest pest native to the north eastern United States and Canada that undergoes epidemic population outbreaks every 30-40 years. During these population outbreaks, lasting for approximately 10 years, millions of hectares of balsam fir (*Abies balsamea*) and spruce (*Picea spp.*) trees are defoliated and killed (Boulanger and Arseneault, 2004; Royama *et al.*, 2005; Sainte-Marie *et al.*, 2015; Burton *et al.*, 2015). Consequently spruce budworm is an economically important defoliator in coniferous forests (Fournier *et al.*, 2010) and has significant effects on forest productivity (MacLean, 1984).

Following the introduction of next generation sequencing technology the ease with which microbial communities can be studied has increased dramatically. This has led to an increased body of literature investigating host-microbe interactions (Kuczynski *et al.*, 2011; Lepage *et al.*, 2013). It is largely accepted that microbes, particularly those associated with the gut, are critical for human health where they interact with the immune system, aid in host nutrient acquisition through metabolic pathways, and in some cases the disruption of the normal microbiota have been linked to diseases such as obesity (Tilg and Kaser, 2011; Turnbaugh *et al.*, 2006, 2009) and neurological disorders (Lepage *et al.*, 2013; Tremlett *et al.*, 2017). The general assumption regarding host-microbe interactions is that they, in some way, have a positive

interaction to their host rather than a negative one. While a large proportion of this research focuses on humans and other mammals, there are growing number of studies investigating the microbial communities of insects (Engel and Moran, 2013; Douglas, 2009; Colman *et al.*, 2012).

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 (Feldhaar, 2011). The collective set of microbial genomes associated with a host, the microbiome, has a much greater functional diversity than the eukaryotic host genome (Lapierre and Gogarten, 2009). Thus the microbiome can act as an extension of the host gut. For example, gut microbial communities can have the capacity to metabolize some compounds that the host cannot. This is particularly important when considering spruce budworm because it feeds on conifer needles which are tough to digest and contain defensive compounds such as terpenes.

Drawing generalizations about the extent to which insect microbiota are important to their host is difficult due in large part to the morphological, physiological, and behavioral variation within insects. Different physiology, life histories, feeding strategies, and levels of social interaction (of which there are many within insects) can all influence how microbes interact with their host (Engel and Moran, 2013). There is evidence to support the assumption that microbial symbiosis is important in a number of insects as well (Engel and Moran, 2013; Kwong *et al.*, 2017; De Souza *et al.*, 2013; Koch and Schmid-Hempel, 2011; Prado *et al.*, 2010; Rosengaus *et al.*, 2011; Emery *et al.*, 2017b; Engel *et al.*, 2012). There is a wide body of evidence showing that termites, which depend on specialized bacteria to allow them to digest cellulose, and various species of bees benefit greatly from their associated microbiota. Bee-associated microbes have been shown to contribute to immune function as well to aid in nutrition through mediating the digestion of cellulose (Engel *et al.*, 2012; Kwong *et al.*, 2017)

The lepidopteran microbiome has been described as a very simple microbial community compared to other insects (Broderick *et al.*, 2004; Mason and Raffa, 2014; Xiang *et al.*, 2006; Tang *et al.*, 2012; Brinkmann *et al.*, 2008; Belda *et al.*, 2011; Robinson *et al.*, 2010; Landry *et al.*, 2015). 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 (Engel and Moran, 2013). 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 (Broderick *et al.*, 2004; Tang *et al.*, 2012; Engel and Moran, 2013; Belda *et al.*, 2011). The highly alkaline gut environment found in lepidopteran larvae would likely be a significant barrier to microbes trying to colonize the gut and would thus provide selective pressures allowing for.

We hypothesized that the eastern spruce budworm has a resident gut microbiome that must be maintained to facilitate 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. The objectives of this study were (1) to determine if the eastern spruce budworm has a resident gut microbiome that is distinct from the microbial assemblages associated with foliage as well as to (2) determine if the gut microbiota associated with spruce budworm larvae, resident or otherwise, influence larval growth rates and survival. We also sought to quantify the effects that antibiotics would have on gut microbial diversity and community composition. Spruce budworm larvae were reared in sterile conditions and exposed to

different diets and antibiotic treatments in order to measure larval growth and survival in each treatment.

1.2 Materials and Methods

1.2.1 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. (NRCAN, 2016).

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 kept fresh by placing the cut stem in a 2ml microcentrifuge tube filled with sterile water, or 50 µg/ml streptomycin for antibiotic treatments, and sealed with parafilm. 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.

1.2.2 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 proxy for health because it is often used as a measure of fitness in pupae and therefore can also be used as a representation for overall health (Hammer *et al.*, 2017). 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 and any growth data collected prior to mortality was not used in the subsequent analysis, as a result we only present data associated with larvae that survived through the entire experiment in this study.

1.2.3 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 which 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 (Fig 1). 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 (Table S1). 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.

1.2.4 DNA extraction and processing

We extracted DNA from the midguts of all surviving larvae (n=96). In addition 10 random 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 guts, foliage, and frass was extracted using the MoBio PowerSoil DNA extraction kit (Qiagen). We used a slightly altered protocol 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) (Chelius and Triplett, 2001) 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. 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.

1.2.5 Amplicon sequencing

16S rRNA gene amplicons were sequenced 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 (Zhang *et al.*, 2014). The resulting paired end sequences were demultiplexed and passed through a quality control workflow using QIIME version 1.9.1 (Caporaso *et al.*, 2010) 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. 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. OTUs were used in our study because they are standard method of identifying bacterial species (Mysara *et al.*, 2017).The most abundant sequence for each OTU was used as a representative sequence that was taxonomically identified to the lowest possible level, i.e species, using a BLAST search of the Greengenes 16S gene database (DeSantis *et al.*, 2006). In addition an alignment of each OTUs representative sequence was used to create a phylogeny using the FastTree 2 software (Price *et al.*, 2010). 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 (R Development Core Team., 2017).

1.2.6 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 (Pinheiro *et al.*, 2017) 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. By defining our model this way we ensured that growth was treated as a repeated measure, meaning that initial differences between larvae are accounted for in the model because the model measures the growth rate of each larva. 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.

1.2.7 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.

Community structure was explored using non-metric multidimensional scaling (NMDS) using two complementary distance measures; unweighted UniFrac, and weighted UniFrac (Lozupone and Knight, 2005). We chose to use these two distance measures in tandem because they each provide slightly different information about community composition. Weighted and unweighted UniFrac are both phylogenetic distances where weighted UniFrac is weighted by abundance. Unweighted UniFrac distance reveals how communities differ in terms of membership whereas weighted UniFrac shows how the relative abundance of microbes change in the community. By using these two distance measures we are able to use both phylogenetic relatedness and abundance to make inferences. 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 (Oksanen *et al.*, 2017).

Gut communities were further analyzed to look 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 to test for

correlations between plant-associated microbial communities and larval growth. 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 (Mandal *et al.*, 2015), which tests for differences in mean relative abundances of taxa between communities. ANCOM utilizes logarithmic transformations, so the method does not perform well with large numbers of zero counts (Weiss *et al.*, 2017). To correct for this prior to analysis, we added a pseudocount of 1 to each value (this is done automatically by the ANCOM package). Because we used relative abundance data, adding 1 to samples with unequal sampling depth would produce unreliable results. We addressed this problem by rarefying samples to 1000 sequences prior to the addition of a pseudocount. With ANCOM, 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. We chose to use this method for calculating differentially abundant OTUs because it has been shown to perform better than other methods (Weiss *et al.*, 2017). A table of all statistical tests used in this study can be found in the supplementary materials (Table S2)

1.3 Results

1.3.1 Effects of diet and antibiotics on larval survival

There was no significant effect of diet (logistic regression; $z= -0.897$, $p=0.3695$) on larval survival rates, however antibiotic treatment tended to favour 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 favouring survival seems to be driven by the difference between larvae feeding on spruce (30%) that was not treated with antibiotics and larvae that fed on synthetic diet (60%) which contained antibiotics. Overall, larvae feeding on artificial diet, which contained antibiotics, had a survival rate of 60%. Larvae feeding on spruce that was not treated with antibiotics had 30% survival. Larvae feeding on spruce and fir treated with antibiotics both had 50% survival and larvae feeding fir without antibiotics had 52.5% survival.

1.3.2 Effects of diet and antibiotics on larval growth rate

The weight of spruce budworm larvae was significantly affected by antibiotic treatment and time (Table 1). In the model, the estimates of time as a main effect represents growth rate. Pairwise comparisons of the least squares means for the time effect show differences in growth rate between individuals feeding on fir and spruce (Table-2)(Table 3)(Fig 2). Comparisons show that individuals feeding on fir treated with antibiotics grew less than those feeding on antibiotic treated spruce (-0.020 ± 0.005(mean $\pm$ SE); $p<0.0001$), and larvae feeding on untreated fir grew less than those feeding on untreated spruce ($-0.017 \pm .006$; $p=0.032$). Comparisons between larvae feeding on antibiotic treated and untreated foliage of the same type (i.e fir or spruce) were not significant however.

The overall differences in growth rate observed between spruce budworm larvae in different experimental groups were due to 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. The only pairwise comparisons that were not found to be significant were those between antibiotic treated fir with untreated fir and antibiotic treated

spruce with untreated spruce. 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.

Redundancy analysis (RDA) of the gut-associated microbial community of spruce budworm larvae feeding on foliage with larval growth rate and the effects of diet and antibiotic treatment as explanatory variables, 9.8% of the variance in spruce budworm gut microbial community structure could be explained. Individually, growth rate explained 4.0 % of the variance, diet explained 4.8 % of the variance, and antibiotic treatment explained 2.3 % of the variance (Fig 3B).

To determine if the plant-associated communities of the foliage were correlated with larval growth, a separate RDA was performed comparing foliage-associated communities with larval growth rates using communities isolated from foliage collected at the first time point. A Procrustes analysis of NMDS ordinations revealed that the community structure of foliage did not significantly differ between collection times (Procrustes; sum of squares = 0.88, p=0.57). Growth rate, tree species, and antibiotic treatment explained 18.7 % of the variance in foliage-associated communities (treatment: 8.86%, diet: 5.55%, growth rate: 3.75 %) (Fig S2)

1.3.3 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) in each treatment group as an additional way to determine if gut community structure impacts spruce budworm larval growth. Gut communities of larvae feeding on antibiotic treated fir were not significantly different between fast and slow growers (PERMANOVA; F=1.89, R^{2}=0.32, p=0.10). Gut communities of larvae feeding on untreated fir foliage were not significantly different among fast and slow growers (PERMANOVA; F=0.74,

R^2=0.15, p=0.86). Gut communities of larvae feeding on antibiotic treated spruce foliage did not differ between fast and slow growing individuals (PERMANOVA; F=1.07, R^2=0.26, p=0.2). Gut communities of larvae feeding on untreated spruce foliage did not differ significantly between fast and slow growing individuals (PERMANOVA; F=2.17, R^2=0.35, p=0.10). Recalculating the aforementioned ordinations with weighted UniFrac did not change the overall results.

1.3.4 Effects of antibiotic treatment on microbial communities

Antibiotic treatment (ANOVA; F=5.834 p=0.019) significantly affected the microbial diversity (Shannon diversity of OTU relative abundances) of the diets (Fig 4A). Foliage that had been treated with antibiotics (1.8 ± 0.12) had slightly lower diversity than untreated foliage (2.20 ± 0.12) however the difference was not significant according to post hoc tests (TukeyHSD; p = 0.051). Shannon diversity in the guts of spruce budworm larvae differed significantly between antibiotic treatments (ANOVA; F=20.5, p<0.001)(Fig 4B). Larvae feeding on synthetic diet that contained antibiotics, had significantly 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) (TukeyHSD; p<0.001)(Fig 4B). The Shannon diversity of microbial communities sampled from spruce budworm frass did not differ among any of the treatments (Fig 4C).

The composition of diet-associated microbial communities (fir foliage, spruce foliage, and synthetic diet) was affected by antibiotic treatment (PERMANOVA; F=1.43, R^2=0.022, p=0.0027) regardless of the ecological distance measure used (Fig 5). However, when just foliage samples were compared antibiotic treatment only had an effect on foliage-associated community composition when UniFrac distances were weighted by relative abundance (Fig 5D-C). Unlike diet communities, in gut-associated communities, antibiotic treatment only affected community structure when synthetic diet fed larvae were excluded and the comparisons were made with

weighted UniFrac distances (PERMANOVA; $F=2.40$, $R^2= 0.060$, $p=0.025$)(Fig 6). There was no effect of antibiotic treatment on microbial community composition associated with the frass of larvae from any treatment group.

Antibiotic treatment had a significant effect on the communities associated with the diets fed to a subset of larvae from 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 membership.

1.3.5 Does spruce budworm have a resident microbiome?

To test if spruce budworm has 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; $F=23.68$, $p<0.001$). Unsurprisingly, a post-hoc test (Tukey's honestly significant difference test; TukeyHSD) revealed that microbial diversity was lower in guts (1.16 ± 0.09 (mean $\pm$ 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; $p=0.986$).

The microbial communities of diets (including artificial diet), guts, and frass were significantly different from each other based on analysis of square root transformed weighted UniFrac distances, based on the phylogenetic relatedness of OTUs among the communities weighted by their relative abundances (PERMANOVA; $F=4.50$,

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 among diet types (PERMANOVA; F=1.66, R^2= 0.052, p<0.001)(Fig 5) The largest difference among diet-associated communities in terms of composition was between foliage communities and the communities in the synthetic diet (Fig 5). Differences between spruce- and fir-associated communities were also evident in our data (PERMANOVA; F=1.27, R^2=0.0255, p=0.031).

Gut-associated communities follow a similar trend but are less pronounced than differences 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 between foliage and artificial diet. Gut community composition on the other hand was only affected by diet type when larvae that fed on synthetic diet were included in the analysis (PERMANOVA; F=12.97, R^2=0.31, p<0.001) (Fig 6). There was no difference in gut-associated community composition between spruce and fir foliage fed larvae (Fig 6). This result indicates that antibiotics affect the relative abundances of gut microbiota in the spruce budworm gut, but difference between host foliage does not. Across all gut samples the most 5 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%).

Frass-associated microbial communities in general were more similar among different diet types and treatments than either the guts or diet samples. Frass communities differed significantly among diets. However when synthetic diet was removed from the model, to analyze just individuals who had been fed foliage, none of the

treatments significantly affected community structure in frass (Fig. S3). Weighted UniFrac with synthetic diet included in the analysis showed that diet had a significant effect on microbial community structure (PERMANOVA; $F=2.052$, $R^2=0.0493$ $p=0.0031$) (Fig.S2), but when only frass of individuals feeding on foliage was no detectable difference in community structure between them. Finally based on unweighted UniFrac distances, a purely phylogenetic distance measure, there was no difference among any of the treatments in frass (Fig S3).

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 a subset of 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.

1.3.6 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 S1). 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. Each OTU identified as being differentially abundant was

more abundant in foliage-associated communities (Fig 7). 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.

There was one differentially abundant OTU (denovo8018: *Halomonas sp.*) between the gut (relative abundance in gut: 0.0003 ± 0.0001) and foliage communities (relative abundance in foliage 0.0 ± 0.0) of larvae feeding on antibiotic treated fir, however *Halomonas sp.* accounts for less than 1% of the gut associated bacteria in larvae feeding on antibiotic treated fir. Comparisons of gut and fir communities from the untreated fir group yielded two differentially abundant OTUs (denovo5188: *Methylocystaceae* and denovo92: *Acetobacteraceae*). Both denovo5188 *Methylocystaceae* and denovo92 *Acetobacteraceae* were more abundant in diet (0.0704 ± 0.031 and 0.026 ± 0.011 respectively) than in guts (0.0 ± 0.0 and 0.0 ± 0.0).

Within the group of larvae being fed spruce foliage, 3 differentially abundant OTUs were detected between gut and foliage communities from the antibiotic group (denovo2429: *Staphylococcus sp.*, denovo4501: *Burkholderiaceae*, and denovo5458 *Enterococcus sp.*). 3 OTUs were found to be differentially abundant in the untreated group (denovo2287: *Erwinia sp.*, denovo1313: *Pseudomonas sp.*, and denovo699 *Oxalobacteraceae*). All of the OTUs identified as being differentially abundant between gut and foliage communities were more abundant in foliage than in guts.

Two differentially abundant OTUs were detected between frass and gut communities in the antibiotic fir group and one differentially abundant OTU was detected between frass and gut communities in the untreated fir group. One of the OTUs detected in the antibiotic fir group (denovo8018: *Halomonas sp.*) and the OTU detected in the fir (denovo3500: *Shewanella sp.*) were both more abundant in guts than in frass. Within the spruce group, one OTU (denovo2429: *Staphylococcus sp.*) was more abundant in

the frass compared to the guts of larvae feeding on both antibiotic treated and untreated foliage. Finally, no OTUs were identified as being differentially abundant between fast and slow growing larvae.

1.4 Discussion

1.4.1 Influence of the gut microbial community on larval growth and survival

The objectives of this study were to determine if spruce budworm has a resident microbiome and if the associated gut microbiota are important for the health, growth and survival, of spruce budworm larvae. One of our hypotheses was that the use of antibiotics would reduce 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. While growth and survival, being general measures of health, are not direct measures of fitness, they are intrinsically linked. Placing our results in the context of fitness is important for studying the ecological impact of spruce budworm, because ultimately spruce budworm fitness is what will have a significant impact on the forest over the course of several years during an outbreak. Larval growth, and by extension pupal weight (not measured), can have important ramifications for adults such that a mal-nourished individual would likely have reduced fitness. Larval survival has an obvious impact on fitness in that increased larval mortality will decrease overall fitness. Our results show that there are some differences in larval spruce budworm gut microbial communities due to diet type and antibiotic treatment. However, none of the changes in gut microbiota as a consequence of antibiotic treatment resulted in significant changes in larval survival or growth.

Similar results were found in a growth experiment where the microbiome of *Manduca sexta* was eliminated via antibiotic treatment and no change in growth was

detected (Hammer *et al.*, 2017). This suggests that the eastern spruce budworm, and perhaps many other lepidopteran species, are not nutritionally dependent on microbial symbiosis. One possible explanation for this could be attributed to the feeding strategy utilized by spruce budworm and many other herbivorous lepidopteran species, i.e bulk feeding. 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 handle the secondary compounds associated with conifer foliage. In fact, one study by Whitaker *et al.* (2016) found that carnivorous and herbivorous larval microbiomes did not differ significantly in composition. This suggests that lepidopteran larvae may not select for bacteria based on their nutritional needs, thus further suggesting that lepidopteran larvae do not rely on microbial symbiosis to extract the necessary nutrients from food.

It is unclear why we observed the lowest survival rate in untreated spruce while also observing higher growth rates in that group compared to larvae in either fir group. One possible explanation is that spruce needles are physically harder for spruce budworm larvae to consume meaning that it is harder for smaller larvae to access those nutrients despite the spruce foliage used could have had a higher nutrient content than the fir foliage, due to the difference between growing in a greenhouse rather than in nature. We attempted to control for this by raising larvae for one week on synthetic diet so that larvae would be large enough to successfully feed on both fir and spruce foliage. It is possible that some larvae were still unable to feed on spruce foliage at the start of the experiment but those that were able to feed on the spruce foliage successfully would have the advantage of the excess nutrients in the greenhouse grown foliage resulting in higher growth rates.

While our data suggest that the gut microbiome is not important for the growth or survival of spruce budworm larvae, there are other aspects to health which our study does not address such as: immune function, development, and reproductive fitness. Further studies will need to attempt to measure these aspects in order to gain a broader understanding of spruce budworm health. It is not possible to obtain an accurate estimate of microbial function from 16S sequences, so we are unable to comment on the potential function of these microbes based on our data. Use of metagenomic or metatranscriptomic sequencing will also help in further elucidating the function of the spruce budworm microbiome.

1.4.2 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 then select for bacteria which could thrive in that environment, but that the community would still reflect the source community originating with the foliage. This pattern would provide evidence that spruce budworm larvae possess a resident gut microbiome, however our data suggest the opposite. This finding is consistent with recent studies (Hammer *et al.*, 2017; Whitaker *et al.*, 2016), that present evidence suggesting that lepidopteran larvae lack resident microbial communities. The main argument for this conclusion is that 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 as previously discussed.

Our results at the community level suggest that as the bacteria moved from foliage to the gut and passed from the gut as frass, the communities became more similar in terms of community composition among treatments at each step. Thus, while we

provide evidence which generally supports the hypothesis that lepidopteran larvae lack a resident microbiome, weak selective pressures seem to be at play in the spruce budworm gut. The selective pressures acting on bacteria in the gut are probably due to the high pH in the lepidopteran larval gut. Looking at finer scales to examine the responses of individual taxa versus a community wide analysis however, we overwhelmingly found that taxa that were differentially abundant between gut- and foliage-associated communities were more abundant in foliage samples.

Our results suggest that spruce budworm larvae have a few bacteria which increase in relative abundance while in the gut. Bacteria will increase in relative abundance in a community if they increase in absolute abundance (i.e are metabolically active and undergo cell division) while other bacteria remain constant or decrease in their absolute abundance. Bacteria can also increase in relative abundance if their absolute abundance remains constant while other bacteria decrease in absolute abundance. These findings support the hypothesis that the dominant taxa in the spruce budworm are food-derived transient microbes but that rare foliage-associated microbes are able to persist in the spruce budworm gut.

There is mounting evidence that lepidopteran larvae lack a resident, or dominant, microbiome and thus are not nutritionally dependent on bacterial symbiosis. Certain rare taxa, however, seem to be able to colonize the gut but their function 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 which 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.

1.4.3 Study limitations

Although we provide evidence which generally supports the hypothesis that lepidopteran larvae lack a resident microbiome, the fact that frass and foliage were not different in microbial diversity but that both frass and foliage had significantly higher microbial diversity than guts raises some questions. This trend suggests that bacteria present in the foliage passed through the gut without colonizing the gut, shifts in community composition from foliage to gut to frass compensated for any potential change in diversity. Or, that these results are partly an artefact of the way frass was collected.

In our study, frass was collected as an aggregate sample that was allowed to accumulate over time. It has been shown previously that human fecal samples can remain stable at room temperature for up to 14 days (Lauber *et al.*, 2010) so it is unlikely that this influenced our results. The close proximity of the frass to the diet in each magenta box could have allowed for horizontal transfer of microbes between the two environments, however the evidence for this is slim because we observed differences in the community composition of frass- and diet-associated communities. 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.

While it is possible that lab reared larvae respond differently to antibiotic treatment, our results generally fall in line with other similar studies suggesting that these are meaningful results. Further studies utilizing both lab reared and field-caught larvae

will be necessary to address discrepancies which could arise from using laboratory reared insects rather than field collected insects in order to confirm our findings. It will also be necessary to carry out similar experiments using only field collected foliage to better control for the differences in nutritional content between plants growing in a natural environment versus in a greenhouse. Finally our study used foliage samples from one field site and from one green house. Previous studies on phyllosphere communities have shown that plants growing in different geographical locations can have significantly different microbial communities (Laforest-Lapointe *et al.*, 2016). Thus, further studies should attempt to control for foliage source by using foliage samples from a consistent environment or site rather than from multiple sources as in our experiment.

1.5 Conclusion

Overall, our results show that the application of antibiotics had a significant effect on the microbial community structure associated with both fir and spruce needles that were fed to spruce budworm larvae. We also show that the community structure of fir foliage was significantly different from that of spruce foliage. Although we observed effects of diet and antibiotic treatment on microbial communities in the guts of spruce budworm; the effects of diet and antibiotic treatment were weaker than those in diet-associated communities. Because the differences between gut communities were less pronounced than between foliage communities, we concluded that some environmental filtering is taking place as bacteria associated with foliage are consumed alongside plant matter and are ingested by the spruce budworm. By the time bacteria passed through the gut as frass, microbial communities were indistinguishable among diet and antibiotic treatments. Selective pressures in the gut, i.e environmental filtering, is noteworthy because it implies that the spruce budworm gut microbiome is not simply food derived but is selected by the gut environment from a regional species pool.

As larvae in our experiment were presented with new-year or budding foliage from both tree species we were able to control for the difference in phylogeny associated with budburst between the two tree species. We provide evidence that suggests that spruce budworm larvae perform better on spruce foliage than on fir foliage when phenology (Quezada García *et al.*, 2015; Fuentealba and Bauce, 2012) is controlled for, suggesting that the preference of spruce budworm for fir foliage in natural ecosystems may be driven by phenology rather than differences in leaf palatability.

Finally, we do not have data to support our hypothesis that alteration of the spruce budworm gut-associated microbial community would result in a reduction of larval growth. We do provide evidence, however, largely in support of the hypothesis that the gut microbial community of lepidopteran larvae is largely transient with one caveat. We show that despite the dominant bacterial taxa in the gut community are unable to colonize the gut and simply pass through the gut, there are selective pressures at play in the spruce budworm gut which select for some rare taxa to colonize the gut.

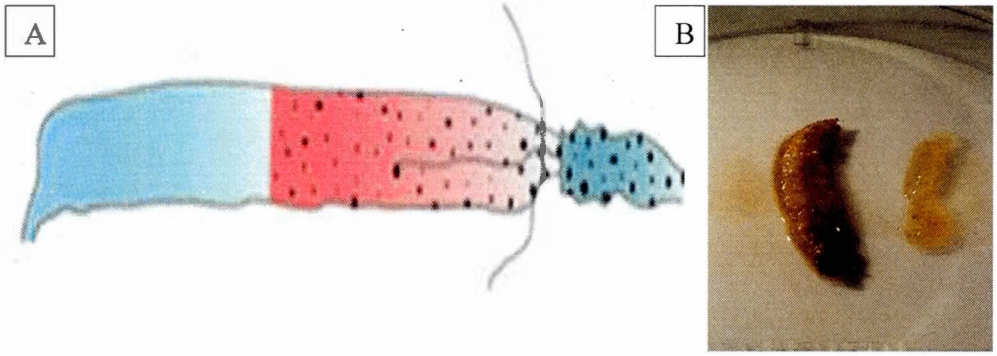

Figure 1.1: A) Schematic of a lepidopteran larval gut adapted from Engel and Moran (2013). From left to right the image shows the hindgut, the midgut, and the foregut. B) Sixth instar larvae with its gut removed (left) and the disected gut (right)

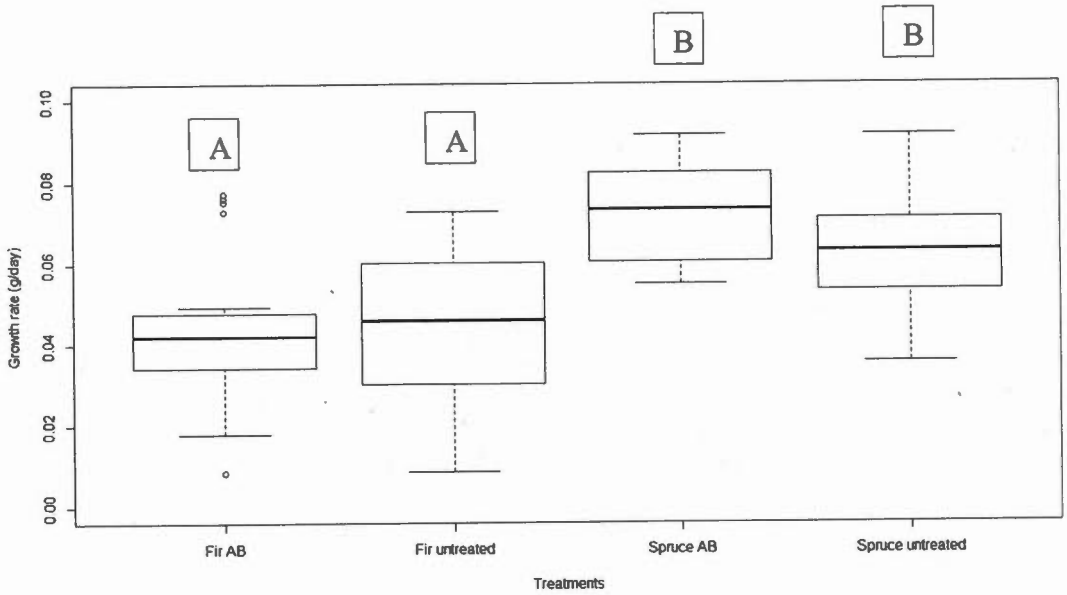

Figure 1.2: Growth rate (grams/day, ± S.E) of spruce budworm larvae among different diets (spruce versus fir foliage) and antibiotic treatments (AB = antibiotic treated). Letters indicate treatment combinations that differed significantly ($p < 0.05$) according to a Tukey's Honest Significant Difference post-hoc test, based on a mixed model (see Methods section for details).

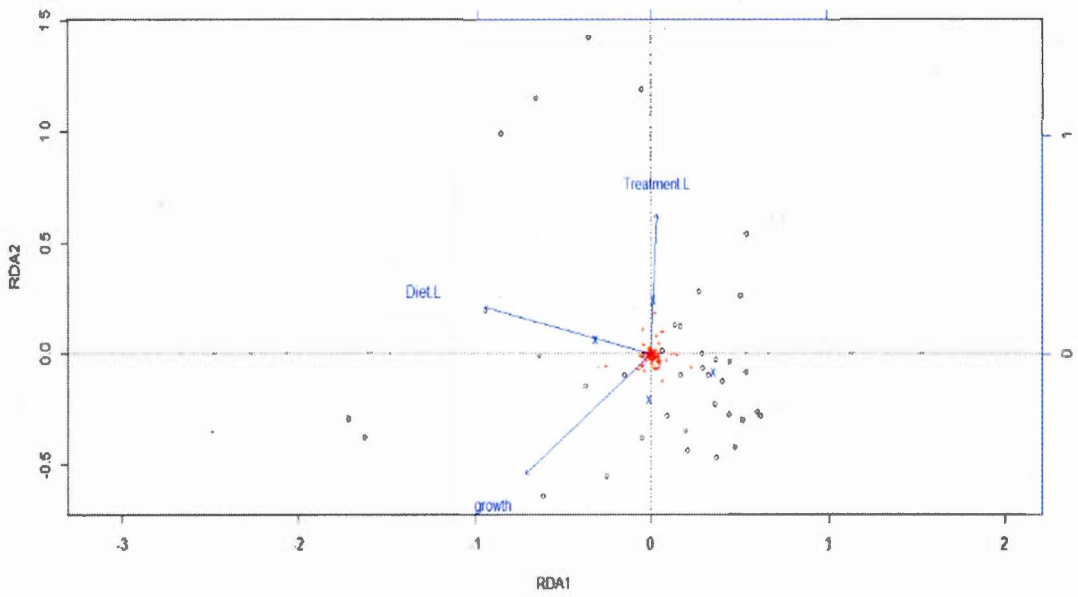

Figure 1.3: RDA biplot of spruce budworm larval gut communities of individuals who were fed foliage with growth rate, diet, and treatment as explanatory variables. The first axis (constrained - 4.9%) and the second axis (unconstrained - 3.0%) account for 7.98% of the total variance.

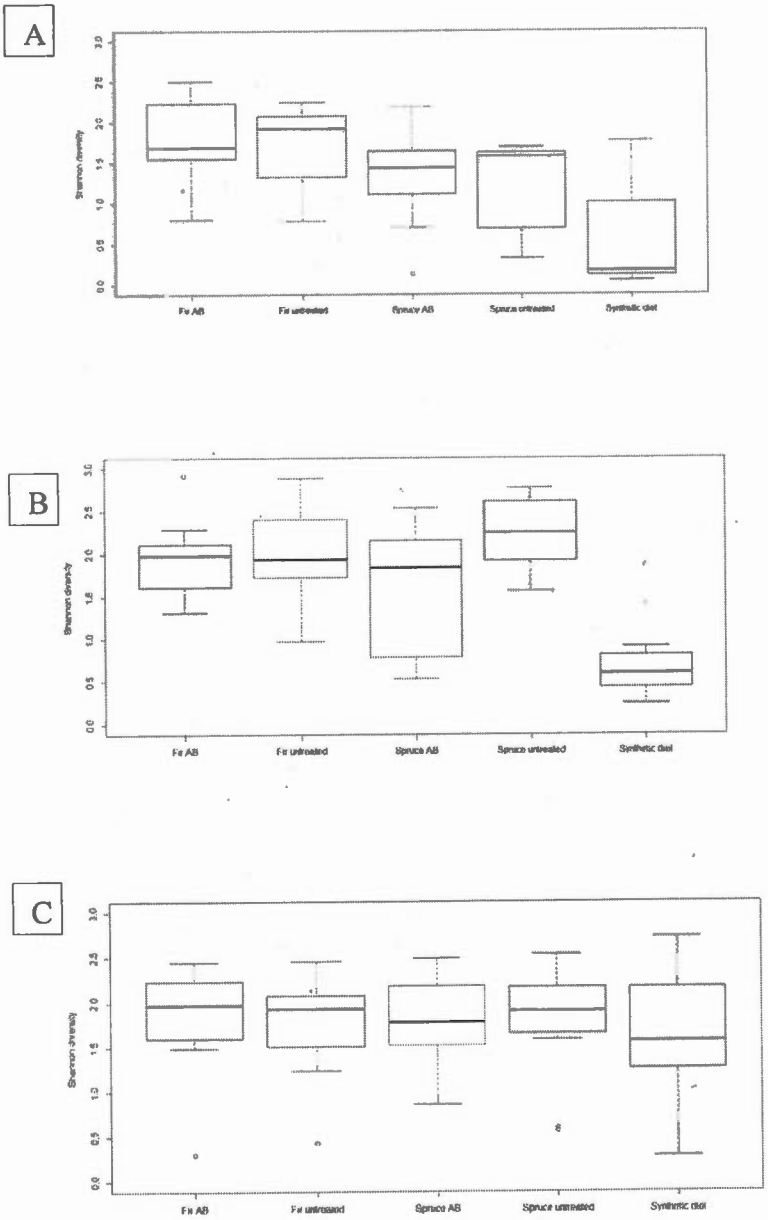

Figure 1.4: Mean (± SE) Shannon diversity of (A) spruce budworm diet- (foliage and synthetic diet) (B) gut- and (C) frass-associated microbial communities among different diets (spruce versus fir foliage and synthetic diet) and antibiotic treatments (AB= antibiotic treated).

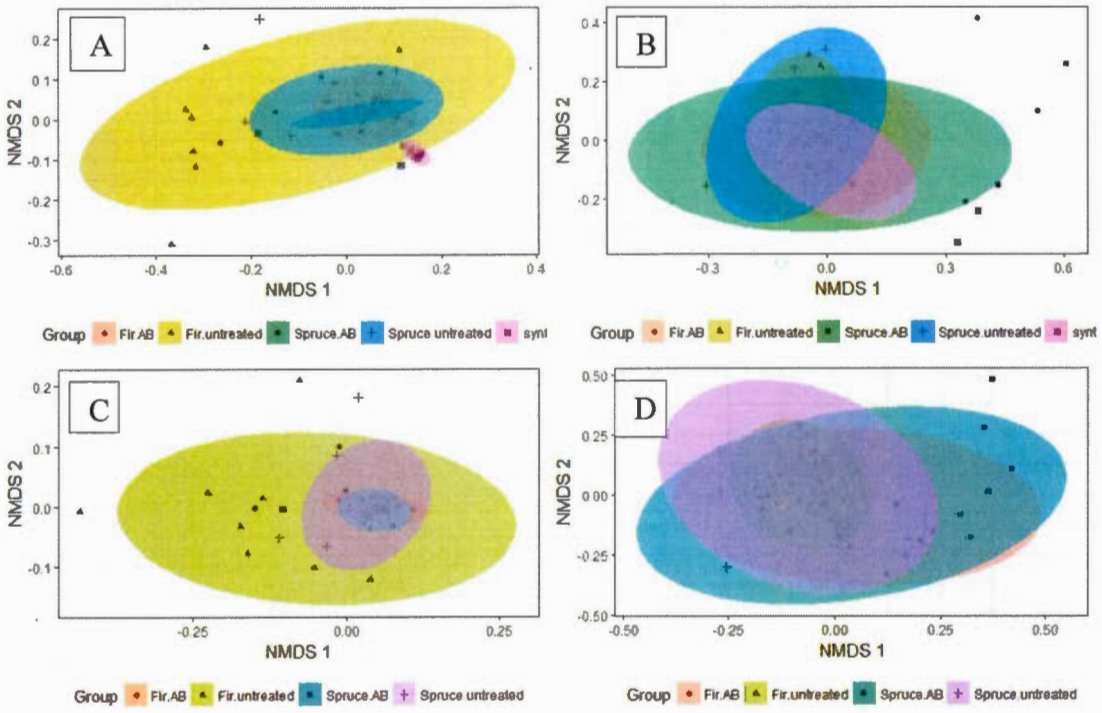

Figure 1.5: NMDS ordinations of diet-associated, foliage and synthetic diet, microbial communities based on weighted and unweighted UniFrac distances. (A: all diets weighted UniFrac stress =0.13, B: All diets unweighted UniFrac stress =0.18, C: only foliage weighted UniFrac stress =0.10, D: only foliage unweighted UniFrac stress =0.20). 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).

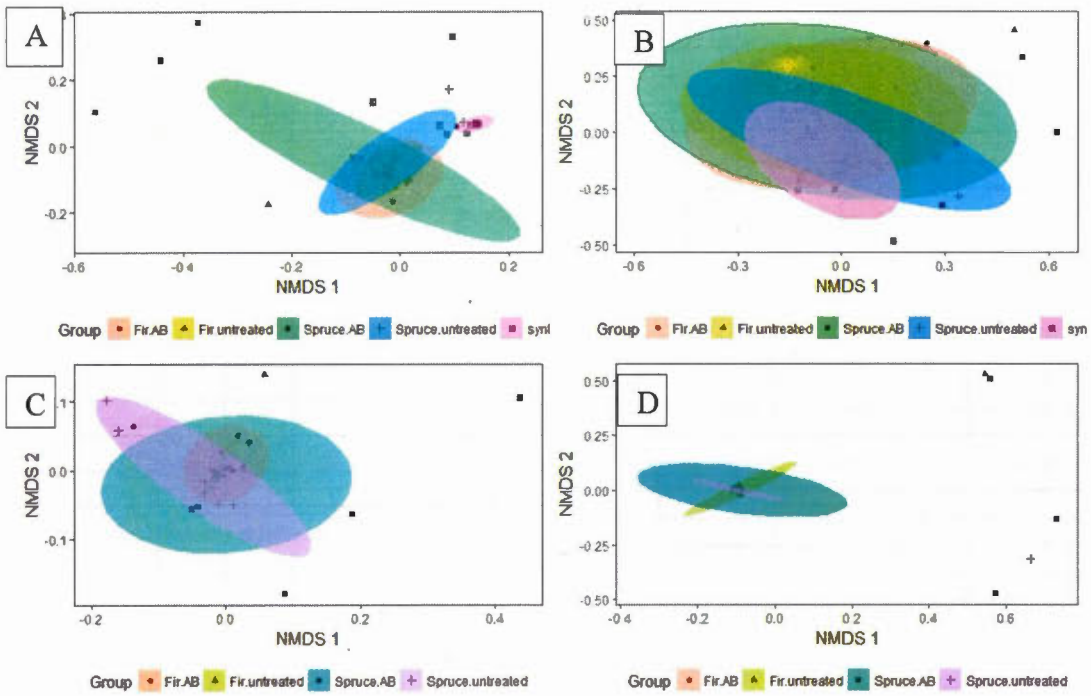

Figure 1.6: NMDS ordinations of gut-associated microbial communities based on weighted and unweighted UniFrac distances. (A: all guts weighted UniFrac stress =0.07, B: All guts UniFrac stress =0.21, C: guts of larvae feeding on foliage weighted UniFrac stress =0.07, D: guts of larvae feeding on foliage 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).

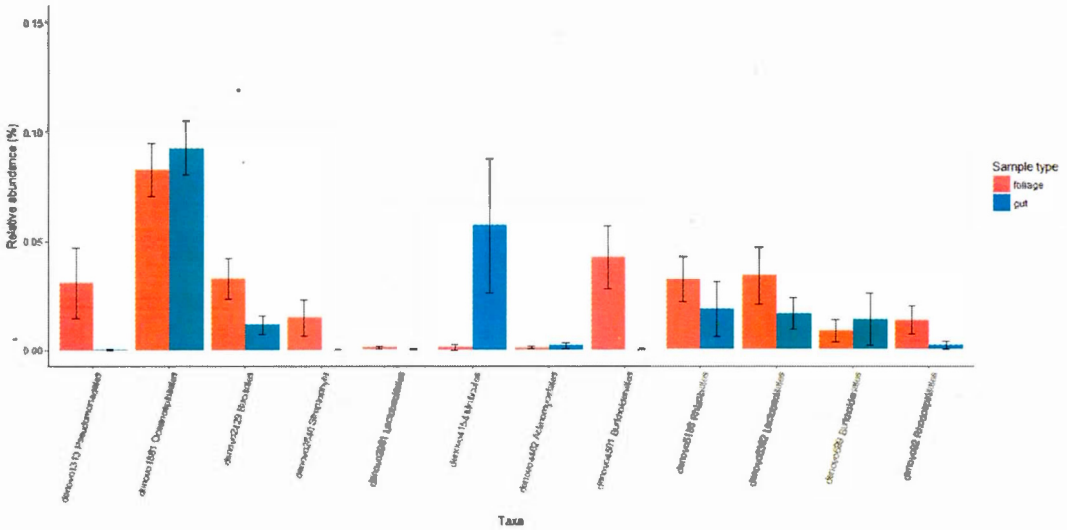

Figure 1.7: Mean relative abundance (± SE) of OTUs identified as being differentially abundant between foliage- and gut-associated communities based on an ANCOM test (ANCOM; adjusted $p < 0.05$). Blue bars represent gut samples and red bars represent foliage samples.

Table 1.1: Estimates of the influence time, antibiotic treatment, and diet on the weight of spruce budworm larvae exposed to different diet and antibiotic treatments. Results represent ANOVA analysis of a mixed effect model 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 and significant interactions between Time and other factors indicate that growth rates differed among treatment combinations.

Variable	F value	P value
Intercept	16637.5	<.0001
Time	751.8	<.0001
Treatment	5.78	0.018
Diet	3.2	0.076
Time:Treatment	3.0	0.081
Time:Diet	33.9	<.0001
Treatment:Diet	3.8	0.056
Time:Treatment:Diet	2.1	0.151

Table 1.2: Pairwise comparisons of growth rate estimates for spruce budworm larvae within treatment groups as determined by a Tukey's Honest Significant Difference post-hoc test based on a mixed effect model with time, antibiotic treatment, and diet as fixed factors and time nested within individual as random factors.

Contrast	Estimate	Standard error	Degrees of freedom	T ratio	P value
Antibiotic fir versus antibiotic spruce	-0.0290	0.0054	483	-5.339	<.0001
Antibiotic fir versus untreated fir	-0.0012	0.0054	483	-0.232	0.9956
Antibiotic fir versus untreated spruce	-0.0184	0.0063	483	-2.92	0.0191
Antibiotic spruce versus untreated Fir	0.0278	0.0053	483	5.165	<.0001
Antibiotic spruce versus untreated spruce	0.0106	0.0062	483	1.693	0.3284
Untreated fir versus untreated spruce	-0.0171	0.0062	483	-2.744	0.0319

Table. 1.3: 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	0.04395	0.003863	0.036359	0.05154
Fir	None	0.045204	0.003779	0.037779	0.05263
Spruce	AB	0.073038	0.003842	0.065489	0.080587
Spruce	None	0.062377	0.004989	0.052574	0.07218

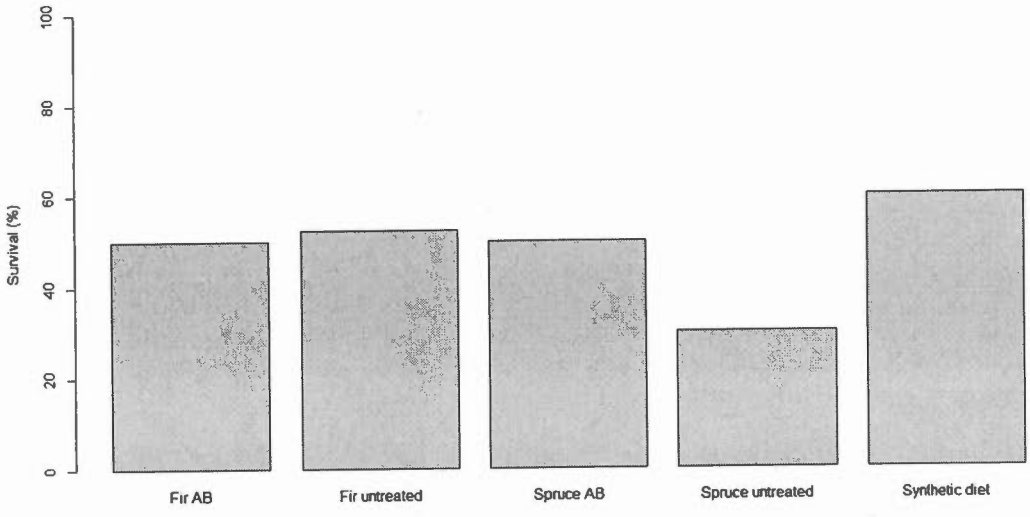

Figure 1.S1: Percent survival of spruce budworm larvae among treatment diets (spruce versus fir foliage and synthetic diet) and antibiotic treatments (AB = antibiotic treatment).

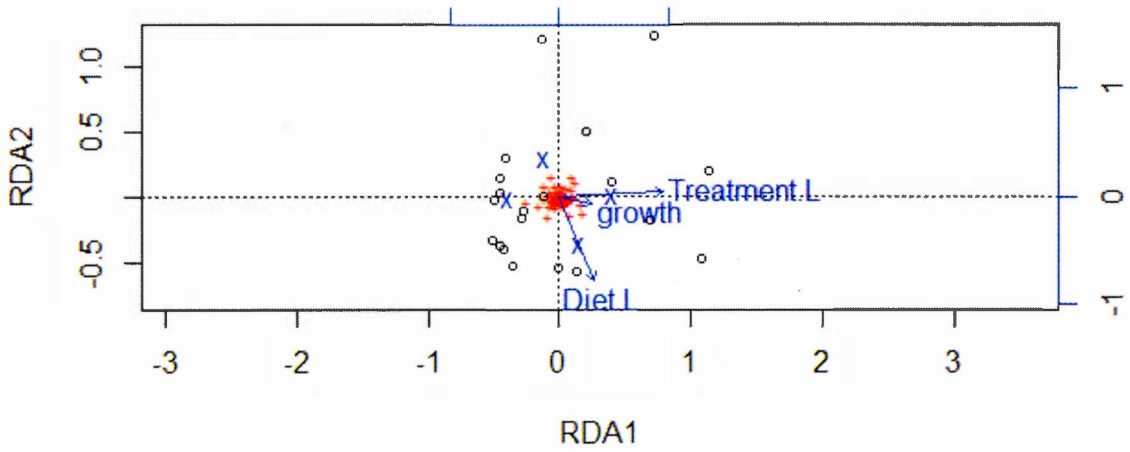

Figure 1.S2: RDA biplot of foliage-associated communities with the growth rate of larvae feeding on foliage, diet, and treatment as explanatory variables. The first axis (constrained - 9.6%) and the second axis (unconstrained - 6.3%) account for 15.9 percent of the total variance.

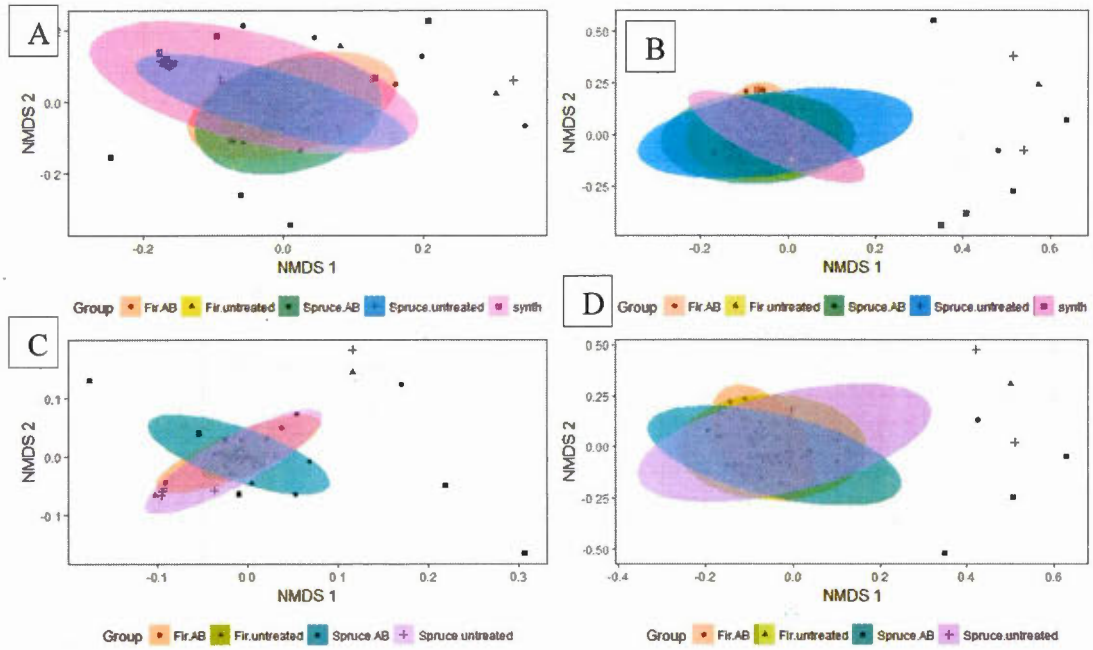

Figure 1.S3: NMDS ordinations of frass-associated microbial communities based on weighted and unweighted UniFrac distances. (A: all frass samples weighted unifrac stress =0.15, B: All frass samples UniFrac stress =0.13, C: frass of larvae feeding on foliage weighted UniFrac stress =0.10, D: frass of larvae feeding on foliage unweighted UniFrac stress =0.19). 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).

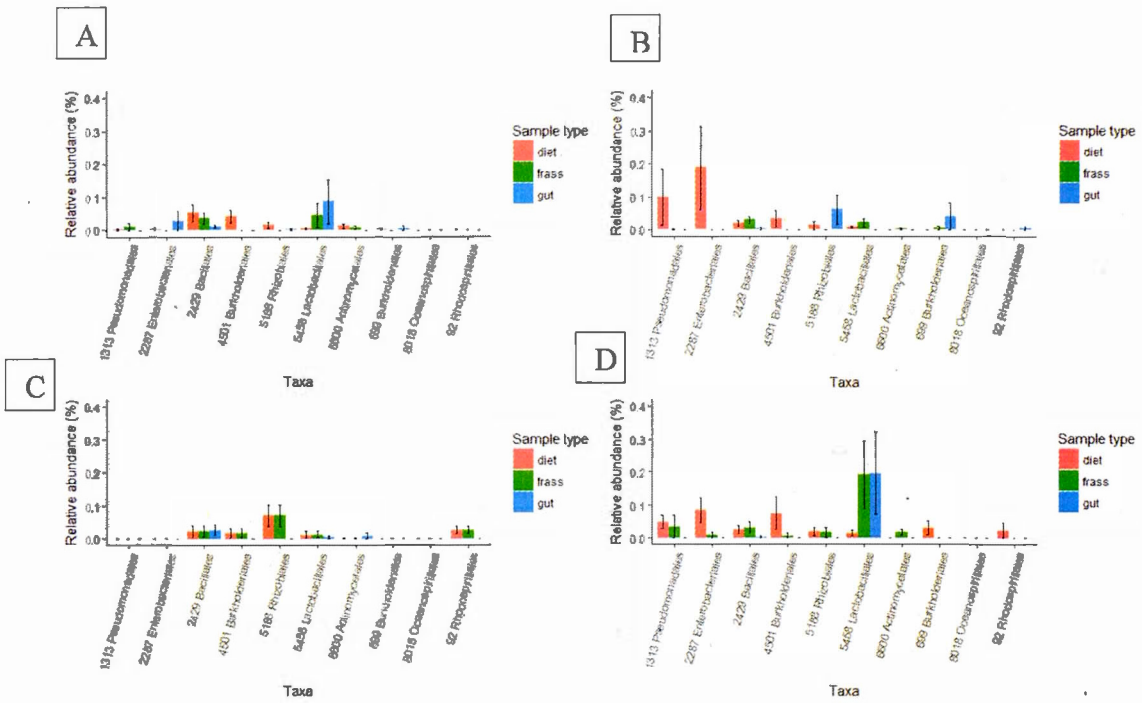

Figure 1.S4: Mean relative abundance (± SE) of OTUs identified as being differentially abundant between comparisons of communities among sample types (diet, guts, and frass) within treatment combinations (fir versus spruce and antibiotic treated vs untreated) based on an ANCOM test (ANCOM; adjusted $p < 0.05$).

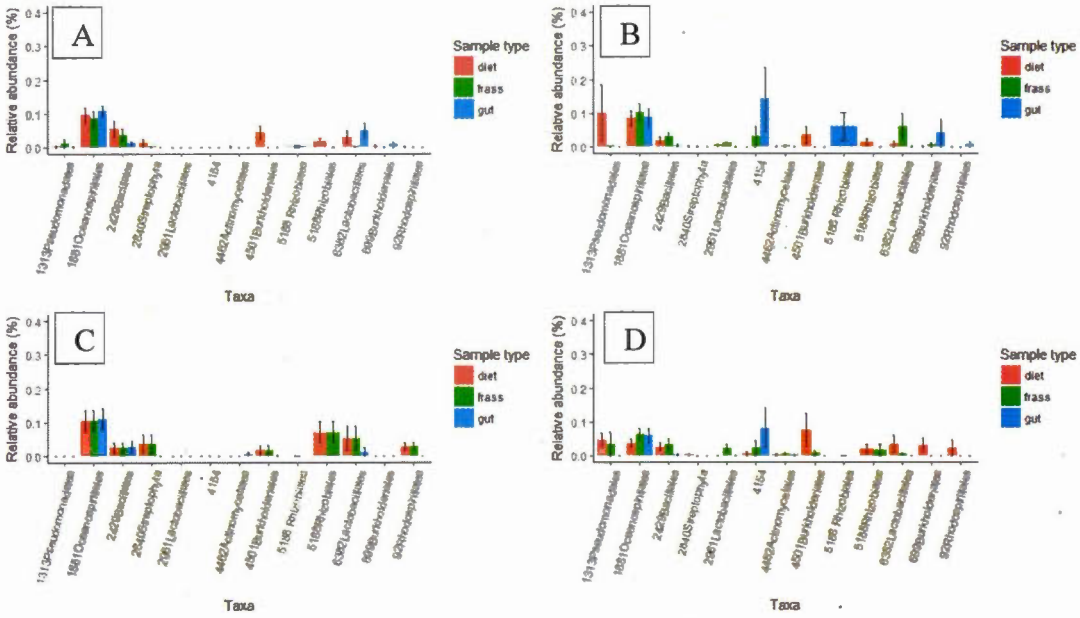

Figure 1.S5: Mean relative abundance (± SE) of OTUs identified as being differentially abundant between sample types among treatment groups i.e a comparison of all guts and all frass samples based on an ANCOM test (ANCOM; adjusted $p < 0.05$). The relative abundances in this figure represent the relative abundance of these taxa within each treatment group.

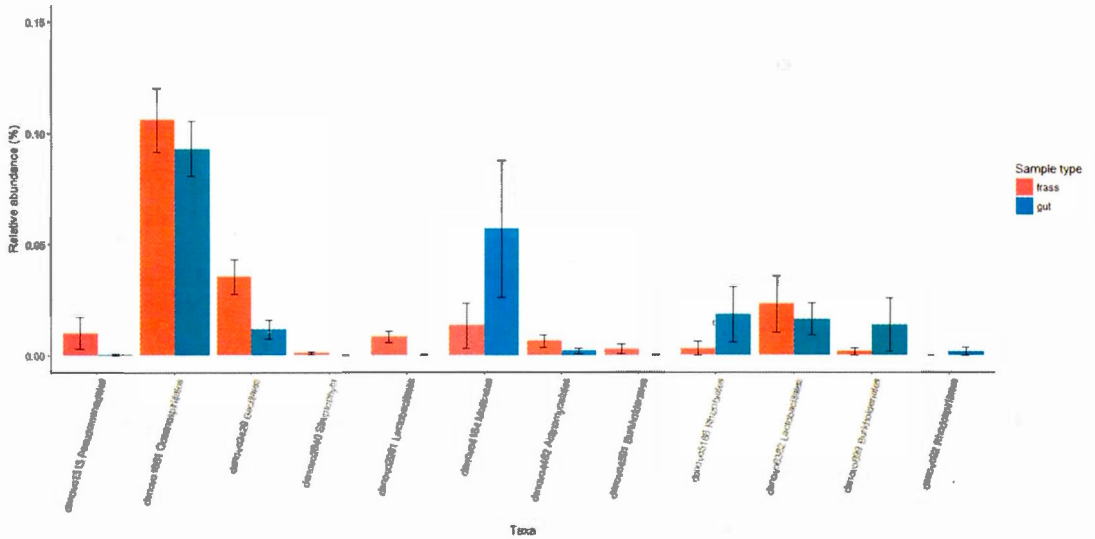

Figure 1.S6: Mean Relative abundance (± SE) of OTUs identified as being differentially abundant between comparisons of all frass samples and all gut samples based on an ANCOM test (ANCOM; adjusted $p < 0.05$). Blue bars represent gut samples and red bars represent frass samples.

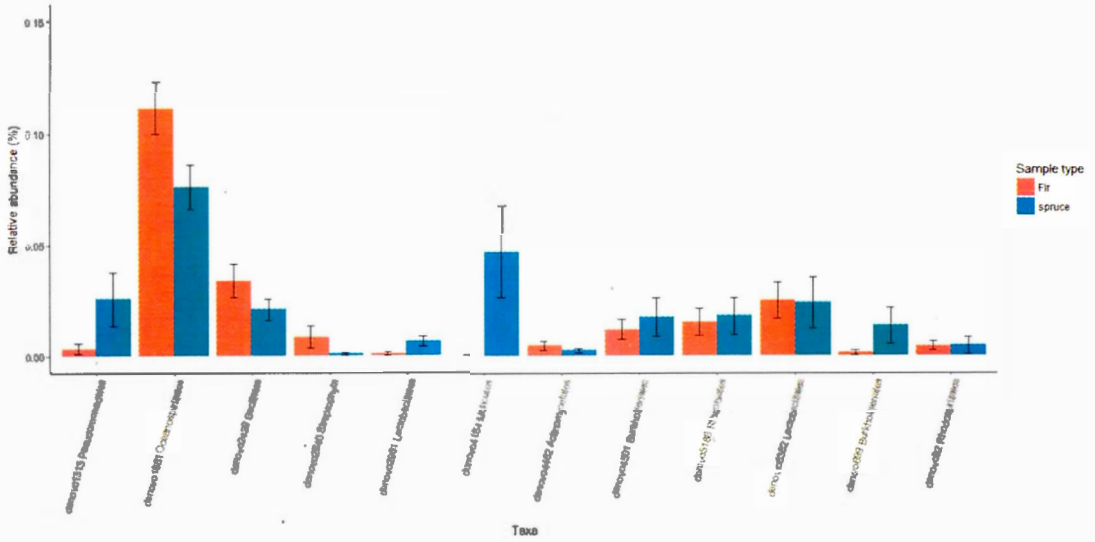

Figure 1.S7: Mean Relative abundance (± SE) of OTUs identified as being differentially abundant between comparisons of all fir samples and all spruce samples based on an ANCOM test (ANCOM; adjusted $p < 0.05$). Blue bars represent spruce samples and red bars represent fir samples.

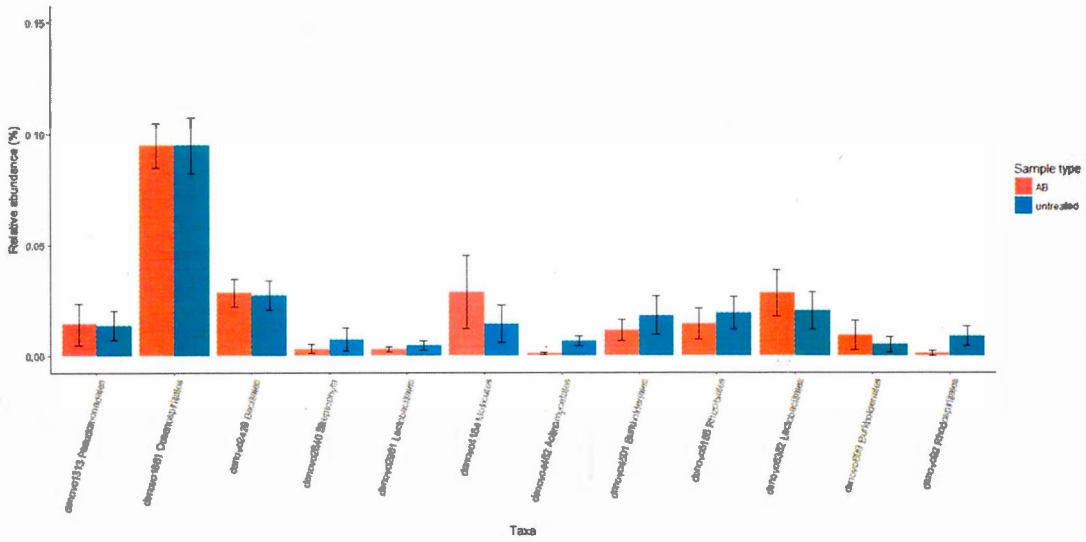

Figure 1.S8: Mean Relative abundance (± SE) of OTUs identified as being differentially abundant between comparisons of all antibiotic treated samples and all untreated samples based on an ANCOM test (ANCOM; adjusted $p < 0.05$). Blue bars represent untreated samples and red bars represent samples that were treated with antibiotics.

Table 1.S1: Total replicates sequenced across all sample types and the time in the experiment samples were collected and frozen.

Sample type	Time after start of treatments	Replicates sequenced
Larval midguts	14 days	96
Foliage	7 days	50
Foliage	14 days	51
Frass	7 days	50
Frass	14 days	49

Table 1.S2: Statistical tests used in this study along with the hypothesis tested, the variables used, and the type of data used to

Test	Hypothesis tested	Variables	Type of data used
Mixed-effects model	Diet and antibiotic treatment will influence growth	Weight , Diet type, Antibiotic treatment, Time	Log transformed weight values of individual larvae recorded every two days for 2 weeks
Logistic regression	Diet and antibiotic treatment will affect larval survival	Mortality	Mortality- Binary response
Redundancy analysis (RDA)	Gut microbial communities are correlated with larval growth	Diet type, Antibiotic treatment, Gut community composition	Growth rates of each larva and relative abundances of OTUs detected in midgut samples.
Redundancy analysis (RDA)	Foliage-associated microbial communities are correlated with larval growth	Diet type, Antibiotic treatment, Foliage-associated community composition	Growth rates of each larva and relative abundances of OTUs detected in foliage samples.
Permutational multivariate ANOVA	Gut community composition is different among fast and slow growing larvae	Gut community composition Growth class(based on growth rate, defined as upper and lower quartiles)	Square root transformed UniFrac distance matrix (weighted by the relative abundance of taxa and unweighted)
ANOVA	Diet and antibiotic treatment will affect microbial alpha diversity	Diet type, Antibiotic treatment	Shannon diversity calculated using relative abundances of OTUs
Permutational multivariate ANOVA	Gut microbial community composition will differ among diets and	Diet type, Antibiotic treatment, Gut community composition	Square root transformed UniFrac distance matrix (weighted by the relative abundance of taxa and unweighted)
Analysis of the composition of microbiomes (ANCOM)	What sample type (guts, foliage, or frass) are individual OTUs associated with	Sample type	Relative abundance of OTUs

Table 1.S3: OTUs identified as being differentially abundant between different comparisons. The first column represents the OTU number as determined during OTU picking (see methods for details). Taxonomic identification is presented for each taxa, if a taxon was not able to be identified to a certain taxonomic level, i.e species, that cell was left blank. Group represents which comparison group that taxon was more abundant in.

Differentially abundant OTUs							
	Phylum	Class	Order	Family	Genus	Species	group
Antibiotic treated fir: guts vs diet							
denovo8018	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Halomonadaceae	Halomonas		gut
Untreated fir: guts vs diet							
denovo5188	Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylocystaceae			diet
denovo92	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae			diet
Antibiotic treated spruce: guts vs diet							
denovo2429	Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Staphylococcus		diet
denovo4501	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae			diet
denovo5458	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	Enterococcus		diet
Untreated spruce: guts vs diet							
denovo2287	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	Erwinia		diet
denovo1313	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas		diet
denovo699	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae			diet
Antibiotic treated fir: guts vs frass							
denovo8018	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Halomonadaceae	Halomonas		gut
denovo6600	Actinobacteria	Actinobacteria	Actinomycetales	Corynebacteriaceae	Corynebacterium		frass
Antibiotic treated fir: guts frass							
denovo2429	Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Staphylococcus		frass
Untreated spruce: guts vs frass							
denovo2961	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus		gut
denovo2429	Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Staphylococcus		frass
denovo4154	Tenericutes	Mollicutes					frass
All samples combined: guts vs foliage							
denovo5188	Proteobacteria	Alphaproteobacteria	Rhizobiales	Methylocystaceae			diet
denovo2840	Cyanobacteria	Chloroplast	Streptophyta				diet
denovo6382	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus		diet
denovo1313	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Pseudomonadaceae	Pseudomonas		diet
denovo2429	Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Staphylococcus		diet
denovo4501	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae			diet
denovo699	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae			diet
All samples combined: guts vs frass							
denovo2961	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus		frass
denovo2429	Firmicutes	Bacilli	Bacillales	Staphylococcaceae	Staphylococcus		frass
denovo4154	Tenericutes	Mollicutes					gut
All samples combined: antibiotic treated vs untreated							
denovo92	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae			untreated
denovo4462	Actinobacteria	Actinobacteria	Actinomycetales	Micrococcaceae	Kocuria	palustris	untreated
All samples combined: spruce vs fir							
denovo1881	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Halomonadaceae	Halomonas		fir

CONCLUSION

I found that despite significant effects of both diet type and antibiotic treatment on diet-associated microbial community composition, there was only a weak effect of antibiotic treatment on the composition of the gut-associated microbial community in spruce budworm larvae. Antibiotic treatment applied to the diet was sufficient to alter the spruce budworm gut microbiome, however we did not observe any resulting effect on either larval growth or survival. Overall we observed that spruce budworm does not need to maintain a resident microbiome.

My results provide evidence to support a growing number of studies suggesting that associations with microbial symbionts may not be as critical to lepidopteran nutrition as previously hypothesized. Although I observed differences in microbial communities due to the use of antibiotics, I was unable to show that the gut microbial community of spruce budworm larvae influenced host growth. Contrary to other studies suggesting that microbial communities of lepidopteran larvae are composed entirely of transient microbes we present evidence suggesting that a select few bacteria are able to persist and increase in relative abundance in the spruce budworm gut. Although the findings of this study suggest that these microbes are not required for the growth or survival of the spruce budworm, one cannot determine what those taxa may be doing in the gut given the limitations in the sequencing technology used.

The main argument for lepidopteran larvae lacking a resident microbiome hinges on the similarity of the gut-associated microbiota to diet-associated microbiota which

can be interpreted as a lack of any host-specific selection. All of the studies supporting this hypothesis, including this one, have exclusively used 16S gene amplicon sequencing. To fully answer this question, differences in absolute abundances and metabolic potential between diet- and gut-associated communities need to be quantified.

The nature of amplicon sequencing requires us to use relative abundances rather than absolute abundances in our community analysis. This is because in amplicon sequencing the target gene, in this case the V4 region of the 16S gene, is amplified using polymerase chain reaction which exponentially increases the amount of genetic material. This, along with interspecific variation in gene copy number, means that it is impossible to get an estimate of absolute abundance using this approach. Absolute abundance is important to consider because it would allow us to properly quantify changes in the microbial community. If, for example, individual taxa increase in relative abundance it is impossible to tell if that is because one taxon increased in absolute abundance or if another taxon's absolute abundance decreased while the first taxon remained the same. Absolute abundance will also provide us with a definitive answer about how the community is affected by antibiotic treatments. It was surprising that antibiotic treatments did not always have major effects on microbial community structure, but with only relative abundance data it is impossible to determine if antibiotic treatment modified the total abundance of the associated microbial community. Absolute abundance can be quantified in future studies using quantitative PCR to determine the number of a single taxa in the community or through methods such as flow cytometry to quantify the number of all bacteria in a sample.

To further understand what functions bacteria provide to spruce budworm, we need information on the metabolic potential of the microbiome and of the genes which are being actively expressed in the gut compared to the foliage. To do this more studies

are needed using metagenomic and metatranscriptomic approaches. By sequencing the metagenome of the spruce budworm gut it will be possible to see the entire genomic potential of the microbial community. Once the entire spruce budworm genome is sequenced, it will be possible to carry out metagenome-wide association studies to determine what effects different bacteria have on the spruce budworm. These studies will be required to elucidate the role that these microbes play in the spruce budworm gut, particularly if they are not immediately involved in nutrient acquisition or promoting host growth. In addition, metatranscriptomic studies are necessary to determine whether or not the spruce budworm microbiome is transient by determining which microbial genes are being actively transcribed in the gut. If, for example, genes associated with microbial metabolism were transcribed in higher frequencies in the spruce budworm gut compared to foliage we would conclude that bacteria in the spruce budworm gut were metabolically active and able to persist in the gut, suggesting that microbes are not transient.

Other limitations of this study involve the design of the experiment. The most pressing of which, is the way that frass was collected for this study. The primary reasoning for collecting frass was that by looking at shifts in the microbial community between diet, the gut, and frass, we would be able to gain a better understanding of the dynamics of the microbial community rather than obtaining a single snapshot of the gut microbial community. For example if a particular bacterium was abundant in foliage and frass, but not in the gut that would suggest that the bacterium in question would likely be transient in nature i.e pass through the gut without colonizing the gut tissues. Furthermore, many studies of animal gut microbiomes have used feces or frass as a surrogate measure of gut microbiome structure.

In an effort to collect enough genetic material to perform a DNA extraction, we let frass accumulate in each magenta box prior to collection. In doing so, we introduced

a number of potential biases such as not collecting immediatley after excretion and the close proximety of the frass to the diet in each container. One way to overcome these limitations in future studies would be to collect frass from a subset of larvae by placing the larvae who have recently fed into separate microcentrifuge tubes without any food. The larvae would then be given time to allow the food that is in their guts to pass through their digestive tract. This would ensure that only fresh frass, within a few hours, would be collected in an isolated environment and immediately frozen.

Another limitation of this study is that differences between the diets used, such as nutrient quality, phenology, and collection source were not fully controlled for. We collected budding foliage of fir trees that were growing in a natural forest, while we collected budding spruce foliage from greenhouse raised seedlings. Because spruce was grown in more favorable conditions (i.e a greenhouse) than fir, our observations suggesting that spruce budworm grows better on fir may be partly explained by this difference. Greenhouse grown seedlings, for example, receive more nutrients and have lower C:N ratios than naturally occurring seedlings. Finally, foliage from each tree species was only collected from one site, or one greenhouse in the case of black spruce, meaning that any variation in plant-associated microbial communities across geographical scales would be lost. Additionally, plants grown in a greenhouse could have different microbiomes compared to members of the same species growing outdoors. In future studies it will be necessary to control for this by only sampling foliage from trees growing in a natural environment and to sample across geographical scales.

There are two potential explanations for the lack of symbiotic relationships between bacteria and spruce budworm. One is that the physiochemical properties of the spruce budworm gut are alkaline. This could allow spruce budworm to effectively digest conifer needles, which are high in lignin and secondary compounds, without the aid of microbially mediated metabolic pathways. If the alkaline environment of the

spruce budworm gut allows spruce budworm to survive without aid from microbial associates, then it is possible that there is not enough evolutionary selection for strong bacteria-spruce budworm associations (Engel and Moran, 2013).

The second possible explanation is that sometime in its evolutionary past, spruce budworm was involved in a horizontal gene transfer event from microbial symbionts that gave them the metabolic capacity to digest conifer needles. Horizontal gene transfer events are typically between two bacterial species, but there have been examples of bacteria transferring genetic material to the genome of a eukaryotic host. The classic example of this is the origin of chloroplasts. There is evidence to suggest that horizontal gene transfer between bacteria and eukaryotes is more common than previously thought (Dunning Hotopp, 2011; Husnik *et al.*, 2013). If horizontal gene transfer occurred between spruce budworm and past microbial symbionts that would mean that spruce budworm could have acquired exogenous genes that aid in digestion or the detoxification of secondary compounds. This hypothesis cannot be tested until the spruce budworm genome is fully sequenced.

This study is the first to directly quantify the link between spruce budworm gut microbes and host growth and survival. The results of this work supports the hypothesis that lepidopteran larvae lack a resident microbiome. The current discussion regarding the lack of a resident microbiome in lepidopteran larvae has important implications into the control of spruce budworm populations, and other lepidopteran pests. My results suggest that because spruce budworm larvae are not nutritionally dependent on a resident gut microbiome, control options directly targeting spruce budworm-microbiome interactions will not be effective. My study also highlights several new questions which must be answered to fully support the hypothesis that lepidopteran larvae lack a resident microbiome. Additional studies, across broader spatial scales, using more replicates, better sampling techniques and sequencing methods will be required to confirm these results.

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