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The Role of Medicinal Food Plants Mediating the Gut Microbiome and Disease Resistance in Monarch Butterflies

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Abstract

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By Erica V. Harris

Animals live in close association with microbes that are largely impacted by host diet and can play a role in modulating host resistance to parasites. Growing evidence across the animal kingdom demonstrates three separate relationships: (i) host diet modulates resistance to parasites; (ii) host diet alters the gut microbiome; and (iii) the gut microbiome modulates resistance to parasites. However, evidence for an indirect link between diet and parasite resistance, through modulation of the microbiome, remains lacking. My dissertation explores the role of environmental factors in the ecological context of host-parasite interactions. I use monarch butterflies (*Danaus plexippus*), their larval food plants (*Asclepias* spp.), and a common natural protozoan parasite, *Ophryocystis elektroscirrha* as a model system to investigate these questions by performing fecal transplants based on alternative plant diets. Before experimentation, I outline the known links between each pairwise interaction. From these two-way interactions, I discuss the potential for the three-way interaction between the diet, microbiome and disease providing the framework for my dissertation. To determine the natural variation of monarch butterfly, I characterize gut microbial communities using 16S sequencing technology and quantify bacterial load using quantitative PCR across the monarch lifecycle when larvae are fed on one of two milkweed species. I find that monarch butterfly gut microbial community composition is relatively consistent throughout development, with the exception of adults. Milkweed diet influences the microbial diversity in early larval instars, but not in later larval instars. To determine if milkweed diet modulates the monarch gut microbiome to an anti-parasitic state, I use a set of fecal transplant experiments and measure disease outcome. I find that lower parasite loads were associated with high microbiome diversity and high microbial abundance. For microbial transplants, lower parasite loads were specifically associated with low relative abundances of *Pantoea* and unclassified Enterobacteriaceae and high relative abundances of *Chryseobacterium*, *Pedobacter* and unclassified *Rhizobiales*. My dissertation demonstrates that diet-mediated microbiome transplants can increase resistance to parasites and that Lepidopteran gut microbiomes can be a crucial functional driver of host fitness.

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My peer network at Emory has been crucial. Lab members that have made my time at Emory unforgettable include Tiff and Tarik, Wen-Hao, Amanda, Signe, Kayla, Sandra and Jacoby. To the postdocs, Leiling, Kandis, Anna, Aileen, Hassan, thank you for challenging and advising me while encouraging me at the same time. You all are going to change many lives through your science. I also want to thank the lab managers, Tarik and Joselyne, for teaching me new protocols and always setting an example of how to manage several projects at once. The undergrads and high school students, Mahal, Ava, Keisha, Arielle, Nancy, Matthew, Itai, in our labs were extremely bright and enthusiastic. I look at you all and see a better version of my

undergraduate self. There were days that I was overwhelmed with life and those worries would disappear when training and working with you all. Because of you all, I am a better scientist and mentor. The Population Biology, Ecology and Evolution Program (PBEE) has been instrumental in my professional development and peer-mentor network. Throughout my time at Emory, PBEE continues to grow larger and more inclusive. I have gained several friends, Carmen, Xorla, Jordan, and Connor, Kathryn, Signe, that continue to inspire me. There are also several faculty, Levi, Tim, Joanna, Berry, and Chris, that gave me written feedback, career advice, teaching opportunities and were collaborators on projects. The staff in the Biology Department, PBEE program and LGS administrators have made this journey possible. Tonya helped me prepare samples for overnight shipping. Malia helped me with room reservations and attend faculty job search and distinguished lecturer lunches. Jan and Scottye were always eager to help with maintenance issues and ideas to improve the lab's workflow. All of the PBEE Program Administrators, Janice, Angela, Roberta, and Chanell, that helped me push paperwork through to the division and graduate school. Emily Morran thank you for being positive, resourceful and an all-around champion with work-life balance. I am richer knowing all of you.

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Chapter 1: Introduction

The impact of diet on health and fitness has been considered in many contexts (Athanasiadou, Kyriazakis, Jackson, & Coop, 2000; Crozier, Jaganath, & Clifford, 2009; Grassi et al., 2005; Riccardi, Giacco, & Rivellese, 2004; Sacks et al., 2001). However, not until recent technological advances in high throughput sequencing, mass spectrometry, and metabolic network models, have we been able to identify and consider how individual dietary components modulate complex gut microbial communities (J. Wang et al., 2014; G. D. Wu et al., 2011). There is increasing evidence that diet modulates gut-associated communities across the animal kingdom (de Filippo et al., 2010; Z. Li et al., 2015; Miyake, Ngugi, & Stingl, 2014; Pinto-Tomás et al., 2011). There is also increasing evidence that the gut microbiome affects disease resistance (Dong, Manfredini, & Dimopoulos, 2009; Fukuda et al., 2011; Kriegel et al., 2011; Lawley et al., 2012). A major limitation of these studies, however, is that they generally examine diet effects on the gut microbiome and the gut microbiome's effects on disease as separate two-way interactions, sometimes inferring the therapeutic potential of treating disease by modifying the gut microbiota through diet without empirical evidence (Claesson et al., 2012; Costello, Stagaman, Dethlefsen, Bohannan, & Relman, 2012). The objective of this dissertation is to examine whether there is a definitive link between host diet, the gut microbiome, and infectious disease using a natural host-parasite-diet system. More specifically, I examine the effects of alternative diets on gut microbial community structure and explicitly test the impact of these gut communities on disease outcome, a question of key importance in many animal systems, including humans (Blaser, Bork, Fraser, Knight, & Wang, 2013; Ponton et al., 2013; Xia et al., 2013). Knowledge of such a three-way interaction could provide a more holistic view of how host and parasites function within a larger community of interacting species (Hall, Duffy, &

Cáceres, 2005; Lafferty, Dobson, & Kuris, 2006). I leverage the tractability of monarch butterflies, insects who as larvae feed exclusively on milkweed plants. In nature, monarchs are frequently infected with a protozoan parasite, *Ophryocystis elektroscirrha*. This natural animal-diet-disease system can be brought into the lab for experimentation. Here, I briefly discuss current knowledge of how diet and microbes mediate animal host defenses. I then discuss the utility of the monarch system in studying questions related to diet and disease. Finally, I provide a brief overview of each chapter of my thesis.

1.1 The Influence of Diet and Microbes on Host Defense

Since parasites are a major threat to host fitness, host have evolved a diverse set of defense mechanisms to prevent infection, or reduce parasite growth and disease symptoms (Parker, Barribeau, Laughton, de Roode, & Gerardo, 2011). These defenses can extend beyond intrinsic host immunity and be influenced by the environment in which the host and parasite interact (Chapuisat, Oppliger, Magliano, & Christe, 2007; Traniello, Rosengaus, & Savoie, 2002). Although some environmental factors can make hosts more vulnerable to disease, it is becoming clear that both abiotic factors and biotic associates can increase resistance to parasites (Jacobus C de Roode, Yates, & Altizer, 2008; Leif L Richardson et al., 2015).

One increasingly recognized environmental factor that influences disease is diet. For example, starved bumblebees have higher parasite-induced mortality compared to those that fight infection with dietary compensatory intake (Brown, Loosli, & Schmid-Hempel, 2000), Egyptian cotton moth larva increase dietary intake of protein when infected with a nucleopolyhedrovirus to increase survival (Lee, Cory, Wilson, Raubenheimer, & Simpson, 2006), and woolly bear caterpillars increase the intake of anti-parasitoid chemicals in their diet to increase survival when infected with tachinid flies (Singer, Mace, & Bernays, 2009). Diets may alter the ability of hosts

to mount an immune response (Moret & Schmid-Hempel, 2000), thus indirectly altering resistance, or they may directly interfere with parasite growth (Müller & Kappes, 2007).

In addition to influencing disease, diet is a strong selective force that is recognized as a major environmental factor in shaping gut microbial community composition in a wide range of hosts (Bäckhed, Ley, Sonnenburg, Peterson, & Gordon, 2005; de Filippo et al., 2010; Tang et al., 2012; G. D. Wu et al., 2011). Diets' effects on gut microbiota occur across the animal kingdom (de Filippo et al., 2010; Miyake et al., 2014; Pinto-Tomás et al., 2011; Turnbaugh et al., 2009a). In laboratory mice, individuals fed a high-fat diet have altered gut microbial communities and blood sugar levels compared to those fed a traditional diet, suggesting that the microbiota may play an energy-harvesting role in diet-induced obesity (Cani & Delzenne, 2009; Turnbaugh, Bäckhed, Fulton, & Gordon, 2008). In insects, the gut microbial community composition of crickets fed on alternative diets varying in protein and carbohydrate ratios differs in bacterial genera abundance (Santo Domingo et al., 1998).

Gut-associated microbes within hosts have been linked to the outcomes of both infectious and non-infectious diseases. While gut-associated microbes may have important consequences for many non-infectious disease outcomes (Mazmanian, Round, & Kasper, 2008; Sokol et al., 2008), their role in mitigating infectious diseases has only recently been considered. A prime example of how the gut microbiota can impact infectious disease outcome is demonstrated by the ability of the bacterium *Clostridium difficile* to cause disease after the long-term use of antibiotics. The clearance, and therefore disturbance, of the gut microbial community favors the growth of *C. difficile* at high densities and is a cause of colitis. Fecal transplants from healthy donors can be used to treat the disease by restoring a diverse community (Brandt, 2013; Lawley et al., 2012; Silverman, Davis, & Pillai, 2010). Hence, the impact of the gut microbiota on *C.*

difficile infection shows that both community composition and ecological dynamics are crucially important in affecting parasite invasion success.

1.2 Monarch Butterflies as a Study System

To broaden our understanding of how environmental factors and host microbiota drive disease resistance in nature, more studies that experimentally alter the gut microbiota and test its impact on disease resistance are needed. However, this requires several criteria to be met concerning diet and infection status manipulation. In terms of diet, i) dietary component(s) of interest should be clearly defined; ii) it should be easy to experimentally manipulate the diet; iii) the gut microbial community should be easily manipulated through the introduction or removal of specific community members so that relationships between host disease resistance and diet-associated microbial communities can be measured. In terms of parasitic resistance, a parasite is needed that: (iv) is easy to introduce and remove, and (v) can be precisely quantified. I am using monarchs and their natural protozoan parasite because together they form an ideal model system to study the links between diet, microbiota, and disease.

Monarch butterflies worldwide are commonly infected with a protozoan parasite, *Ophryocystis elektroscirrha* (Altizer, Oberhauser, & Brower, 2000; Leong, Yoshimura, & Kaya, 1997). This parasite exists as dormant spores on adult butterflies and is transmitted to offspring and surrounding milkweed food plants during oviposition. Infection occurs when hatching caterpillars ingest their spore-covered eggshell and spore-covered milkweed food plants (Fig. 1.1).

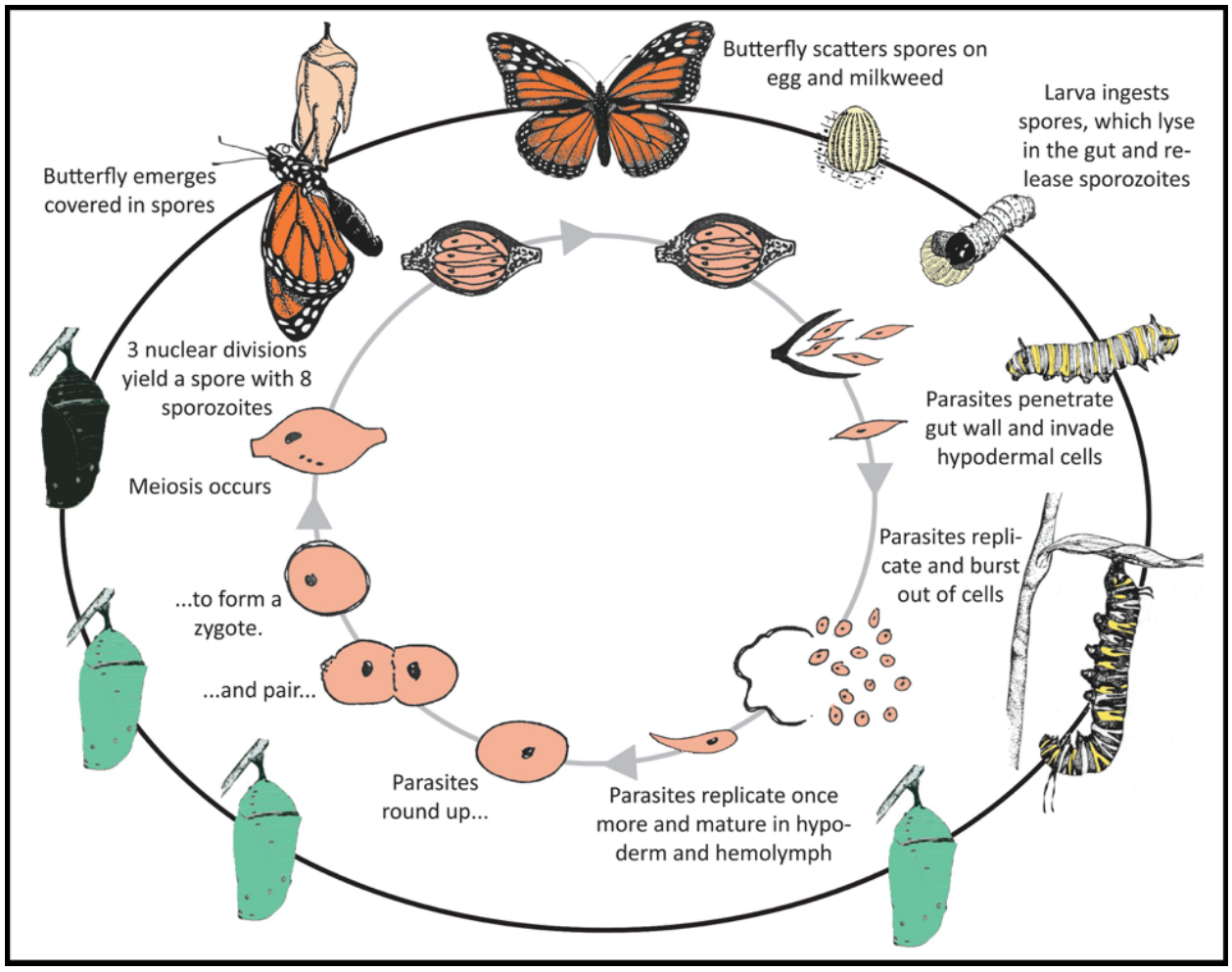

Figure 1.1. Life cycle of *Ophryocystis elektroscirrha* in the monarch butterfly. The monarch life cycle, from egg to adult is shown on the outer ring, and the life cycle of its protozoan parasite, *O. elektroscirrha*, is shown on the inner ring. Image credit: Jacobus C. de Roode

O. *elektroscirrha* spores then migrate to the midgut, release encapsulated parasites, and pass through the intestinal wall to the hypodermal tissues, where parasites heavily replicate asexually and then form sexual spores on the outside of the newly emerging butterfly (McLaughlin & Myers, 1970). Parasite infection only occurs at the larval stage; adults can no longer become infected. Parasites reproduce at high rates in caterpillars, with a single spore giving rise to

hundreds of thousands of parasite progeny on the adult butterfly (D. Roode, Gold, & Altizer, 2007). This high replication is detrimental to monarch butterfly fitness, with higher spore loads resulting in reduced larval survival, adult body mass, mating success, and longevity (Jacobus C de Roode et al., 2008; D. Roode et al., 2007). Lower infection rates and parasite spore loads indicate greater host resistance (Lefèvre, Williams, & de Roode, 2011).

Monarch butterflies are specialist herbivores on species of milkweed, mostly in the genus *Asclepias* (Ackery & Vane-Wright, 1984). Milkweeds produce secondary chemical compounds, named cardenolides. Monarchs have co-evolved with milkweeds and have the ability to sequester these cardenolides within their bodies (Reichstein, Euw, Parsons, & Rothschild, 1968). Previous research has shown that feeding on milkweeds with higher concentrations of cardenolides (medicinal milkweeds) reduces parasite infection and spore load, thus increasing host resistance and reducing parasite virulence (Sternberg, Lef, et al., 2012). Infected monarchs, therefore, benefit from feeding on high-cardenolide milkweeds. It is currently unknown whether this effect is a direct result of cardenolides or is due to some correlated feature of the milkweed diet. Therefore I will refer to anti-parasitic plants as medicinal, regardless of whether these effects are due to toxic chemicals, nutrition or some other factor (Raubenheimer & Simpson, 2009). Infected monarch caterpillars do not preferentially consume medicinal milkweed when given a choice (Lefèvre et al., 2012; Lefèvre, Oliver, Hunter, & de Roode, 2010), but infected female butterflies preferentially lay eggs on medicinal milkweed (Lefèvre et al., 2010), thereby reducing parasite infection and disease in their offspring. Medicinal milkweeds only confer protection to monarch caterpillars when consumed just before or during parasite inoculation, but not after infection has occurred. This suggests that consumption of medicinal milkweeds reduces the effective infectious dose - and consequently the resulting spore load on adult butterflies -

instead of parasite growth post-infection (J C de Roode, de Castillejo, Faits, & Alizon, 2011). It is unknown whether cardenolides are directly toxic to parasites or whether they are indirectly linked to parasite resistance, possibly through alteration of the host gut-microbiota to an anti-parasitic state. Importantly, because parasite spores release sporozoites into the gut lumen, these sporozoites will directly encounter gut microbes before traversing the mid gut wall.

1.3 Overview of Dissertation

The main goal of this dissertation is to explore how host medicinal diet shapes the gut microbiota and the role that the microbiota plays in disease resistance. In Chapter 2, a commentary previously published in *PLoS Pathogens*, I outline the known links between diet and infectious diet outcome, diet and the gut microbiome, and the gut microbiome and infectious disease. From these two-way interactions, I discuss the potential for the three-way interaction between the diet, microbiome and disease. I then outline experimental approaches needed to verify this link. In Chapters 3 and 4, I then focus on the monarch butterfly system. Because little is known about the composition of the monarch gut microbiome previously, in Chapter 3, I study how the monarch gut microbiome changes over development and is influenced by the milkweed species on which a larva is reared. Importantly, I do find that the milkweed species on which a monarch is reared does influence gut microbiome diversity, setting the stage for Chapter 4, in which I test whether altering the gut microbiome of monarchs can alter their resistance to infectious disease. Finally, in Chapter 5, I provide a brief conclusion and call for future work. Using a natural host-parasite-diet system, this work is an important step in advancing our understanding of how environmental factors and host microbiota drive disease resistance in nature.

Chapter 2: Diet-microbiome-disease: Investigating diet's influence on infectious disease resistance through alteration of the gut microbiome

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Abstract

Abiotic and biotic factors can affect host resistance to parasites. Host diet and host gut microbiomes are two increasingly recognized factors influencing disease resistance. In particular, recent studies demonstrate that: (1) particular diets can reduce parasitism; (2) diets can alter the gut microbiome; and (3) the gut microbiome can decrease parasitism. These three separate relationships suggest the existence of indirect links through which diets reduce parasitism through an alteration of the gut microbiome. However, such links are rarely considered and even more rarely experimentally validated. This is surprising because there is increasing discussion of the therapeutic potential of diets and gut microbiomes to control infectious disease. To elucidate these potential indirect links, we review and examine studies on a wide range of animal systems commonly used in diet, microbiome, and disease research. We also examine the relative benefits and disadvantages of particular systems for the study of these indirect links, and conclude that mice and insects are currently the best animal systems to test for the effect of diet-altered protective gut microbiomes on infectious disease. Focusing on these systems, we provide experimental guidelines and highlight challenges that must be overcome. Although previous studies have recommended these systems for microbiome research *per se*, here we specifically recommend these systems because of their proven relationships between diet and parasitism,

between diet and the microbiome and between the microbiome and parasite resistance. Thus, they provide a sound foundation to explore the three-way interaction between diet, the microbiome and infectious disease.

2.1 Introduction

Parasites can severely reduce host fitness, and host defenses against parasites are under strong selection. Hosts and parasites are often studied as pair-wise interactions (Lambrechts, Fellous, & Koella, 2006) without considering the environment in which they interact (Lafferty et al., 2006). This is problematic because biotic and abiotic factors can have strong effects on host resistance to parasitic infection (Lazzaro & Little, 2009; Wolinska & King, 2009). One increasingly recognized environmental factor that influences disease is host diet (Fig. 2.1). Host diet also importantly shapes the gut microbiome in a wide range of hosts (Fig. 2.2).

The gut microbiome, in turn, can be a crucial driver of infectious disease. The complex community of microorganisms inhabiting an animal's digestive tract constitutes the gut microbiota, and their collective genetic content constitutes the gut microbiome. Changes in gut-associated microbial community composition and diversity have been associated with *Clostridium difficile* infection in humans (van Nood et al., 2013) and malaria infection in mosquitoes (Dong et al., 2009).

Current understanding thus shows three important relationships: (1) diet can alter disease resistance; (2) diet can affect the gut microbiome; and (3) the gut microbiome can reduce or increase disease resistance. The potential link between these relationships remains understudied and poorly understood. Specifically, while these relationships suggest that diets could increase or reduce disease resistance by altering the host gut microbiome, there are no existing studies to

support this. Instead, most studies have independently investigated the relationships between diet and disease resistance, diet and the gut microbiome and the gut microbiome and disease resistance (Fig. 2.1). For example, studies have shown separately that diet affects the gut microbiome, and that the gut microbiome affects parasitic resistance in both mice and mosquitoes infected with *Plasmodium* spp. (Linenberg, Christophides, & Gendrin, 2016; Villarino et al., 2016a). Whether this increased resistance is a result of the diet-altered microbiome is unknown. Similarly, honeybees fed aged mixed pollen diets have an increased relative abundance of *Frischella perrara*, and these diets also increase resistance to bacterial and microsporidian parasites; whether this increased resistance is the result of a diet-altered microbiome is also unknown (Maes, Rodrigues, Oliver, Mott, & Anderson, 2016). It is also important to note that host immunity could play a key role in directly or indirectly modulating interactions between diet-microbiome-disease (Sansone et al., 2015). For example, *F. perrara*, the same gut microbe that is correlated with aged mixed pollen diets, also activates the honeybee immune system (Emery, Schmidt, & Engel, 2017) making it difficult to determine the sequence of events between host diet metabolism, host immunity activation, and parasitic infection inhibition. In this review, we focus on the interaction between host diet, the gut microbiome, and parasites without specific consideration of the role of host immunity in most cases, but ultimately how immunity changes with changes in diet and changes in the microbiome should be investigated where feasible.

The potential for diet to alter infectious disease resistance by altering the gut microbiome is relevant to a wide variety of animal systems, including humans. In particular, given increasing calls to create personalized diets to augment human gut microbiomes (Derrien & Veiga, 2017), it is crucial to determine how such changes in diet will make hosts more or less susceptible to

infectious disease agents. Since our focus is on infectious diseases, we define parasites as microorganisms that can cause infectious disease (bacteria, fungi, protozoa, and viruses). The goal of this review is to provide guidelines to study how diets indirectly change infectious disease resistance by altering the gut microbiome, and to suggest suitable model systems to address this question. Using key references that are essential across taxa, we begin by reviewing the above-mentioned, two-way relationships. We then discuss the challenges that need to be overcome to specifically integrate these separate relationships into a cohesive framework. Finally, we synthesize methods by which we can empirically test this potential three-way interaction. Our review and recommendations are not meant to be exhaustive but rather to provide a step towards advancing our understanding of how a host's diet and gut microbiome interact to drive infectious disease resistance.

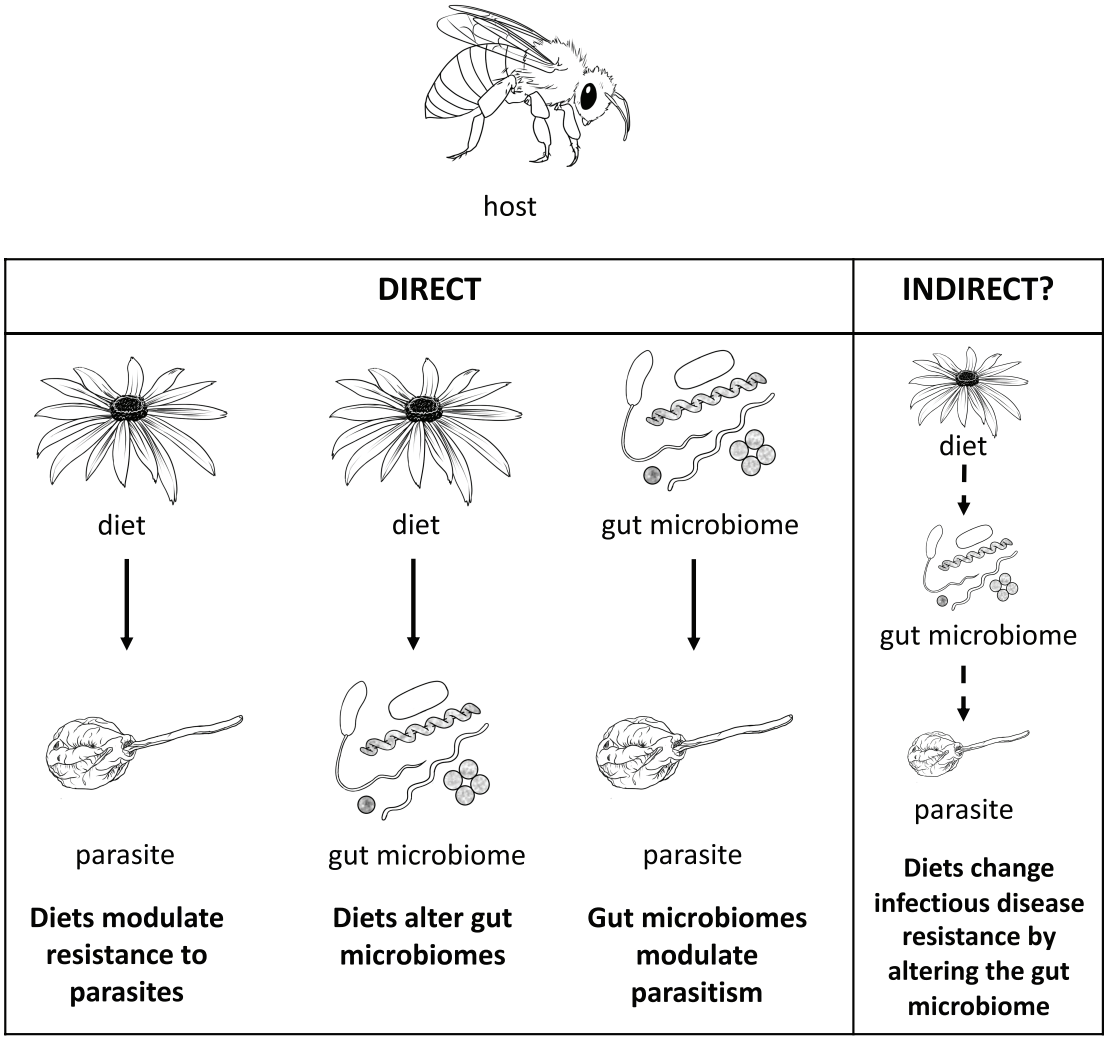


Figure 2.1. Direct and indirect relationships between host diet, host gut microbiome, and parasites. In bees**,** studies have independently shown that diets increase resistance to parasites [9,21], diets alter the gut microbiome [75], and gut microbiomes reduce parasitism [90,107]. However, it is not known whether there is an indirect link between the three based on these direct relationships Alternatively, the host immune system can indirectly alter this potential three-way interaction by modulating antimicrobial peptides or pattern recognition receptors via diet or the gut microbiome to fight parasites [11,110].

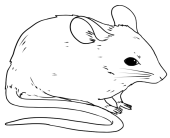
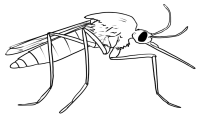
Study system	Diets modulate resistance to parasites	Diets alter gut microbiomes	Gut microbiomes modulate parasitism	Advantages	Disadvantages
 Humans	Shankar *et al.* (1999)	De Filippo *et al.* (2010) Wu *et al.* (2011) Claesson *et al.* (2011)	Silverman *et al.* (2010) Yooseph *et al.* (2011) Morton *et. al.* (2015)	• Results directly applicable to human health • Characterized immune system	• Sub-optimal system with confounding factors • Difficult to experimentally control diet for extended time
 Non-human primates	Huffman *et al.* (1997)	Kisidayová *et al.* (2009)	Hensley-McBain *et al.* (2016)	• Close phylogenetic relationship to humans	• Many endangered species • Time intensive sample collection • Small samples sizes
 Mice	Nagajyothi *et al.* (2014) Meadows *et al.* (2015) Villarino *et al.* (2016)	Turnbaugh *et al.* (2009) Hildebrandt *et al.* (2009) Villarino *et al.* (2016)	Yilmaz *et al.* (2014) Villarino *et al.* (2016)	• Controlled host diets • Tractable parasites and microbiome • Easy to genetically manipulate • Characterized immune system • Human microbiome colonization	• Small sample size • Most populations well-adapted to lab environment
 Bees	Alaux *et al.* (2010) Foley *et al.* (2012) Anthony *et al.* (2015) Richardson *et al.* (2015) Maes *et al.* (2016)	Martinson *et al.* (2011) Billiet *et al.* (2016) Maes *et al.* (2016)	Evans and Armstrong (2005) Forsgen *et al.* (2009) Yoshiyama and Kimura (2009) Koch and Schmid-Hempel (2011) Mockler *et al.* (2018)	• Controlled host diets • Tractable parasites and microbiome • Relatively simple microbiome • Agricultural applications	• Not directly related to human health • Holometabolous insect that undergoes metamorphosis
 Mosquitoes	Linenberg *et al.* (2016)	Wang *et al.* (2011) Linenberg *et al.* (2016)	Dong *et al.* (2009) Boissiére *et al.* (2012)	• Controlled host diets • Tractable parasites and microbiome • Relatively simple microbiota • Characterized immune system • Epidemiological applications	• Not directly related to human health • Holometabolous insect that undergoes metamorphosis
 Fruit flies	Howick and Lazzaro (2014)	Sharon *et al.* (2010) Chandler *et al.* (2011) Wong *et al.* (2011) Broderick and Lemaitre (2012) Fink *et al.* 2013 Vacchini *et al.* (2017)	Sansone *et al.* (2015)	• Controlled host diets • Tractable parasites and microbiome • Relatively simple microbiota • Easy to genetically manipulate • Characterized immune system	• Not directly related to human health • Most populations well-adapted to lab environment
 Lepidoptera	de Roode *et al.* (2008) Singer *et al.* (2009) Kangassalo *et al.* (2015) Kelly and Bowers (2018)	Broderick *et al.* (2004) Pinto-Tomás *et al.* (2011) Belda *et al.* (2011) Gayatri *et al.* (2012) Staudacher *et al.* (2016)	Johnston and Rolff (2015) Caccia *et al.* (2016)	• Controlled host diets • Tractable parasites and microbiome • Relatively simple microbiota • Agricultural applications	• Not directly related to human health • Holometabolous insect that undergoes metamorphosis

Figure 2.2. Animal systems showing three separate relationships between diet, parasites, and the gut microbiome. Mice and insects are ideal systems to study the potential indirect, three-way link due to the systems' controlled host diets, tractable and relatively simple microbiota, and tractability of parasites.

2.2 Diets modulate resistance to parasites

Certain diets have been shown to confer protection against infectious diseases in multiple animal systems. Specifically, many animals can obtain anti-parasitic diets by eating plants with toxic defensive chemicals. Nematode-infected chimpanzees, for example, eat bitter plants with nematocidal compounds (Huffman, Gotoh, Turner, Hamai, & Yoshida, 1997; Page, Huffman, Smith, & Towers, 1997), and woolly bear caterpillars infected with parasitoid flies increase their consumption of diet alkaloids reducing infection (Singer et al., 2009). Similarly, monarch butterfly larvae suffer less protozoan infection when feeding on milkweed plants with high concentrations of cardiac glycosides (de Roode, Pedersen, Hunter, & Altizer, 2008; Gowler, Leon, Hunter, & de Roode, 2015a; Sternberg, Lefèvre, et al., 2012a; Tao, Hoang, Hunter, & de Roode, 2016), Anicia checkerspot butterflies are more immunocompetent when fed plants with higher concentrations of iridoid glycosides (Kelly & Bowers, 2018), and bumblebees that consume alkaloid-rich nectar experience reduced infection with trypanosome gut parasites (Anthony, Palmer-Young, Leonard, Irwin, & Adler, 2015; Leif L Richardson et al., 2015). Thus, many herbivores exploit plant defensive chemistry to reduce parasite infection and growth.

Animals can also increase parasitic resistance by increasing the quality and types of foods that they eat. For example, honey bees with a diverse pollen diet are more immunocompetent than individuals fed on a monofloral diet (Alaux, Ducloz, Crauser, & Le Conte, 2010). Similarly, lab-reared honey bee larvae gain resistance to fungal pathogens when nutrient-poor diets are supplemented with polyfloral pollens (Foley, Fazio, Jensen, & Hughes, 2012). Fruit flies fed low-sugar diets have lower bacterial pathogen load and reduced mortality than when fed on high-sugar diets (Howick & Lazzaro, 2014). Mice infected with protozoan parasites that cause Chagas disease have reduced parasitemia numbers when fed high-fat diets (Nagajyothi et al., 2014a). As

with other animals, the diet of humans is a strong driver of parasite infection. Human malnutrition is a global concern that is associated with micronutrient deficiencies and is linked to immunodeficiency. For example, malnourished children in Papua New Guinea are at higher risk of malaria infection. Supplementing their diets with Vitamin A reduces both *Plasmodium falciparum* density and disease symptoms, including fever (Shankar et al., 1999).

Diets can have a complex effect on a host's ability to fight infection. The addition of a dietary component may not always positively correlate with parasitic resistance; the effect of diet on parasites can be negatively correlated with an increase in dietary components being correlated with a decrease in parasitic resistance. For example, mice infected with protozoan parasites, that cause murine malaria, and fed folate-supplemented diets have decreased survival and decreased resistance to parasitemia compared to mice fed the standard dose of recommended folate (Meadows, Bahous, Best, & Rozen, 2015). Similarly, greater wax moths infected with a fungal parasite and fed high nutrition diets were more susceptible and experienced a higher mortality rate than infected individuals raised on low-nutrition diet or uninfected hosts (Kangassalo et al., 2015).

Thus, diets can confer protection against infectious diseases by direct interference through chemical inhibition of parasites or modulation of available resources to fight pathogens. Alternatively, diets may confer protection through alteration of microbial competition, which until recently has been largely overlooked, and which we will address below.

2.3 Diets alter gut microbiomes

As with other ecological communities, gut microbial communities are groups of interacting species that occur together at the same time in a defined place. Recent technological advances have increased the feasibility of studying gut community composition and function (Cho & Blaser, 2012; Waldor et al., 2015). Gut microbial communities have a structure that is characterized by species richness (the number of species), species evenness (the relative abundance of each species), and species diversity (a metric accounting for both species richness and evenness). Because different microbial species can have diverse roles, the overall function of these communities is typically characterized by assaying total genetic content (metagenomics) and gene expression (transcriptomics).

Different host species have different microbiomes, driven by host genetics, evolutionary history and evolved dietary specialization (Colman, Toolson, & Takacs-Vesbach, 2012; Martinson et al., 2011; Moeller et al., 2014). Termites, for example, are consumers of cellulose-based plant materials but cannot directly break down cellulose; instead they harbor vertically transmitted microbial gut symbionts – bacteria, protists, and archaea - that contain cellulose-digesting genes (Warnecke et al., 2007). Termites that specialize in different feeding groups (*e.g.,* wood, grass, humus, soil, and fungus) harbor significantly different assemblages of gut microbes (Mikaelyan, Dietrich, et al., 2015a), a signature of evolved microbiome specialization.

The microbiome, however, is also plastic, and changes in diet can alter gut microbial community composition (David, Maurice, et al., 2014; Gary D Wu et al., 2011), and thus have the potential to importantly shape community function. For example, in wood-feeding termites of the same feeding group, changes in diet are accompanied by shifts in the dominance of protist species (Tarayre et al., 2015a). In humans, major shifts in diet (*i.e.,* shift from high fat/low fiber

to low fat/high fiber diet) also significantly influence the gut community composition over short time periods (David, Materna, et al., 2014; David, Maurice, et al., 2014). However the human gut microbiome is relatively stable over time (David, Materna, et al., 2014; Faith et al., 2013), with long-term diet strongly correlating with bacterial enterotype, the classification of microbiome samples based on clustering in ordination analyses (Arumugam et al., 2011; Claesson et al., 2012; Gary D Wu et al., 2011). After a dietary perturbation, communities tend to shift back towards their original community composition and stabilize. While such plastic changes of the gut microbiome in response to dietary shifts have been observed across the animal kingdom (de Filippo et al., 2010; Kišidayová et al., 2009; Miyake et al., 2014; Pinto-Tomás et al., 2011; Turnbaugh et al., 2009a; Y. Wang, Gilbreath, Kukutla, Yan, & Xu, 2011a), it is not clear whether diets change the microbiome through similar mechanisms across systems, and whether these changes are generally stable or transient.

Supplements added to the diet can also modulate the gut microbiota. Prebiotics are dietary supplements that once consumed by the host act as food or substrates for the host microbiota. More specifically, the "prebiotic effect" is the selective stimulation of growth and metabolic activity of a single or limited number of taxa in the gut microbiome that confers health benefits to the host (G R Gibson & Roberfroid, 1995; Roberfroid et al., 2010). A common prebiotic for humans is inulin and its chemical derivatives (Glenn R. Gibson, 1999). Inulin is a soluble fiber found in many plants naturally occurring in foods such as chickory root, garlic, and onions (Jovanovic-Malinovska, Kuzmanova, & Winkelhausen, 2014) and is also commercially produced. In clinical studies, healthy humans administered inulin-containing foods over the span of weeks show a change in microbial community composition, with significant increase in *Bifidobacteria* (Kleessen et al., 2007; Tuohy, Kolida, Lustenberger, & Gibson, 2001). In turn,

Bifidobacteria and *Lactobacilli* are common genera used as probiotics in several hosts (Aly, Abdel-Galil Ahmed, Abdel-Aziz Ghareeb, & Mohamed, 2008; Villarino et al., 2016a). Probiotics are non-native live microorganisms orally consumed by hosts and beneficial to host health. Probiotics naturally occur in fermented foods such as yogurt. The combined synergistic effect of prebiotics and probiotics is synbiotics (Boger, Lammerts van Bueren, & Dijkhuizen, 2018). *Bifidobacteria* and *Lactobacilli* bacteria may play a role in the treatment or prevention of several human infections, including the infection of the human digestive tract caused by *Clostridium difficile* and human vaginal bacterial infections (Falagas, Betsi, & Athanasiou, 2007; Sazawal et al., 2006). However, it can difficult to elucidate the efficacy and mechanisms of prebiotics and probiotics in humans. Interestingly, use of prebiotic and probiotic supplements in more tractable model systems, such as bees, shrimp, and fish, suggests that such supplements can confer antimicrobial activity, increase immune gene expression, and decrease load of bacterial pathogens and intestinal parasites in these systems (El Khoury et al., 2018; Y. Li, Liu, Dai, Li, & Ding, 2018; Piazzon et al., 2017).

A major issue with elucidating the effects of diet on the human gut microbiome is the occurrence of confounding factors. For example, human children from rural Africa and modern western Europe fed on plant- and animal-based diets respectively exhibit significant differences in bacterial communities: *Prevotella*, *Xylanibacter, and Treponema* genera are abundant in rural Africans but absent in western Europeans (de Filippo et al., 2010). The bacteria in these genera contain genes involved in cellulose hydrolysis and are associated with the capacity to metabolize indigestible polysaccharides commonly found in plants. Despite the apparent link between diet and microbiota composition, factors other than diet, such as host genetics, race, ethnicity, variation in antibiotic use and geographically varying environmental factors, could also play a

role. Human microbiome research is also hampered by logistical constraints, such as inconsistent self-assessments on dietary questionnaires and budget limitations that prevent supplying large cohorts with controlled diets for an extended period of time (Sonnenburg & Bäckhed, 2016; Turnbaugh et al., 2009a). Ironically, what this means is that despite the fact that human health is the primary focus of diet-gut microbiome research, humans are a sub-optimal system to understand how diet shapes microbial community dynamics. Therefore, to better understand the mechanistic links between diet and the gut microbiome, it is beneficial to study systems in which confounding factors can be more easily controlled (Dillon & Dillon, 2004; Engel & Moran, 2013; Ley et al., 2005; Pernice, Simpson, & Ponton, 2014).

Mice are the most common animal model used to translate gut microbiome research to human health, in part because human fecal microbial communities can successfully colonize germ-free, inbred mouse strains (Turnbaugh et al., 2009a). Major dietary shifts from low fat/high fiber to high fat/high sugar diets in such mice cause rapid changes in microbial community structure and function (Hildebrandt et al., 2009; Turnbaugh et al., 2009a). Thus, as with humans, diet is a major driver of microbiome composition in mice.

Insects also provide excellent systems to study the effects of diet on the gut microbiome (Pernice et al., 2014). Similar to termites, mentioned above, microbial communities of fruit fly species vary with the different fruits and flowers on which these species are specialized to feed. Fly microbial communities are also plastic, changing with dietary shifts (Chandler, Lang, Bhatnagar, Eisen, & Kopp, 2011). For example, within a single population of the fly *Drosophila elegans,* feeding on two different flowering plant genera (*Alpinia* and *Brugmansia*) results in different abundances of the dominant bacterial families. Similarly, feeding *Drosophila suzukii* fruit-based natural and non-fruit artificial diets results in altered communities (Vacchini et al.,

2017). Diet also influences *Drosophila melanogaster* gut microbial community composition (Broderick & Lemaitre, 2012; Sharon et al., 2010; C. N. A. Wong, Ng, & Douglas, 2011). For example, altering the fat content, particularly from high-fat to no fat (*i.e.,* starvation), of *D. melanogaster* diet results in changes in the abundance of some bacteria as well as changes in the overall number of microbes in the community (Fink, Staubach, Kuenzel, Baines, & Roeder, 2013).

Diet also strongly influences microbial communities of bees, butterflies and moths. Bee gut microbial communities are dominated by eight dominant bacterial phylotypes, bacterial clusters based on sequence similarity, that can be modified with alternative syrup and pollen diets (Billiet et al., 2016a; Martinson et al., 2011). Similarly, the dependence of gut microbial community composition on alternative larval host plants is widespread in lepidopteran species (Belda et al., 2011; Priya, Ojha, Kajla, Raj, & Rajagopal, 2012a; Robinson, Schloss, Ramos, Raffa, & Handelsman, 2010; Staudacher et al., 2016). For example, tobacco budworm larvae fed three alternative host plants have significantly different bacterial families (Staudacher et al., 2016), and there is variation in bacterial phylotypes in the gypsy moth microbiome based on alternative plant diets (Broderick, Raffa, Goodman, & Handelsman, 2004). While these examples demonstrate that diet affects the gut microbiome in many animal systems, the mechanisms by which this occurs are largely unknown (Box 2.1).

Box 2.1. Crucial Considerations in the Study of Diet-Microbiome-Disease Interactions

1. ***Comparing microbial communities***. A major challenge plaguing the field of microbiome research is defining what variation to quantify and what variation matters [30,111,112]. While it is becoming relatively simple to characterize a gut microbial community, it is more difficult to conclude what variation between experimental groups is biologically significant. Differences that may impact host phenotypes may lie in the presence and diversity of the microbial community, the presence of particular taxa, the abundance of particular taxa, or microbial gene expression, regardless of the genome of origin. Technological approaches vary in the degree to which they can characterize these differences. Furthermore, in the case of differences at the taxonomic level, studies define community composition differently at the phylum (Wu et al., 2011), genus [45], species [113], and strain [114,115] levels. This inconsistency demonstrates that there is no workbook for which of these to quantify, requiring a thorough investigation of each system studied.
2. ***Accounting for individual microbiome variation***. There is substantial individual variation in gut microbiome composition, which may be due to genetics, abiotic or biotic factors, or stochasticity. Furthermore, gut microbial communities change over development, with sometimes high species turnover, adding more variation to an animal system [116,117]. Because of the many sources of microbiome variation, studying the link between diet, the microbiome and disease can be difficult, as the microbiome may vary for reasons other than diet. Thus, the key is to determine the relevant variation due to changes in diet, and to determine how those particular changes correlate with disease resistance.
3. ***Defining which dietary components influence the microbiome and disease susceptibility***. Diets have many components. Therefore, it is imperative that studies first clearly define which dietary component(s) or dietary supplement(s) are considered when assessing the influece of diet on diease resistance or on the microbiome. To date, several different dietary components have been implicated in influencing the gut microbiome in animals, including fiber, protein, plant secondary metabolites, types of fat, foodborne bacteria, and prebiotics [12,45,118]. The dietary component(s) of interest may be nutritious or toxic depending on the study [119]. If a dietary shift is observed to modulate the gut microbiome or disease resistance, then the exact nature of what components of that diet are shifting should be characterized. Systems in which diet can be experimentally manipulated are ideal, as controlled diets eliminate confounding dietary variables, making it possible to observe the direct effect of a single dietary component on gut microbiota composition and on disease susceptibility. Furthermore, such diets can be standardized, providing the opportunity for comparisons across studies. However, one drawback of such controlled diets is that they are not generalizable to natural diets [111]. Coupling a chemically well-defined diet with a natural diet in animal systems should provide novel insights as to diet's role in altering the microbiome and disease [47].

2.4 Gut microbiomes modulate parasitism

Microbial symbionts, microbes that form a long-term association with hosts, can play important roles in animal health, particularly in mitigating infectious diseases. For example, aphids harbor non-gut associated bacterial symbionts that protect them against fungal pathogens and parasitoid wasps (Oliver, Russell, Moran, & Hunter, 2003; Scarborough, Ferrari, & Godfray, 2005). Similarly, beewolf wasps incorporate symbiotic bacteria into their larval cocoons for protection against pathogenic fungi (Kaltenpoth, Göttler, Herzner, & Strohm, 2005; Kroiss et al., 2010); and salamanders have skin bacterial symbionts that produce antifungal metabolites against chytrid fungus (Brucker et al., 2008). It is now clear that gut-associated microbial symbionts can also play major roles in infectious disease dynamics, with changes in microbial community structure and function being correlated with parasite infection in several systems. These community structure changes can be caused by dysbiosis, or disruption of the "healthy" microbiome, or parasite infection. While both states have the potential to shift parasite resistance, their mechanisms can be different. In the case of dysbiosis, gut pathogens may exploit an empty niche or host physiological stress to successfully colonize the gut. Systemic parasites may exploit organism stress to disseminate and replicate throughout the body. A well-known example is microbial-conferred protection against the bacterium *Clostridium difficile*, which is a leading cause of chronic diarrhea following the long-term use of antibiotics in humans. Antibiotic-induced disturbance of the gut microbial community favors the increased growth of *C. difficile* and recurrent infection. Clinical microbiome transplants via feces (*i.e.,* fecal transplants) from healthy donors can be used to treat the disease in infected recipients by restoring the gut community (Brandt et al., 2012; Hensley-McBain et al., 2016; Silverman et al., 2010; Youngster et al., 2014). Hence, *C. difficile* infection exploits dysbiosis by proliferating in the gut bacterial

community and shows that community composition and potentially the number of bacteria is crucially important in affecting parasite invasion success. Similarly, sterile sugar-fed and antibiotic-treated bees suffer increased trypanosome infection relative to bees with a complete gut microbiome, and fecal transplants restore the bees' gut microbiota and increase resistance (Koch & Schmid-Hempel, 2011a). Although the protective effect of the gut microbiome against parasites is evident in these and other systems, the properties of the microbiome that reduce parasitism are rarely known.

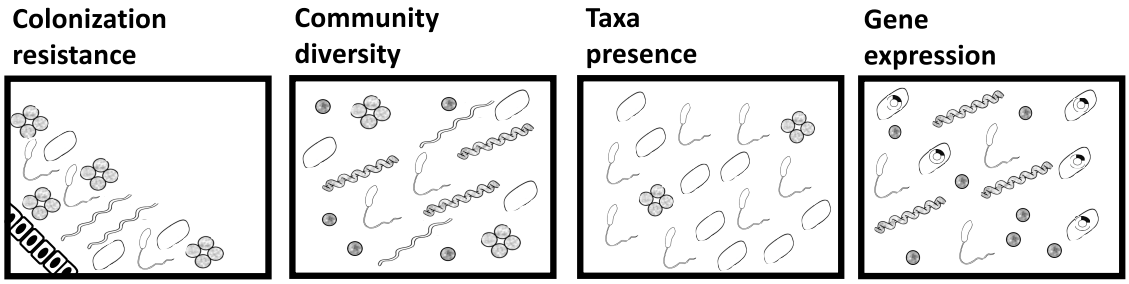
The protective effects of the gut microbiome may result from the presence and diversity of the microbial community, the presence of particular taxa, or the presence of particular genes within the microbial community (Fig 2.3). Several examples illustrate the importance of the community. As mentioned above, the gut microbiome of bees provides protection against trypanosome infection (Koch & Schmid-Hempel, 2011a; Palmer-Young, Raffel, & McFrederick, 2019); however, consumption of a single bacterial class does not reduce trypanosome burdens. Similarly, a diverse bee gut community is also protective against a bacterial pathogen *Paenibacillius larvae*, the causative agent of American foulbrood (Evans & Armstrong, 2005; Forsgren, Olofsson, Vásquez, & Fries, 2009; Yoshiyama & Kimura, 2009); while eleven isolated, cultured bacterial phylotypes differentially inhibit the growth of parasite strains *in vitro*, only the microbial cocktail of all eleven bacterial phylotypes completely inhibits the growth of *P. larvae in vitro* and *in vivo.* Desert locusts also have decreased pathogen colonization with increased numbers of gut bacterial species (Dillon, Vennard, Buckling, & Charnley, 2005): specifically, the presence of two and three bacterial species provides more protection against *Serratia marcescens* than the presence of only one species. The importance of the microbial community may result from the complementary and synergistic anti-parasitic effects of different

microbes. While the benefits of a diverse microbial community are widely accepted, the mechanism of protections are poorly understood in animal models (Heintz-Buschart & Wilmes, 2018; Kešnerová et al., 2017). Potential mechanisms include high functional diversity (Carrara, Giometto, Seymour, Rinaldo, & Altermatt, 2015), increased functional redundancies (Moya & Ferrer, 2016), and metabolic cross-feeding (Hoek & Merks, 2017; Kešnerová et al., 2017).

The presence of particular taxa can also be a protective property of the gut microbiome. Several malaria-associated studies across animal systems find a correlation between particular bacterial taxa and *Plasmodium* infection. Malian children with a lower risk of infection by the malaria parasite *Plasmodium falciparum* have a higher proportion of *Bifidobacterium* and *Streptococcus* genera compared to higher risk individuals (Yooseph et al., 2011). This example like others merely presents a correlation between the presence and absence of particular gut bacteria taxa and parasites (Boissière et al., 2012; Morton et al., 2015). Demonstrating a causative link, in *Anopheles* mosquitoes, the ingestion and colonization of *Chromobacterium* results in induction of immune genes and decreased susceptibility to *P. falciparum* infection and dengue virus (Ramirez et al., 2014). Similarly, antibiotic-treated mice inoculated with a cultured microbial cocktail containing *Bifidobacterium* and *Lactobacillus* display a decreased malaria parasite burden compared to control mice, suggesting that these taxa have a modulatory effect on parasitism (Villarino et al., 2016a).

Sometimes it is not the presence of the gut microbial community or presence of particular taxa in that community, but rather the expressed genes of the community that have a modulatory effect on protection. For example, laboratory mice can harbor gut bacteria that express glycan surface proteins. These glycan surface proteins elicit glycan-specific antibodies that attack *Plasmodium* spp. during transmission from *Anopheles* mosquitoes to mice lacking the glycan-

surface protein gene (Yilmaz et al., 2014). Similarly, mice colonized with *Bifidobacterium breve* bacteria expressing exopolysaccharides have significantly less colonization and persistence of a murine bacterial pathogen compared to mice without bacteria expressing an exopolysaccharide gene (Fanning et al., 2012). The protective effect of *B. breve* is linked to a gene cluster responsible for the expression of exopolysaccharides. These two studies demonstrate that protection can be induced, or pathogenesis inhibited, by manipulating the gene expression of gut microbes. Importantly, given that bacteria can horizontally transfer genes, protection against parasitic infection conferred by expressed genes has the potential to persist in a microbial community independent of the presence of particular taxa. However, these scenarios are not exhaustive nor mutually exclusive. For example, in bees, high community diversity, high bacterial abundance, and taxa presence all contribute to protection against a trypanosome parasite (Mockler, Kwong, Moran, & Koch, 2018).



Community A. The community is generally protective.
Endogenous gut microbiota at the epithelium interface provide physical niche protection against invading pathogens, regardless of membership.

Community B. Taxa diversity is protective.
Higher species richness, and maybe evenness, confers protection, possibly because of synergistic effects of multiple species.

Community C. Taxa presence is protective.
The presence of certain taxa is important for disease resistance.

Community D. Gene expression is protective.
Microbiota contain genes that facilitate or inhibit parasite infection directly through metabolite production or indirectly through immune modulation.

Figure 2.3. Properties of the gut microbiome that could reduce parasitism. The protective effects of the gut microbiome may derive from colonization resistance, the abundance and evenness of one or more species at various taxonomic levels, the presence or absence of particular species, or the presence or abundance of certain genes. These scenarios are not exhaustive nor mutually exclusive [107].

2.5 Experimental Approaches to Study Diet-Microbiome-Disease Interactions

As is clear from the examples above, diets alter both parasite resistance and gut microbiomes in a range of animals. Because the microbiome is an important driver of parasite resistance, these relationships suggest that diets may change parasite resistance through their effects on the gut microbiome. However, to our knowledge, the effect of the diet on infectious disease susceptibility through their impact on the microbiome has not been unequivocally demonstrated in any system. Non-human animal systems that have separately demonstrated that diet alters resistance to parasites, diet alters the gut microbiome, and the gut microbiome alters parasitism, are ideal systems to test explicitly for the potential of diet altering disease resistance by modulating the gut microbiome. To fully explore this link, researchers must study diet, the microbiome and disease in tandem in a controlled, experimental setting. The best case studies, based on current literature, appear to be experimentally tractable insect and mouse systems (Fig 2.2, Box. 2.2).

The need to study diet, the microbiome and disease together is clear when one tries to connect the three across separate studies. For example, bee studies have shown that diets rich in alkaloids increase resistance to a variety of parasites, including trypanosomes, fungi, and microsporidia (Foley et al., 2012; Maes et al., 2016; Leif L Richardson et al., 2015). Separate studies have shown that pollen supplemented with nectar diet alters the gut microbial community composition of bee larvae (Billiet et al., 2016a), and, as highlighted above, other studies have shown that bee gut microbes can increase resistance to pathogens and parasites (Forsgren et al., 2009; Koch & Schmid-Hempel, 2011a). Complications arise when trying to link these studies. First, the dietary components considered were different across studies (Box. 2.1). Second, the two-way relationships were studied in different life stages: while the effect of alkaloid diets on

the trypanosome *Crithidia bombi* was investigated in bee adults (Leif L Richardson et al., 2015), and the effect of gut microbes on *C. bombi* was also studied in adults (Koch & Schmid-Hempel, 2011a), the effect of protein and sugar-rich diets on bee microbial communities was investigated in bee larvae (Billiet et al., 2016a). Similarly, in mosquitoes, a particular larval aquatic diet increases resistance to *Plasmodium* spp. and also increases the relative abundance of two bacterial families (Linenberg et al., 2016). Separate studies have demonstrated that mosquito gut microbes reduce parasitism with *Plasmodium* (Dong et al., 2009; Ramirez et al., 2014). Ideally, researchers would study all three interactions across life stages since diet differences across immature stages could have lifelong effects on adult individuals. Similar to bees, the dietary components and life stages between these mosquito studies were different –fish flakes for aquatic larvae, versus sugars and blood in adults. We are aware of only a single study using mice in which all three separate components were considered (Villarino et al., 2016a). However, even within this study it is not clear that diet mediated its anti-parasitic effects by modulating the gut microbiome (Box. 2.2).

In order to study the potential effect of diet-altered protective gut microbiomes on infectious disease, we propose several recommendations. First, studies should only use animal systems in which host diet increases resistance to tractable parasites. Second, host genetics should be carefully controlled. Ideally, host genetics can be controlled by testing individuals with identical or similar genetic backgrounds, such as monozygotic twins or full siblings. Alternatively, unrelated individuals can be partitioned across treatments to reduce confounding factors. Third, it is important to determine whether alternative diets are associated with alterations of the microbial community, and if so to try and elucidate if the same dietary components are responsible for altering parasite resistance. Fourth, it should be demonstrated

directly, through a unified study, that gut microbial community variation caused by alternative diets correlates with disease susceptibility. A particularly powerful approach for this test is to use fecal transplants (Koch & Schmid-Hempel, 2011a; Turnbaugh et al., 2009a; Villarino et al., 2016a). Beyond demonstrating that the actual altered microbiome provides resistance to parasites, carefully manipulated fecal transplants can also be used to elucidate whether the presence of the entire gut community is needed for protection, or whether the presence and abundance of particular taxa are more important. To tease apart the protective mechanism of species presence and interactions, cultivated microbial transplants of specific community members have been effective in bees, mosquitoes, and mice (Forsgren et al., 2009; Ramirez et al., 2014; Villarino et al., 2016a). Silencing microbial community members' genes is also an effective way to resolve whether the presence and expression of certain genes are responsible for the protective mechanism of the gut microbiome, as shown in mice (Fanning et al., 2012; Yilmaz et al., 2014). Finally, for animal systems with robust genetic tools, including mosquitoes and moths, both host immune genes and microbiome toxin genes can be silenced to determine their interplay (Caccia et al., 2016; Dong et al., 2009; Johnston & Rolff, 2015).

2.6 Conclusion

Existing studies suggest that diets can alter host resistance to parasites by modulating the gut microbiome, but conclusive studies remain lacking. While an understanding of diet-microbiome-disease interactions is critical for humans, we propose alternative animal model systems to test fundamental properties of this potential interaction. These animals are relevant to agriculture and epidemiology, and they allow for carefully controlled experiments with few constraints on sample size. Most importantly, they are tractable systems that have strong

evidence of each separate interaction: diet increases resistance to parasites, diet affects the gut microbiome, and the gut microbiome reduces parasitism (Fig. 2). Existing experimental tools now allow researchers to build on the separate, direct relationships to determine if there is an indirect link between host diet, host gut microbiome, and parasite infection. Elucidation of the importance and ubiquity of such a link will help us better understand the therapeutic potential of diets and gut microbiomes to control infectious disease.

2.7 Acknowledgements

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Box 2.2. Case Study: Composition of Gut Microbial Community Modulates Severity of Malaria in Mice

One study on mouse malaria investigated the three relationships that are the focus of this review: (1) diet alters disease resistance; (2) diet affects the gut microbiome; and (3) the gut microbiome changes disease resistance [7]. This study first found that genetically inbred mice (C57BL/6) infected with *Plasmodium* significantly differed in parasite burden based on mouse vendor source. Mice from Jackson Laboratory (Jax) and Taconic (Tac) had a significantly lower number of parasites and no mortality compared to National Cancer Institute/Charles River (NCI) and Harlan (Har) mice. To test whether diet increases resistance to malarial infection, Jax (resistant) and NCI (susceptible) mice were fed two commercial chow diets: NIH-31 and Teklad 22/5. Although parasitemia was not affected in susceptible NCI mice fed these diets, the Teklad 22/5 diet significantly increased parasitemia and mortality in resistant Jax mice compared to the NIH-31 diet. This study also demonstrated that the alternative diets affect the gut microbial community composition: Jax mice fed the Teklad 22/5 diet had lower relative abundance of the bacterial family Peptostreptococcaceae compared to Jax mice fed the NIH-31 diet. The researchers then used fecal transplants, microbial supplementation and immune assays to demonstrate that the gut microbiome reduces parasitism. However, instead of carrying out this study with mice of similar origin fed on alternative diets, the researchers used mice that varied in resistance due to different vendor origin (Jax and Tac versus NCI and Har). Thus, while suggestive of an indirect link, this study did not yet unequivocally demonstrate that diets altered disease resistance by modulating the gut microbiome.

Chapter 3: Alternative diets affect the monarch butterfly microbiome in early development

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Abstract

Animal traits such as development and diet greatly impact the composition and dynamics of associated microbial communities. However, we still lack a comprehensive view of how these factors interact to drive the formation of the animal gut microbiome and how microbiome composition varies in relation to host traits and ecological factors. This is particularly true for Lepidoptera (butterflies and moths), in which the results of previous studies of the structure, stability and function of gut microbial communities have varied greatly. Here, we use monarch butterflies (*Danaus plexippus*), a specialist herbivore with a chemically well-defined diet of milkweed plants, to investigate the impacts of host plant species and development on the gut microbial community. We characterize gut microbial communities and quantify bacterial load across the monarch lifecycle (parent adults, offspring eggs, larvae, pupae, and offspring adults) when larvae are fed on one of two milkweed species. We find that monarch butterfly gut microbial community composition is relatively consistent throughout development, with the exception of adults. Indeed, we provide the first evidence that female parent adults have significantly different microbial communities than their oviposited eggs. Interestingly, milkweed diet influences the microbial diversity in early larval instars, but not in later larval instars. This study demonstrates that the monarch butterfly microbiome is highly dynamic throughout the life

cycle and that ecological factors such as host diet and ontogenetic development can, in tandem, impact host-microbiome associations.

3.1 Introduction

Animals form symbiotic relationships with microbial communities. Across a wide range of animal taxa, gut bacterial communities in particular have been shown to play essential roles in development (Coon, Vogel, Brown, & Strand, 2014; Sommer & Bäckhed, 2013), digestion (Brune, 2014; Marcobal et al., 2011), behavior (Heijtz et al., 2011; A. C.-N. Wong et al., 2017), and defense against natural enemies (Koch & Schmid-Hempel, 2011a; Ramirez et al., 2014). Amongst other factors, such as host genetics and antibiotic exposure, ontogenetic development and host diet have been implicated as major drivers of gut microbial community composition (Carmody et al., 2014; Voreades, Kozil, & Weir, 2014).

Newly formed gut microbial communities in young hosts undergo ecological succession, through which bacteria colonize, cooperate, and compete to occupy the same physical space (Gillilland et al., 2012; Peterfreund et al., 2012). These communities typically change during development until they reach a steady state (Costello et al., 2012). This process of change in the microbiome across animal development has been seen in humans (Yatsunenko et al., 2012), in other mammals (Zhang et al., 2019), in birds (Awad et al., 2016), and in insects (Coon et al., 2014; Hammer, McMillan, & Fierer, 2014).

Diet affects microbial community composition in the gut of many animal taxa (Billiet et al., 2016b; Broderick et al., 2004; de Filippo et al., 2010; Mikaelyan, Dietrich, et al., 2015b; Turnbaugh et al., 2009b). Interactions between diet and the gut microbiome can be driven by evolved specializations or by plastic associations influenced by alternative diets. For example,

termites and cockroaches are specialized to feed on cellulose-based plant materials and have more similar gut bacterial communities compared to other non-cellulose-based eating insects (Boucias et al., 2013; Mikaelyan, Köhler, et al., 2015; Warnecke et al., 2007). However, within termites and cockroaches, changes in diet can alter gut microbial community composition. For example, the microbial communities of dung-feeding and wood-feeding termites differ in their abundant phyla composition and metabolic activities (He et al., 2013). Furthermore, wood-feeding termites have distinct gut microbial community compositions when fed artificial wood-based diets differing in cellulose and xylan composition (Tarayre et al., 2015b). Similarly, cockroaches fed diets differing in protein composition have distinct bacterial communities mainly due to abundances of a few key bacterial families (Pérez-Cobas et al., 2015).

Although development and diet are increasingly recognized as important drivers of microbiome composition and function, we still lack a comprehensive view of how these factors interact to drive the formation of the animal gut microbiome. Insects make an ideal study system to better understand the underlying mechanisms that drive microbiome composition due to their well-defined development and diets as well as their experimental tractability (Colman et al., 2012; Pernice et al., 2014). Lepidoptera provide a particularly suitable system to study the relationship between diet, development, and gut microbial communities. As holometabolous insects, they have four distinct developmental life stages (egg, larva, pupa, and adult), of which the two feeding stages (larva and adult) use drastically different diets — generally, solid plant foliage and liquid nectar respectively. Furthermore, the plant diets of larvae are compositionally well-characterized, consisting of essential nutrients and secondary metabolites such as alkaloids, phenolics and cardiac glycosides (L. L. Richardson et al., 2015; Tao, Berns, & Hunter, 2014a; Vilanova, Baixeras, Latorre, & Porcar, 2016). Although many lepidopteran larvae are specialist

herbivores, they can frequently utilize closely related plants that vary widely in primary and secondary chemicals, enabling a careful study of the role of specific classes of plant chemicals in shaping gut microbial communities.

The existing studies of insect gut microbiomes indicate that these communities are relatively simple in composition, with significantly fewer dominant taxa present compared to those of mammals (Martinson et al., 2011; Suenami, Konishi Nobu, & Miyazaki, 2019; Yun et al., 2014). However, insect gut community composition is highly variable compared to mammals (Yun et al., 2014). Lepidoptera in particular also have significantly lower abundances of bacteria than other animals (Hammer, Janzen, Hallwachs, Jaffe, & Fierer, 2017). Despite this simplicity, the gut microbial communities of lepidopteran species do vary. What drives this variation is not fully understood (Paniagua Voirol, Frago, Kaltenpoth, Hilker, & Fatouros, 2018). In part, some variation may be driven by a lack of a core microbiome and weak associations with bacteria in general, at least in some species (Hammer et al., 2017). However, studies differ. Recent work has revealed that lepidopteran gut community composition can be highly variable between and within individuals of the same species (Chen et al., 2016; Staudacher et al., 2016), or it can be relatively stable (Anand et al., 2010; Broderick et al., 2004). In some species, but not in others, microbial communities are distinct between larvae and adults (Hammer et al., 2014; Phalnikar, Kunte, & Agashe, 2018a). And, in some species but not in others, feeding on alternative larval diets leads to colonization of distinct gut communities (Broderick et al., 2004; Phalnikar et al., 2018a; Pinto-Tomás et al., 2011; Staudacher et al., 2016; Whitaker, Salzman, Sanders, Kaltenpoth, & Pierce, 2016). These differences across studies and systems could be driven by how samples are collected, how microbes are acquired, how dietary components and plant

chemistry differ and how evolution has shaped the maintenance of associations. All this variation could have functional importance, though this is yet unclear.

Given the differences across studies and systems, detailed, controlled investigations are needed to better understand how microbiome composition varies in relation to host traits and ecological factors. Here, we focus on two important factors, developmental stage and larval diet, in a specialized butterfly host that has been a model in studies of herbivore-plant evolution, migration and disease ecology (Agrawal et al., 2009; Bradley & Altizer, 2005; Ehrlich & Raven, 1964; Gowler, Leon, Hunter, & de Roode, 2015b; D. Roode et al., 2007; Zhan, Merlin, Boore, & Reppert, 2011). Monarch butterfly (*Danaus plexippus*) caterpillars are specialist herbivores, feeding mostly on milkweed plants (*Asclepias* spp.). *Asclepias* spp. vary in their concentrations of cardenolides, toxic secondary chemicals that monarchs can sequester to make themselves unpalatable to predators (Brower & Calvert, 1985; Holzinger, Frick, & Wink, 1992; Martin, Lynch, Brower, Malcolm, & Van Hook, 1992). High-cardenolide milkweeds also provide protection against the common and virulent parasite *Ophryocystis elektroscirrha* (de Roode et al., 2008; Gowler et al., 2015a; Lefèvre et al., 2012, 2010), and infected monarchs preferentially oviposit on high-cardenolide plants, reducing infection in their offspring (Lefèvre et al., 2012, 2010). Because the parasite is ingested and must pass through the gut lumen before crossing the midgut wall and infecting hypodermal tissues, it is likely interacting with the microbes within the gut.

The monarch microbiome remains poorly characterized. In the 1970s, a study using culture-dependent methods suggested that there were no egg-associated bacteria and that dominant cultivable bacteria families such as Bacillaceae, Corynebacteriaceae and Enterobacteriaceae fluctuated during larval, pupal and adult stages (Kingsley, 1972). However,

sampling using culture-dependent methods is limited by culture conditions and by sample depth, limiting the inferences that can be drawn. Here, we characterize gut microbial communities and quantify bacterial load across the monarch lifecycle, including parental adults (F_1) and their offspring eggs, five larval instars, pupae and offspring adults (F_2). Moreover, we rear monarchs on two species of milkweed food plants that vary widely in their concentrations of cardenolides, while being similar in nutrient content: low-cardenolide *Asclepias incarnata* and high-cardenolide *A. curassavica* (Tao, Berns, & Hunter, 2014b). This study is the first comprehensive monarch gut microbiome characterization using culture-independent methods.

3.2 Methods

3.2.1 *Insect rearing*

Ten adult individuals from four lineages were fed on sterile 20% sucrose solution; these adults are referred to hereafter as the F_0 generation. F_0 adults were mated with the opposite sex of another lineage. Once mated, five F_0 females of the same lineage were placed in a single butterfly cage (two cages per lineage) maintained in the greenhouse and oviposited on either milkweed food plant species, *A. incarnata* or *A. curassavica*. Their eggs (the F_1 generation) were collected and hatching caterpillars were fed on respective host plants and reared to adulthood. F_1 adults were fed sterile 20% sucrose solution and placed in a single butterfly cage (two cages per lineage) maintained in the greenhouse. They were allowed to oviposit on either one of two milkweed species, *A. incarnata* or *A. curassavica*. Oviposited eggs seeded the F_2 generation, the focus of our study. F_2 eggs were then moved to individual plastic, lidded cups, where they were placed on leaves of either *A. incarnata or A. curassavica*. Once the eggs hatched, leaves were replaced daily. Overall, there were four F_2 diet treatments that differed in parent (F_1)-offspring

(F_2) larval diet combinations: (1) parents fed on *A. incarnata* and offspring fed on *A. incarnata* (n = 26), (2) parents fed on *A. incarnata* and offspring fed on *A. curassavica* (n = 27), (3) parents fed *on A. curassavica* and offspring fed on *A. curassavica* (n = 29), and (4) parents fed on *A. curassavica* and offspring fed on *A. incarnata* (n = 22) (Fig. 3.1).

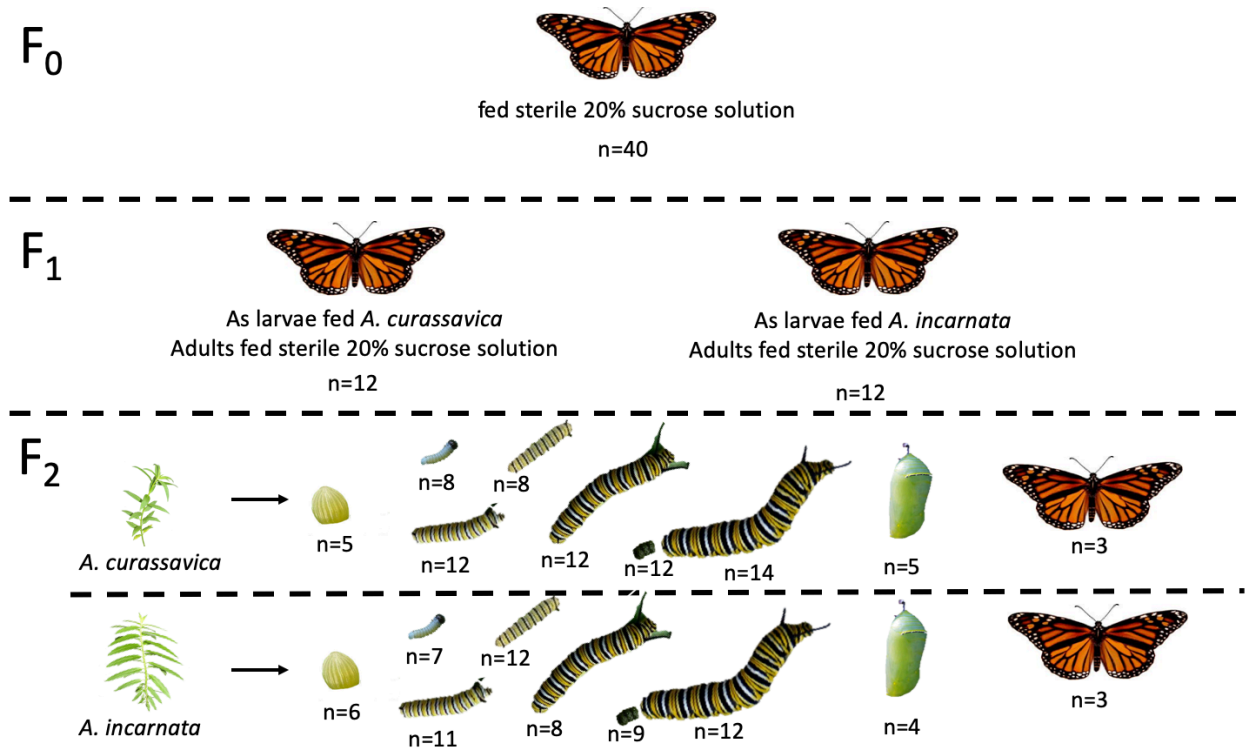

Figure 3.1. Schematic of experimental design. Egg sample is a pool of 20 individual eggs. Frass samples were collected from fifth instar larvae. Each frass sample is a pool of five frass pellets.

3.2.2 Sample collection and gut dissections

We stored parental F_1 adults after oviposition. Resulting F_2 offspring, once hatched, fed on either *A. incarnata* or *A. curassavica*. These were collected at all developmental stages (egg, larva, pupa, and sucrose-fed adults). Each egg sample consisted of a pool of 20 eggs. From all larval instars, we dissected out guts for further analysis. Prior to dissection, larvae were euthanized with CO_2, then whole bodies were surface-sterilized with molecular grade ethanol for 3 minutes. Seven-day old pupae were handled similarly though not euthanized. For adults, we clipped off the wings at the thorax, then surface sterilized them in molecular grade 99% ethanol for 3 minutes. Guts were dissected with sterile instruments and immediately frozen. To test if larval frass microbial communities are reflective of their gut microbial communities, we collected frass excreted from 21 fifth instar larvae. Each frass sample consisted of five frass pellets. Frass samples were not surface-sterilized. All samples were frozen at -80°C until DNA extraction. Sample sizes are indicated in Figure 3.1.

3.2.3 Gut Microbiome Community Profiling

DNA was extracted using the Qiagen DNeasy PowerSoil kit, following the manufacturer's protocols. Extractions were sent to the University of Michigan's Center for Microbial Systems for PCR amplification, amplicon library preparation, and 16S rRNA sequencing. The 16S rRNA gene was amplified with barcoded dual-indexed primers 515F and 806R specific to the V4 region. The PCR cycle consisted of 2 min at 95°C, followed by 30 cycles of 95°C for 20 s, 55°C for 15 s, and 72°C for 5 min, followed by 72°C for 10 min. PCR reactions were normalized, pooled, and quantified for amplicon library preparation. Libraries were sequenced on an Illumina MiSeq platform with 250bp paired ends.

Bacterial sequences were processed and analyzed in Mothur v 1.40.3 (Kozich, Westcott, Baxter, Highlander, & Schloss, 2013). Sequences were paired and trimmed. Sequences less than 250bp or greater that 289bp in length were removed from analysis. Reads were aligned to the V4 region of the 16S rRNA gene using the SILVA reference database. Chimeras and non-bacterial 16S rDNA (i.e., archaea, chloroplasts, and mitochondria) were removed. A mock community was co-sequenced (ZymoBIOMICS™ Microbial Community DNA Standard) to determine the sequencing error rate, which was 0.0082%. Operational taxonomic units (OTUs) were clustered based on 97% sequence similarity. Taxonomic assignments were determined using a Bayesian classifier and mapping sequences against the Ribosomal Database Project (RDP). Samples with less than 136 total reads were removed from the analysis; this minimum total read number optimized sequencing depth and sample size for the various treatment groups. Rarefaction curves confirmed at least 97% coverage of each OTU analyzed. Visualizations and all statistical tests of sequence data were performed in R v3.3.3 (R Development Core Team 2012) using packages phyloseq (McMurdie & Holmes, 2013) and vegan (Dixon, 2003).

3.2.4 Quantitative PCR

To determine differences in bacterial sequence abundance between developmental stages and larval instars fed on *A. incarnata* and *A. curassavica*, mean copy numbers of 16S rRNA genes in a subset of samples were estimated using qPCR (n = 138). Each sample was amplified in triplicate, except for four samples amplified in duplicate due to lack of DNA, with the same 16S rRNA primers used for PCR amplification (515F and 806R). Primers and reaction conditions are described in Cariveau, Elijah Powell, Koch, Winfree & Moran (2014). Standard curves were calculated using purified genomic *E. coli* DH10B cells (ThermoFisher Scientific). To calculate

the starting copy number for the standard curve, we used the copy number calculator for real time PCR on scienceprimer.com, and generated the standard curve in relation to the serial dilution of 1:10. The standard copy number started at 1.6×10^{11} and was diluted down to ~1.6×10^{4}. No samples were considered out of range. The estimated mean absolute copy number across triplicates, and in four cases duplicates, was used for analyses.

3.3 Results

3.3.1 Membership of the Monarch Gut Microbiome

After quality filtering and preprocessing, 1,600,731 sequences were retrieved from 160 samples. These were clustered into 1,305 operational taxonomic units (OTUs) with 97% sequence similarity. Dominant genera were *Asaia, Pantoea, Paenibacillus, and Bacillus,* and many OTUs were considered as unclassified Enterobacteriaceae (Fig. 3.2). Of note, *Asaia* was common in adults but rare in all other life stages.

3.3.2 Changes in Community Composition across the Monarch Lifecyle

When considering all life stages, there was a significant effect of monarch life stage on the composition of the microbial gut community based on a Bray-Curtis dissimilarity matrix (PERMANOVA with 10,000 permutations; $p < 0.05$; Fig. 3.3). Consistent with *Asaia* dominating the gut community of adults only, adult butterfly guts had a significantly different membership than other life stages, which did not significantly differ from one another based on pairwise comparisons (Table 3.1). Given this, it is not surprising that F_1 female adults had significantly different gut microbial communities than their oviposited eggs (PERMANOVA with 10,000 permutations; $p < 0.05$; Fig S3.1). Shannon diversity also significantly differed

across life stages (ANOVA, $F = 4.2$, $p = 0.02$), which was driven by a decrease in diversity in pupae and adults (pairwise stats: Table 3.2; Fig. 3.4), suggesting that microbial diversity decreases in later life stages.

Focusing on larvae only, there was little difference in microbial community composition across the five larval instars based on a Bray-Curtis dissimilarity matrix (PERMANOVA with 10,000 permutations; $p > 0.05$). Shannon diversity did significantly differ across larval instars (ANOVA, $F = 3.5$, $p = 0.01$). Pairwise comparisons revealed that this difference was largely driven by the fifth instar larvae, which had the lowest diversity of all instars (pairwise stats: Table 3.3).

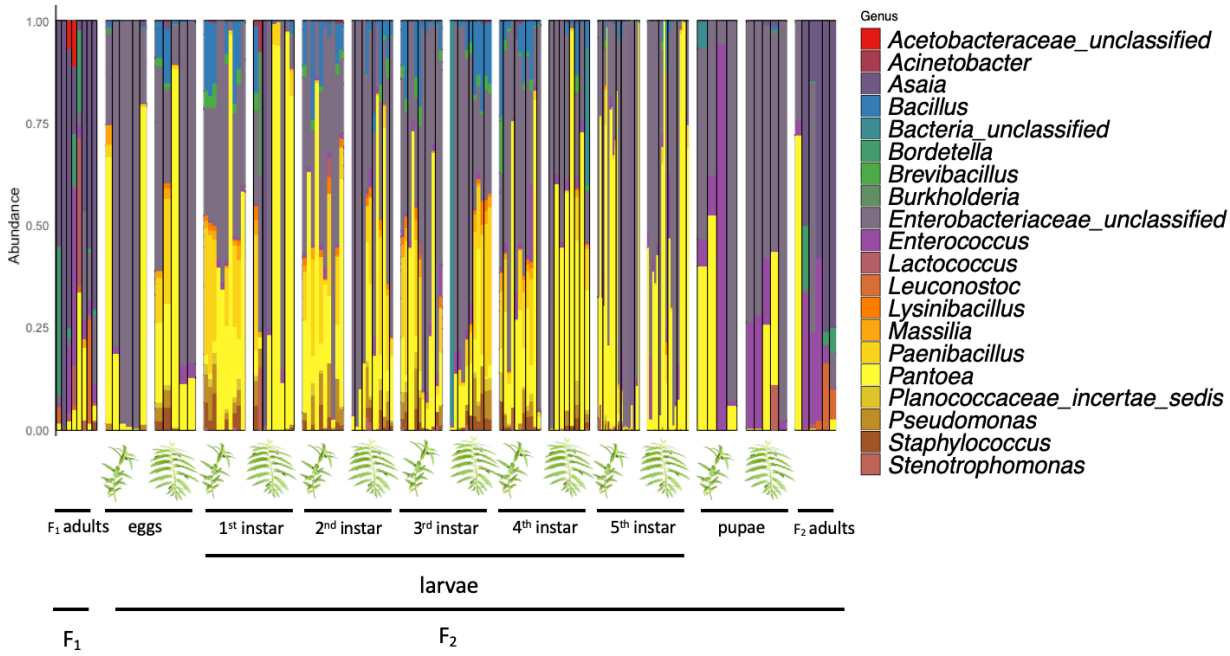

Figure 3.2. Microbial community composition across developmental stages, based on top 25 most abundant OTUs. Monarchs were reared on two host plants, *A. incarnata* and *A. curassavica* indicated by plant icons. Host plant, for eggs, indicated the host plant on which their F_1 parents fed. For all other development stages, the host plant species indicates what that individual fed on as a larva. Larvae are indicated by instar (1^{st} to 5^{th}). With the exception of egg samples, which were pooled, each column represents the microbial community within the gut of one individual.

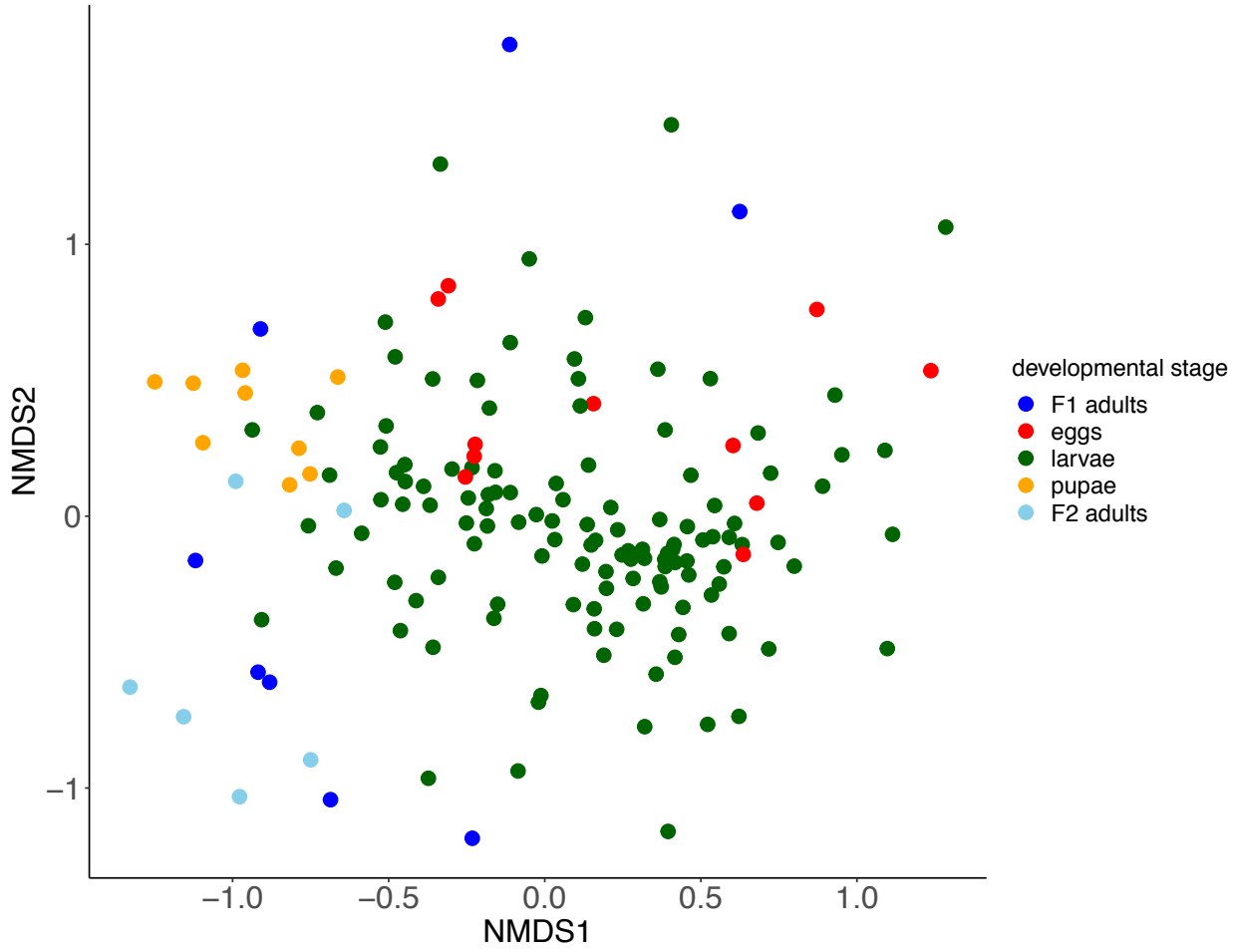

Figure 3.3. Composition of adult butterfly gut communities are different than those found in other life stages. Here, all larval stages (first to fifth) are considered together. Adults were fed sterile 20% sucrose water prior to dissection. PERMANOVA with 10,000 permutations: $p < 0.05$.

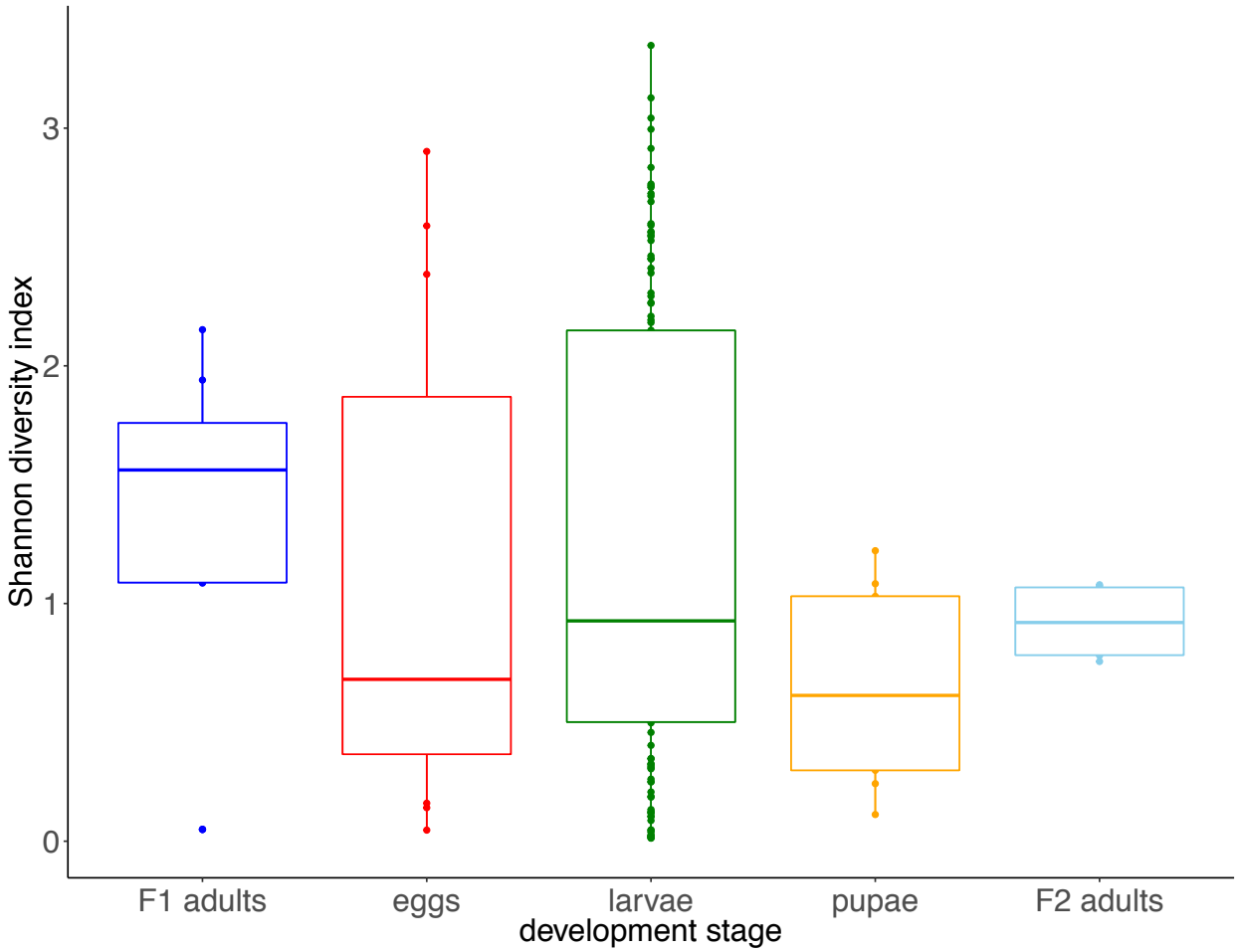

Figure 3.4. Shannon diversity of the microbial community differs across development stages, with pupae having the least diverse microbial gut communities. Here, all larval stages (1st to 5th) are considered together.

Table 3.1. Pairwise comparison results of community composition between monarch developmental stages. Pairwise ADONIS using Tukey's post hoc test for multiple comparisons for each possible pair (see Fig. 3). Asterisks indicate significant differences between treatments.

	F_1 adults	**eggs**	**larvae**	**pupae**	**F_2 adults**
F_1 adults		0.003*	0.003*	0.003*	0.76
eggs			0.834	0.390	0.006 *
larvae				0.090	0.006 *
pupae					0.006 *
F_2 adults					

Table 3.2. Pairwise comparison results of Shannon diversity between monarch developmental stages. One-way ANOVA with pairwise comparisons using Tukey's post hoc test for multiple comparisons for each possible pair (see Fig. 4). Asterisks indicate significant differences between treatments.

	F_1 adults	**eggs**	**larvae**	**pupae**	**F_2 adults**
F_1 adults		0.13	0.32	0.37	0.25
eggs			0.61	0.045*	0.036*
larvae				0.01*	0.015*
pupae					0.019*
F_2 adults					

Table 3.3. Pairwise comparison results of Shannon diversity between larval instars. One-way ANOVA with pairwise comparisons using Tukey's post hoc test for multiple comparisons for each possible pair (see Fig. 5). Asterisks indicate significant differences between treatments.

	1st	**2nd**	**3rd**	**4th**	**5th**
1st		0.29	0.067	0.78	0.001*
2nd			0.088	0.49	0.001*
3rd				0.26	0.098
4th					0.001*
5th					

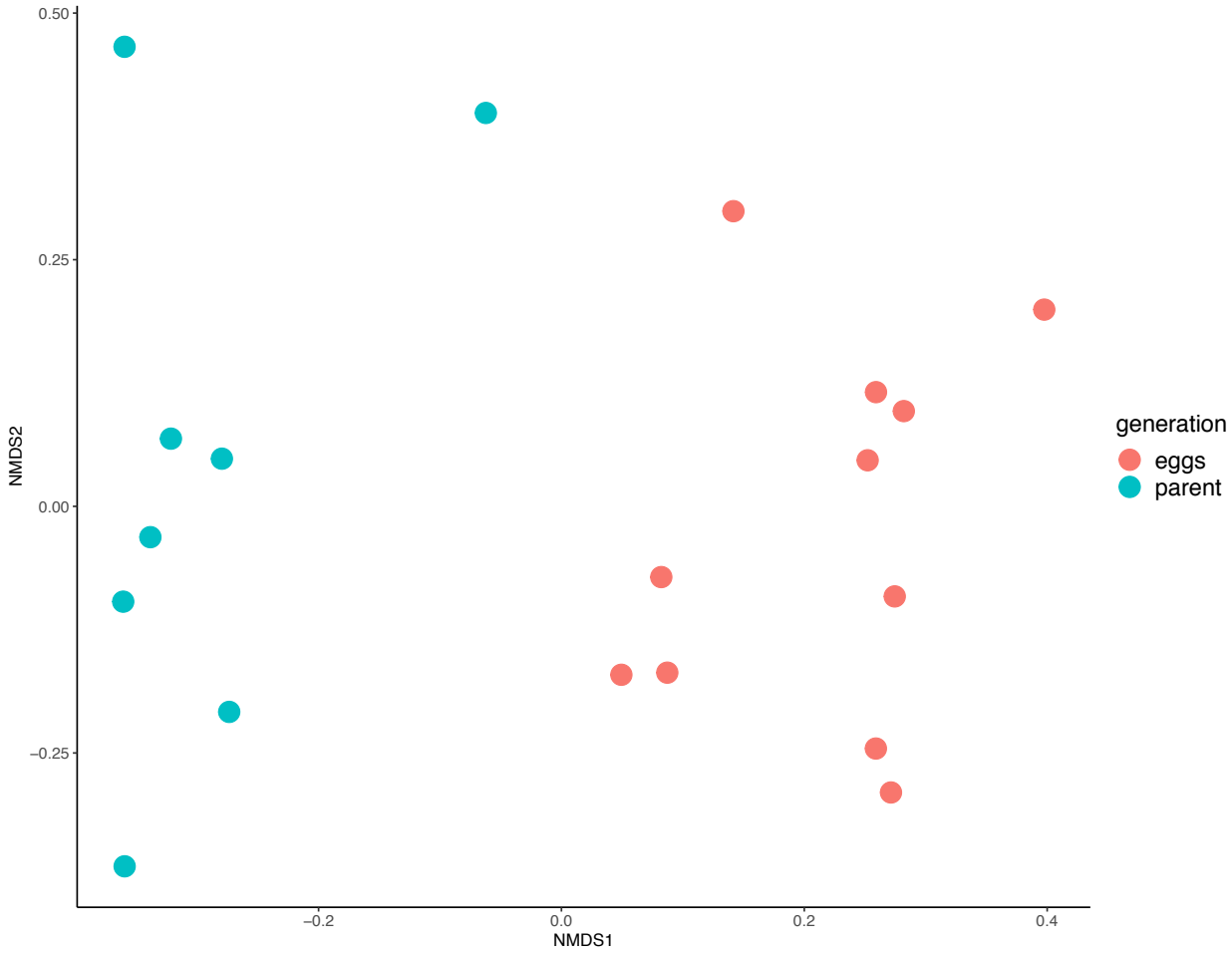

Figure S3.1. Female parent adults have different communities than their oviposited eggs. Parents are F_1 female adults that were fed sterile 20% sucrose water prior to dissection. Egg samples are a pool of 20 eggs each. PERMANOVA with 10,000 permutations: $p < 0.05$.

3.3.3 Influence of Host Plant Diet on the Larval Gut Microbiome

Host plant diet had little effect on community composition of larval gut microbiomes (PERMANOVA with 10,000 permutations; $p > 0.05$; Fig. S3.2). However, Shannon diversity did differ significantly between larvae fed on *A. curassavica* and *A. incarnata* (two-way ANOVA, $F = 4.1$, $p = 0.001$; Fig 3.5). Specifically, first and second instar larvae fed on *A. curassavica* had a more diverse gut microbiome that those fed on *A. incarnata* (first instar: ANOVA, $F = 6.7$, $p = 0.001$; second instar: ANOVA, $F = 3.5$, $p = 0.01$).

3.3.4 Comparison of Larval Frass and Gut Microbial Community Composition and Load

To better understand whether larval frass communities are reflective of larval gut communities, we compared the microbial community composition of 5th instar larval guts and frass excreted by 5th instars close to pupation. To better understand the microbial dynamics before and during pupation, we also compared the microbial communities of mature pupae. We found that there was significant overlap of larval frass and larval gut community composition (PERMANOVA with 10,000 permutations; $p > 0.05$). Mature pupae gut microbial community composition, however, differed from both other groups (PERMANOVA with 10,000 permutations; $p < 0.001$; Fig. S3.3).

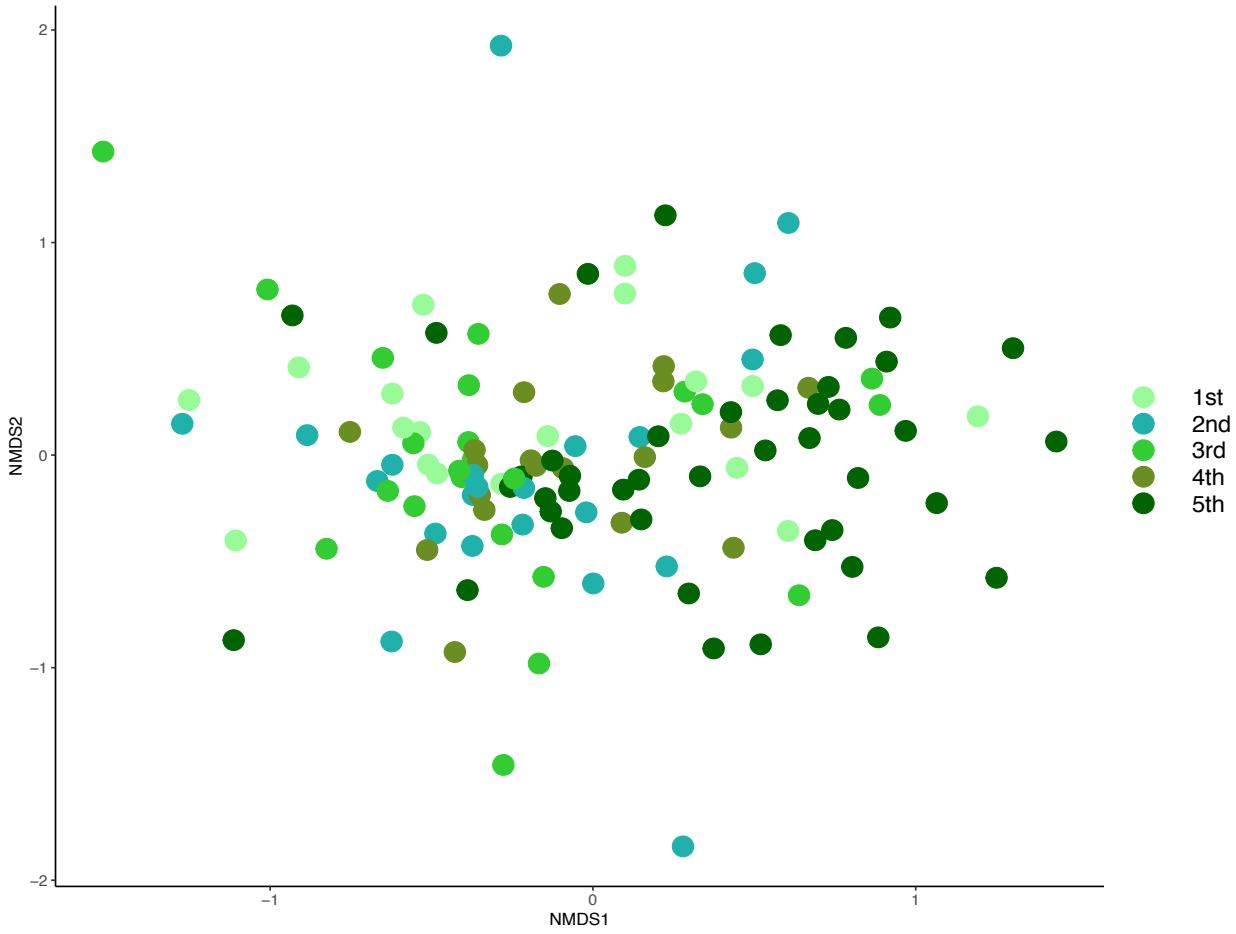

Figure S3.2. Composition of larval instar gut communities are not different from one another. Individuals are not distinguished by larval milkweed diets, *A. incarnata* and *A. curassavica*. permutational multivariate PERMANOVA with 10,000 permutations: $p > 0.05$.

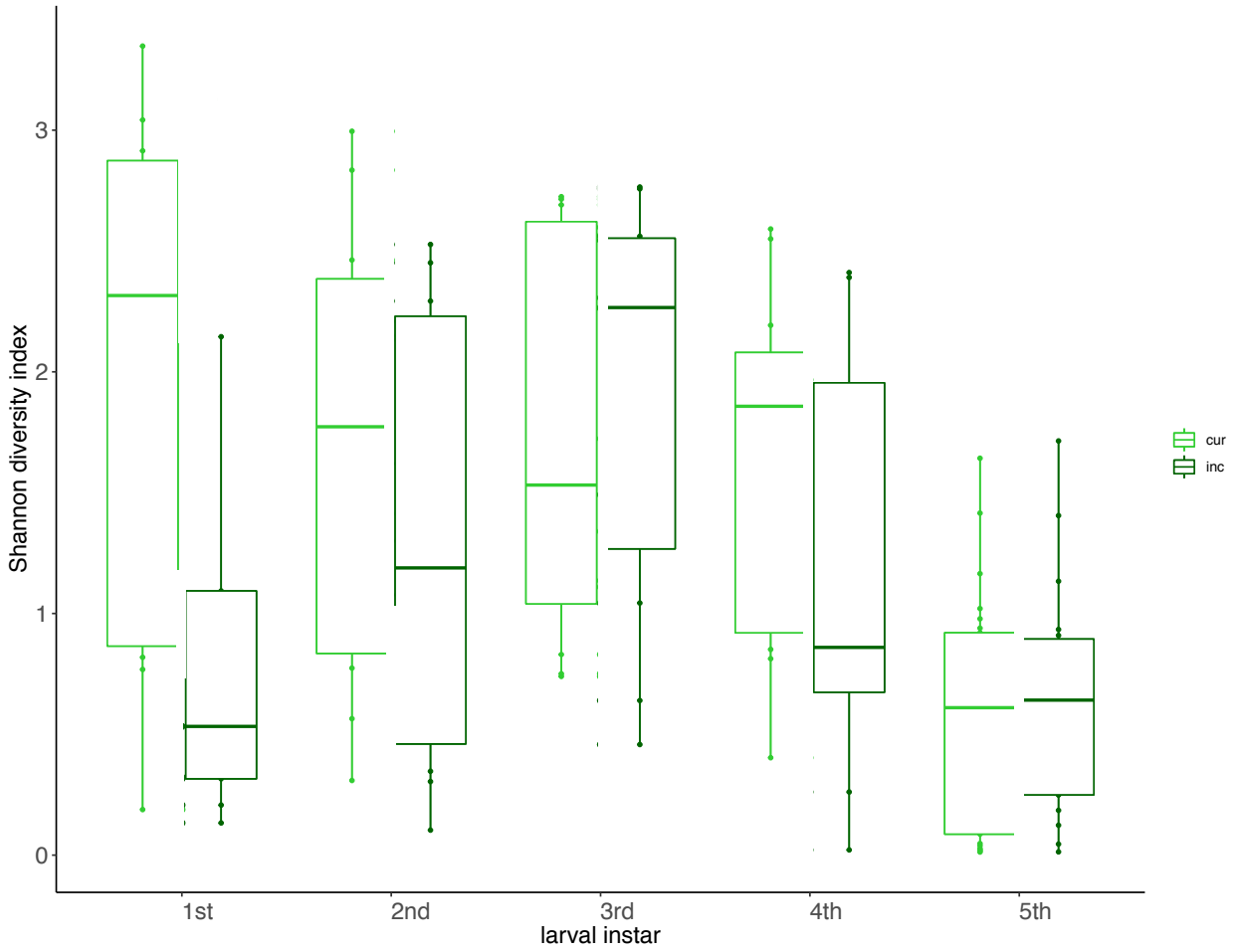

Figure 3.5. Shannon diversity across larval instars fed on both milkweed species. Gut microbial diversity is higher in earlier larval instars fed on *A. curassavica* compared to those fed on *A. incarnata*.

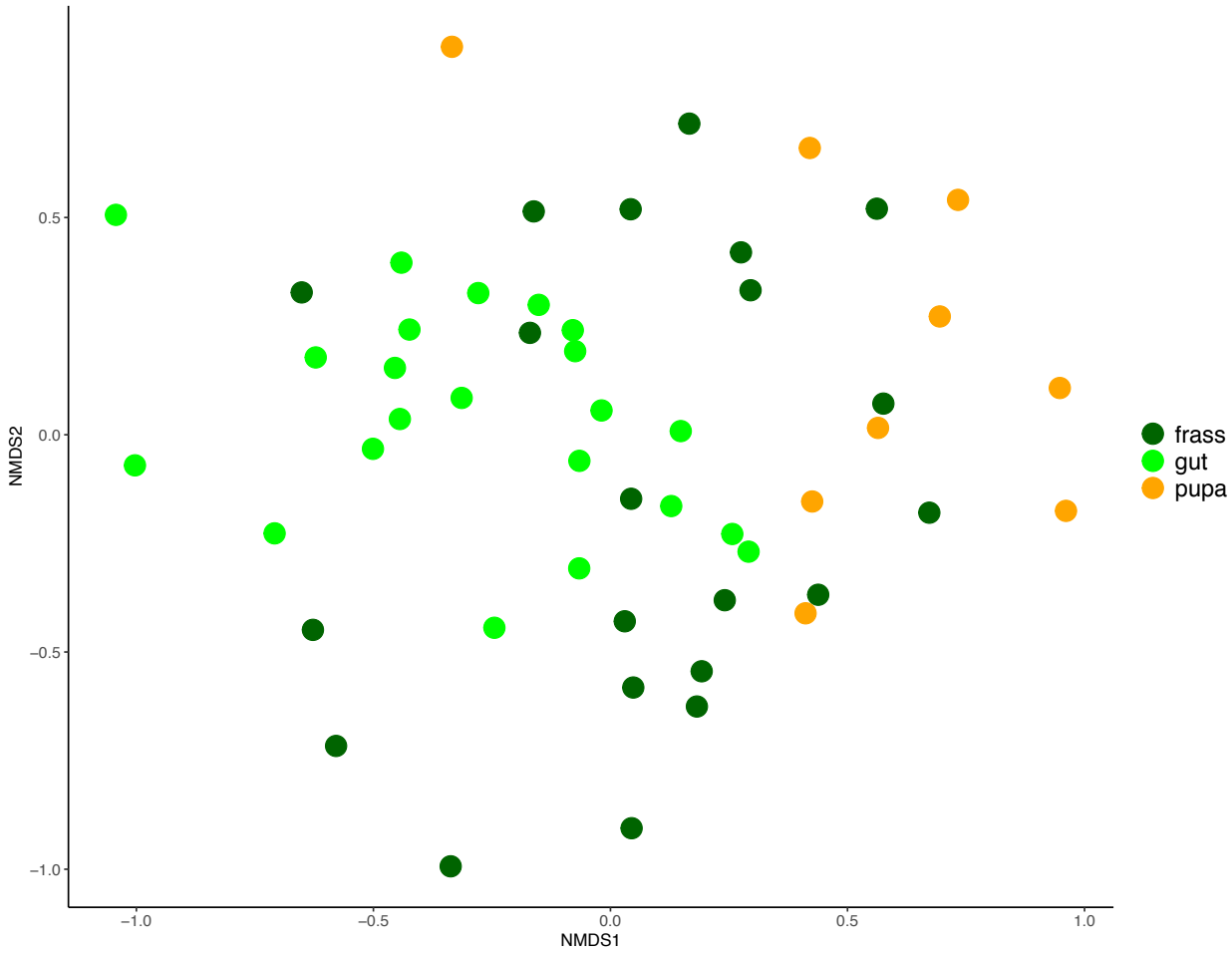

Figure S3.3. Larval gut and frass microbial communities are similar but distinct from pupae. Guts and frass were collected from 5th instar larvae. PERMANOVA with 10,000 permutations: $p < 0.05$.

3.3.5 Quantitative PCR

When estimating the bacterial loads of each life stage using qPCR-based quantification of 16S rRNA copy number, as expected, eggs were estimated to have significantly fewer bacteria relative to larvae, pupae and adults, which did not differ significantly between groups (one-way ANOVA, $F = 5.4$, $p < 0.0001$; Fig. 3.6A). For larval instars, first instars had significantly fewer bacteria than all other larval instars (one-way ANOVA, $F = 4.13$, $p = 0.004$; Fig. 3.6B). There was no difference in bacterial load for each larval instar based on larval milkweed diet (two-way ANOVA, $F = 0.78$, $p = 0.54$; Fig. 3.6C). When we compared bacterial load between larval guts, larval frass samples, and pupal guts, we found that the load of bacteria in frass was significantly higher than that in the larval gut and pupal samples (one-way ANOVA, $F = 12.95$, $p = 3.45 \times 10^{-5}$; Fig 3.6D).

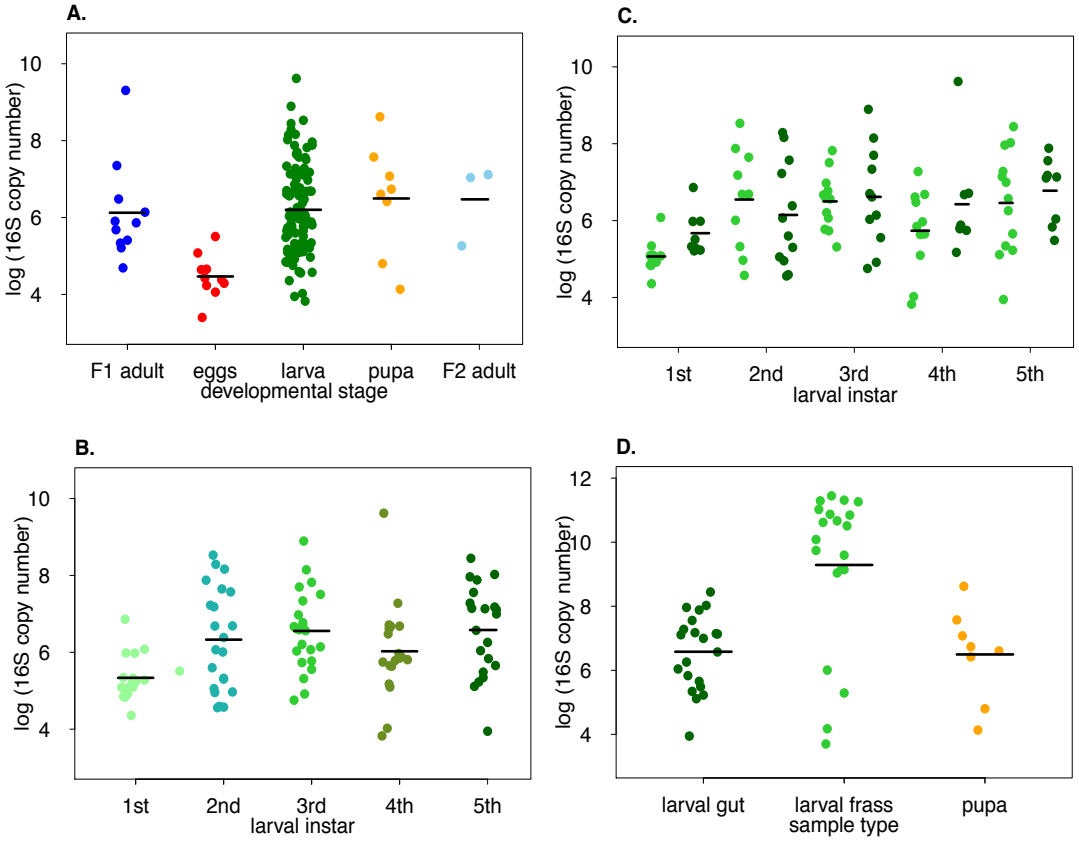

Figure 3.6. Microbial abundance across developmental stages. Shown are the $\log_{10}$ 16S rRNA copy number per sample. Sample type is specified on the x-axis. Points represent individual samples, and horizontal bars represent means. (A) Eggs have lower microbial abundance than other developmental stages. (B) First instars have lower microbial abundance than other larval instars. (C) Larval diet does not affect microbial abundance across larval instars. For each larval instar, dots represent individual guts. Light green dots are larvae fed *A. curassavica*. Dark green dots are larvae fed *A. incarnata*. (D) Larval guts have higher microbial abundance than larval frass and pupae.

3.4 Discussion

Here, we observe the microbial community composition dynamics of the monarch butterfly across its lifecycle using culture-independent methods. Building on culture-dependent methods (Kingsley, 1972), our results broaden our understanding by including the characterization of microbial communities of monarch adult parents and their eggs, quantifying bacterial loads of all developmental stages, and assessing the impact of alternative larval diets during development.

The most striking development pattern is that adult communities differ from those of all other instars, similar to other studies of microbial community dynamics across developmental stages (Hammer et al., 2014; Phalnikar, Kunte, & Agashe, 2018b). These differences are most likely due to drastic dietary shifts across development, from feeding on foliage as larvae to feeding on liquid (here, sugar water) as adults. Interestingly, gut microbial communities of adult *Heliconis* butterflies that feed exclusively on pollen differ from adult *Heliconis* butterflies that do not, highlighting that diet matters also within life stages (Hammer et al. 2019).

We find that adults have gut bacteria dominated by *Asaia*, an unclassified member of the *Acetobacteraceae* and unclassified *Enterobacteriaceae*. These taxa are commonly found in insects including Lepidoptera. For example, *Asaia* is commonly found in healthy mosquitoes throughout development and in various geographic locations (Favia et al., 2007; Hughes et al., 2014). Interestingly, *Asaia*, dominating the sucrose fed monarch adults in this study, is a dominant taxon found in nectar fed to bumblebees (Schaeffer, Rering, Maalouf, Beck, & Vannette, 2019). In mottled willow moths, *Asaia* is present in egg-associated microbial communities (Gao et al., 2019) although we find no evidence of *Asaia* in monarch eggs.

In contrast to the Kingsley 1972 study, we find that monarch eggs do indeed have a bacterial community associated with them, which is dominated by *Pantoea* and unclassified

Enterobacteriaceae. Monarch egg-associated communities are more similar to the larval gut communities than those of their female mothers, suggesting that some microbes that are rare in adults may be transmitted from the eggs into later stages. In contrast, in the moth species *Spodoptera exigua*, there are significant similarities between adult parent gut and whole egg microbial communities (Gao et al., 2019). Since there is no comprehensive understanding of when or how microbes are transmitted between stages, the horizontal and vertical acquisition of microbes needs to be further explored.

In terms of monarch larval plant diet, alternative milkweed species affected larval microbial community composition in early instars (first and second), suggesting that the composition of the plants modulate the gut microbial communities. Since the milkweed species used here, *A. curassavica* and *A. incarnata*, are similar in nutrients but differ greatly in toxic compounds called cardenolides (Tao et al., 2016), we hypothesize that the chemical properties of these plants are central to the changes in gut microbial community composition. The fact that these differences are only seen in early instars may suggest that the newly forming larval gut community is highly sensitive to the introduction of new microbes, a pattern observed in other insects such as cockroaches and honey bees (Anderson et al., 2016; Carrasco et al., 2014).

Variation in Shannon diversity across the life cycle is not uncommon in butterflies, with pupae in particular having significantly lower richness and diversity (Hammer et al., 2014). This could be explained by the drastic reorganization of body tissues and non-feeding state of this life stage. It is important to note that our study demonstrates that microbes do persist throughout the pupal stage, providing evidence that Lepidoptera do not void themselves of their microbes as pupae (Hammer et al., 2014; Johnston & Rolff, 2015; Phalnikar et al., 2018b). This is in contrast to honeybees, which do void all microbes as part of the pupal stage (Engel, Martinson, & Moran,

2012; Martinson et al., 2011). Interestingly, although having high microbial abundance, the gut microbial communities of fifth instar larvae have lower diversity than those of other larval instars. One explanation for this is that fifth instar larvae have larger guts in terms of volume, which could facilitate colonization by microbes that are good competitors and suppress the growth of other microbes (Kingsley, 1972). Another explanation is that fifth instar larvae excrete large amounts of frass before morphing into pupae. The excretion of gut contents may decrease the overall gut microbial diversity in fifth instar larvae. This is supported by our microbial abundance data, which demonstrate that fifth instar larval frass has a high concentration of microbes.

We provide the first comprehensive, culture-independent characterization of monarch-associated microbial communities. We demonstrate that the monarch butterfly microbiome is highly dynamic throughout the life cycle. Although the community composition of larval guts appears to be relatively stable throughout the larval developmental stage, the microbial diversity decreases and microbial abundance increases during larval development. Furthermore, larval gut microbial diversity is significantly influenced by milkweed diet in early larval instars, a development stage that is ecologically relevant in terms of natural enemies that colonize the gut at this stage. While we demonstrate that dynamic gut microbial community properties exist in the monarch-milkweed system, further studies are needed to analyze the role of these communities in driving host fitness.

3.5 Acknowledgements

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Chapter 4: Diet indirectly alters parasite resistance through the gut microbiome in monarch butterflies

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Abstract

Animal hosts live in close association with microbes that are largely impacted by host diet and can play a role in modulating host resistance to parasites. Growing evidence across the animal kingdom demonstrates three separate relationships: (i) host diet modulates resistance to parasites; (ii) host diet alters the gut microbiome; and (iii) the gut microbiome modulates resistance to parasites. However, evidence for an indirect link between diet and parasite resistance, through modulation of the microbiome, remains lacking. We tested for such a link using monarch butterflies (*Danaus plexippus*) and their virulent protozoan parasite, *Ophryocystis elektroscirrha*. Monarch caterpillars are specialist feeders on milkweed plants, and milkweeds with high concentrations of toxins reduce parasite growth, thus acting as medicinal plants. Here we show that monarchs reared on medicinal milkweed have different microbiomes than those reared on non-medicinal milkweed. Moreover, using a set of fecal transplant experiments, we were able to feed gut microbes from monarchs reared on medicinal milkweed to monarchs reared on non-medicinal milkweed, altering the microbiome and reducing parasite growth. Lower parasite loads were associated with high microbiome diversity and high microbial abundance. For microbial transplants, lower parasite loads were specifically associated with low relative abundances of *Pantoea* and unclassified Enterobacteriaceae and high relative abundances of *Chryseobacterium*,

Pedobacter and unclassified *Rhizobiales*. This study demonstrates that diet-mediated microbiome transplants can increase resistance to parasites and that Lepidopteran gut microbiomes can be a crucial functional driver of host fitness.

4.1 Introduction

Animals live in close association with microbes, which are essential drivers of animal fitness. In particular, microbes that inhabit the gut and their collective genomes - the gut microbiome - regulate digestion, development, immunity, and disease resistance (Brestoff & Artis, 2013; Koch & Schmid-Hempel, 2011b; McFall-Ngai et al., 2013). While the gut microbiome is partly shaped by host genetics and immunity, the environment in which animals live plays a major role in structuring microbiome composition and function. One significant environmental factor is host diet, which has been shown to play a crucial role in shaping the gut microbial community in a range of animals, including mice and moths (Abnous et al., 2009; Carmody et al., 2014; Priya, Ojha, Kajla, Raj, & Rajagopal, 2012b; J. Wang et al., 2014). In addition to evolved dietary specializations of hosts (Muegge et al., 2011; Russell et al., 2009), changes in diet can alter the gut microbiome (Mikaelyan, Dietrich, et al., 2015a; Turnbaugh et al., 2009a). More specifically, alternative diets differing in nutritional components, probiotics, and plant secondary metabolites all can greatly alter gut microbial community composition (Daisley et al., 2020; Hildebrandt et al., 2009; Kohl & Dearing, 2012; Mason, Rubert-Nason, Lindroth, & Raffa, 2015). Diets do not only shape microbiomes but also resistance to parasites. In particular, hosts can alter disease outcomes by consuming antiparasitic diets that capitalize on plant defensive chemistry (Gowler et al., 2015b; L. L. Richardson et al., 2015), by nutrient supplementation (Meadows et al., 2015; Shankar et al., 1999), and by enriching food quality

(Foley et al., 2012; Nagajyothi et al., 2014b). Recently, the gut microbiome has been shown to modulate parasitism in several animals, including mice and bees (Fanning et al., 2012; Koch & Schmid-Hempel, 2011b; Mockler et al., 2018). These observed relationships suggest that there may be an indirect link between diet, microbiome and disease resistance, through which animals could increase their resistance by choosing diets that create an anti-parasitic microbiome (Harris, de Roode, & Gerardo, 2019).

The idea that animals could alter their diet choice to increase parasite resistance through modulation of the gut microbiome is not only of scientific interest, but may also have therapeutic potential. Indeed, there is an increased call for creating personalized diets to augment gut microbiomes to improve health, both in humans and agricultural animals (Daisley et al., 2020; Derrien & Veiga, 2017; Kolodziejczyk, Zheng, & Elinav, 2019). However, empirical evidence of this potential three-way interaction is lacking mainly because previous studies have not tested the separate pairwise interactions (diet-microbiome; diet-parasite resistance; microbiome-parasite resistance) in tandem using tractable animal systems in which host diet increases resistance to tractable parasites. We suggest that insect gut microbiomes are particularly powerful animal systems to study gut microbiome and diet interactions given the ease of controlling their diets, their tractable and relatively simple microbiota, and strong evidence of each separate pairwise interaction in several insect systems (Dillon & Dillon, 2004; Douglas, 2019; Engel & Moran, 2013; Harris et al., 2019; Pernice et al., 2014). Here, we empirically test for the three-way interaction between diet, gut microbiomes and disease using a natural insect system.

Monarch butterfly caterpillars (*Danaus plexippus*) are specialist herbivores of milkweed plants, mostly in the genus *Asclepias*. Monarchs can feed on multiple milkweed species, which vary in in nutrient and defensive chemistry composition (Tao et al., 2016). In particular,

milkweeds vary in the concentration of cardenolides, toxic chemicals that monarchs sequester as an anti-predator defense (Brower & Calvert, 1985). Recent work has shown that these chemicals also play a role in anti-parasite defense, with high-cardenolide milkweeds reducing parasite infection, growth and virulence of the common and naturally occurring protozoan parasite, *Ophryocystis elektroscirrha* (de Roode et al., 2008; Gowler et al., 2015a; Sternberg, Lefèvre, et al., 2012b). Monarchs reared on the high-cardenolide *Asclepias curassavica* suffer lower infection rates and parasite growth, and consequently experience greater pre-adult survival and longer adult lifespan than those reared on the low-cardenolide *A. incarnata* (Lefèvre et al., 2011; W.-H. Tan, Tao, Hoang, Hunter, & de Roode, 2018; Tao et al., 2016). Across a wide range of milkweed species, the medicinal effects of milkweed are correlated with cardenolide concentration (de Roode et al., 2008; Gowler et al., 2015b; Sternberg, Lefèvre, et al., 2012a), but whether these effects are due to direct toxicity for the parasite or mediated by the gut microbiome, remains unknown. *O. elektroscirrha* forms dormant spores on the outside of adult monarch butterfly bodies (McLaughlin & Myers, 1970). When monarchs lay eggs on milkweed, some of these spores are passively transferred to the egg and the milkweed, and hatching caterpillars ingest these spores when consuming the egg chorion and milkweed. Spores then release sporozoites in the midgut lumen, which traverse the midgut wall and undergo asexual and sexual reproduction in the hypodermal tissues during the caterpillar and pupal stage to form new spores in the newly emerging butterfly. Thus, parasites can interact directly with plant toxins and gut microbes during the infection process. Given the ease of administering a controlled, natural diet and tractable parasites (Jacobus C. de Roode, Chi, Rarick, & Altizer, 2009b; de Roode et al., 2008; Harris et al., 2019), monarchs provide an ideal system to test whether milkweed diet affects parasite infection through alteration of the caterpillar microbiome. As such, this system

provides the ideal test case to determine the potential indirect link of diet affecting parasite resistance through modulation of the gut microbiome.

In addition to testing this important indirect link, our study also provides crucial insights into the potential role of microbes driving Lepidopteran fitness. Lepidopteran gut microbiomes have been viewed as an exception to typical animal-microbe symbioses: existing studies indicate a loose relationship between Lepidopteran hosts and their gut microbiota with highly variable bacterial community composition seen within species and a lack of a core, resident microbiome (Chen et al., 2016; Minard, Tikhonov, Ovaskainen, & Saastamoinen, 2019; Staudacher et al., 2016). Instead, support for a transient microbiome, consisting of dead and dormant microbes that are present in the gut for a short period of time, is more widely accepted (Hammer et al., 2017; Paniagua Voirol et al., 2018; Staudacher et al., 2016). In addition, the existing literature paints the Lepidopteran microbiome as one without functional relevance. For example, when disrupted by antibiotics or deprived of nutrients, the Lepidopteran gut microbial community does not seem to be essential for host development, survival, or reproduction (Hammer et al., 2017; Phalnikar, Kunte, & Agashe, 2019; Ravenscraft, Kish, Peay, & Boggs, 2019). However, without exception, these studies have focused on intrinsic fitness measures that can be measured in the lab, without a view to test the Lepidopteran microbiome's role in the context of natural enemies. Thus, while the microbiome may not be important for Lepidopteran survival in the absence of predators or parasites, it could potentially provide crucial protection against such natural enemies.

Here, we leveraged the strong interaction between the monarch caterpillar's medicinal milkweed diet and parasite infection to alter the gut microbiome of monarch caterpillars by feeding them fecal-supplemented natural milkweed diets. We used culture-independent sequencing and quantification to characterize gut community composition and abundance, and

performed disease resistance assays to quantify parasite load. For the first time, we demonstrate that diet-mediated microbiome transplants can increase resistance to parasites, and demonstrate that Lepidopteran gut microbiomes can have a crucial functional role.

4.2 Methods

4.2.1 Plant and insect rearing

Milkweed seeds were purchased from Seed Needs U.S.A. (myseedneeds.com). Two milkweed species were used, *Asclepia curassavica* and *A. incarnata*. These species were chosen for their similarity in nutritional nitrogen and phosphorous concentrations, marked differences in cardenolide concentrations and medicinal effects, with the high -cardenolide *A. curassavica* reducing parasite infection and growth compared to the low-cardenolide *A. incarnata* (de Roode et al., 2008; Sternberg, Lef, et al., 2012; Tao et al., 2016). Plants were grown in a greenhouse with natural lighting and weekly fertilization. Monarchs (*Danaus plexippus*) were obtained from four outcrossed lineages in a laboratory stock of outbred descendants of wild-caught monarchs from the Eastern North American migratory population, collected at their migration stopover site St. Marks, Florida. There were two groups of caterpillars: donors and recipients. Donor caterpillars (one lineage) excreted frass pellets, which were either supplemented directly to milkweed leaves (Experiment 1, untreated frass) or treated to separate microbes from plant materials and chemicals (see below) and then supplemented onto milkweed leaves (Experiment 2, treated frass) for subsequent feeding to recipient caterpillars (three lineages) during frass- and microbe- supplementations.

4.2.2 Overview of Experimental Design

We conducted two microbiome transplant experiments of a similar design, differing only in transplant preparation. Both experiments used the host plants *A. curassavica* and *A. incarnata*. Feeding on *A. curassavica* increases both monarch resistance and tolerance to parasite infection relative to feeding on *A. incarnata* (de Roode et al., 2008; Sternberg, Lefèvre, et al., 2012b). Thus, *A. curassavica* acts as a medicinal plant diet, whereas *A. incarnata* does not. In both experiments, there were four treatment groups: caterpillars reared on *A. curassavica*; caterpillars reared on *A. incarnata*; caterpillars reared on *A. incarnata* and receiving a microbiome transplant from caterpillars reared on *A. curassavica*; and caterpillars reared on *A. incarnata* and receiving a microbiome transplant from caterpillars reared on *A. incarnata*. We hypothesized that if the parasite protection conferred by feeding on *A. curassavica* is due to alteration of the gut microbiome, recipients receiving microbes from donors fed on *A. curassavica* should have higher resistance to *O. elektroscirrha* than those given microbes from donors fed on *A. incarnata*.

In Experiment 1, frass from donors was used to directly inoculate the surfaces of hatching eggs and *A. incarnata* leaf disks, which were fed to recipient caterpillars (Fig. S4.1). Control caterpillars received a sham transplant and were fed either *A. incarnata* or *A. curassavica*. Then, recipients were either inoculated with *O. elektroscirrha* or left uninfected. In Experiment 2, we treated the frass from donor caterpillars to isolate microbes from other fecal components, including plant material and cardenolides. In both experiments, we specifically chose to rear all transplant-recipient caterpillars on *A. incarnata* to avoid confounding pre-existing medicinal effects from protection conferred by *A. curassavica*.

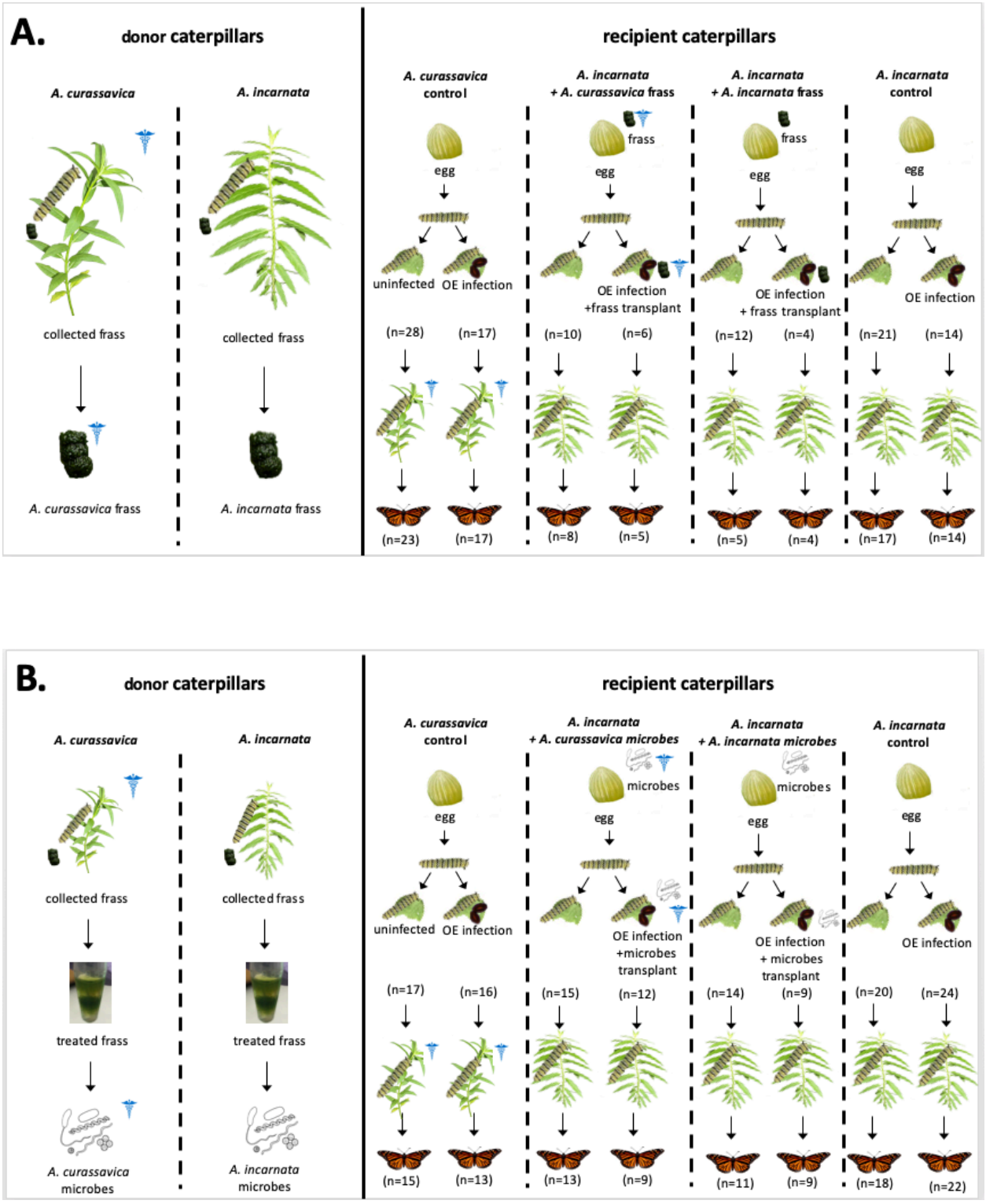

Figure S4.1. Schematic experimental design. (A) Experiment 1, (B) Experiment 2

4.2.3 Transplant Preparation

To ensure that recipient caterpillars received fresh transplants, frass samples were collected from donors the same day that transplants were administered to recipients. Recipients received transplants from the same individual donor throughout the duration of the experiment (i.e. the same donor was used for egg and leaf disk supplementation). For Experiment 1, five frass pellets were collected directly from donor caterpillars and mixed with 1ml sterile phosphate buffered saline (PBS). Fecal material is known to have many components, including plant secondary metabolites (Bojanova & Bordenstein, 2016; Dillon & Charnley, 1995). Therefore, for Experiment 2, in order to minimize the potential confounding effects of cardenolides contained within donor frass pellets, we separated soluble and insoluble components of frass with density centrifugation using modified methods described in Hevia et al. (Hevia, Delgado, Margolles, & Sánchez, 2015) prior to microbe supplementation of eggs and leaf disks (Fig. S4.2). Briefly, five frass pellets were added to 860 μl of sterile 0.9% NaCl (w/v) and 300 μl of sterile 80% Nycodenz® (w/v) (Sigma-Aldrich, Darmstadt, Germany) and centrifuged for 40min at 10,000 rpm at 4°C. The entire soluble layer containing live bacteria was extracted and added to 1ml of sterile phosphate buffered saline (PBS), hereafter referred to as microbe solution. This method decreased the cardenolide concentration per transplant to a negligible amount (Fig S4.3). We visually confirmed the presence of live bacteria with microscopy.

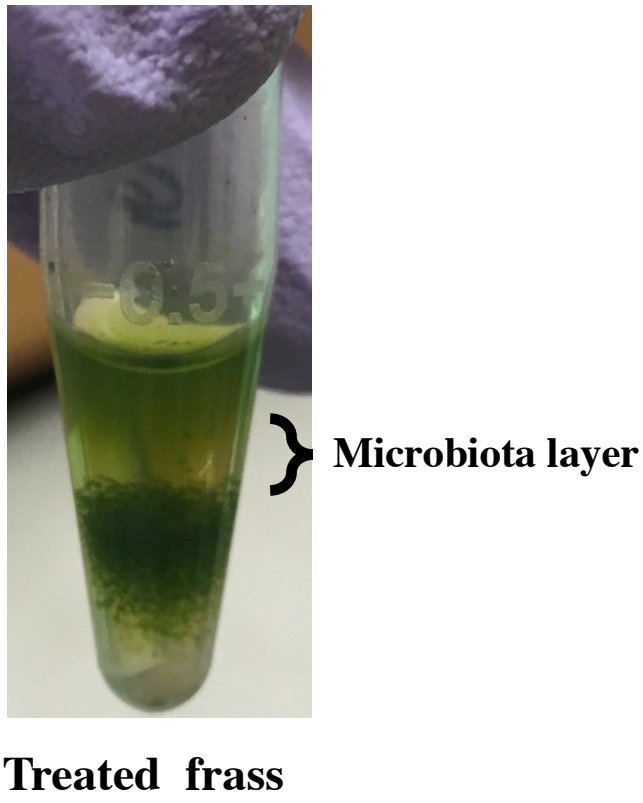

Figure S4.2. Schematic of treated frass with density centrifugation.

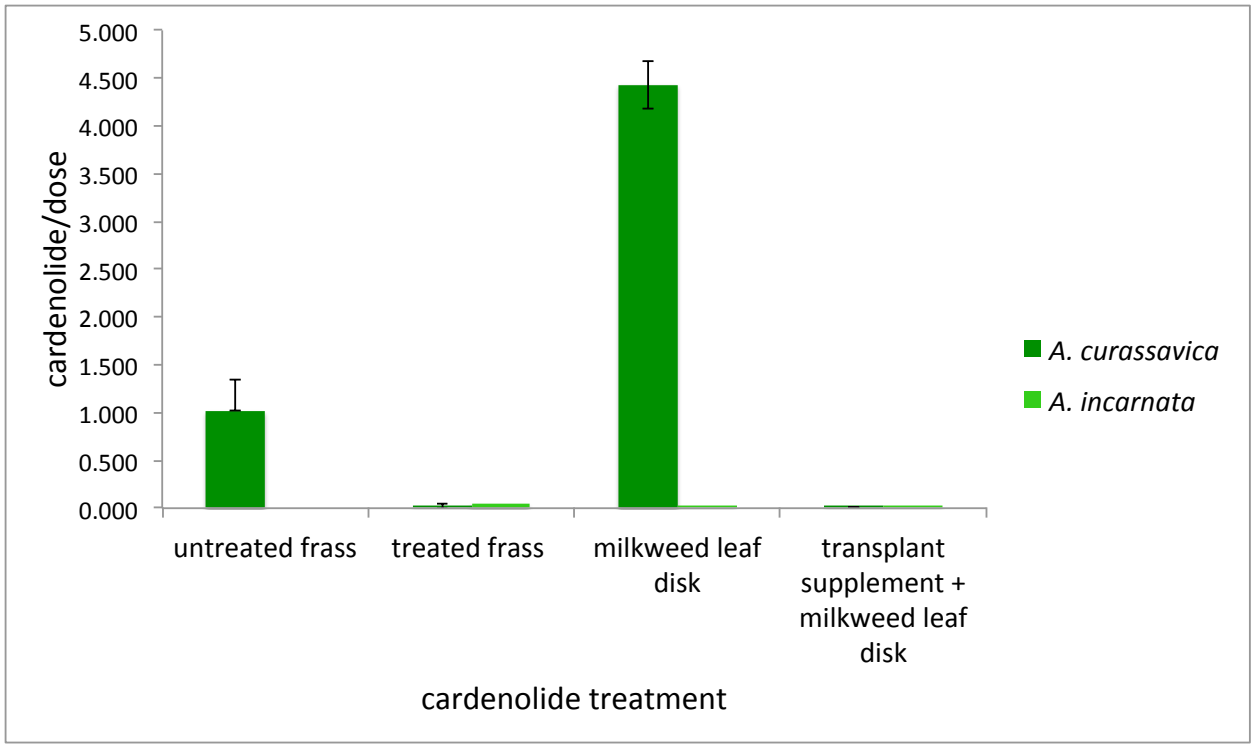

Figure S4.3. Cardenolide dosages for transplants and host plants. We compared the amount of cardenolides per dose of untreated frass, treated frass, milkweed leaf disks, and the transplant dose for Experiment 2. Transplant doses consist of a milkweed leaf disk with an inoculum of treated frass. For both experiments, the concentration of cardenolides significantly differed between the two host plant species (one-way ANOVA, $p = 0.006$), confirming previous findings (35). As expected, the total cardenolide concentration of *A. curassavica* foliage was 10-fold higher than *A. incarnata* foliage (one-way ANOVA, $p = 0.003$). In Experiment 1, the untreated frass from caterpillars reared on *A. curassavica* had 5-fold higher cardenolide concentration than the frass from caterpillars reared on *A. incarnata* (one-way ANOVA, $p = 0.02$), confirming that some cardenolides were carried over to recipient caterpillars during egg- and milkweed-supplementation with untreated frass. In Experiment 2, by treating the frass solution and extracting the microbes, we removed the transfer of cardenolides, with the concentration of cardenolides in the microbe supplements being negligible (one-way ANOVA, $p = 0.58$). This validates that treating frass with density centrifugation reduces the transfer of cardenolides, particularly for high-cardenolide *A. curassavica* plants, to recipient caterpillars.

4.2.4 Transplant Supplementation

Since the understanding of Lepidoptera microbiome dynamics is still in its infancy (Paniagua Voirol et al., 2018), we had little prior information about the appropriate time for microbial community transplants. To increase the likelihood of the microbial community transplant establishing in the gut, recipient caterpillars received two supplementations, first as an emerging hatchling from the eggshell (egg supplementation) and second as a second instar caterpillar feeding on a milkweed leaf disk (milkweed supplementation).

For egg transplant supplementations, recipient eggs were randomly assigned as one of two control types (*A. curassavica* control or *A. incarnata* control) or one of two transplant-supplemented treatments (*A. incarnata* with *A. incarnata* transplant or *A. incarnata* with *A. curassavica* transplant). Prior to hatching, control eggs with dark spots (indicating imminent hatching) were surface-sterilized with 20% bleach for 10s, 70% ethanol for 20s, and distilled water for 20s, then washed with 3 μl sterile PBS. Similarly, transplant-supplemented eggs were surface-sterilized with 20% bleach for 10s, 70% ethanol for 20s, and distilled water for 20s, but washed with 3 μl of the corresponding transplant solution. Hatched caterpillars were then reared on either *A. incarnata* or *A. curassavica* until maturing into second instars.

For the second transplant supplement, second instar larvae consumed a circular 0.8 cm diameter milkweed leaf disk coated with 3 μl transplant solution. Second instar larvae in the control treatments (i and ii) consumed a circular 0.8 cm diameter milkweed leaf disk coated with 3 μl sterile PBS.

4.2.5 Parasite Inoculation

Parasite (*Ophryocystis elektroscirrha)* inoculations occurred on the same day, but immediately prior to the transplant supplementations onto the same milkweed leaf disk. For the infected treatments, a standardized dose of 10 parasite spores was manually placed onto leaf disks, as described previously (de Roode, Gold, & Altizer, 2006). The parasite clone (C_1-E_{25}-P_3) was collected from an infected wild-caught monarch from the same population that we collected monarchs from. Uninfected caterpillars were fed leaf disks without parasites (Fig. S1). After caterpillars consumed their entire leaf disk (up to 48 hours), and thus their entire parasite inoculum and microbe transplant supplement, they were transferred to individual milkweed host plants enclosed in transparent tubes (5 inches x 22.5 inches) with mesh lids, maintained in a greenhouse with natural lighting. Caterpillars in the *A. curassavica* control treatment (i) were placed on *A. curassavica* plants; all other caterpillars were placed on *A. incarnata* plants. Monarchs were either reared to adulthood for parasite resistance assays or dissected as second instars for 16S rRNA analysis and qPCR-based quantification of the gut microbiome. One day following pupation, pupae were glued into 16 ounce solo cups and transferred to a climate-controlled room, maintained at 26°C and a 16L:8D cycle.

4.2.6 Parasite Resistance

Upon adult eclosion, monarchs were placed in 8.9 x 8.9 cm glassine envelopes, and maintained in a 12°C incubator (16L:8D) until death. After death, the parasite spore load of each butterfly was measured by methods described in (Jacobus C. de Roode, Chi, Rarick, & Altizer, 2009a). Briefly, monarch butterfly bodies were vortexed in 5ml sterilized water for 5min, and total spore loads in the water were estimated using a hemocytometer slide. Parasite spore load provides a

measure of parasite infection and growth, and is strongly negatively correlated with resistance (de Roode et al., 2008). We used linear models (one-way ANOVA) with normal error distributions to analyze the impact of treatment on monarch adult parasite spore load. Parasite spore load data was log 10-transformed to meet the assumption of normality. All statistical analyses were performed using R version 3.3.3 (Team, 2017).

4.2.7 Gut Microbiome Community Profiling

Caterpillars were surface-sterilized with 99% ethanol for 3 minutes. Guts were dissected using sterile instruments, then stored at -80°C. DNA was extracted using the Qiagen DNeasy PowerSoil kit (cat# 12888-100) and samples stored at -80°C. Extractions were sent to the University of Michigan's Center for Microbial Systems for PCR amplification, amplicon library preparation, and 16S rRNA sequencing. The 16S rRNA gene was amplified with barcoded dual-indexed primers 515F and 806R specific to the V4 region. The PCR cycle consisted of 2 min at 95°C, followed by 30 cycles of 95°C for 20 s, 55°C for 15 s, and 72°C for 5 min, followed by 72°C for 10 min. PCR reactions were normalized, pooled, and quantified for amplicon library preparation. Libraries were sequenced on an Illumina MiSeq platform with 250bp paired ends.

Bacterial sequences were processed and analyzed in mothur v1.40.3 (Kozich et al., 2013). Sequences were paired and trimmed. Sequences less than 250bp or greater that 289bp in length were removed from analysis since the targeted length for our method is 250bp. Reads were aligned to the V4 region of the 16S rRNA gene using the SILVA reference database. To further improve sequence quality, chimeras and non-bacterial 16S rDNA (i.e., Archaea, chloroplasts, and mitochondria) were removed. A mock community was co-sequenced (ZymoBIOMICS™ Microbial Community DNA Standard, cat# D6306) to determine the sequencing error rate,

which was 0.0082%. Operational taxonomic units (OTUs) were clustered based on 97% sequence similarity. Taxonomic assignments were determined using a Bayesian classifier and mapping sequences against the Ribosomal Database Project (RDP). Samples with less than 136 total reads were removed from the analysis. This minimum total read number optimized sequencing depth and sample size for the various treatment groups. Rarefaction curves confirmed the at least 97% coverage of each OTU analyzed. Visualizations and all statistical tests of 16S sequence data were performed in R v3.3.3 (R Development Core Team 2012) using packages phyloseq (McMurdie & Holmes, 2013) and vegan (Dixon, 2003).

4.2.8 Quantitative PCR

To determine differences in abundance of bacteria among recipient caterpillars fed on alternative milkweed-supplemented diets, mean copy numbers of 16S rRNA genes in a subset of samples were calculated (n=31). Each sample was amplified in triplicate, except for four samples amplified in duplicate due to lack of DNA, with the same 16S rRNA primers used for PCR amplification, 515F and 806R. Primers and reaction conditions are described in Hammer et al. (Hammer et al., 2017). Standard curves were calculated using purified genomic *E. coli* DH10B cells (ThermoFisher Scientific, cat #18290015). To calculate the starting copy number for the standard curve, we used the copy number calculator for real time PCR on scienceprimer.com, and generated the standard curve in relation to the serial dilution of 1:10. Standard copy number started at 1.6×10^{11} and was diluted to 10^{4}. No samples were considered out of range. We estimated the absolute number of 16s copies in each sample based on the standard curve. The mean of triplicates, and in four cases duplicates, was used for analyses.

4.2.9 Transplant and foliar chemical analyses

We measured the concentration of cardenolides for four types of samples: (1) untreated frass solution in PBS, (2) treated microbe solution in PBS, (3) milkweed plant (*A. curassavica* and *A. incarnata*) only and (4) transplant supplement (frass solution and microbe solution) plus the milkweed leaf disk it was transferred onto (Fig. S4.3).

Transplant and foliar cardenolides were measured using reverse-phase ultra-performance liquid chromatography (UPLC; Waters Inc., Milford, MA, USA) with methods described previously (Tao & Hunter, 2012; Zehnder & Hunter, 2007). On an absorbance range of 200 to 300 nm, cardenolides were determined by symmetrical peak absorption maxima between 216 and 222 nm. Total cardenolide concentration was calculated by the sum of all separated cardenolide peaks, corrected by the concentration of the internal standard (digitoxin) and sample mass.

4.3 Results

4.3.1 Influence of Diet on Parasite Sporeload

4.3.1.1 Experiment 1

Parasite sporeload was measured for each diet treatment: *A. curassavica* control, *A. incarnata* + *A. curassavica* frass, *A. incarnata* + *A. incarnata* frass, and *A. incarnata* control (Fig. 4.1A). There was an effect of diet treatment on parasite sporeload (one-way ANOVA, $p = 0.005$). To determine which diets were driving these differences in parasite sporeload, we performed Tukey's Test with pairwise comparisons and adjusted the p-values. As expected based on previous studies, control caterpillars reared on medicinal *A. curassavica* experienced lower parasite growth than control caterpillars reared on non-medicinal *A. incarnata*, confirming the

medicinal effects of *A. curassavica* ($p_{adjusted}$= 0.006). Supplementing the non-medicinal *A. incarnata* with frass reduced parasite growth, thus increasing parasite resistance. Interestingly, this effect occurred for all caterpillars receiving a frass transplant, whether the transplant derived from caterpillars reared on the medicinal *A. curassavica* ($p_{adjusted}$= 0.031) or the non-medicinal *A. incarnata* ($p_{adjusted}$= 0.044). Since *A. curassavica* control, *A. incarnata* + *A. curassavica* frass, and *A. incarnata* + *A. incarnata* frass treatments all had lower parasite growth in comparison to *A. incarnata* control, these three diet treatments are hereafter referred to as having a low mean parasite sporeload (low) in comparison to the *A. incarnata* control (high).

4.3.1.2 Experiment 2

Similar to Experiment 1, there was an effect of diet treatment on parasite sporeload (one-way ANOVA, p = 0.013, Fig. 4.1B). To better understand the drivers of these differences in parasite sporeload, we again performed Tukey's Test and adjusted the p-values. Similar to Experiment 1, control caterpillars reared on *A. curassavica* experienced lower parasite growth than control caterpillars reared on *A. incarnata* ($p_{adjusted}$= 0.05). However, unlike Experiment 1, only one microbe transplant diet treatment resulted in reduced parasite growth: supplementing the non-medicinal *A. incarnata* plant with microbes from caterpillars reared on *A. curassavica* reduced parasite growth (one-way ANOVA, p= 0.025), but no such effect occurred for microbes derived from caterpillars fed on *A. incarnata* (one-way ANOVA, p= 0.88). Since *A. curassavica* control and *A. incarnata* + *A. curassavica* microbes treatments had lower parasite growth in comparison to *A. incarnata* control and *A. incarnata* + *A. incarnata* microbes treatments, we will refer to these two sets of treatments as "low" and "high" sporeload from hereon.

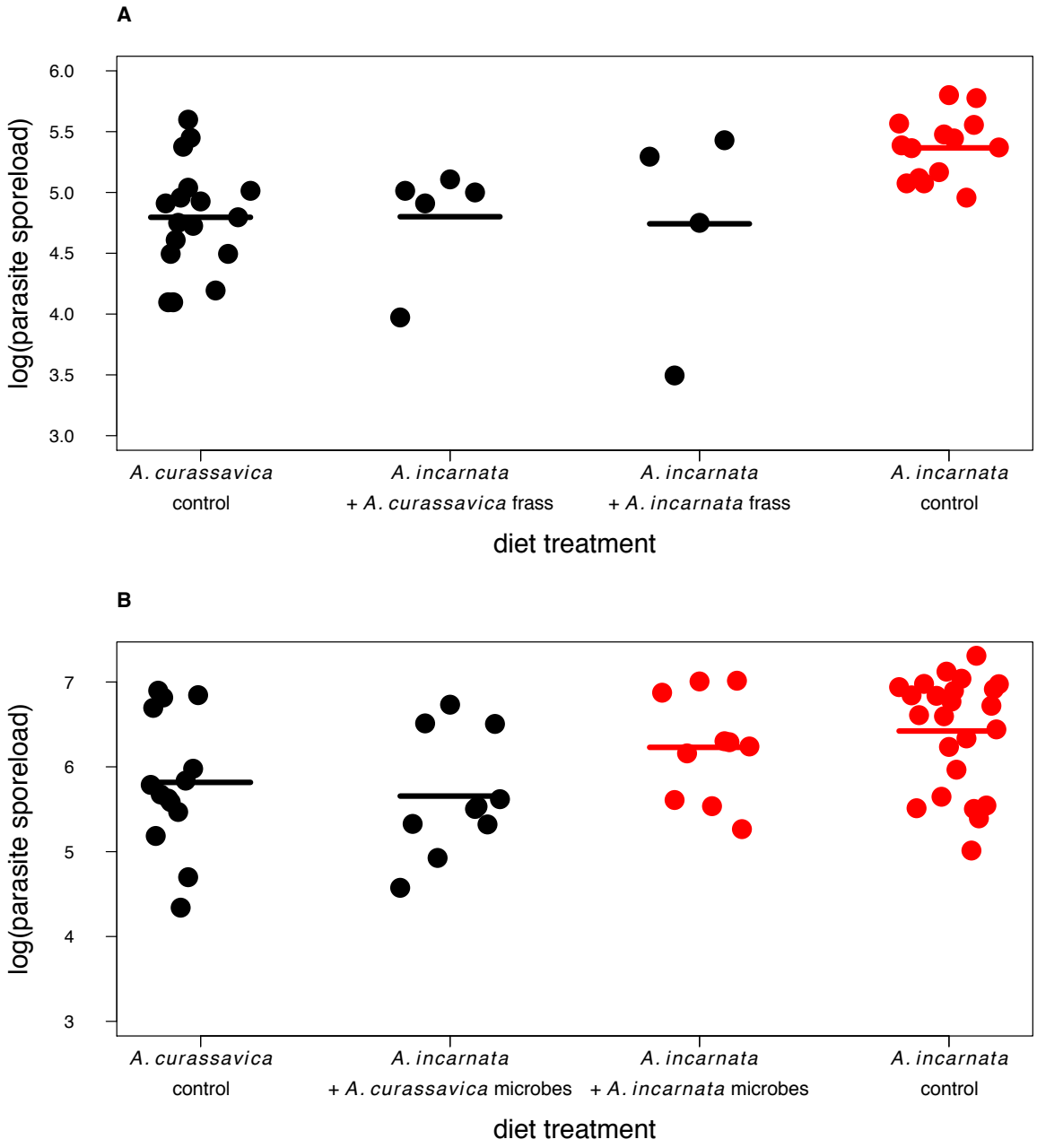

Figure 4.1. Parasite sporeload for monarchs reared on alternative diets. (A) In Experiment 1, caterpillars were either fed a control diet of *A. curassavica* foliage ('*A. curassavica* control') or *A. incarnata* foliage ('*A. incarnata* control'), or were fed *A. incarnata* foliage supplemented with frass obtained from caterpillars reared on *A. curassavica* or on *A. incarnata*. (B) In Experiment 2, similar treatments were used, except that supplemented caterpillars received purified gut microbes as opposed to whole frass. Data points represent individual monarch adults, and horizontal bars represent means. Red indicates treatments with significantly higher parasite spore loads than other treatments, which are in black. In the remainder of the paper these treatments are referred to as "high" (red) and "low" (black) respectively.

4.3.2 Membership of the Monarch Gut Microbiome

4.3.2.1 Experiment 1

After quality filtering and preprocessing, 28,937 sequences were retrieved from 22 caterpillar samples. Sequences were clustered into 401 operational taxonomic units (OTUs) with 97% sequence similarity. Dominant taxa, or taxa accounting for at least 75% of a sample's composition, included *Pantoea*, *Asaia*, *Chryseobacterium, Pseudomonas* and unclassified Enterobacteriaceae (Fig. 4.2A). *Pantoea* and unclassified Enterobacteriaceae were the most prevalent, being present in 100% of the samples, suggesting that there is a core microbiome. These taxa were present in only 30% of our negative sequencing controls, confirming that these two prevalent taxa were not a sequencing error.

4.3.2.2 Experiment 2

After quality filtering and preprocessing, 28,937 sequences were retrieved from 17 caterpillar samples. Sequences were clustered into 435 operational taxonomic units (OTUs) with 97% sequence similarity. Dominant taxa were similar to those in Experiment 1, including *Pantoea*, *Asaia*, *Chryseobacterium, Pseudomonas, Sphingobacterium* and unclassified Enterobacteriaceae (Fig. 4.2B). *Pantoea* and unclassified Enterobacteriaceae were again present in 100% of the samples, strengthening the argument that there is a core microbiome for monarchs.

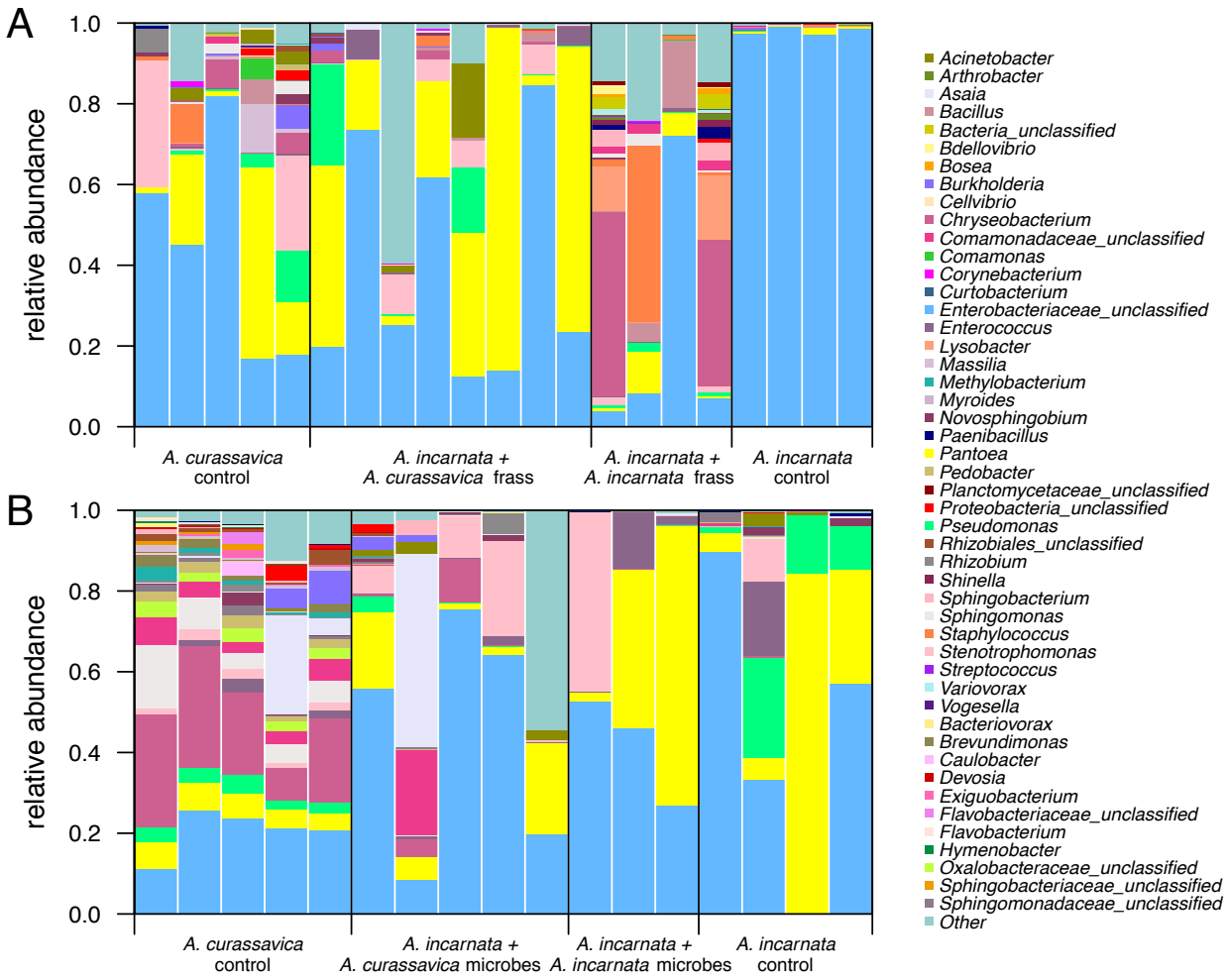

Figure 4.2. Gut bacterial community composition for caterpillars reared on alternative diets. Stacked bar plots show the relative abundance of the top 50 genera. Each bar represents the bacterial community composition of an individual caterpillar gut in Experiment 1 (A) or Experiment 2 (B). OTUs corresponding to the same taxa are grouped together.

4.3.3 Changes in Community Composition Based on Alternative Diets and Parasite Load

4.3.3.1 Experiment 1

There was no difference between diet treatments and bacterial community composition (PERMANOVA p = 0.67, Fig S4.4A). However, once control and transplant-supplemented diets were grouped based on whether they were associated with a low or high mean parasite spore load, there was a significant difference (PERMANOVA, p = 0.013, Fig S4.4B).

To tease apart the properties of the gut microbiome that may influence the overall community structure between low- and high-spore load treatments, we measured alpha diversity, OTU relative abundance, and OTU presence and absence. Alpha diversity was quantified with the Shannon diversity index, taking into account both OTU richness and evenness (Fig. 4.3A). High parasite spore load treatments had significantly lower Shannon diversity in comparison to the low spore load treatments (t = -4.018, p = 0.0007). Furthermore, there were differences in means within the low parasite sporeload treatments. Specifically, the *A. incarnata* + *A. incarnata* frass treatment was more diverse than *A. incarnata* + *A. curassavica* frass treatment (one-way ANOVA, $p < 0.001$) and *A. curassavica* control treatment (one-way ANOVA, $p < 0.02$).

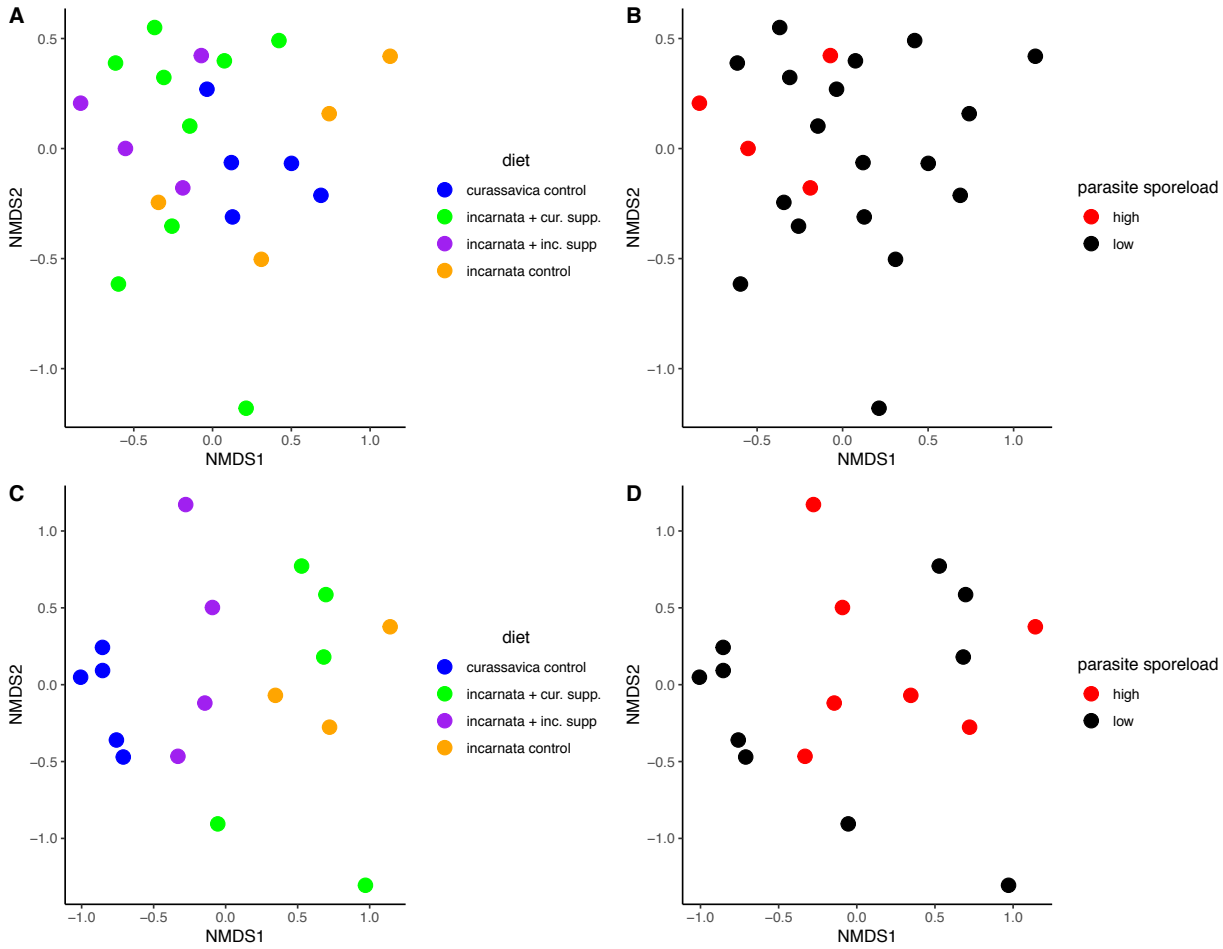

Figure S4.4. Effect of diet and parasite load on community structure. (A) For Experiment 1, there is no significant relationship between diet treatment and the overall gut community composition (PERMANOVA p = 0.67). (B) For Experiment 1, there is a significant relationship between parasite sporeload, which was influenced by diet (Fig. 1), and gut community composition (PERMANOVA, p = 0.013). (C) For Experiment 2, there is again no significant relationship between diet treatment and the overall gut community composition (PERMANOVA p = 0.097). (D) For Experiment 2, as with Experiment 1, there is significant relationship between parasite sporeload, which was influence by diet (Fig. 1), on gut community composition (PERMANOVA, p=0.05).

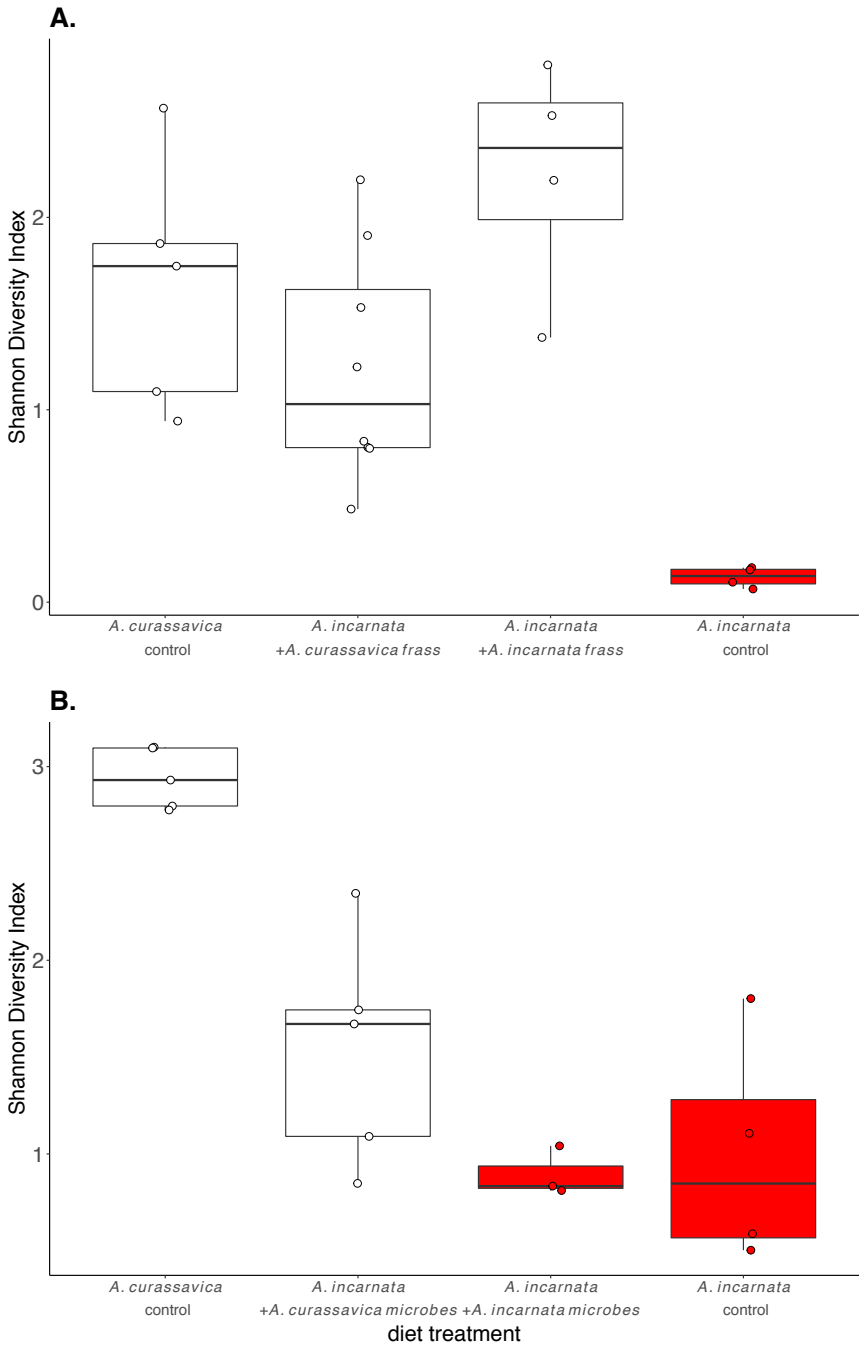

Figure 4.3. High microbial diversity corresponds to a lower mean parasite sporeload. Shannon diversity of gut microbial communities of caterpillars fed on four alternative diets: *A. curassavica* foliage ('*A. curassavica* control'), *A. incarnata* foliage with a transplant of frass or microbes from *A. curassavica*-fed donors ('*A. incarnata* + *A. curassavica* transplant'), *A. incarnata* foliage with a transplant of frass or microbes from *A. incarnata*-fed donors ('*A. incarnata* + *A. incarnata* transplant.') and *A. incarnata* foliage ('*A. incarnata* control'). Box plots in red indicate diet treatments associated with significantly lower parasite spore loads (Fig. 1). (A) Experiment 1, (B) Experiment 2.

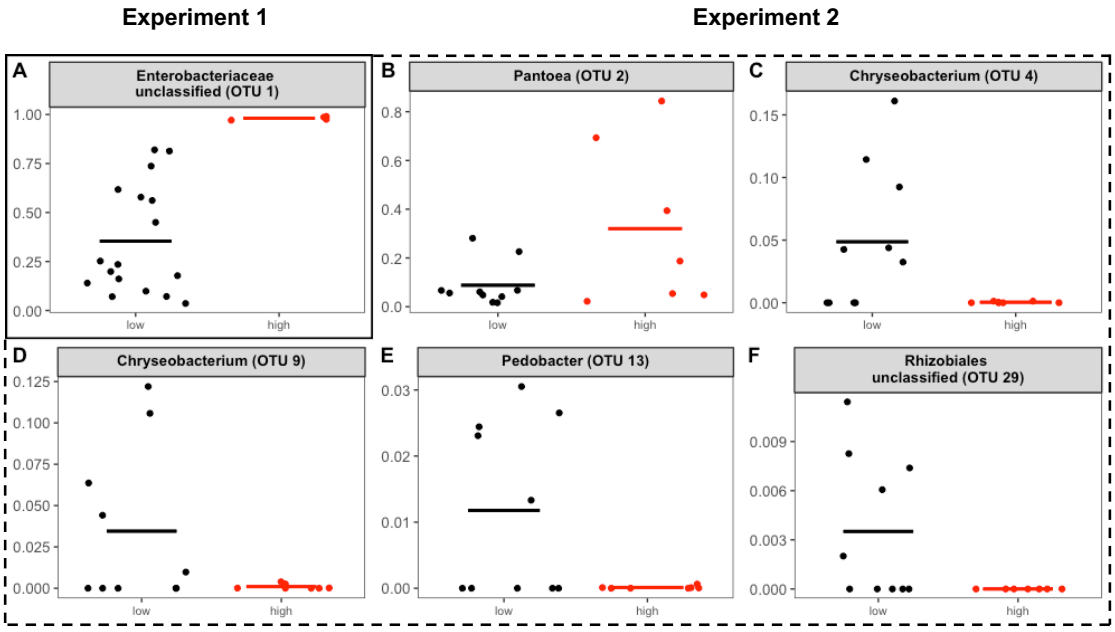

Figure 4.4. Relative abundance of some OTUs in caterpillar gut microbial communities differs in relation to parasite sporeload. OTUs from the top 50 most abundant genera whose relative abundance significantly differed across treatments associated with low (black) and high (red) parasite sporeloads (diet treatments associated with low and high parasite sporeloads are indicated in Fig. 1). (A) Experiment 1, (B – F) Experiment 2. Points represent individual gut samples, and horizontal bars represent means.

We measured the relative abundance of the top 50 OTUs (Fig 2). There were no specific OTUs that were influenced by diet treatments. However, there was one OTU, *Enterbacteriaceae_unclassified* (OTU 1), whose relative abundance was correlated with the mean parasite spore load between high and low treatments (LME, p = 0.0001; Fig 4.4A). For high parasite spore load treatments, *Enterbacteriaceae_unclassified* was highly abundant ranging from 90-100%. Having *Enterbacteriaceae_unclassified* in large abundance is thus associated with lower parasite resistance. With respect to absence/presence of microbes, we found that the presence – but nor relative abundance – of Comamonadaceae (OTU 22) (LME, p = 0.05) and *Corynebacterium* (OTU 50) (LME, p = 0.05) was significantly associated with lower parasite spore load.

4.3.3.2 Experiment 2

Similar to Experiment 1, control and microbe transplant supplemented diets did not significantly alter overall bacterial communities (PERMANOVA, p = 0.097, Fig S4.4C), but once again, there was a significant difference in bacteria community composition for high- and low-parasite sporeload treatments (PERMANOVA, p = 0.053, Fig S4.4D). It is important to note that there was an increase in microbial diversity of the non-medicinal *A. incarnata* control treatment compared to the same treatment in Experiment 1. This diversity increase in *A. incarnata* control may contribute to more similarities between this diet treatment and the other diet treatments.

For alpha diversity, similar to Experiment 1, high parasite sporeload treatments had significantly lower Shannon diversity compared to low sporeload treatments (t = -3.033 , p = 0.008, Fig. 4.3B). Furthermore, there were differences in means within the low parasite sporeload treatments. For example, the medicinal *A. curassavica* control treatment had a higher

Shannon diversity index than the *A. incarnata* + *A. curassavica* microbes treatment (t = -1.53 , p = 0.01). These findings suggest that microbes supplemented from *A. curassavica*-fed caterpillars contributed to the increase in gut community diversity of recipient caterpillars fed on non-medicinal *A. incarnata*.

Specific OTUs that are correlated with the difference between high and low parasite load treatments were *Pantoea* (OTU 2), *Chryseobacterium* (OTU 4 and OTU 9), *Pedobacter* (OTU 13), and *Rhizobiales_unclassified* (OTU 29) (Figure 4.4B-4F). The mean proportion of *Pantoea* (OTU 2) was significantly higher for high mean parasite sporeload treatments than low sporeload treatments (t = 5.6, p =0.02) suggesting that an abundance of this microbe may result in lower parasite resistance. However, this trend was reversed for *Chryseobacterium* (OTU 4 and OTU 9), *Pedobacter* (OTU 13), and *Rhizobiales_unclassified* (OTU 29), suggesting that a higher proportion of these microbes may confer protection against parasite infection (t = 3.1, p = 0.002, t = 1.7, p = 0.04; respectively).

With respect to presence/absence of microbes, presence of *Rhizobiales_unclassified* (OTU 29), *Brevundimonas* (OTU 36), and *Hymenobacter* (OTU 42) was significantly associated with lower parasite sporeload. *Rhizobiales_unclassified* is the only OTU for which both the presence and relative abundance were associated with lower parasite sporeload. The relative abundance of neither *Brevundimonas* nor *Hymenobacter* was significantly associated with disease outcome. All three of these OTUs were present in very low abundances.

4.3.4 Changes in Microbial Abundance across Treatments

4.3.4.1 Experiment 1

The mean copy 16s rRNA copy number was significantly lower in samples within the *A. incarnata* control treatment than in all other diet treatments (one-way ANOVA, $p = 0.019$, Fig. 4.5A). Interestingly, *A. incarnata* control is the same diet treatment that was associated with the highest parasite spore load. This suggests that low microbial abundance in the gut microbial community is a property that is associated with low parasitic resistance.

4.3.4.2 Experiment 2

The mean 16s rRNA copy number of sampled in the *A. incarnata* control and *A. incarnata* + *A. incarnata* treatments was significantly lower than those of the *A. curassavica* control and *A. incarnata* + *A. curassavica* diet treatments (one-way ANOVA, $p = 8.3 \times 10^{-5}$, Fig 4.5B). Coincidentally, the two diet treatments with the lowest microbial abundance are also associated with the high parasite sporeloads. These results are in agreement with Experiment 1 and suggest that low microbial abundance is associated with low parasite resistance in this monarch-milkweed-parasite system.

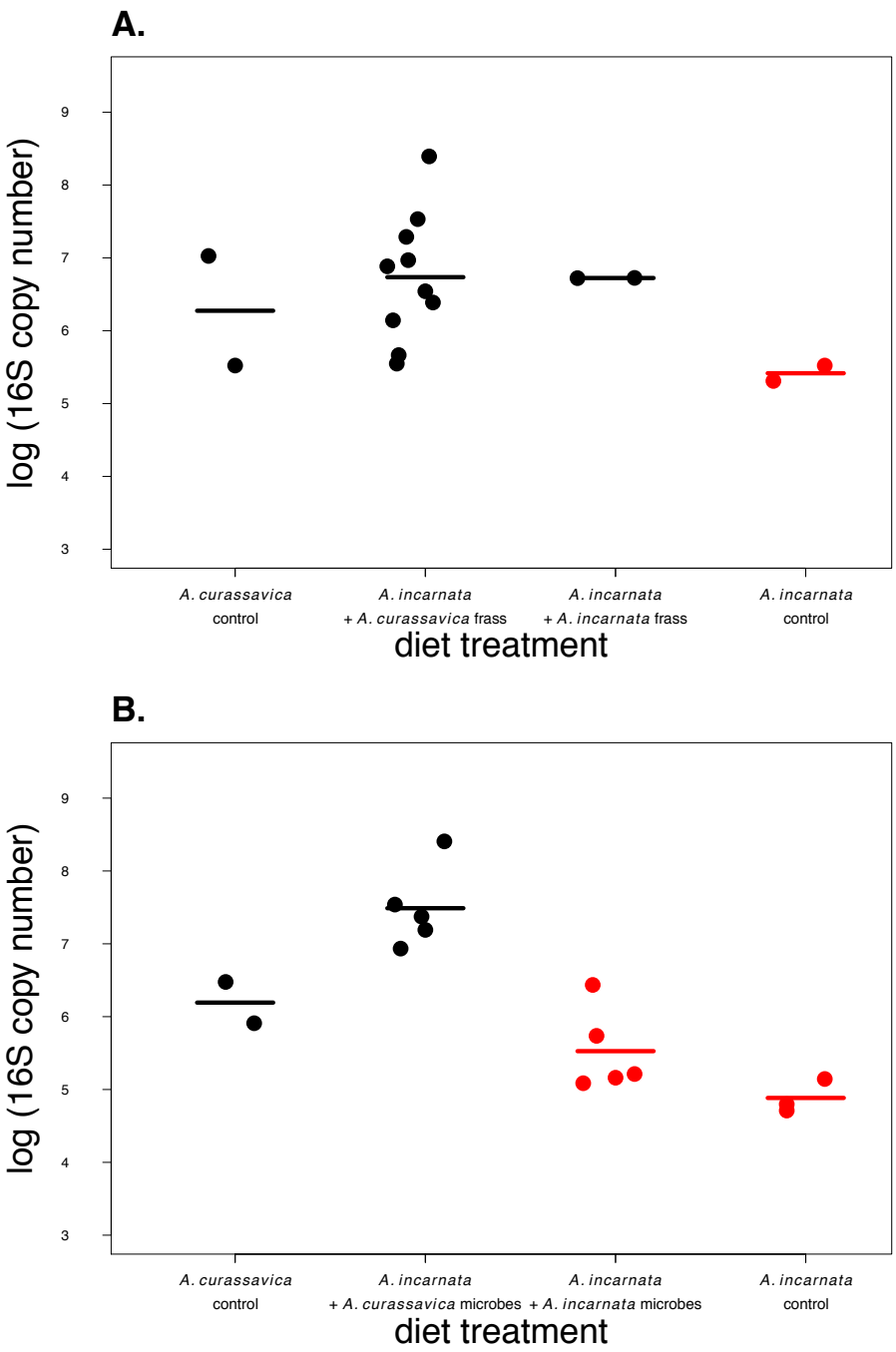

Figure 4.5. Higher microbial abundance corresponds to a lower mean parasite sporeload. Shown are the $\log_{10}$ 16S copy number per sample. Each sample is a second instar gut. Treatments are as in Figs. 4.1 and 4.3. Points represent individual samples, and horizontal bars represent means.

4.4 Discussion

In contrast with prevailing views, our study suggests that the gut microbiome can be an important driver of Lepidopteran fitness. While existing studies have investigated the role of gut microbes on life history traits, such as growth, survival and development time (Hammer et al., 2017; Phalnikar et al., 2019; Ravenscraft et al., 2019), we studied the role of the microbiome in the context of natural enemies. Focusing on monarch butterflies and their prevalent protozoan parasite *Ophryocystis elektroscirrha*, we found that both microbiota composition and microbial abundance were significantly associated with parasite resistance. By supplementing larval frass or microbes derived from frass onto milkweed diets, we altered recipient caterpillars' gut microbiomes and thereby increased parasite resistance. These findings are in line with a growing appreciation of indirect relationships between host diet, the gut microbiome, and disease resistance (Daisley et al., 2020; Linenberg et al., 2016; Maes et al., 2016; Villarino et al., 2016b). Indeed, our work provides some of the first evidence for the existence of indirect diet effects on disease resistance through modulation of the gut microbiome (Harris et al., 2019). Although other studies have shown the three, separate relationships (diet altering the gut microbiome; diet modulating parasite resistance; gut microbiome modulating parasitism), our study demonstrates how these three relationships can interact to provide parasite resistance based on diet-induced microbiome modulation.

The anti-parasitic effects of high-cardenolide milkweeds are well established in the monarch-parasite system (Gowler et al., 2015a; Lefèvre et al., 2010; Sternberg, Lefèvre, et al., 2012a; Tao et al., 2016), but the mechanism by which these medicinal effects occur have remained unknown. In principle, medicinal milkweeds' protective mechanism on parasite growth could be direct or indirect; milkweed chemicals could directly interfere with parasite infection or

growth, could stimulate anti-parasitic immunity or could alter the microbiome to an anti-parasitic state. Direct toxic effects have been hard to study, because *O. elektroscirrha* cannot be cultured outside live monarchs. However, previous work has investigated the effect of medicinal milkweeds on monarch immunity. While diets have been shown to enhance immunity in other Lepidoptera (Cotter et al., 2019), we have not found such evidence in monarchs. Indeed, when rearing monarch caterpillars on the non-medicinal *A. incarnata* and the medicinal *A. curassavica*, we did not find upregulation of immune genes in the monarchs reared on *A. curassavica*. In contrast, we observed down-regulation of four immune genes, suggesting that the use of medicinal milkweed can in fact reduce investment in canonical immunity (Smilanich & Nuss, 2019; W. Tan et al., 2019).

In this study, we tested the third hypothesis: that medicinal milkweeds can modulate monarch gut microbial communities to an anti-parasitic state. Similar to previous monarch-milkweed-parasite studies, we found that monarch caterpillars reared on the medicinal *A. curassavica* experienced lower parasite growth than caterpillars reared on the non-medicinal *A. incarnata* (Jacobus C. de Roode et al., 2009a; de Roode et al., 2008; Gowler et al., 2015b; Sternberg, Lefèvre, et al., 2012a; W.-H. Tan et al., 2018). Importantly, we show that these alternative milkweed diets are also associated with distinct larval gut microbial community composition, and that supplementing diets with microbes from caterpillars fed on medicinal milkweed can transfer those medicinal properties. Diet-microbiome relationships have been observed in several other Lepidoptera species. Gypsy moths feeding on four alternative species of deciduous trees and an artificial diet had distinct gut microbial communities (Broderick et al., 2004). Similarly, corn earworm moths' gut microbial communities varied between the alternative diets of eight host plant species and an artificial diet (Priya, Ojha, Kajla, Raj, & Rajagopal,

2012c). Furthermore, the tobacco budworm is another moth species in which alternative diets of tobacco, cotton, and chickpea all result in a distinct gut microbial community in both field and lab reared groups (Staudacher et al., 2016). However, not all Lepidopteran gut microbial communities are associated with different larval diets (Whitaker et al., 2016).

While our study is the first to show that microbe transplants can reduce parasitism in Lepidoptera, previous studies have shown similar effects in other animals. For example, feeding bumblebees fecal matter from nest mates can reduce infection with trypanosomes (Koch & Schmid-Hempel, 2011b), and supplementing diet with isolated microbes can decrease infection of *Paenibacillus* and *Nosema* in honeybees (Daisley et al., 2020; Maes et al., 2016) and *Plasmodium* in mosquitoes and mice (Linenberg et al., 2016; Villarino et al., 2016b). In the current study, one property of the gut microbiome that was associated with disease resistance was the relative abundance of specific microbes, including *Pantoea*, *Chryseobacterium* and *Pedobacter,* and unclassified Enterobacteriaceae and Rhizobiales. Unclassified Enterobacteriaceae and *Pantoea* are commonly found in other Lepidopteran gut microbiomes (Minard et al., 2019; Whitaker et al., 2016). When present in high abundance, these taxa made monarchs more susceptible to parasite growth. It is possible that these taxa are good competitors that outcompete other microbes during early colonization of the gut. *Pantoea* is a genus of Enterobacteriaceae that is found in Lepidoptera (Whitaker et al., 2016), stinkbugs (Duron & Noël, 2016; Kashkouli, Fathipour, & Mehrabadi, 2020), and environmental plant and soil samples (Berg et al., 2002; Kuklinsky-Sobral et al., 2004; von Bodman, Bauer, & Coplin, 2003). Another key taxon in the monarch microbiome was *Chryseobacterium*; when present and in relatively high amounts, this taxon was associated with a lower parasite sporeload. *Chryseobacterium* is commonly found in other insects such as midges (Campbell, Mummey,

Schmidtmann, & Wilson, 2004), tsetse flies (Geiger et al., 2011), cockroaches (Dugas, Zurek, Paster, Keddie, & Leadbetter, 2001) and other Lepidoptera (Eski, Demir, Güllü, & Demirbağ, 2018; Secil, Sevim, Demirbag, & Demir, 2012). The specific role these taxa play in monarch butterfly fitness needs to be explored. Future diet-gut microbiome-parasite studies using monarchs should use these OTUs, if cultivable, as candidate taxa to selectively supplement onto milkweed diets and measure disease outcome.

An important remaining question is how monarch microbiomes are assembled, and what the origin is of the microbes inhabiting the monarch gut. Microbes could be vertically transmitted from mothers during oviposition or horizontally acquired from their surrounding environments. Extracellular symbiont transmission routes for several insects orders including Hemiptera, Hymenoptera, Diptera, and Coleoptera, are well-known (Salem, Florez, Gerardo, & Kaltenpoth, 2015). While symbiont transmission routes for Coleoptera and Diptera are primarily vertical with egg and oviposition site inoculation, Hemiptera and Hymenoptera utilize a variety of vertical and horizontal transmission routes. However, microbial transmission in Lepidoptera is still unresolved (Paniagua Voirol et al., 2018). Currently, there is little evidence of egg-associated bacteria being present in the gut lumen (Brinkmann, Martens, & Tebbe, 2008), but this is mainly because there is less data available about Lepidoptera egg-associated communities (Hammer et al., 2014; Phalnikar et al., 2018a). In terms of horizontal transmission, milkweed-associated microbes could also be the microbes that ultimately colonize and persist in the gut lumen. Recently, there has been evidence suggesting that Lepidoptera gut-associated microbial community composition greatly overlaps with larval food plant-associated communities in several butterfly species (Phalnikar et al., 2018b). More specifically, for three butterfly species, 80% of the larval diet OTUs comprised the dominant bacteria in larval guts including *Prevotella*

copri and Methylobacteriaceae. Furthermore, a comparison between six Lepidopteran host species revealed that plant microbial DNA sequences account for the majority of microbial sequences in the gut and fecal microbial communities (Hammer et al., 2017). Another scenario is that the gut microbes are not derived from a particular source, but that milkweeds instead modulate existing gut communities to an alternative state. For example, in the case of the Glanville fritillary butterfly, the microbial communities between host plants and caterpillars gut were very different (Minard et al., 2019), but plant metabolite composition was a determinant driver of key OTU presence and abundance.

Numerous studies have characterized the microbial communities associated with hosts across animal taxa (Huttenhower et al., 2012; Runckel et al., 2011) and examined how insect microbial communities respond to alternative diets (Billiet et al., 2016a; Broderick et al., 2004; Chandler et al., 2011), how insect diets modulate parasite resistance (Kelly & Bowers, 2018; Linenberg et al., 2016; Leif L Richardson et al., 2015), and how manipulating insect gut microbial communities can affect disease outcome (Ramirez et al., 2014). Our study unifies these separate, pairwise interactions by demonstrating that diets can affect parasite resistance through modulation of the gut microbiome. Using a natural Lepidopteran system, we demonstrate that butterfly-associated gut microbial communities have a functional role in Lepidopteran fitness by increasing host resistance to natural parasites when fed medicinal plant diets. Future studies with other animal systems should test if this indirect three-way link is a general relationship in nature. Our study emphasizes the importance of considering host fitness traits in the context of their surrounding environment and natural enemies.

4.5 Acknowledgements

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Chapter 5: Conclusions and Future Directions

In this dissertation, I studied monarch butterfly associated microbial communities in the context of disease resistance. In chapter 3, I studied how ontogenetic development and larval milkweed diet influence variation in monarch microbial communities. In chapter 4, I studied how larval milkweed diet affects disease resistance through modulation of the gut microbiome. Here, I summarize the main findings and discuss the future directions for each chapter.

5.1 Discussion of Chapter 3

We observed the microbial community composition dynamics of the monarch butterfly across its lifecycle using culture-independent methods. Building on culture-dependent methods (Kingsley, 1972), our results broaden our understanding by including the characterization of microbial communities of monarch adult parents and their eggs, as well as the bacterial loads of all developmental stages. More specifically, we show that the microbial community of eggs is not similar to their parents, suggesting that the core of the gut microbiome is not vertically transmitted during oviposition (Hammer et al., 2017; Paniagua Voirol et al., 2018; Salem et al., 2015). Furthermore, we observed that adult gut microbial communities are distinct from those of all other developmental stages, similar to other studies comparing microbial community dynamics across developmental stages (Hammer et al., 2014; Phalnikar et al., 2018b). This is most likely due to drastic dietary shifts across development, from feeding on foliage as larvae to feeding on liquid as adults.

An important remaining question is how monarch microbiomes are assembled, and what the origin is of the microbes inhabiting the monarch gut. Microbes could be vertically

transmitted from mothers during oviposition or horizontally acquired from their surrounding environments. Extracellular symbiont transmission routes for several insects orders, including Hemiptera, Hymenoptera, Diptera, and Coleoptera, are well-known (Salem et al., 2015). While symbiont transmission routes for Coleoptera and Diptera are primarily vertical, with egg and oviposition site inoculation, Hemiptera and Hymenoptera utilize a variety of vertical and horizontal transmission routes. However, microbial transmission in Lepidoptera is still unresolved (Paniagua Voirol et al., 2018). Currently, there is little evidence of egg-associated bacteria being present in the gut lumen (Brinkmann et al., 2008), but this may be because of the paucity of data available on egg-associated microbial communities of Lepidoptera. Given that we show that there is little overlap between microbial communities of adult and eggs, and that the plant species on which they feed impacts the gut microbial community of early instar larvae, we hypothesize that much of the monarch microbiome is environmentally acquired from the foliage on which they feed.

5.2 Discussion of Chapter 4

Our study is the first to show that microbe transplants can reduce parasitism in Lepidoptera. Previous studies have shown similar effects in other animals. For example, feeding bumblebees fecal matter from nest mates can reduce infection with trypanosomes (Koch & Schmid-Hempel, 2011b), and supplementing diet with isolated microbes can decrease infection of *Paenibacillus* and *Nosema* in honeybees (Daisley et al., 2020; Maes et al., 2016) and *Plasmodium* in mosquitos and mice (Linenberg et al., 2016; Villarino et al., 2016b). In the current study, one property of the gut microbiome that was associated with disease resistance is the relative abundance of specific microbes, including bacteria in the genera *Pantoea*, *Chryseobacterium, Rhizobiales* and

Pedobacter, and other bacteria in the *Enterobacteriaceae*. Bacteria in the *Enterobacteriaceae*, and *Pantoea* spp. specifically, are commonly found in other Lepidopteran gut microbiomes (Minard et al., 2019; Whitaker et al., 2016). It is possible that these taxa are good competitors that outcompete other microbes during early colonization of the gut. Another key taxon in the monarch microbiome was *Chryseobacterium*; when present and in relatively high amounts, this taxon was associated with a lower parasite sporeload. *Chryseobacterium* is commonly found in other insects, such as midges (Campbell et al., 2004), tsetse flies (Geiger et al., 2011), cockroaches (Dugas et al., 2001) and Lepidoptera (Eski et al., 2018; Secil et al., 2012). The specific role that these taxa play in monarch butterfly fitness needs to be explored. Future diet-gut microbiome-parasite studies using monarchs should use cultivable representatives of these OTUs as candidate taxa to selectively supplement milkweed, or, if available, artificial diets, and measure disease outcome.

In Chapter 4, beyond specific taxa whose abundances were correlated with disease resistance, other properties of the microbiome that were associated with higher disease resistance included higher community diversity and higher microbial abundances. This work sets the stage to try to disentangle how these various characteristics of the microbiome mediate disease resistance.

5.3 Potential Mechanisms of Resistance

While it is well supported that alternative milkweed diets differing in cardenolide concentration provide resistance to *Ophryocystis elektroscirrha* in monarch butterflies (de Roode et al., 2008; Gowler et al., 2015a; Sternberg, Lefèvre, et al., 2012a; Tao et al., 2016), this dissertation strongly suggests that gut microbial communities are also contributing to parasitic resistance. More

specifically, we demonstrate that by transferring microbial communities associated with a medicinal milkweed diet, we consequentially transfer the resistance properties of that diet. However, the mechanism by which medicinal milkweed interacts with gut microbes to increase host resistance is unknown. The gut microbiome may directly interact with parasites during infection of the gut lumen or indirectly modulate parasite resistance through the immune system or modulation of nutritional resources. The modulation of nutritional resources is probably not the case since both milkweed host plants, *A. curassavica* and *A. incarnata*, have similar foliar phosphorous and nitrogen levels and differ substantially in cardenolide concentration (de Roode et al., 2008; Tao et al., 2016). The monarch immune system is modulated in response to the two host plant species. Surprisingly, there are only a small number of immune genes were down-regulated in monarchs fed medicinal, *A. curassavica* (W. Tan et al., 2019). Although only a few genes, these activated immune genes may still have a role in parasite inhibition. For example, a few mosquito host immune lectin and protease genes are up-regulated when mosquitoes infected with *Plasmodium* are colonized with a certain strain of *Serratia marcescens* (Bai et al., 2019).

Alternatively, the microbiome may interact with parasites directly during infection. In mosquitoes, gut bacteria directly inhibit parasite growth and development with secretions of metabolites, reactive oxygen species and proteins (Gao et al., 2019; Romali & Gendrin et al., 2018). In monarchs, one possibility to test this hypothesis would be to perform *in vitro* competition assays between the protozoan parasites and cultivable gut microbes induced by milkweed host plant. A limitation to the monarch system is that the parasite has not yet been cultivated independent of the host. To work around this limitation, another method to test for a direct interaction of the gut microbiome with the parasite could be the use of metagenomics, metatranscriptomics and metabolomics which are feasible in insect systems (Malacrino 2018;

Quinn et al., 2020). A possible future experiment would be to collect gut samples of caterpillars fed alternative diets and taken at different time points of infection to observe the microbial genes present and being expressed. The results would identify candidate microbial taxa and genes associated with parasitic resistance. Candidate genes can be manipulated in future experiments to elucidate a mechanism. This mechanism would inform other insect systems on the host diet-gut microbiome-parasite interactions.

In the context of evolution, there could possibly be coevolution between the host diet, parasite and gut microbiome. The monarch butterfly-milkweed system has a commonly occurring gut-associated parasite. There may be stronger selection on the host to maintain association with a protective gut microbiome. This selection, in turn, could shape the host's use of immunity to protect itself from natural enemies. This could also explain why it has been difficult for previous Lepidoptera-gut microbiome studies to observe functional effects of gut microbial community in these hosts. This avenue should be further explored with coevolution passage experiments.

5.4 Broader Impacts

Although we utilize a monarch-milkweed-parasite system, our findings can be generally applied to other host-diet-parasite systems. We still have much to learn about how intrinsic host factors and the environment influence the microbiome. This dissertation is a step towards that understanding. A major challenge moving forward for researchers will be knowing what characteristics of both the microbiome and the host to consider when trying to understanding microbiome variation and its phenotypic effects. For example, there is substantial individual

variation in gut microbiome composition, which may be due to host genetics, abiotic or biotic factors, or stochasticity. Additionally, gut microbial communities change over development, adding more complexity (Hammer et al., 2014; Y. Wang, Gilbreath, Kukutla, Yan, & Xu, 2011b). To further complicate gut microbiome studies, there have been extensive studies showing that sample collection and preparation can bias results (Blaser et al., 2013; Hammer, Dickerson, & Fierer, 2015).

Technological advances in sequencing technology have enabled the characterization of microbial communities in many organisms, ranging from small sponges, microscopic worms, and insects (Engelberts et al., 2020; Pinto-Tomás et al., 2011; Vega & Gore, 2017) to larger reptiles, mice, and humans (Hildebrandt et al., 2009; Keenan, Engel, & Elsey, 2013; Turnbaugh et al., 2009a). This dissertation, along with other recent studies, moves beyond simply assessing correlation between gut microbial community composition and host phenotypes to taking an experimental approach, in which manipulative studies elucidate causative links. Manipulating diets and gut microbiomes to influence disease outcome has mainly been used in mice and humans (Claesson et al., 2012; de Filippo et al., 2010; Hildebrandt et al., 2009) to explore human health. In many ways, humans are sub-optimal systems to study the interaction between diet, gut microbiome and disease (Harris et al., 2019). More recently (Mockler et al., 2018), and in this dissertation, insects have provided a powerful system to examine this interaction. We form a better understanding of diet-microbe-disease dynamics and address questions that link known pairwise relationships into a more comprehensive three-way interaction. Although there is substantial variation in Lepidopteran gut microbial communities, monarch butterflies provide a foundation to study diet-microbe-disease dynamics in an ecological framework. Future studies

can begin to explore the role of particular microbes and address how specific chemical properties of diet shape disease.

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