Microbiome analysis of monarch butterflies reveals effects of development and diet

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ABSTRACT

Diet profoundly influences the composition of an animal's microbiome, especially in holometabolous insects, offering a valuable model to explore the impact of diet on gut microbiome dynamics throughout metamorphosis. Here, we use monarch butterflies (*Danaus plexippus*), specialist herbivores that feed as larvae on many species of chemically well-defined milkweed plants (*Asclepias* sp.), to investigate the impacts of development and diet on the composition of the gut microbial community. While a few microbial taxa are conserved across life stages of monarchs, the microbiome appears to be highly dynamic throughout the life cycle. Microbial diversity gradually diminishes throughout the larval instars, ultimately reaching its lowest point during the pupal stage and then recovering again in the adult stage. The microbial composition then undergoes a substantial shift upon the transition from pupa to adult, with female adults having significantly different microbial communities than the eggs that they lay, indicating limited evidence for vertical transmission of gut microbiota. While diet did not significantly impact overall microbial composition, our results suggest that fourth instar larvae exhibit higher microbial diversity when consuming milkweed with high concentrations of toxic cardenolide phytochemicals. This study underscores how diet and developmental stage collectively shape the monarch's gut microbiota.

INTRODUCTION

Like most animal and plants, insects form symbiotic relationships with microbial communities. The microbial residents can be seamlessly integrated into insect biology and ecology, and microbes play an essential role in the lives of the majority of insect species (Douglas, 2022). Insect gut microbes, for example, affect insect development (Sommer and Bäckhed, 2013; Li *et al.*, 2023), digestion (Marcobal *et al.*, 2011; Brune, 2014), behavior (Heijtz *et al.*, 2011; Wong *et al.*, 2017) detoxification of harmful substances (Berasategui *et al.*, 2017; Siddiqui *et al.*, 2022) and defense against natural enemies (Piel, 2002; Ramirez *et al.*, 2014). Gut microbiota function relies on the specific composition of microbes, which is composed of beneficial symbionts, as well as pathogens and commensals (Dillon and Dillon, 2004). Several factors influence composition, including insect host species, environmental conditions, genetics, social interactions, immune responses, maternal transmission, diet and exposure to antibiotics (Douglas, 2011; Hasan and Yang, 2019). While less explored, insect development also influences the composition and function of the gut microbiota (Pernice *et al.*, 2014; Hammer and Moran, 2019).

For microbes, the insect gut can be a hostile environment. This may especially be the case for holometabolous insects, which undergo complete metamorphosis through egg, larval, pupal, and adult stages. Each developmental transition involves shedding of the cuticle (ecdysis) and often substantial turnover and transformation of the inner gut cuticle (foregut and hindgut) (Cracraft and Donoghue, 2004). As such, metamorphosis may radically remodel the morphology, biochemistry and chemical attributes of the digestive system (Engel and Moran, 2013). Consequently, in early developmental stages, the gut microbiomes of insects undergo ecological succession and turnover, during which bacteria establish colonies, engage in cooperative

interactions and compete for spatial dominance (Hammer and Moran, 2019; Figueiredo and Kramer, 2020). These dynamics eventually result in the establishment of a more stable microbial community in the adult stage, often differing significantly from that in the larval stages (Hu *et al.*, 2013; Zhang *et al.*, 2018; Augustinos *et al.*, 2019; Wang *et al.*, 2019, 2023; Yao *et al.*, 2019; Xue *et al.*, 2021; Li *et al.*, 2022, 2023).

In conjunction with developmental metamorphosis, alterations in diet can exert significant influence on the gut microbiota, leading to the elimination of certain microbes and the promotion of others (Luo *et al.*, 2021; Brunetti *et al.*, 2022). The profound impact of diet on the gut microbiome has been observed in numerous insects, including Indian fruit flies (*Anastrepha obliqua*) (Cárdenas-Hernández *et al.*, 2023), cereal leaf beetles (*Oulema melanopus*) (Wielkopolan *et al.*, 2021), rainbow stag beetles (*Phalacrognathus muelleri*) (M. Wang *et al.*, 2020), dung beetles (*Copris incertus*) (Suárez-Moo *et al.*, 2020), and European firebugs (*Pyrrhocoris apterus*) (Sudakaran *et al.*, 2012). Furthermore, the synergistic effect of diet and life stage has been observed in several lepidopteran species, where the two feeding stages (larva and adult) have drastically different diets—generally, solid plant foliage for larvae and liquid nectar for adults—resulting in distinct microbial communities (Hammer *et al.*, 2014; Phalnikar *et al.*, 2018; Gohl *et al.*, 2022).

Lepidoptera usually have simple guts, comprising a midgut protected by a peritrophic matrix, which fosters a relatively uncomplicated and nonspecific microbiome (Paniagua Voirol *et al.*, 2018; Mason, 2020). Despite this simplicity, gut microbial communities in lepidopteran species vary not only across species but also between populations, individuals, and even sexes

(Chen *et al.*, 2016; Staudacher *et al.*, 2016; Paniagua Voirol *et al.*, 2018; X. Wang *et al.*, 2020; Fu *et al.*, 2023). Some of this variation is driven by diet, and particularly larval host plant diet, which can vary both within and between insect species. For several lepidopteran species, alternative larval diets lead to the colonization of distinct gut communities (Broderick *et al.*, 2004, 2004; Pinto-Tomás *et al.*, 2011; Staudacher *et al.*, 2016; Whitaker *et al.*, 2016; Phalnikar *et al.*, 2018). While some of this variation may be imposed by nutritional differences, it may also result from changes in plant phytochemistry, particularly for insect species that sequester and accumulate toxic secondary metabolites from plants. These metabolites, including alkaloids, phenolics, and cardiac glycosides, impose not only dietary challenges to their microbiota but also select for survival and detoxification in close proximity to these poisonous chemicals (Shikano *et al.*, 2017). Despite the potential importance of life stage and diet in shaping the microbiota of lepidopteran species, studies to test their combined effects remain lacking (Smilanich and Muchoney, 2022).

Here, we focus on how developmental stage and larval diet influence gut microbiome composition of a specialized butterfly that has been a model for studies of herbivore-plant interactions, secondary metabolite sequestration, migration, and disease ecology (Ehrlich and Raven, 1964; Bradley and Altizer, 2005; de Roode *et al.*, 2008; Agrawal *et al.*, 2009; Zhan *et al.*, 2011; Gowler *et al.*, 2015). Monarch butterfly (*Danaus plexippus*) caterpillars are specialist herbivores, feeding exclusively on milkweed plants (mostly in the genus *Asclepias*). Milkweed species vary in their concentrations of cardenolides, toxic secondary chemicals that monarchs can sequester to make themselves unpalatable to predators (Brower and Calvert, 1985; Holzinger *et al.*, 1992; Martin *et al.*, 1992). High-cardenolide diet also provide protection against the virulent protozoan parasite *Ophryocystis elektroscirrha* (Hoogshagen *et al.*, 2023), and infected monarchs

129 preferentially oviposit on high-cardenolide plants, reducing infection in their offspring (Lefèvre *et*
130 *al.*, 2010, 2012). Consuming cardenolides boosts monarch butterfly survival against the parasite
131 but simultaneously suppresses their immunity, as evidenced by decreased melanization,
132 phenoloxidase activity, hemocyte numbers, and downregulation of immunity-related genes (Tan
133 *et al.*, 2019; Decker *et al.*, 2021). This dual effect may involve direct toxicity to the parasite, but
134 the suppression of the pathogen could also be driven by alteration to the microbiota, as observed
135 in other lepidopterans, such as common buckeyes (*Junonia coenia*) (Smilanich *et al.*, 2018) and
136 Melissa blue butterflies (*Plebejus melissa*) (Yoon *et al.*, 2019).
137
138 Until now, there have been limited efforts to understand the diversity and composition of
139 monarchs' microbiota. Recent comparisons of microbiota of second instar monarch larvae feeding
140 on different plants (*A. curassavica* and *A. syriaca*), revealed no host plant-related differences in
141 microbial diversity but did reveal differences in microbial composition (Hansen and Enders, 2022).
142 The study also highlighted the similarity between the microbiota of the second instar larvae and
143 the rhizosphere microbiome of milkweed plants, suggesting an environmental influence on
144 monarch gut microbiota. Beyond these findings, there is a knowledge gap as to how monarch
145 microbiota change during and across life stages and how diet influences these changes.
146
147 Here, we characterize gut microbial communities and quantify bacterial load across the
148 monarch lifecycle, encompassing parental adults (F_1) and their offspring eggs, all larval instars,
149 pupae, and offspring adults (F_2), as well as larval frass. We reared larval monarchs on two species
150 of milkweeds that vary widely in their concentrations of cardenolides but are similar in nutrient
151 content: low-cardenolide *A. incarnata* and high-cardenolide *A. curassavica* (Tao *et al.*, 2014). In

light of other studies on lepidopteran species conducted thus far, we discuss the significance of our findings, indicating the role of the environment and host plant chemicals in shaping the monarch's gut microbial community.

RESULTS

Membership of the Monarch Gut Microbiome

After quality filtering and preprocessing, we obtained 3,352,317 reads representing 1319 ASVs from a total of 160 samples, including 11 egg samples (20 eggs/sample), 105 larval guts, 21 larval frass samples, 9 pupal guts, and 14 adult guts (see Figure 1 and Table S1 for sampling scheme). Filtering out Archaea, mitochondria, and chloroplast sequences resulted in 1071 remaining ASVs representing the bacterial microbiota. Dominant families were Acetobacteraceae, Alcaligenaceae, Bacillaceae, Brevibacillaceae, Enterococcaceae and Erwiniaceae. Of note, Acetobacteraceae and Alcaligenaceae were common in adults but rare in all other life stages, while Enterobacteriaceae and Erwiniaceae dominated the immature stages (Figure 2A).

Considering all life stages, a strain of *Enterobacter* was the most prevalent ASV in our dataset, present in all egg samples (11/11) and majority of larval (104/105), frass (20/21), and pupal (8/9) samples, albeit often at low relative abundance. The relative abundance of *Enterobacter sp.* increases in the later larval instars and becomes dominant during the pupal stage. This strain also exhibited high prevalence, though low relative abundance, in adult samples (12/14).

Pantoea sp. is also highly prevalent and extremely abundant, making it very dominant especially across immature stages samples (Figure 2B-C). It was detected in all eggs (11/11) and

177 the majority of larval (98/105), frass (21/21), and pupal samples (5/9), but was relatively rare in
178 adults (7/14).
179
180 In fifth instar larvae, while many microbial taxa observed in previous instars seem to be
181 lost or present in a very low abundance almost all samples show a high abundance of either
182 *Enterobacter* sp., *Pantoea* sp., or a co-occurrence of both, with one strain often dominating the
183 other. Additionally, a few samples exhibit a high abundance of *Enterococcus sp.* (3/22) (Figure
184 2B). This pattern is also observed in frass samples from fifth instar larvae.
185
186 *Asaia* appear characteristic of the adult microbiota. In all adults (F_1 and F_2), three strain
187 belonging to the genus *Asaia* (Family Acetobacteraceae) was consistently observed, irrespective
188 of sex (Figure 2B-C). *Asaia* sp1. prevalence was notably lower in eggs (5/11), larvae (27/105),
189 frass (1/21), and pupae (2/9), with very low relative abundance when detected. This suggests that
190 this strain may persist, albeit in reduced numbers, throughout the developmental life cycle.
191 Additionally, it may be acquired by adults from the environment, thus increasing its relative
192 abundance and our ability to detect it in adult life stages. *Asaia* sp2. and *Enterococcus* sp. were
193 two other dominant taxa found in adults (Figure 2B).
194
195 **Changes in Microbial Community Composition and Diversity across the Monarch Lifecyle**
196 For comparative analysis of microbial communities across various life stages and diets,
197 samples were rarefied to 700 reads per sample, leading to a reduced data set of 105 samples (Table
198 S1B) with a total of 672 ASVs. There was a significant effect of monarch life stage on the
199 composition of their microbial communities based on Bray-Curtis dissimilarity (PERMANOVA

with 10,000 permutations; $p < 0.001$, $R^2 = 0.26$). According to Adonis pairwise comparison, no significant difference was observed between the gut microbial communities of larvae and eggs. However, pupae and adult butterflies possessed their own distinguishable communities, albeit with slight overlap with that of other development stages (Table 1A, Figure 3A). This dissimilarity is evident, with statistical support, when comparing F1 female adults and their oviposited eggs (PERMANOVA with 10,000 permutations; $p < 0.001$, $R^2 = 0.54$; Figure 3D). No significant difference was found between female and male adults (PERMANOVA with 10,000 permutations; $p = 0.342$, $R^2 = 0.08$) nor between F_1 and F_2 adults (PERMANOVA with 10,000 permutations; $p = 0.932$, $R^2 = 0.04$). Taken together, these results emphasize differences between the microbial community composition of mature stages (adults) and immature stages (eggs and larvae).

The DESeq2 results (Table S2) show that strains with a mean abundance over 30, including *Enterobacter* sp., *Asaia* sp., and *Pantoea* sp., exhibit significant differences across life stages, underscoring the role of these strains in shaping microbial community shifts (Figure 2). In addition, the presence, absence, and changing abundance of less abundant strains (with mean abundance between 25-30 reads) also contribute to the distinctiveness of microbial profiles at certain life stages (Figure S2-S5, Table S2). Notably, there is a significant higher abundance of *Pantoea* sp., *Enterobacter* sp., *Bacillus* sp., *Enterococcus* sp1 and *Paenibacillus*, and *Staphylococcus* genus in egg and larval samples compared to adults (Figure S2). During the transition to the pupal stage, *Enterobacter* sp. becomes dominant. Moreover, pupae have significantly lower abundances of *Bacillus* sp., *Staphylococcus* sp., and *Paenibacillus* sp. compared to larvae, and lower abundance of *Asaia* sp. compared to adults. In the adult stage, the genus *Asaia* is dominant, with *Enterococcus* sp2 and *Serratia* sp. also present to a lesser extent (Figure S2).

Bray-Curtis dissimilarity testing revealed non-significant community differences among larval instars (PERMANOVA with 10,000 permutations; $p = 0.12$, $R^2 = 0.07$). Adonis pairwise comparisons indicated that significant dissimilarities were observed only in the fifth instar, which differed significantly from all instars except the fourth (Table 1B, Fig 3B). The emergence of very low abundance of *Serratia* sp. and the increasing abundance of *Enterobacter* sp. in the fourth and fifth instars could explain the differences observed between later and earlier instars (Figure S2).

To better understand whether the bacterial communities within larval frass reflect those in larval guts and/or predict those in pupal guts, we compared the microbial community composition of frass excreted by fifth instars close to pupation to that of late fifth instar larvae and pupae (PERMANOVA with 10,000 permutations; $p < 0.001$, $R^2 = 0.18$, see Figure 3D). There was apparent overlap between the larval frass and larval gut community compositions (Adonis pairwise comparison, 10,000 permutations; $p = 0.7$, $R^2 = 0.02$). The fifth instar larvae share the same dominant strains as the frass, but with different abundances (Figure S4). There were significant differences between the microbial communities of larval frass and pupae (Adonis pairwise comparison, 10,000 permutations; $p = 0.001$, $R^2 = 0.41$), which is consistent with the fact that there were also significant differences between the microbial communities of fifth instar larvae and pupae (Adonis pairwise comparison, 10,000 permutations; $p = 0.003$, $R^2 = 0.38$). In the pupal stage, some larva-specific strains, such as *Bacillus* sp and *Paenibacillus*, as well as the genus *Staphylococcus*, are lost. However, a few adult-specific strains, such as *Enterococcus* sp1 and *Asaia* sp3, appear (Figure S2-S4).

The measurement of microbial Shannon diversity revealed a pattern wherein eggs exhibit high diversity (Figure 4A). However, it is important to note that egg samples were pooled, so their diversity cannot be independently compared with other stages. Microbial diversity fluctuated throughout the larval stages but tended to decrease overall from the first instar to the fifth instar (Figure 4B), reaching a minimum in the pupal stage and then recovering in adults (Figure 4A). While this pattern of changing microbial diversity is visually evident in the graphs, comparison of Shannon diversity by pairwise post-hoc tests indicated that these differences in diversity were only significant for comparisons between pupa and F_1 adults (Table 1A) and between third and fifth instar larvae (Table 1B). Consistent with Shannon diversity, the results for bacterial richness exhibit a similar pattern. Pairwise comparisons of bacterial richness indicate that pupae have the lowest richness, which is significantly different from that of larval instars (Table 1A). However, no significant differences were observed in microbial richness among any of the larval instars (Table 1B).

Influence of Host Plant Diet on the Larval Gut Microbiome

When considering all larval instars, host plant diet had no significant effect on the community composition of gut microbiomes (PERMANOVA with 10,000 permutations; p = 0.110, R^2 = 0.05; Figure 3C). The analysis of Shannon diversity among developmental stages comparing host plants was conducted using the Kruskal-Wallis test. Shannon diversity did not show a significant difference between larvae fed on *A. curassavica* and *A. incarnata* (p = 0.1 Figure 4C). However, when considering individual larval instars, fourth instar larvae fed on *A.*

curassavica had more diverse gut microbiomes than those fed on *A. incarnata* (Figure 4C; Table 1C).

DESeq2 analysis indicates that none of the strains show significant differences in abundance between larval stages when comparing the two diet groups, with a threshold of mean abundance set at 25. However, visual examination of their microbial profiles reveals some descriptive differences between the two groups, such as variation in microbial prevalence, although these differences are not statistically significant (Figures 2B and S3). For example, larvae feeding on *A. incarnata* appear to exhibit a higher prevalence of *Pantoea* sp across all stages (expect third instar), with an apparent increase in *Asaia* sp during the first and second instars. In contrast, larvae feeding on *A. curassavica* tend to show a greater dominance of *Enterococcus* sp and *Paenibacillus* sp2 in the later stages, particularly the fifth instar. Additionally, *Massilia* sp is consistently present in all larval instars of the *A. curassavica* group but absent in the *A. incarnata* group.

Overall Microbial Densities

The quantification of 16S V4 rRNA copy numbers was conducted for all life stages individually, including each egg sample (rather than pooling of 20 eggs per sample). The results revealed a significant difference in 16S rRNA copy numbers across different life stages (one-way ANOVA, $F = 5.346$, $p < 0.001$; see Figure 5A). Further pairwise comparisons indicated significant differences between the egg and all other life stages, with the eggs exhibiting the lowest bacterial abundance (Figure 5A, Table 1D).When focusing on larvae, quantification of 16S copy number revealed a significant difference among instars (one-way ANOVA, $F = 4.126$, $p = 0.004$), with

significantly lower bacterial abundances in the first instar compared to the second, third, and fifth instars (Table 1E; Figure 5C).

There was no significant difference in bacterial load among larval instars based on the larval milkweed diet (one-way ANOVA, F = 1.048, p = 0.308; Figure 5D). However, when comparing each instar separately, first instar larvae fed on *A. curassavica* had a lower bacterial load compared to those fed on *A. incarnata* (Table 1C). Additionally, when comparing the bacterial load between fifth instar guts, fifth instar frass samples, and pupal guts, we found that the bacterial load in frass samples was significantly higher than that in larval and pupal gut samples (one-way ANOVA, F = 12.95, $p < 0.001$; Figure 5D). There was no significant difference between pupal gut and frass samples (T-test, p = 0.91).

DISCUSSION

High fluctuations in microbial composition and diversity among individual lepidopterans (Robinson *et al.*, 2010; Hammer *et al.*, 2014; Minard *et al.*, 2019) and other insects (Gupta and Nair, 2020; Muratore *et al.*, 2020; Suenami *et al.*, 2023) emphasize the need for robust and ample sampling in microbiota research. Our study, based on 160 samples from eggs, larvae (including frass), pupae, and adults, serves as a robust case study, allowing a comprehensive exploration of microbial shifts and diversity patterns in monarchs with relation to two key potential drivers of microbiota composition: host development and host diet. We find that both developmental stage, and diet influence key measures of microbiota composition, diversity, and abundance in this tractable animal model.

Overall pattern of microbial diversity across monarch development

In monarchs, the most apparent influence of microbiome development is that composition of adult gut microbial communities differ from those of all other life stages. These differences likely are driven by the drastic dietary shift that comes with adulthood, from feeding on milkweed foliage as larvae to feeding on liquid (here, sucrose solution) as adults. This has been seen in other lepidoptera, including Western bean cutworms, (*Striacosta albicosta*) (Ayayee *et al.*, 2022), European corn borers (*Ostrinia nubilalis*) (Belda *et al.*, 2011), domesticated silkworms (*Bombyx mori*) (Chen *et al.*, 2018) and red postmen (*Heliconius erato*) (Hammer *et al.*, 2014). Consistent with the hypothesis that dietary shifts drive changes in the microbiota, similar shifts are not seen in species where adults do not feed, such as the Indianmeal moth (*Plodia interpunctella*) (Mereghetti *et al.*, 2019).

Patterns of changes in microbial community diversity across lepidopteran development differ across species. Several previous studies of lepidopteran microbiome diversity have revealed a 'U-Shaped' pattern of diversity, with diversity decreasing from egg to pupal stages before recovery upon maturation to adult. This trend has been reported for the European corn borer (Belda *et al.*, 2011), the Western bean cutworm, (Ayayee *et al.*, 2022) and the Fall armyworm (*Spodoptera frugiperda*) (Fu *et al.*, 2023). However, some lepidopterans exhibit a different pattern, in which diversity tends to drop in adulthood, as seen in the domesticated silkworm (Chen *et al.*, 2018), Oriental fruit moth (*Grapholita molesta*) (X. Wang *et al.*, 2020) and greater wax moth (*Galleria mellonella*) (Gohl *et al.*, 2022).

For monarch's gut microbiome, our data exhibit a general U-shaped pattern of diversity, wherein the lowest diversity is observed in pupae (Figure 3). However, caution is needed when interpreting

the diversity of eggs compared to other stages. Each egg sample consisted of a pool of 20 individuals, potentially introducing a bias toward increasing diversity in these samples.

A focus on eggs

In contrast to Kingsley (1972), who suggested that monarchs lack egg microbiota, our research reveals that monarch eggs do indeed host bacteria. This difference is not surprising, as Kingsley used culture-based methods to quantify microbes, whereas we used sequencing to capture both culturable and unculturable microbes. However, the source of these microbes is not clear. The first common assumption is that all or part of this microbiota is maternally transferred to eggs, as reported in some lepidopteran species (Freitak *et al.*, 2014; Mereghetti *et al.*, 2019). This is often indicated by the similarity between the microbiota compositions of female adults and eggs, and the presence of common microbial taxa, a phenomenon observed in various species, including silkworms (Chen *et al.*, 2018), greater wax moths (Gohl *et al.*, 2022), beet armyworms (Gao *et al.*, 2019) and fall armyworms (Fu *et al.*, 2023). It has also been observed through experimental study of the transmission of fluorescently tagged bacteria; in one such study, labeled *Enterococcus* were consistently observed in all life stages and generations of Egyptian cotton leafworm (*Spodoptera littoralis*) (Teh *et al.*, 2016). In contrast, other studies have found environmental transmission to be more important than maternal transfer, such as in *S. albicosta*, where the microbiota of eggs are very similar to those on leaves of their corn host plant (Ayayee *et al.*, 2022). Our findings indicate that monarch egg-associated communities, primarily dominated by Erwiniaceae and Enterobacteriaceae, exhibit greater similarity to larval gut communities than those of their mothers. This suggests that environmental acquisition may be common. This observation may also account for the low microbial load in monarch eggs compare to other stages (Table 1D). We did, however,

find that *Enterobacter sp*., was consistently present across all stages (although in low relative abundance, especially in egg and larva stages), suggesting potential vertical transmission throughout the dynamics monarch lifecycle. Additionally, *Pantoea sp.*, prevalent in the egg, larval, and pupal stages at high relative abundance, and *Asaia* sp1., prevalent in the adult stages, were both found in all other stages, albeit at low frequency of occurrence and relative abundance. Altogether, these findings suggest the existence of a prevalent microbiome whose members' abundances change with the developmental and dietary shift associated with adulthood.

Across larval instars

As monarch larvae progress through five instar stages, they dramatically increase in size and consume more food, likely creating a different niche for bacterial intake and growth. As such, drastic changes between microbial communities of early and later larval instars have been shown in several lepidopteran case studies (Mason and Raffa, 2014; Chen *et al.*, 2018; Gohl *et al.*, 2022). However, our findings reveal a nuanced picture. While the microbial community composition of monarch larval guts appears to remain relatively stable throughout larval development (Figure 3B), we observed contrasting trends in microbial diversity and abundance (Figure 4B and 5C). Specifically, microbial diversity tends to decrease, while microbial abundance tends to increase from the first to the fifth larval instar. It is noteworthy that these changes occur gradually and, when considering the broader context, are statistically supported only between the first and fifth instars.

Fifth instar larvae excrete large amounts of frass before morphing into pupae, which may partly explain the overall reduction in gut microbial diversity observed in fifth instar larvae (Figure 4).

Frass samples contained a higher microbial load compared to fifth instar larvae and other stages, possibly due to the frass environment being less stringent and more conducive to microbial proliferation than the gut environment. The microbial composition of frass may also provide insights into the level of dependency of gut microbiota on diet and environment. For instance, the frass of the Melissa blue butterfly includes microbiota that significantly differs from larval microbiota and is more similar to plant microbial communities (Chaturvedi *et al.*, 2017). This suggests a higher stability and lesser dependence on diet for the Melissa blue microbiota compared to monarchs, where the microbial community found in frass resembles that of the gut. Future work, of course, should assess the microbial communities of milkweed plants for comparison to the microbial communities associated with monarchs across their lifecycle.

The final steps: from pupa to adult

In almost all existing lepidopteran studies, the lowest diversity of microbiota is observed in pupae (Johnston and Rolff, 2015; Phalnikar *et al.*, 2018). This could be explained by the purging of gut contents before pupation, the drastic reorganization of body tissues and the non-feeding state of this life stage. Upon enclosing, most adult lepidoptera begin to feed, which is expected to lead to changes in microbial composition and increases in both diversity and abundance. In our study, pupae had the lowest diversity of gut microbes, with a shift in microbial community from that of larvae characterized by a dominance of *Enterobacter* sp over *Pantoea* sp (Figure 2C). As expected, diversity recovered in adults. This increase in diversity is in tandem with a change in composition (Figure 3A), and may be driven by increases in the abundance of specific taxa, such as three strains of *Asaia*. Since *Asaia* is common in plant nectars (Lenaerts *et al.*, 2017; Bassene *et al.*, 2020), as well as in insects that feed on sugary nectar and plant sap ((Bassene *et al.*, 2020)

(Gonella *et al.*, 2012, 2012; Li *et al.*, 2019) and even in several lepidopterans (Robinson *et al.*, 2010; Gao *et al.*, 2019; X. Wang *et al.*, 2020), a substantial part of this shift may be driven by differences in the nutritional composition of leaves and sugary liquids (Shao *et al.*, 2024). While in our study adults were fed a diet consisting only of sugar, in natural populations, adults feed on various nectars, and are therefore expected to have different compositions and diversity of microbiota.

The effect of plant diet, with a focus on plant chemistry

Even though the composition of lepidopteran's prevalent microbiota can exhibit independence from diet (Whitaker *et al.*, 2016), certain lepidopteran species display notable variations in microbiota composition when consuming different host plant diets. Examples include corn earworm (*Helicoverpa zea*) (Jones *et al.*, 2019), cotton leafworm (*Spodoptera littoralis*) and cotton bollworm *(Helicoverpa armigera)* (Tang *et al.*, 2012) and European gypsy moth *(Lymantria dispar)* (Mason *et al.*, 2015). This trend becomes more pronounced when multiple generations of insects are exposed to a controlled diet, as seen in rice leaffolder (*Cnaphalocrocis medinalis*) feeding on either rice or maize (Yang *et al.*, 2022), and *S. littoralis* larvae feeding on either cabbage or cotton (Roy *et al.*, 2023).

Our results indicate that the gut microbiota of fourth instar larvae feeding on *A. curassavica,* which contains higher concentrations of cardenolides, are more diverse than those of individuals feeding on *A. incarnata*. Additionally, first instar larvae feeding on *A. curassavica* exhibited significantly lower microbial abundance compared to those feeding on *A. incarnata* (Fig 5D). Changes in the presence of particular strains or the prevalence of common strains due to the

feeding groups are also detectable; for instance, *Massilia* sp is present only in the *A. curassavica* group, while *Pantoea* sp shows a higher prevalence in the *A. incarnata* group (Figure S3).Since the milkweed species used here are similar in nutrients but differ greatly in toxic cardenolides (Tao *et al.*, 2016), it is possible that the chemical properties of these plants contribute to slight alterations in gut microbial diversity and composition. In a similar study comparing the influence of different milkweed species (*A. syriaca* and *A. curassavica*) on the microbiota of second instar monarch larvae, caterpillars feeding on these plants exhibited similar microbial diversity but different microbial composition (Hansen and Enders, 2022). The study reported different dominant bacterial families than we observed in our study, and identified Enterobacteriaceae as a rare family (Hansen and Enders, 2022). In contrast, Enterobacteriaceae, which includes the genera *Enterobacter* and *Pantoea*, was the most prevalent family in our dataset. Variability in findings may stem from differences in study design, original monarch populations, and plant species. Our study design included plants growing in the same soil sample, which may contribute to greater similarity in the microbial environment of the two groups. Additionally, this suggests that other unknown factors might influence the shaping of the monarch's microbiome. These results highlight the need for more comprehensive studies in controlled environments to elucidate the underlying mechanisms and emphasize the necessity of studying microbiomes across diverse populations to obtain a clearer picture.

In conclusion, our study significantly advances our understanding of the dynamic monarch gut microbiome by characterizing microbial communities, quantifying bacterial loads across developmental stages, identifying prevalent microbiota, and assessing the impact of alternative larval diets. We found that adults, eggs/larvae, and pupae form three distinct microbial

communities, with pupae exhibiting the lowest diversity and adults the highest. Our findings suggest that while environmental factors influence microbiota shifts, certain microbial taxa may persist, indicating potential maternal transfer and maintenance within the monarch population. Furthermore, diets rich in cardenolides have the potential to reduce bacterial loads in early larval development and to increase gut microbiome diversity in later larval stages. Further studies are needed to determine the transmission route of monarch microbes, how monarch microbiota varies in nature and the importance of the microbiota for monarch life history traits and protection against pathogens (Smilanich *et al.*, 2018).

MATERIALS AND METHODS

Insect rearing

The monarchs used in this study were descendants of individuals collected from St Marks, Florida, US. Several generations had been lab reared prior to this experiment. To minimize the possibility of carryover effects from the parental diet, ten adult individuals from four lineages were fed on sterile 20% sucrose solution, a common lab diet; these adults are referred to hereafter as the P generation. P adults were mated with the opposite sex of another lineage. Once mated, five P 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 once hatched, the larvae were fed on either of the two 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 ovipositing on either one of two milkweed species, *A. incarnata* or *A. curassavica*. Oviposited eggs, recorded to be collected from either *A. incarnata* or *A. curassavica*, seeded the

F_2 generation, which is the focus of our study. F_2 eggs were then moved to individual plastic, lidded cups, where they were placed on leaves of either greenhouse grown *A. incarnata or A. curassavica.* Once the eggs hatched, leaves were replaced daily. Similar to P and F_1, F_2 adults from both diets were again placed in a single cage and provided with a sterile 20% sucrose solution. "Overall, there were four F_2 diet treatments differing in the plant species fed to larvae of their parents (F_1) and to them (F_2) when they were larvae. The four treatments included: (1) F_1 and F_2 both fed on *A. incarnata* (n = 27); (2) F_1 fed on *A. incarnata*, and F_2 fed on *A. curassavica* (n = 27); (3) F_1 and F_2 both fed on *A. curassavica* (n = 29); and (4) F_1 fed on *A. curassavica*, and F_2 fed on *A. incarnata* (n = 22) (see Figure 1 and Table S1.A)."

Sample collection and gut dissections

We collected parental F_1 adults after oviposition. Resulting F_2 offspring, once hatched, fed on either greenhouse grown *A. incarnata* or *A. curassavica*. F_2 individuals were collected at all developmental stages (egg, larva, pupa, and sucrose-fed adults). Due to the difficulty of extracting DNA from individual eggs, the egg samples consisted of a pool of 20 eggs each. Sample sizes for each developmental stages are indicated in Figure 1. From all larval instars, larvae were euthanized with CO_2, then whole bodies were surface-sterilized with 95% molecular grade ethanol for three minutes. Then, we dissected out guts of second, third, fourth and fifth instars for further analysis; first instars were not dissected because of their small size, which prevented removal of the gut from the rest of the animal tissue. Seven-day old pupae were handled similarly to later larval instars though not euthanized. For adults, we clipped off the wings at the thorax, then surface sterilized as described for larvae. Guts from larvae, pupae and adults were dissected with sterile instruments and immediately frozen. In addition, to test if larval frass microbial communities are reflective of their gut microbial communities, we collected frass excreted on sterilized Petri dishes

503 from 21 fifth instar F_2 larvae (12 fed on *A. curassavica* and 9 fed on *A. incarnata*). Each frass
504 sample consisted of five frass pellets. Frass samples were not surface-sterilized. All samples were
505 frozen at -80° until DNA extraction.
506
507 **Gut Microbiome Community Profiling**
508 DNA was extracted using the Qiagen DNeasy PowerSoil kit, following the manufacturer's
509 protocols. Extractions were sent to the University of Michigan's Center for Microbial Systems for
510 PCR amplification, amplicon library preparation, and high-throughput 16S rRNA sequencing. The
511 16S rRNA gene was amplified with barcoded dual-indexed primers 515F and 806R specific to the
512 V4 region. The PCR cycle consisted of two min at 95°C, followed by 30 cycles of 95°C for 20 s,
513 55°C for 15 s, and 72°C for five min, followed by 72°C for 10 min. PCR reactions were
514 normalized, pooled, and quantified for amplicon library preparation. Libraries were sequenced on
515 an Illumina MiSeq platform with 250bp paired ends. A mock community was co-sequenced
516 (ZymoBIOMICS^{TM} Microbial Community DNA Standard) to determine the sequencing error rate,
517 which was 0.0082%.
518 Raw bacterial sequences were processed and analyzed in qiime2 v 2019.7 (Hall and Beiko,
519 2018). Once the primers were removed the reads were merged and trimmed, sequences less than
520 250bp or greater that 289bp in length were removed from analysis. Quality filtering was performed
521 using DADA2 (Callahan *et al.*, 2016) and subsequently a Bayesian V4 specific classifier was
522 designed to taxonomically identify the amplicon sequence variants (ASV's) using the SILVA v132
523 reference database (Quast *et al.*, 2013). Visualizations and all statistical tests of sequence data were
524 performed in R v4.2.1 (R Core Team, 2022) using packages *phyloseq* (McMurdie and Holmes,
525 2013), *vegan* (Dixon, 2003), *pairwiseAdonis* v0.4 (Martinez Arbizu, 2020), and *qiime2R* (Bisanz,

2018). For generating figures and conducting statistical analysis, and particularly to minimize the effect of sample size bias, samples were normalized to 700 reads per sample, resulting in a reduction of sample size (Table S1). Based on the rarefaction curve plot generated in R using *phyloseq* and *vegan* packages (Figure S1), rarefying to 700 reads indicates a negligible loss of diversity. The curves show that increasing sequencing depth beyond 700 reads yields few new types of ASVs, suggesting that rarefying to 700 reads is sufficient for detecting high-abundance microbial taxa.

Analysis of Community Structure and Diversity

For the analysis of taxonomic composition of each sample, the ASV's in the datasets were classified using a qiime2 V4 classifier. Bray-Curtis distances between all communities were calculated and the significance of clustering at the community level was tested for both metrics using the Adonis function implemented in the VEGAN package, including taxonomy, life stage and diet as possible variables. The PERMANOVA (Permutational Multivariate Analysis of Variance) non-parametric test implemented in R was used to assess differences in microbial community composition between developmental stages (egg, larva, pupa, and adult). The primary objective was to understand broader microbial dynamics throughout the life cycle, addressing questions such as how microbial diversity changes between stages, indications of plausible vertical transmission and/or environmental acquisition. For the focused analysis on larval instars, PERMANOVA examined differences across the five larval instars to monitor progressive changes and identify potential microbial shifts linked to larval development stages. To identify strains driving these differences, DESeq2 (R package) (Love *et al.*, 2014) was used for differential abundance analysis, performing pairwise comparisons between developmental stages and filtering

549 significant results with a p-value cutoff of <0.05. Strains with a baseMean (average abundance)
550 greater than 25 were included in order to focus on biologically relevant features, excluding low-
551 abundance strains.

552 Microbial diversity within each sample was estimated using the Shannon diversity index at
553 the ASV level, calculated with the 'phyloseq' package in R. Due to the non-normal distribution of
554 the data, as determined by Shapiro-Wilk test implemented in R ($p > 0.05$), non-parametric tests
555 were employed for comparisons. The analysis of Shannon diversity among developmental stages
556 was conducted using the Kruskal-Wallis test to assess overall differences in Shannon diversity
557 values among groups. Subsequently, pairwise post-hoc tests with Holm correction were performed
558 to identify specific differences between pairs of developmental stages. In addition to Shannon
559 diversity, bacterial richness was also estimated to provide a more comprehensive understanding of
560 the microbial community structure. Using both Shannon diversity and bacterial richness allows us
561 to capture the complexity of the microbial community (richness and evenness) as well as the actual
562 number of distinct microbial taxa. Bacterial richness was estimated by counting the number of
563 observed ASVs within each sample, also using the 'phyloseq' package in R. Similar to the Shannon
564 diversity analysis, the richness data were tested for normality using the Shapiro-Wilk test, and due
565 to non-normal distribution, non-parametric tests were applied. For the larval stages, the Kruskal-
566 Wallis test was used to evaluate overall differences in observed richness among larval instars,
567 followed by pairwise Wilcoxon tests with Holm correction to identify specific differences between
568 pairs of larval instars.

569

570 **Quantitative PCR and Analysis of Bacterial Load**

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 *et al.*, 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 (Science Primer online platform, www.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 analysis of bacterial load. To estimate individual samples from pooled egg samples, the 16S rRNA copy numbers were initially divided by 20. Subsequently, the log10 values of each resulting sample were calculated. These log10 values were then utilized for statistical analysis. Shapiro-Wilk normality tests did not reject the null hypothesis, indicating that the data approximated a normal distribution ($p > 0.05$). Therefore, parametric tests were employed for comparison. Specifically, One-way ANOVA followed by T-tests with corrected p-values using the Holm method were used to assess differences in bacterial loads among developmental stages, larval instars, and diets.

Data Availability

Raw sequence reads are available on NBCI's Sequence Read Archive under project PRJNA816827.

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Figure 1. Schematic of experimental design, depicting the diets received for each group in colored circles (red for *A. curassavica* and pink for *A. incarnata*). The numbers within each circle represent the sample size for microbial metabarcoding. F_2 diet treatments include (1) F_1 and F_2 on *A. incarnata* (n = 27), (2) F_1 parent on *A. incarnata* and F_2 offspring on *A. curassavica* (n = 27), (3) F_1 and F_2 on *A. curassavica* (n = 29), and (4) F_1 parent on *A. curassavica* and F_2 offspring on *A. incarnata* (n = 22). Frass samples (not shown in figure) were collected from fifth instar larvae, and each egg sample consists of a pool of 20 eggs.

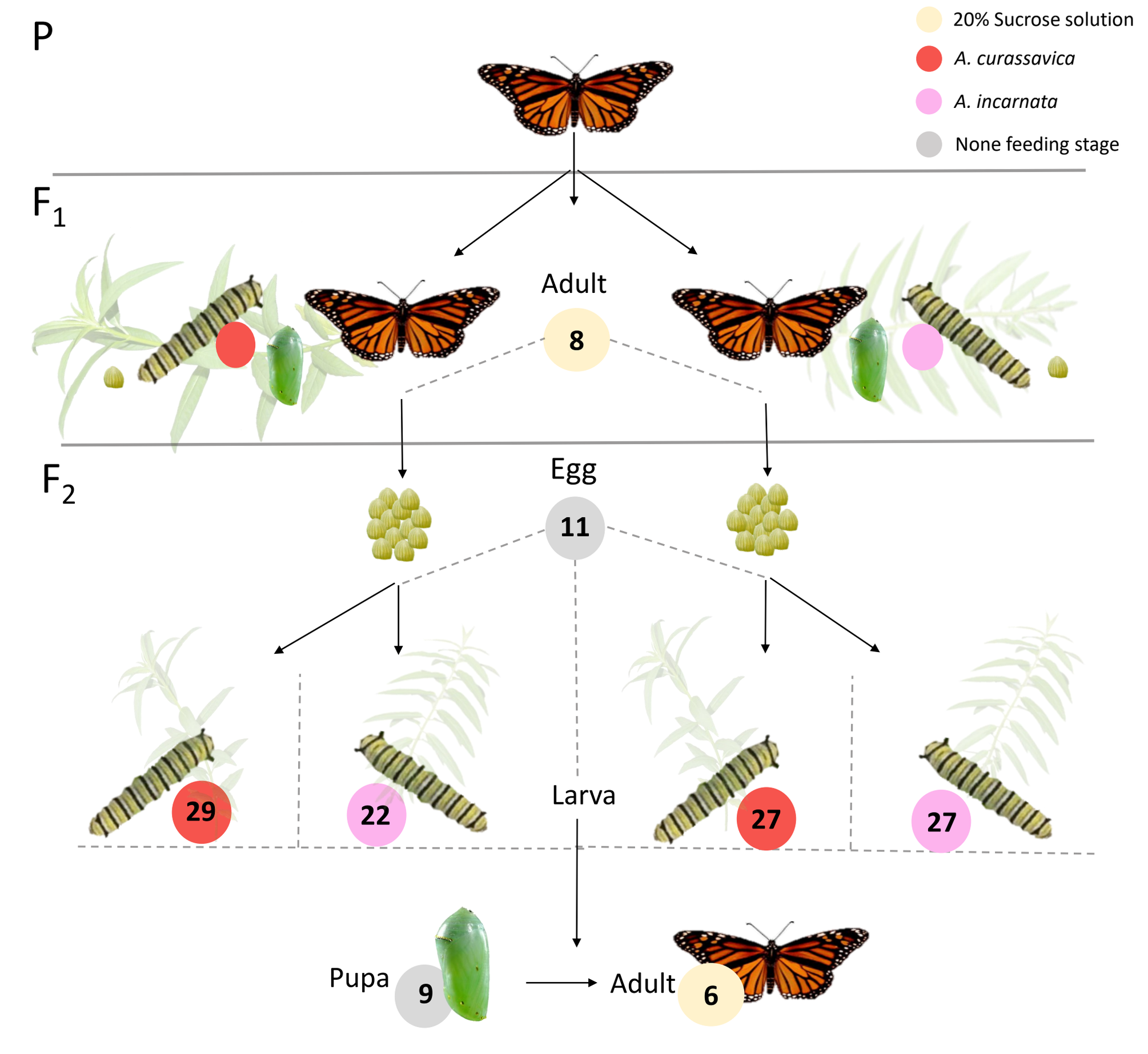

P
20% Sucrose solution
A. curassavica
A. incarnata
None feeding stage
F1
Adult
8
F2
Egg
11
29
22
Larva
27
27
Pupa
9
Adult
6

Figure 2. Microbial community composition across developmental stages, **A.** based on top 25 most abundant families, and **B.** based on the top strains (ASVs) with a mean abundance of over 0.1% across all samples. For the top 20 strains, see Figure S2. Monarch larvae were reared on two host plants, *A. incarnata* and *A. curassavica*. 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. **C.** Mean abundance of selected strains across developmental stages (strain colors as in B).

A

B

C

F_1 Adult
Egg
A. inc
A. cur
Pupa
F_2 Adult
1st instar
2nd instar
3rd instar
4th instar
5th instar
Abundance
1.00
0.75
0.50
0.25
0.00
Family
Acetobacteraceae
Alcaligenaceae
Bacillaceae
Brevibacillaceae
Burkholderiaceae
Enterococcaceae
Erwiniaceae
Exiguobacteraceae
Leuconostocaceae
Opiliones
Oxalobacteraceae
Paenibacillaceae
Planococcaceae
Staphylococcaceae
Streptococcaceae
Weeksellaceae
Xanthomonadaceae
Yersiniaceae
Strain
Asaia sp1
Asaia sp2
Asaia sp3
Enterobacter sp
Enterococcus sp
Pantoea sp
Other strains
0.8
0.6
0.4
0.2
0.0

Figure 3. Comparative analysis of gut microbial community variations (PERMANOVA with 10,000 permutations: $p < 0.05$): **A.** Across all developmental stages, revealing differences in the composition of adult butterfly gut communities compared to other life stages. Except for eggs, which are pools of whole eggs, all samples are from single individuals, and all larval stages (first to fifth) are considered together. Eggs and first instars are based microbial community profiling of whole individuals, while all other life stages are based on gut tissue only. **B.** Across the five larval instars. **C.** Between two larval feeding diets, considering all larval stages together. **D.** Between eggs and their F_1 female parent. Notably, the gut microbial communities of female adults differ from those of their oviposited eggs, with parents being F_1 female adults fed sterile 20% sucrose water before dissection, and egg samples consisting of a pool of 20 eggs each. **E.** Between the gut and frass microbial communities of the fifth instar larva and pupa, showing overall overlap and similarity.

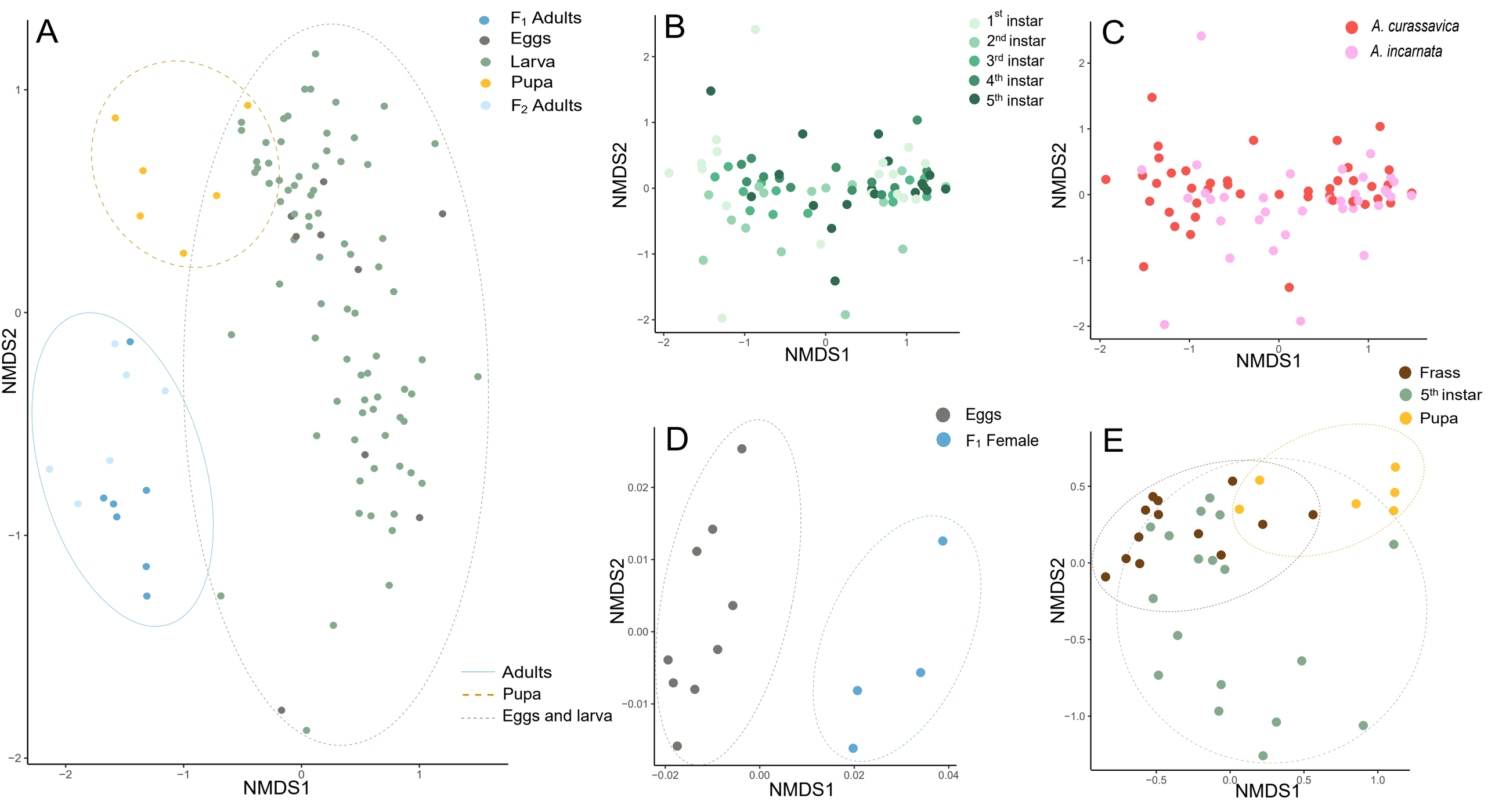

A
F1 Adults
Eggs
Larva
Pupa
F2 Adults
Adults
Pupa
Eggs and larva
NMDS1
NMDS2
B
1st instar
2nd instar
3rd instar
4th instar
5th instar
NMDS1
NMDS2
C
A. curassavica
A. incarnata
NMDS1
NMDS2
D
Eggs
F1 Female
NMDS1
NMDS2
E
Frass
5th instar
Pupa
NMDS1
NMDS2

Figure 4. Shannon diversity of the microbial community across **A.** All developmental stages, including larvae (1st to 5th) analyzed collectively, with each point representing one sample (pools of 20 eggs per egg sample, whole individual first instars, and individual guts for all other life stages). **B.** Larval instars, considering those fed on both milkweed species, revealing higher diversity in earlier instars. **C.** Larval instars based on their diets, distinguishing between *A. curassavica* (high cardenolides) and *A. incarnata* (low cardenolides).

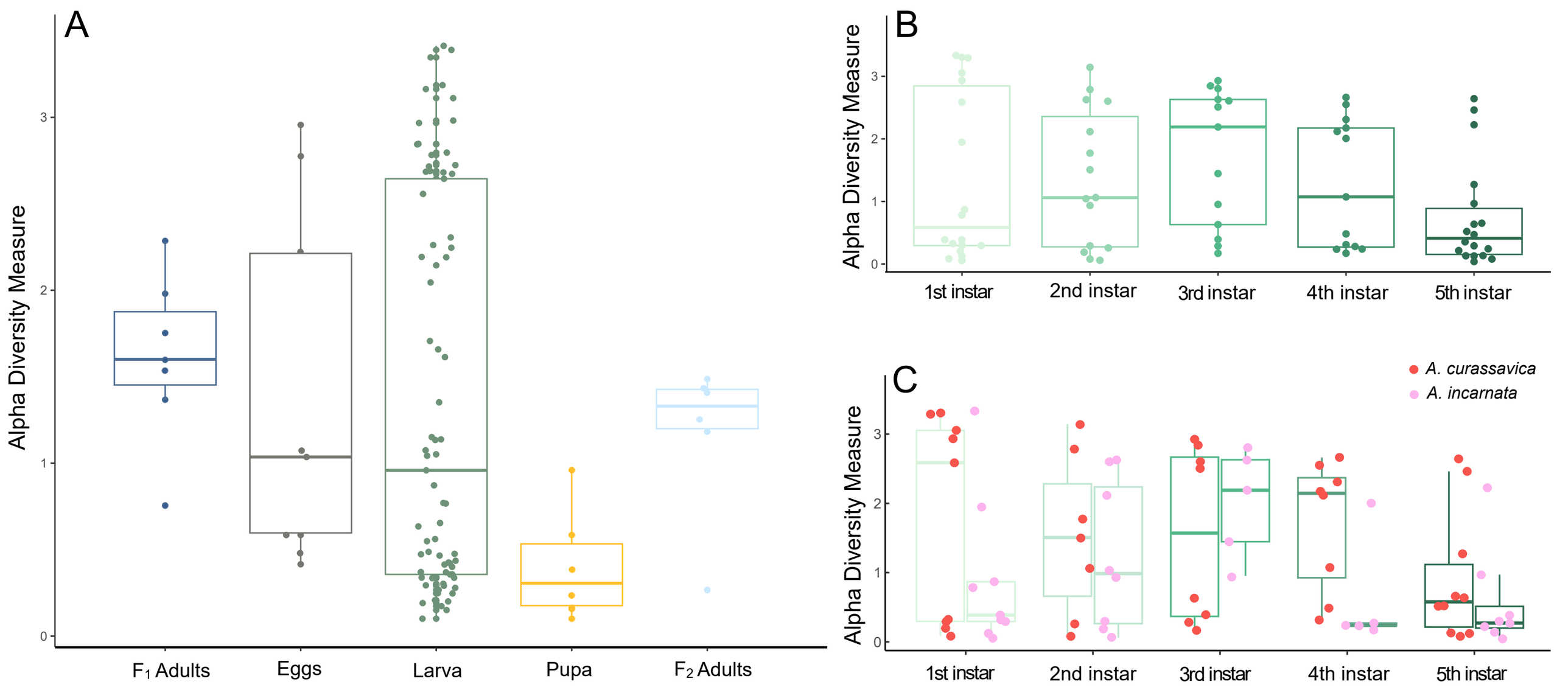
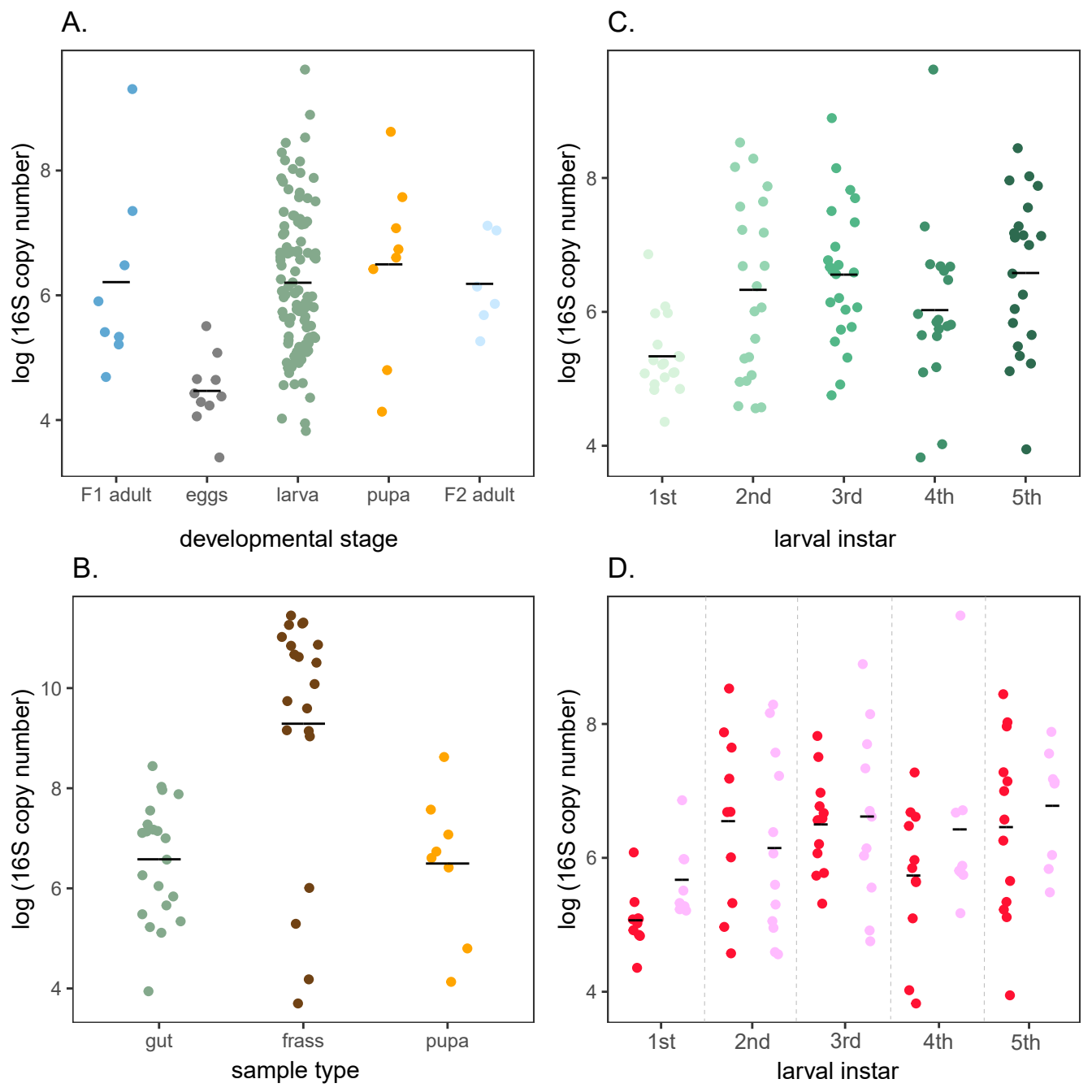

A
Alpha Diversity Measure
0
1
2
3
F1 Adults
Eggs
Larva
Pupa
F2 Adults
B
Alpha Diversity Measure
0
1
2
3
1st instar
2nd instar
3rd instar
4th instar
5th instar
C
Alpha Diversity Measure
0
1
2
3
A. curassavica
A. incarnata
1st instar
2nd instar
3rd instar
4th instar
5th instar

Figure 5. Bacterial abundance across developmental stages, as estimated by quantitative PCR. **A.** Egg samples (20 eggs per sample) have fewer bacteria than individual guts of other developmental stages. All larval instars combined for this comparison. **B.** Larval frass samples have more bacteria than larval and pupal guts. **C.** First instar guts have fewer bacteria than other larval instars. **D.** Larval diet does not affect microbial abundance across larval instars. Red dots (left) are larvae fed *A. curassavica,* and pink (right) are larvae fed *A. incarnata*. Points represent individual samples, and horizontal bars represent means.


A.
log (16S copy number)
8
6
4
F1 adult
eggs
larva
pupa
F2 adult
developmental stage
C.
log (16S copy number)
8
6
4
1st
2nd
3rd
4th
5th
larval instar
B.
log (16S copy number)
10
8
6
4
gut
frass
pupa
sample type
D.
log (16S copy number)
8
6
4
1st
2nd
3rd
4th
5th
larval instar