On the influence of gut bacteria on host biology: insights from ecological and evolutionary studies on *Drosophila melanogaster*

विद्या वाचस्पति की
उपाधि की अपेक्षाओं की आंशिक पूर्ति में प्रस्तुत शोध प्रबंध

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द्वारा / By

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Certificate

Certified that the work incorporated in the thesis entitled “On the influence of gut bacteria on host biology: insights from ecological and evolutionary studies on *Drosophila melanogaster,*” submitted by Akshay Malwade, was carried out by the candidate, under my supervision. The work presented here or any part of it has not been included in any other thesis submitted previously for the award of any degree or diploma from any other University or institution.

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Date: 27/10/2024

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Title of Thesis: On the influence of gut bacteria on host biology: insights from ecological and evolutionary studies on *Drosophila melanogaster*

I declare that this written submission represents my idea in my own words and where others' ideas have been included; I have adequately cited and referenced the original sources. I declare that I have acknowledged collaborative work and discussions wherever such work has been included. I also declare that I have adhered to all principles of academic honesty and integrity and have not misrepresented or fabricated or falsified any idea/data/fact/source in my submission. I understand that violation of the above will be cause for disciplinary action by the Institute and can also evoke penal action from the sources which have thus not been properly cited or from whom proper permission has not been taken when needed.

The work reported in this thesis is the original work done by me under the guidance of Prof. Sutirth Dey.

Date: 27/10/2024

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I dedicate this thesis to my family, teachers, and friends who make me who I am.

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Synopsis

Title: On the influence of gut bacteria on host biology: insights from ecological and evolutionary studies on *Drosophila melanogaster*

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Chapter 1: Introduction

Gut microbes are increasingly implicated in modulating host biology (McFall-Ngai et al. 2013; Rosenberg 2021). The ability of the microbiome to contribute to diverse host phenotypes and, in turn, alter host fitness, raises the possibility that the presence of such microbes can influence the host's evolution (Alberdi et al. 2016; L. P. Henry et al. 2021; Hoang et al. 2021; Macke et al. 2017; Moran et al. 2019; Rosenberg and Zilber-Rosenberg 2016). However, empirical studies on the evolutionary aspects of host-microbe relationships are rare (L. P. Henry et al. 2021; Kolodny et al. 2020; Macke et al. 2017).

In this chapter, we first provide a brief overview of the historical events that played an important part in the development of the microbiome field. Then, we discuss the potential role of microbes in host evolution and the tenets of the holobiont theory. We also cover the debate surrounding the idea of a holobiont and place ourselves in this debate. We end with a brief overview of the questions that we have asked in the subsequent chapters. Briefly, our primary aim in this thesis is to carefully study the "host + microbes" unit (i.e., holobiont) in detail by finding out what happens to diverse host phenotypes when microbes are taken away

from the host on a short timescale of a single host generation and on a longer timescale of multiple host generations.

Chapter 2: Composition of outbred *Drosophila* microbiome and how it changes with age, sex, and diet of the host

Before starting the experiments to take away the microbes to know the microbe's contribution towards the host, as a foundational step, we spent some initial effort on characterizing the microbiome of our outbred *Drosophila melanogaster* populations and how it changes with important factors such as age, sex, and diet of the flies. This is needed as each host-microbiome system can be unique, and most of the fly microbiome composition studies are on inbred strains. We found that the microbiome of our outbred *Drosophila* is also as simple as seen in inbred strains. 16S rRNA sequencing at the population level and Sanger sequencing of bacterial colonies showed that the microbiome is dominated by *Acetobacter* spp. and *Lactobacillus* spp. By plating fly homogenates, we found that the *Acetobacter* spp. abundance goes down to undetectable levels in the older flies.

When we looked at the total bacterial count by plating fly homogenates, we observed that females had a higher bacterial load than males, which can be partially explained by the difference in the body weight of the two sexes. We also found that the bacterial load in the flies increases when the protein content in the diet is increased with a concomitant decrease in the diet's carbohydrate content.

Chapter 3: Standardization of bleaching method and short-term microbiome removal studies

In the first part of this chapter, to know the microbiome's contribution to host biology, we standardized a protocol to generate flies without microbes and flies with reconstituted microbes. By comparing the phenotypes in these two types of flies, we can look at the contribution of microbes to host phenotype. To generate microbe-free (or axenic) flies, we standardized the bleach treatment by varying the bleach concentration and time. To create a corresponding control for this, we used native-microbiome-community reconstituted flies whose microbiome was reconstituted on eggs just after the bleach treatment. We also used an assay design where the assay flies have not experienced the bleach in their lifetime. The rationale for choosing these aspects is discussed in detail in this chapter.

In the second part of this chapter, we employed the two types of flies discussed above to see what happens when the microbiome is removed for a short time period of over a single generation. We found that most of the traits (a mix of life-history, stress-related, and behavioral traits) that we checked showed differences when the microbiome was removed completely, reducing the host fitness. The phenotypic changes after microbiome removal were sexually dimorphic in the case of locomotor activity and desiccation resistance. We also speculate that the body's resource allocation pattern might have changed in response to the microbiome removal due to corresponding changes in the life-history traits.

Chapter 4: Experimental evolution of *Drosophila* without its microbiome for 54 generations

We used experimental evolution on laboratory populations of *D. melanogaster* to understand how a host can evolve without its microbiome. For this, we compared replicate *Drosophila* populations reared without the microbes (labeled as MBL_{1-4}, for "Microbiota-less") for 54 host generations with their corresponding controls (labeled as MB_{1-4}), whose microbiome was reconstituted every generation. This comparison was done in two common-garden assay environments: with microbes and microbe-free. We found that contrary to what we expected, only modest adaptations were seen in MBL populations. When looking at the phenotypic plasticity of MB-MBLs across with-microbe and microbe-free environments, we saw that MBLs had lesser plasticity than MBs. This might hint at MBLs reducing their dependence on microbes.

We performed RNA-Seq on MBL_1 and MB_1 populations to see if there were changes in gene expression patterns. We found that a cluster of anti-microbial peptides (AMPs) was up-regulated, and another cluster of heat shock proteins (HSPs) was down-regulated in MBL_1 compared to MB_1. The RNA-Seq results also hinted that the peritrophic matrix could be a site of interest for further investigations related to these populations. We propose that a hygiene-hypothesis-like process in flies might explain the upregulated AMP response in flies without microbes for many generations.

Overall, these results show that while the host's homeostasis is perturbed by microbiome manipulation on short timescales, the host-microbiome integration seems more labile on longer timescales, with hosts showing potential for adaptation without the microbes. The "evolutionary addiction" hypothesis can explain our observations (Angela E. Douglas 2018a; T. J. Hammer 2023; Moran et al. 2019).

Chapter 5: Contribution of microbiome to outbred vs. inbred *Drosophila* host

While most of the studies on *Drosophila*-microbiome interactions are done on inbred host strains (i.e., strains with reduced genetic variation than the outbred strains), our results on short timescales were obtained on outbred strains. Therefore, we asked if the reduced genetic variation of the host through inbreeding affects the extent of microbiome modulation of the host phenotypes. We found that the overall modulation of the host by microbiome was less pronounced in the inbred flies than in their outbred counterparts. Inbred populations had more variation in the traits than the outbred populations. The traits with higher variation in outbred flies further increased their variation in inbred flies. Also, while both males and females showed microbiome modulation in outbred flies, only female inbred flies showed modulation by microbiome. These results reveal that outbred strains of *Drosophila* might differ in their response to microbiome than the inbred strains.

Chapter 6: Conclusion, future perspective, and implications

In this final chapter, I summarize our results, discuss potential new directions based on this work, and end with the implications of our results.

Manuscripts in preparation that are associated with this thesis:

1. Malwade, A., Koner, A., Vibishan, B., Gadkari, C., Saini, S., Khodake, V., & Dey, S. (2024, October 25). Experimental evolution of *Drosophila* without its microbiome. bioRxiv. https://doi.org/10.1101/2024.10.25.620247
2. Malwade, A., Koner, A., Vibishan, B., Khodake, V., & Dey, S. Microbes modulate multiple phenotypes of outbred *Drosophila* affecting its fitness (in prep.)
3. Malwade, A., Koner, A., Lhamo, Y., Gadkari, C., Sattaru, KC., Khodake, V., & Dey, S. Reduced host genetic variation via inbreeding leads to decreased microbiome-mediated modulation of host traits (in prep.)

Manuscripts during PhD period that are not associated with this thesis topic:

1. Lall, S., Mudunuri, A., Santhosh, S., Malwade, A., Thadi, A., Kondakath, G., & Dey, S. (2019). Adult crowding induces sexual dimorphism in chronic stress-response in *Drosophila melanogaster*. bioRxiv, 702357. https://doi.org/10.1101/702357

2. Vibishan, B., Malwade, A., Gupta, S. R., Koner, A., Khodake, V., & Dey, S. (2023, October 31). Selection for dispersal under larval malnutrition results in a non-monotonic kernel in *Drosophila melanogaster*. bioRxiv. https://doi.org/10.1101/2023.10.26.564216
3. Vibishan, B., Malwade, A., Gupta, S. R., Koner, A., Khodake, V., & Dey, S. Malnourished dispersers show sexually-dimorphic trait correlations under dispersal selection (in prep.)

Chapter 1
Introduction

Microbes are omnipresent. In nature, no animal or plant lives in isolation without coming in contact with microbes in their lifetime. In fact, multicellular organisms originated and evolved alongside the prokaryotic ancestors of microbes that we encounter today (Hooper and Gordon 2001; McFall-Ngai et al. 2013). In this light, it is no surprise that the metazoan hosts might have started outsourcing some of the services from microbes, thus developing interactions with varying degrees of dependence. Congruent with this notion, several studies across different taxa have credibly established that perturbations in microbes associated with the hosts (i.e., microbiota) can lead to alterations in the host phenotypes (McFall-Ngai et al. 2013; Moran et al. 2019; Rosenberg 2021; Sommer and Bäckhed 2013).

From initial efforts to finding the host's microbiota profile, the field has moved to characterize the mechanistic details of these interactions (Surana and Kasper, 2017). As these details continue to emerge, we are also becoming aware of the multitude of possibilities by which microbes can influence the evolutionary aspects of the hosts (L. P. Henry et al. 2021; Macke et al. 2017; O'Brien et al. 2024; Rosenberg and Zilber-Rosenberg 2016; Shapira 2016; Vega 2019). To appreciate this current understanding of host-associated microbiomes, one needs to appreciate the contribution of past efforts that helped the field to grow. Therefore, in the following sections, we briefly highlight the studies and technological aspects that were crucial in the development of the field of microbiome studies.

Early pioneering studies of microbiome: a very brief description

In the 1680s, Antonie van Leeuwenhoek fabricated a microscope and described bacteria from rainwater and his oral microbiome (dental plaque), which he called "animalcules" (Kutschera 2023; Lane 2015). However, it was not until the work of Robert Koch, Louis Pasteur, Elie Mechnikoff, Escherich, and others a century ago that we started to understand the influence of microbes on metazoan development (Savage 2001). One of the first and most important milestones in understanding the impact of microbes on metazoans was the development of the germ theory of disease (Pariente 2019). The theory established that specific microorganisms could cause particular diseases, which can be controlled by eliminating the cause, i.e., pathogenic microbes. While the role of microbes in pathogenesis was being studied, researchers also looked at the other side of the coin by looking at the positive effects of microbes on their hosts. For example, while investigating why the silkworm industry suffered heavy losses due to silkworm mortality, Pasteur discovered that alteration in

silkworm's gut microbiome can affect its health (W.-J. Lee and Brey 2013). This observation implied that some microbes are indispensable for the normal functioning of the host.

Along with Pasteur, Elie Metchnikoff investigated the role of the microbiome on digestion and nutrition. Metchnikoff et al., using common fly (*Calliphora vomitoria)* axenic larvae (i.e., larvae devoid of microbiome), showed that certain trypsin-producing bacteria were essential for normal fly development. Axenic flies without these bacteria produced smaller adults, and this phenotype could be rescued by supplementing trypsin in the media (Metchnikoff 1911). This is one of the first documented demonstrations that gut microbes produce metabolites needed in normal host development and that the axenic hosts can be used to study the effect of microbiome elimination on the host (W.-J. Lee and Brey 2013). In later sections, we will see how maintaining axenic flies has tremendously contributed to microbiome research. After these initial pioneering studies, the field of microbiome research was still limited by the techniques to culture the gut microbes.

Advances in the anaerobic culture methods

Human pathogens that can grow in aerobic conditions were well studied. Still, the gut microbiome members that needed anaerobic culture conditions received less attention due to the unavailability of robust anaerobic culture techniques. This scenario changed around the 1950s, mainly due to the roll-tube method described by Robert. E. Hungate (Hungate 1950). Others subsequently modified this method, and several methods that we still use, such as GasPak and the anaerobic glove box, were devised around this period that simplified culturing anaerobes (H. Clark 2019). With the advent of these methodologies, previously unculturable gut microbes were cultured and studied for various ways in which they influence the host.

The efforts to develop new culture methods capable of growing a vast majority of diverse environmental microorganisms are still underway. But despite these efforts, it was clear that plate counts almost always grossly underestimated the number of existing cells in the sample. This is also known as "the great plate count anomaly" (Lagier et al. 2018). This observation meant a more robust approach was required to study uncultivable and unidentifiable microbiomes. This problem was later solved by developing the DNA-sequencing-based approaches that utilized the 16S rRNA gene as a taxonomic barcode.

Rise of the DNA-sequencing-based technology

Building on the work of researchers like Carl Woese, George Fox, and Norman Pace, who used 16S rRNA sequences for phylogeny construction, the field of microbiome research received an impetus to identify and discover microbes from the gut microbiome by comparing 16S rRNA sequences across different bacteria (Zhulin 2016). Maxam-Gilbert and Sanger DNA sequencing helped speedily build 16S rRNA databases, significantly enhancing this comparison. Conserved regions in about 1.5kb long 16S rRNA gene serve as a binding site for the universal bacterial primers, and rapidly evolving regions facilitate high-resolution classification of a given strain when compared with the existing 16S rRNA database. With the renewed interest in microbiome research, facilitated by further technological development in sequencing methodology, the use of PCRs, and improvement in the associated algorithms that analyze data, the microbiome field has grown exponentially over the last few years.

Microbes affect the host in various ways

Today, a lot is known about how host and microbiota interact. While pathogenic bacteria can harm the hosts, we now know that certain microbes associated with the hosts can offer a variety of services to the hosts, such as the provision of nutrients, protection against pathogens, breakdown of complex polysaccharides and toxins that can otherwise be detrimental to the host (Moran et al. 2019). The microbiome can educate the immune system of the hosts (Blackwell 2022) and affect developmental, physiological, and even behavioral processes that are thought to be solely under host control (Cryan and Dinan 2012; Gilbert et al. 2012; Macke et al. 2017; Sommer and Bäckhed 2013). We are learning that the host-microbe interactions form a broad spectrum that ranges from pathogenicity to mutualism (Drew et al. 2021; Hooper and Gordon 2001), and certain microbes can transition within the spectrum, changing the type of association they have with hosts (Drew et al. 2021). These insights are obtained in various model organisms that continue to expand our view of the host-microbiome interactions. We discuss these models briefly below.

Current model organisms in the microbiome field

Along with sophisticated technological breakthroughs, using suitable model organisms has given new dimensions to the burgeoning field of microbiome research. These model organisms include hydra, bobtail squid, pea aphids, *Drosophila*, *C. elegans*, zebrafish, and

mice (Hoang et al. 2016; Kostic et al. 2013; W.-J. Lee and Brey 2013). Each model organism has its pros and cons. One must choose the model organism best suited for the question under consideration, even in the subfields of microbiome research (Kostic et al. 2013). For example, studying correlates of adaptive immunity at the embryo or larval stages in zebrafish will be futile because only adult zebrafish have fully developed adaptive immunity (Lam et al. 2004). We have chosen *Drosophila* for our studies for the reasons discussed below.

What makes *Drosophila* unique for microbiome studies

Drosophila has been one of the models of choice for studies on developmental biology, immunity, neurobiology, behavior, and disease phenotypes (Broderick and Lemaitre 2012; Ren et al. 2007). The information obtained from these studies over several decades and the newly available 'omics datasets for the *Drosophila* model have given rise to a toolkit that can be used for genetic manipulations needed to decipher mechanistic details of host-microbe interactions. In addition, the microbiota of laboratory-reared *Drosophila* is simple (<70 taxa) as compared to other vertebrate model systems such as zebrafish and mice with complex microbiota (200 to 500 taxa) (Broderick and Lemaitre 2012; Erkosar et al. 2013; W.-J. Lee and Brey 2013). Microbe-free (i.e., axenic) flies can be easily produced in large numbers with established protocols at low cost, making *Drosophila* an ideal choice even for ecological and evolutionary studies, in addition to the other current areas of exploration (Angela E. Douglas 2018b).

Early studies by Marion Bakula on *Drosophila* microbiome

One of the earliest accounts of how *Drosophila* gets its microbial flora post-embryogenesis comes from the pioneering work of Marion Bakula. To check if the association of the fly with microbes (specifically, bacteria) is fortuitous, she did a series of elegant investigations (Bakula 1969). She showed that eggs are sterile inside females' bodies until they come in contact with the feces. During egg laying, the egg's outer chorion layer is contaminated with the mother's fecal bacteria. Further, to check if larvae obtained these bacteria from the outer chorion layer of the egg, she stained the chorion layer with non-penetrating methylene blue dye just before egg hatching. Within 1 hour of hatching, all the larvae had the methylene blue stain in their alimentary tract, showing that larvae indeed ingest the chorion layer, thus obtaining the fecal deposits from the mother. She also showed that the egg itself is sterile

inside. This means the only source of the transmission of microbes from mother to egg is through the external chorion layer. Taking these results into account, she also demonstrated that if the egg chorion is made microbe-free or removed altogether (called dechorionation) using bleach and if these eggs are grown in a sterile environment after that, then one can have microbe-free flies. The same method has been used in several recent microbiome research investigations exploring the genetic and molecular players involved in host-microbe crosstalk.

Apart from maternal transfer of the microbiota as described by Bakula, several other factors such as age, strain, developmental stage, and diet are known to influence the fly microbiome (Chandler et al. 2011; Han et al. 2017; Adam C-N Wong et al. 2013). The emerging insight in the field is that every population of *Drosophila*, lab-reared or natural, has its characteristic microbiota composition that is decided by a suit of factors including, but not limited to, those mentioned above. A more detailed discussion of these factors is presented in Chapter 2.

Microbes affecting *Drosophila* phenotypes

Using *Drosophila* as a model and using a method similar to Bakula's to render flies microbe-free, there is a rich literature available on how microbes of *Drosophila* affect the host phenotypes (Broderick and Lemaitre 2012; Buchon et al. 2013; Angela E. Douglas 2018b; Erkosar et al. 2013; W.-J. Lee and Brey 2013; Ludington and Ja 2020). While we discuss this in detail in Chapter 3, here we note that different microbes in different labs have been shown to affect diverse phenotypes of *Drosophila*, in turn affecting their fitness.

Role of microbes in host evolution

Any process or entity affecting individual fitness is of evolutionary importance. For example, dispersal is a well-studied process affecting organism fitness (positively or negatively, depending on the context). This change in fitness can cause allele frequencies to change, thus playing an essential role in evolution by natural selection. Similarly, in the past few decades, numerous host-associated microbiomes have been shown to affect host fitness. These microbiomes have a vast gene pool that is over and above the host's genotype. This expanded gene pool can provide the hosts with essential services (e.g., amino acids, short-chain fatty acids, and protection from pathogens). Therefore, host-associated microbiomes can be drivers of host evolution (L. P. Henry et al. 2021). Consider a hypothetical scenario where an

environmental fluctuation reduces the availability of the essential amino acids in the host diet to very low levels. Now, if a few of the amino acid-deprived hosts, by chance, get associated with a set of microbes producing these essential amino acids, then all the other hosts without the supply of these amino acids will eventually perish as symbiotic hosts through their improved fitness, take over the population. This assemblage of host plus amino acid producer microbes will be selected over time. This is one hypothetical way in which microbes could aid their host's evolution.

The thought that symbiosis can be a mechanism of evolution is old (Margulis 1975; Margulis and Fester 1991; Sagan 1967; Sapp 1994) but has gained newfound importance recently as we study diverse types of symbioses across the tree of life (Morris 2018; O'Malley 2017). Of particular interest has been the symbiosis between hosts and their associated microbes, which is considered to be a legitimate unit of selection. This unit, i.e., host and its microbes together, is called the holobiont, and the total genetic material of the holobiont is called the hologenome. Hologenome consists of the host's own genetic material, including mitochondrial/chloroplast genomes and genomes of all the associated microbes (Rosenberg 2021).

In the following sections, we discuss the hologenome theory and its critiques in detail.

The hologenome theory of evolution was proposed by Ilana Zilber-Rosenberg and Eugene Rosenberg (Rosenberg 2021; Rosenberg and Zilber-Rosenberg 2016, 2018; Zilber-Rosenberg and Rosenberg 2008) although the term was informally coined in 1994 by Jeff Richardson (Arnold 2013). Initial studies on the effects of microbes on coral bleaching (Kushmaro et al. 1996) were followed by the observation that the coral could become resistant to one of the pathogens (Reshef et al. 2006). This suggested that the interaction between the hosts (corals in this case) and their accompanying microbes can be bidirectional. This insight was ultimately generalized to the hologenome theory (Zilber-Rosenberg and Rosenberg 2008), which, in its current form, has four principles. We are reproducing the original text from their 2018 paper, which contained a refined version of their original arguments, to avoid any confusion that paraphrasing might entail. These four principles are:

1. *All animals and plants harbor abundant and diverse microbiota and are thus considered holobionts.*
2. *The host with its microbiome, the holobiont, functions generally as a distinct biological entity anatomically, metabolically, immunologically, during development*

and in evolution. (An entity is defined as "an independent thing; that which contains in itself the conditions essential to individuality; that which forms of itself a complete whole.")

3. *A significant fraction of the microbiome genome together with the host genome is transmitted from one generation to the next and thus can propagate unique properties of the holobiont.*
4. *Genetic variation in the hologenome can be brought about by changes in the host genome as well as by changes in the microbiome genome. Since the microbiome genome can adjust to environmental dynamics more rapidly and by more processes than the host genome, it can play a fundamental role in the adaptation and evolution of the holobiont.*

We agree with the fourth principle, but we think there are issues with the other three principles of the theory (highlighted in red ink). Most of these shortcomings have already been pointed out by several authors (Angela E. Douglas and Werren 2016; Moran and Sloan 2015; Morris 2018; Rodrigo 2023; Skillings 2016) and we present a summary of these objections.

The first principle says that "all" animals and plants are associated with "abundant and diverse" microbes. In his article "Not all animals need a microbiome," Tobin Hammer illustrates that many organisms do exist without microbiomes (T. J. Hammer et al. 2019). Rodrigo et al. (2023) also made this same observation as ours (Rodrigo 2023). Moreover, how does one set a benchmark for "abundant and diverse"? What are we comparing here, i.e., "abundant and diverse" with respect to what? No prescription is provided in this respect. We begin to see that the wording of the theory is not precise.

The definition of holobiont itself needs to be more precise from the practical standpoint (Morris 2018). Organisms interact with various microbes from their surroundings – some are physically closer to the host than others. How do we decide where to draw the boundary? In other words, where does the holobiont end? Drawing such a line in ecosystems with complex interactions is challenging, as these interactions may be hidden and difficult to discern. It is known that some microbes can form stable associations with the host, while others are merely transient partners. When partners are transient, will they be part of the holobiont, even if a transient lifestyle lowers the probability of co-transmission?

The second principle has faced the most criticism. It says that the holobiont (host plus all of the microbiome) is a distinct entity and unit of selection. This unit is also described as an independent "superorganism" (Morar and Bohannan 2019; Zilber-Rosenberg and Rosenberg 2008). Morar and Bohannan define a superorganism as "a collection of organisms (from the same or from different species) that interact closely and from which functions emerge that do not exist at the level of individual organisms" (Morar and Bohannan 2019). Douglas and Werren have criticized the claim that hosts and microbes evolve as a single cooperative selection unit (Angela E. Douglas and Werren 2016). The criticism stems from the observation that for the holobiont to be a unit of selection, the interests of all the constituent entities of the holobiont must be aligned, suppressing any conflict between host and microbes and between different microbes. Douglas and Werren argue that such an alignment might exist in a few cases but will be rare, and evidence for such alignment of interest is lacking. Also, they argue that by ignoring conflicts and antagonistic interactions and focusing on only cooperative aspects, the hologenome theory of evolution remains restrictive. The authors suggest that the existing "microbiome as an ecological community" approach is well suited to understand the host-microbiome interactions and their evolutionary implications – a new framework is not needed. Moran et al. also pointed out similar issues in their critique of the theory. We generally agree with the points made in these two studies (Angela E. Douglas and Werren 2016; Moran and Sloan 2015).

In the third principle, the theory says a "significant" portion of the microbiome is transmitted to the next generation. Indeed, for the holobiont to be considered as a unit of selection, faithful reconstitution of the holobiont across generations is needed. However, the most common modes of microbiome transmission are not that faithful (Shapira 2016), introducing substantial noise in the holobiont reconstitution process. Also, in many symbiotic systems, the modes of transmission are not yet well established, clouding our ability to comprehend what portion of the microbiome is faithfully transmitted. Even if a part of the microbiome is transmitted with high fidelity, the probability that all the microbes in the microbiome will achieve this is likely low. This further lowers the chance that holobiont will likely act as a unit of selection.

In summary, we agree with what Douglas and Werren observed, "diverse transmission modes, metapopulation structures, and styles of interaction are the norms in complex host-microbiome assemblages. Therefore, it is highly unlikely that the entire microbiome will evolve as a "holobiont" with its host (Angela E. Douglas and Werren 2016)."

What we have done in this thesis

The concept of ubiquitous and tightly integrated host-microbiome cross-talk has raised several interesting possibilities about how we view these tight associations and their biological implications if we define the system of interest as "host + microbes" (see Morar and Bohannan, 2019 for a detailed discussion). For example, as discussed in the last section, the proponents of the hologenome concept claim that the host and associated microbes together can be considered a cooperative unit on which selection acts (Rosenberg and Zilber-Rosenberg, 2014). To test such claims and to get conceptual clarity on what roles microbes can play in host evolution, one needs carefully designed experiments on a system that can be studied for multiple generations and whose microbiota can be easily manipulated. While we do not take on these claims head-on in this thesis, we decided to carefully study the "host + microbes" unit as a foundational step towards that goal. We thought one way to study this unit was to dismantle it one part at a time. While several single-generational short-term studies have demonstrated the utility of this approach, long-term studies are limited. The insights from these long-term studies can help build on the short-term studies to give us a better view of how integrated the host-microbiota system really is.

Before directly embarking on the long-term studies, we took several steps that guided us in designing the long-term aspects. We first studied the composition of microbes in our fly populations and how fly age, sex, and diet composition affect these microbes (Chapter 2). In the first part of Chapter 3, we adopted and standardized a method to generate flies without their microbes and the control flies that will have their microbes. The difference between the phenotypes of these two types of flies is taken as a contribution of these microbes to the host phenotype studied. In the second part of Chapter 3, we studied how these microbes influence host biology over a timescale of a single host generation. This gave us a handle on the nature of the symbiosis (ecological principles) at work in our outbred lab flies. We then dismantled the "host + microbes" unit over the long term by maintaining fly hosts without their microbes for 54 generations and studying flies' adaptation without their microbes (Chapter 4). Thus, Chapters 3 and 4 describe what happened when we manipulated the "microbe" part of the unit, the former on a single-generation timescale while the latter on a multi-generation timescale. We then decided to tinker with the "host" part (Chapter 5). One cannot completely remove the host from the picture, as studying microbes in isolation would not yield meaningful insights about the unit. Then, the question is, how do we tinker with the host side without taking the host out of the picture entirely? We achieved that through inbreeding. We

used ten generations of inbreeding via full-sib matings with a population size of two to reduce the genetic diversity of the host. We created four inbred lines from four outbred populations and then subsequently studied the microbe's contribution to inbred vs. outbred hosts using the same experimental framework as described in Chapter 3. By doing this, we wanted to know if there are any differences in how the microbiome plays its part when the host is inbred vs. outbred. Would the microbiome part increase its contribution to the host's fitness when the host is losing its genetic variation and fitness through inbreeding? Finally, we put together all our insights in the last chapter (Chapter 6) and point toward interesting avenues for future work.

Chapter 2
Composition of outbred *Drosophila* microbiome and how it changes with age, sex, and diet of the host

2.1 Introduction

Microbes have successfully lived on Earth for about ~3.5 billion years (Knoll 2015). Metazoans that followed them originated and evolved in the niche where microbes were already present (McFall-Ngai et al. 2013). This co-occurrence continues to the present day and is likely to continue for times to come. As described in Chapter 1, advances in sequencing and culturing techniques have allowed us to ask many important questions in the context of host-microbiota interactions. Are some groups of microbes better at colonizing particular hosts? If yes, what factors determine their colonization success? (Ganesan et al. 2022; Vega 2019) How complex are the communities that exist in various parts of organisms? Does host-associate microbiota go through cycles of succession (C. Martino et al. 2022)? Is the assembly of the microbiota a deterministic or a stochastic process (Obadia et al. 2017)? Are there priority effects (Debray et al. 2022; Fukami 2015)? How stable (Faith et al. 2013; Revel-Muroz et al. 2023) and heritable (Grieneisen et al. 2021; Opstal and Bordenstein 2015) are the microbiota? The inquiry into all such questions begins with knowing what microbes are present in the host microbiome and how this composition changes with various factors.

The composition of the *Drosophila* microbiome is well-studied (Adair et al. 2018; Bost, Franzenburg, et al. 2018; Broderick et al. 2014a; Broderick and Lemaitre 2012; Chandler et al. 2011; Corby-Harris et al. 2007; Cox and Gilmore 2007; Erkosar et al. 2013; Mazzucco and Schlötterer 2021; I. S. Pais et al. 2018; Ren et al. 2007; Ridley et al. 2013; Staubach et al. 2013; Téfit et al. 2018; Adam C-N Wong et al. 2013; C. N. A. Wong et al. 2011). The laboratory-reared strains of *Drosophila* are generally dominated by Firmicutes (high abundance on a complex polysaccharide diet) and Proteobacteria (high abundance on a sugar-rich diet) (Angela E. Douglas 2018b). It is also known that the microbiomes of the wild-caught *Drosophila* tend to be more diverse than their laboratory counterparts (Adair et al. 2018; Bost, Martinson, et al. 2018; Corby-Harris et al. 2007). The genera *Lactobacillus* and *Acetobacter* are found in most laboratory and wild populations. They have been used extensively in studies unravelling molecular-level details of the host-microbe interactions (Blum et al. 2013; Dodge et al. 2023; Erkosar et al. 2013; Fast et al. 2018; Jia et al. 2021; M. E. Martino et al. 2018; Shin et al. 2011; Storelli et al. 2011; Suyama et al. 2023). Along with the genus *Enterococcus*, *Lactobacillus* and *Acetobacter* form members of the *Drosophila* "core" gut microbiome (Erkosar et al. 2013). Unlike mammalian gut microbiota, most of the taxa in the *Drosophila* gut microbiome are aerobic or aerotolerant (Angela E. Douglas

2018b). This means these microbes can easily be grown in the lab in a normal oxic environment. The low diversity of the fly microbiome also makes this system tractable in microbiome manipulation studies (Angela E. Douglas 2018b; Erkosar et al. 2013; C. N. A. Wong et al. 2011).

Although these aspects of the core gut microbiome in *Drosophila* are well-known, many host and environmental factors can affect the microbiome composition (Benson et al. 2010; Hasan and Yang 2019; Li et al. 2020). For example, the genotype of the host can affect what kind of microbiomes it accommodates (Blekhman et al. 2015; Bonder et al. 2016; Dzierozynski et al. 2023; Goodrich et al. 2014). Using genome-wide association studies (GWAS) on *Drosophila* genetic reference panel (DGRP) lines, a set of host genes was shown to affect the abundance of *Acetobacter tropicalis* (Chaston et al. 2016). While host genotype is broadly invariant as host ages, the expression of host genes can change with age (Kadoguchi et al. 2022). Along with the changes in gene expression patterns, age-specific changes in host physiology could remodel the gut microbiomes of hosts (Ghosh et al. 2022; Wu et al. 2021). These gene expression patterns and physiological changes could be sex-specific, and hence, along with host genotype and host age, host sex can affect microbiome composition (Kim et al. 2020; Valeri and Endres 2021; Yoon and Kim 2021). Among the environmental factors, the host diet can have a significant effect on microbiome composition (Chandler et al. 2011; Dapa et al. 2022; David et al. 2014; Heiman and Greenway 2016; Heras et al. 2022; Turnbaugh et al. 2009).

Given that microbiome composition can vary with host-related and environmental factors, as mentioned above, and given that these factors differ between different labs, almost every host-microbiome system can be unique – one needs to be very careful while extrapolating any results to their own system. Therefore, before starting our studies, it was imperative to establish the nature of microbiota in our own flies and how it changes with various factors.

We wanted to know what microbes are associated with our outbred baseline laboratory populations of *Drosophila* that trace their ancestry to wild-caught IV lines (Ives 1970; Rose 1984). While we know the microbiomes of inbred strains such as *Oregon-R*, *Canton-S*, *yw*, and w^{1118} (Broderick and Lemaitre 2012; Han et al. 2017), we have not come across any study that has reported on the microbiomes of the outbred laboratory populations of *Drosophila* kept at a large population size.

When we sequence the microbiomes at only one time point, we get a snapshot of the microbiome that is generally shown to be labile and which is probably influenced by the most recent biological milieu of the host. For the host, aging is inevitable; as the host ages, this milieu will change (Aleman and Valenzano 2019). What happens to the composition of the microbiome then? Is it kept constant by the host, indicating that the associated microbes are crucial for host fitness throughout its life, or does the microbiome change in response to processes that happen during host aging? Studies on many model organisms, including *Drosophila*, have shown that the microbiome changes with the host age (Bana and Cabreiro 2019), and in general, the abundance of microbes increases with the age of the fly (Brummel et al. 2004; R. I. Clark et al. 2015; Guo et al. 2014; H.-Y. Lee et al. 2019; C. N. A. Wong et al. 2011). However, it is possible that these patterns can change based on the diets or genotypes of the flies. Thus, it was important for us to understand how the microbiome changed over the lifetime of our outbred fly populations raised on the banana-jaggery food medium in our lab.

Along with age, the sex of organisms can also influence their microbiome (Elderman et al. 2018). *Drosophila* shows sexual dimorphism in various traits like body size (Bharathi et al. 2004; Carreira et al. 2009), development time (Bharathi et al. 2004), stress resistance (Matzkin et al. 2007; Tower et al. 2020), and behavioral traits (Asahina 2018) including social interactions (Leech et al. 2021; Panos et al. 2024), foraging and locomotion (Shu et al. 2021). This can affect the carrying capacity of different microbes, in turn affecting the total bacterial load each sex can harbor. While Han et al. reported on the role of sex in two different *Drosophila* strains (*Canton-S* and w^{1118}), the study only looked at the bacterial composition but not the bacterial abundance (Han et al. 2017). This study showed that apart from the *Enterococcus* that only differentially showed up in females of both strains, the overall composition was similar in males and females.

The diet of the host can determine the microbiome composition of *Drosophila* (Chandler et al. 2011; Erkosar et al. 2018; Y. Henry et al. 2020). Studies have shown that adapting to the fly's diet might be the foremost obstacle microbes have to tackle to get stably associated with the hosts (M. E. Martino et al. 2018). The central role of diet in deciding the host's microbiome composition makes intuitive sense as the microbes actively metabolize the host's diet components, making these metabolites available not only to the host but also to the other microbes in the community. If the source of these dietary components changes (e.g., a shift

from protein-based to carbohydrate-based diets), then that could affect the composition of the microbiome in the gut.

In laboratory stocks, fly-associated microbes may have a phase where they need to persist on the flies' food. Therefore, it is fair to assume that dietary changes could reshape the host microbiome. As a proof of principle, we wanted to see how the microbiome abundance might respond to systematically varied protein-to-carbohydrate ratio, as we already had a system in place to test this. In addition, the *Drosophila* studies where microbes were shown to help the host had a nutrient limitation as a common stressor (Grenier and Leulier 2020a; Shin et al. 2011; Storelli et al. 2011). Specifically, diets with poor protein content were shown to capture the microbe's role in modulating host physiology effectively. We wanted to find out how the varied protein-to-carbohydrate ratio changes the host microbiome abundance in general and in a particular scenario where diet protein content is low (i.e., low protein-to-carbohydrate ratio).

In summary, in this chapter, we asked what microbes are associated with our flies and how this association might change with age, diet, and sex of the *Drosophila* host. We found that our laboratory populations of outbred *Drosophila* have a simple gut microbiome dominated by *Acetobacter* and *Lactobacillus* spp. This microbiome changes as the host ages and *Acetobacter* fraction reduces in old flies. The bacterial load is higher in females than in males, but normalization by the body weight can explain this result. When we varied the protein-to-carbohydrate ratio (achieved by changing the yeast-to-sugar ratio), we observed a reduction in the bacterial load as the protein content in the diet decreased.

2.2 Methods

2.2.1 *Drosophila* NDB stocks and rearing

Wolbachia-free *Drosophila melanogaster* used in the single-generation study were derived from four large, outbred laboratory populations named New Dey Baselines (NDB_{1-4}). These fly lines were maintained on the standard banana-jaggery food (see Table 2.2) with a 14-day discrete generation cycle at 25^0C under constant light conditions with a breeding population of ~2400 individuals. NDBs trace their ancestry to the DB populations (see section 2.2.3 for the details), which in turn trace back to the wild-caught IV lines (Ives 1970; Rose 1984) via the JB_{1-4} populations (Sheeba et al. 1998).

2.2.2 Maintenance regime of baseline *Drosophila* populations (NDB_{1-4})

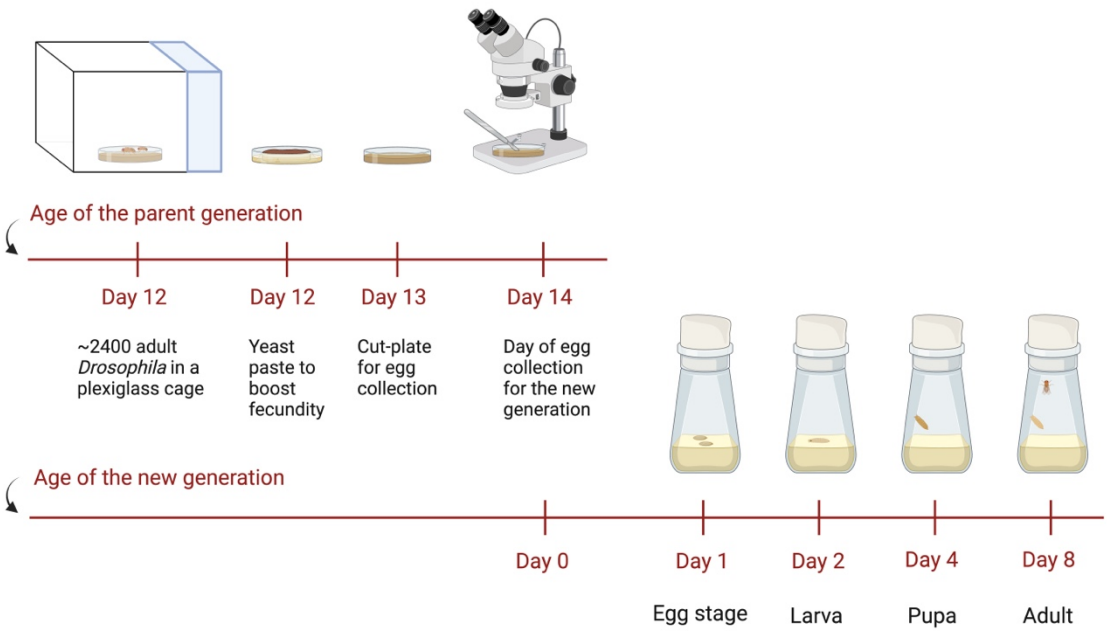

Figure 2.1 Maintenance regime of baseline *Drosophila* populations (NDB_{1-4}). This image is created using www.biorender.com.

In the lab, adult *Drosophila* of four baseline populations (NDB_{1-4}) are maintained in plexiglass cages (25 cm x 21 cm x 16 cm) at a large population size of ~2400 (Figure 2.1) per population. On day 12 (after egg deposition), the fly population is provided with a moist yeast paste on top of the banana-jaggery food to boost egg output. This yeast paste also contains a few drops of glacial acetic acid to attract the flies to the paste. At the end of day

13, a food plate, with vertical edges of food exposed for oviposition, is provided for 12-16 hrs. On day 14, deposited eggs are collected by cutting thin food strips from the exposed surface, under a binocular stereo microscope. The size of the strips is adjusted under the microscope using a clean scalpel such that each strip contains about 400 eggs. These strips are then transferred to plastic and translucent *Drosophila* milk bottles containing ~50-60ml of banana-jaggery food at the bottom. This marks the new generation's 0^{th} day after egg deposition (Figure 2.1). Adult flies arising from six such bottles are transferred to fresh plexiglass cages on the 12th day (post egg collection). This gives rise to a new generation with a population size of ~2400 individuals, which do not coexist with the adults of their parental generation at any stage.

2.2.3 *Drosophila* DB stocks and rearing

NDB_{1-4} were derived from their most recent ancestors in our lab, Dey Baselines (DB_{1-4}) (Sah et al. 2013). These four DBs were maintained in our lab for 220 generations and were used as baselines for this period. We pooled an equal proportion of eggs from each DB population and created four independent NDBs. We maintained NDBs separately for ~10 generations without any assays for them to stabilize after this intermixing. We give these details as initial bleaching standardization (Chapter 3) happened on these populations. The maintenance of DBs was similar to NDBs except for two differences: (a) DBs were maintained in *Drosophila* 8-dram vials at an egg density of ~ 60 per vial with ~ 6ml of food as opposed to NDBs that are reared in milk bottles (b) DBs were yeasted on 18^{th} day, provided with a cut plate on 20^{th} day, and their eggs were collected for next generation on day 21. Thus, DBs followed a 21-day discrete generation cycle as opposed to a 14-day discrete generation cycle followed by NDBs.

2.2.4 Fly-associated bacterial load analysis

2.2.4.1. Culture-dependent method

Flies were handled aseptically inside a Level II biosafety cabinet (make: Microfilt, India). Adult flies (day 12 after egg deposition, unless stated otherwise) were sorted by sex under mild CO_2 anesthesia. Five male and five female flies under anesthesia were surface sterilized with 70% ethanol in 1 ml microcentrifuge tubes. These flies were then homogenized in 200 µl of autoclaved dH_2O and plated on the nutrient agar (NA)(HiMedia) or the de Man, Rogosa, and Sharpe (MRS) medium (HiMedia) and incubated for 72 hrs and 48 hrs, respectively, at 25^0C under aerobic conditions. NA is a general purpose media that supports

the growth of a wide variety of microbes and MRS is conducive for the growth of fly associated microbes such as *Lactobacillus* and *Acetobacter*. 100 µl of fly homogenates were spread-plated using 5-6 glass beads to check the microbe-free state, and 20 µl of drops were spotted on the media for the bacterial load. Dilutions with 10-100 colonies were counted, and the counts were multiplied by the overall dilution factor to estimate colony-forming units (CFUs) per fly.

2.2.4.2. Culture-independent method

To test the bacterial load using PCR, total DNA was extracted from eight to ten adult flies using the QIAamp DNA Mini kit (Qiagen, Catalogue No. 51304) as per the manufacturer's instructions. We modified three steps of the manufacturer's protocol in line with the protocol (Jehrke et al. 2018). First, we used mechanical homogenization (with a homemade handheld motorized pestle mixer) to lyse the tissue in 1.5ml Eppendorf tubes instead of just Proteinase-K treatment. Second, 20 µl of 20 mg/ml RNase A (HiMedia, Catalogue No. DS0003-5ML) was added to each sample after tissue lysis. Third, the sample was incubated with Buffer AL at 70^0C for 30 min instead of 10 min. We avoided any vortex step after tissue lysis to minimize the shearing of the DNA. The quality of total DNA was checked on the agarose gel. This DNA, composed of the fly and microbial genomes, was used as a template for PCR using 16S bacterial universal primers with a product size of about 1.5kb. The details of the primers are given below in Table 2.1. The primers were ordered from Sigma-Aldrich, India. The PCR reactions were set up using GoTaq® Flexi DNA Polymerase (Promega, Catalogue No. M829) with 40 ng gDNA input. The initial denaturation was at 94^0C for 2 min, followed by 30 cycles of (denaturation at 94^0C for 30s, annealing at $T_m = 60^0$C for 1 min, extension at 72^0C for 1 min), with final extension 72^0C for 10 min. The PCR products were visualized on 1% agarose gel with 1kb plus DNA ladder (NEB, Catalogue No. N3200S) as the molecular weight marker.

Table 2.1 16S bacterial universal primers used for PCR

Primer	Sequence	Label	Reference
Forward	AGAGTTTGATCCTGGCTCAG	fD1	(Weisburg et al. 1991)
Reverse	ACGGCTACCTTGTTACGACTT	rP2	(Weisburg et al. 1991)

2.2.5 A note on the gut bacteria as a primary focus area of this study

Like many other studies in the literature, when investigating the microbiome using plating of fly homogenates or 16S rRNA sequencing, we have gotten rid of the surface microbes by surface sterilizing the flies (Gould et al. 2018; Kietz et al. 2018; Koyle et al. 2016). This means our results pertain to the microbiome composition from the internal tissue of the whole fly, and excludes the outer surface. Thus, we make the implicit assumption that the dominant portion of the microbiome related to the fruit flies comes from their internal tissues, particularly the gut (Storelli et al. 2011; Adam C-N Wong et al. 2013). This assumption is supported by an experimental study that showed no significant differences in CFU counts between isolates from the whole body and the gut tissue in both laboratory and wild-caught flies (M. Pais 2011).

In a strict sense, we could have sequenced microbiomes from only the gut tissue by dissecting the gut out of the fly. But, the scale at which this needs to be done for our multiple baseline and derived populations makes it a difficult task to achieve. In literature, studies have relied on using fly full-body homogenates instead of doing gut dissections (Corby-Harris et al. 2007; Cox and Gilmore 2007; Jehrke et al. 2018; Ren et al. 2007; Ridley et al. 2013; Téfit et al. 2018; C. N. A. Wong et al. 2011). In that light, for purely logistic reasons, we made a choice to avoid gut dissections.

In addition, we have not studied the roles of microbes other than bacteria, such as yeasts, viruses, and archaea. Along with the other microbes, we have also not investigated how endosymbionts such as *Wolbachia* or *Spiroplasma* (if present) affect fly hosts.

2.2.6 Identification of fly-associated bacteria by Sanger sequencing

Isolated bacterial colonies on agar plates were used as a DNA source for colony PCR using 16S bacterial universal primers. The PCR product (~1.5kb) was column purified using the QIAquick PCR purification kit (Qiagen, Catalogue No. 28104) and sent to a commercial vendor (1st BASE laboratories, Malaysia), for Sanger sequencing. The template was sequenced with forward and reverse primers (Table 2.1). The sequences obtained were aligned against the NCBI database using BLAST to identify the bacterial strains with the highest sequence similarity.

2.2.7 Population-level 16S rRNA amplicon sequencing

To know the relative microbiota composition in *Drosophila* populations, DNA was extracted as described in section 2.2.4.2. DNA was run on 1% agarose gel to see the extraction quality. After the integrity check on agarose gel, DNA was sent for 16S rRNA V3-V4 region

amplicon sequencing to a commercial vendor (1st BASE Laboratories, Malaysia). Sample quality was again confirmed by the vendor using agarose gel, spectrophotometer, and fluorometric method. All samples passed DNA quality check and were subjected to amplicon PCR followed by a quality check. These samples were then processed to prepare amplicon libraries using 2-step PCR according to Illumina's 16S metagenomic library preparation guidelines (Link to the guidelines - https://sapac.support.illumina.com/downloads/16s_metagenomic_sequencing_library_preparation.html). These libraries were processed using Illumina's MiSeq platform using 2x300bp chemistry.

Data analysis of amplicon sequencing

Sequence adapters and low-quality reads were removed from the paired-end reads before the first 2,00,000 raw reads were extracted using BBTools. Then, the forward and reverse reads were merged using QIIME. DADA2 pipeline (https://benjjneb.github.io/dada2/) was used to remove low-quality regions and chimeric errors. The resulting data was in the form of amplicon sequence variants (ASVs). The taxonomic classification was done using scikit-learn (https://scikit-learn.org/stable/) and the Naive Bayes classifier against the SILVA database (release 132). This part of the analysis was performed by the commercial vendor (1st BASE Laboratories, Malaysia).

2.2.8 Diet of the *Drosophila*: banana-jaggery medium

In this thesis, we have used the *Drosophila* diet with the following ingredients for routine maintenance and for experiments unless stated otherwise.

Table 2.2 Composition of the banana-jaggery medium

Component	Per Liter (L)
Banana (g)	205
Barley (g)	25
Jaggery (g)	35
Yeast (g)	36
Agar (g)	12.4
Water (mL)	120

Ethanol (mL)	22
Benzoate (g)	2.4
Ethanol for Benzoate (mL)	23

2.2.9 Cornmeal diet with different P:C ratios (i.e., different Yeast: Sugar ratios)

To look at the effect of different protein-to-carbohydrate ratios in a fly diet, we used a previously standardized cornmeal-based fly diet instead of a banana jaggery medium. In this diet, the main protein source was yeast, and the main carbohydrate source was sugar. By varying the amount of these components, we could generate the cornmeal diet with different Yeast: Sugar ratios, as mentioned in the table below.

Table 2.3 Composition of the cornmeal diet with different P:C ratios

Component	**1:1**	**3:1**	**1:3**	**4:0**
Yeast (g)	40	60	20	80
Sugar (g)	40	20	60	00
Corn (g)	100	100	100	100
Agar (g)	12	12	12	12
Water (L)	1.12	1.12	1.12	1.12
Ethanol (mL)	10	10	10	10
Benzoate (g)	1	1	1	1
Propionic acid (mL)	10	10	10	10

2.2.10 Statistical analysis

Statistical analysis was performed using STATISTICA v5 (StatSoft Inc, Tulsa, Oklahoma) and Past 4 (Ø. Hammer and Harper 2001). For the data in the form of CFU counts, we used log transformation before the ANOVAs (Zar 1999). The details of ANOVAs are given in the respective results sections. For significant main effects in ANOVAs, pairwise differences were analyzed using Tukey's HSD test. For interpreting effect sizes using Cohen's d, the following criteria were followed: $d > 0.8$ (high); $0.8 > d > 0.5$ (medium); $d < 0.5$ (low). The graphs are plotted in GraphPad Prism (v10.2.1). The figures drawn using BioRender have that specification in their legend.

2.3 Results

2.3.1 Laboratory fly populations are associated with *Acetobacter* and *Lactobacillus spp.*

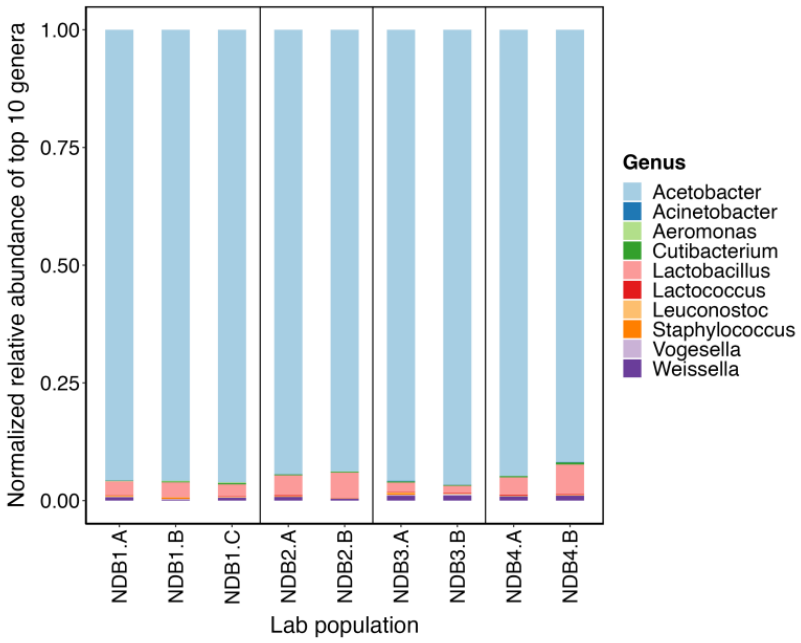

Figure 2.2 Composition of the outbred laboratory-reared *Drosophila* microbiome. Bacterial 16S rRNA amplicon sequencing showed that Genus *Acetobacter* dominates the baseline lab populations NDB_{1-4}. Genus *Lactobacillus* is present in all samples as well. The alphabets in the label (A, B, C) indicate biological replicates. This sequencing was done on full-body DNA extracted from female flies collected on day 12 (after egg collection).

To understand the bacteria associated with our four outbred baseline populations NDB_{1-4}, we extracted genomic DNA from surface sterilized flies and subjected it to 16S rRNA amplicon sequencing (see Method section 2.2.7 for details). All the samples showed the presence of *Acetobacter* and *Lactobacillus* (Figure 2.2). This observation is shared by several other studies reporting the widespread presence of these taxa in natural and laboratory *Drosophila melanogaster* populations (Adair et al. 2018; Broderick and Lemaitre 2012; Angela E. Douglas 2018b; Erkosar et al. 2013; Adam C-N Wong et al. 2013).

To further validate this observation, we plated fly homogenates of surface sterilized flies onto Nutrient Agar (NA) and de Man–Rogosa–Sharpe (MRS) medium (see Method section 2.2.4.1 for details). The colony morphology of different isolated colonies on MRS-agar plates resembled *Acetobacter* and *Lactobacillus* spp. *Acetobacter*-like colonies were toffee-colored, and *Lactobacillus*-like colonies were yellowish or white with a curd-like appearance (Figure 2.3). This agrees with morphological characteristics reported by others (Koyle et al. 2016;

Mokeev et al. 2021). To confirm this identification, individual colonies were subjected to colony PCR using bacterial 16S rRNA universal primers (see Method section 2.2.6 for details). Purified PCR products were Sanger sequenced using bacterial 16S rRNA universal primers. These Sanger sequencing results showed that colony morphology-based identification was correct.

Taken together, these observations established that the microbiome composition of our outbred laboratory-reared baseline *Drosophila* populations is simple, with the dominance of *Acetobacter* and *Lactobacillus* spp. This result is shown using culture-dependent as well as culture-independent methods.

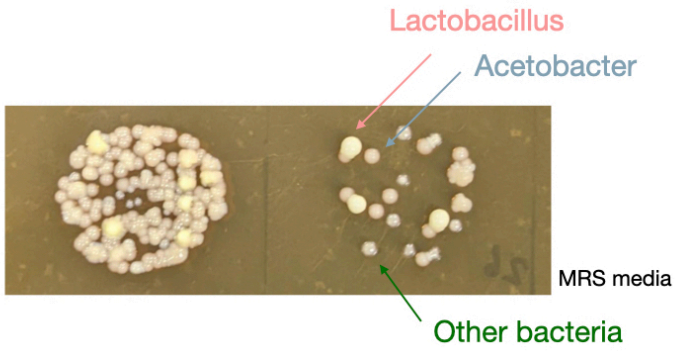

Figure 2.3 Two major types of colonies on MRS-agar. Colony morphology of *Acetobacter* and *Lactobacillus* on MRS. The left spot has a 10x lower dilution than the right spot. Both are from the same sample.

2.3.2 The microbiome composition changes with fly age

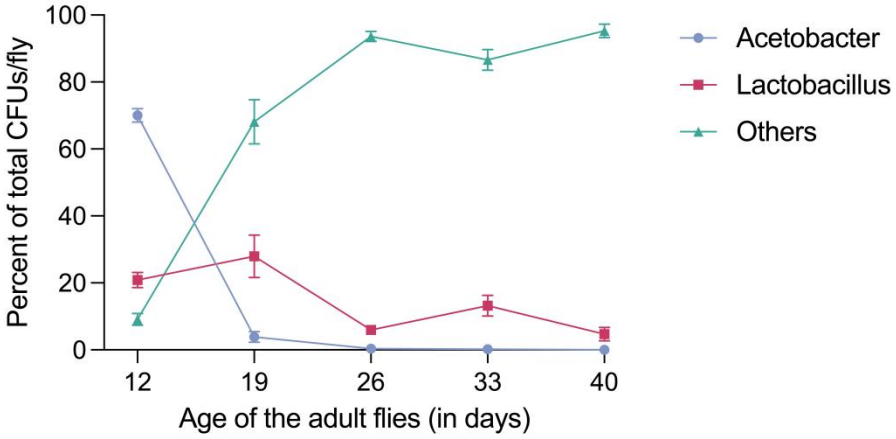

Figure 2.4 Change in the composition of the *Drosophila* microbiome with age. Percent of total CFUs/fly as a function of age. This data is pooled over four baseline lab populations NDB_{1-4}. Each population had four biological replicates. The points indicate the mean, and the error bars indicate SEM over four population replicates NDB_{1-4}.

Having identified the colonies of two dominant genera, we used the distinct colony characteristics of *Acetobacter* and *Lactobacillus* on MRS-agar to study microbiome changes with host age. We plated fly homogenates of ten surface sterilized flies (comprising five males and five females per replicate) at different fly ages. This was done with four baseline lab populations NDB_{1-4}. The results from the four populations were pooled together as individual populations showed similar trends (as also evidenced by the small error bars) (Figure 2.4).

We found that the profile of colony-forming units (CFUs) per fly on day 12 after egg collection matches the 16S rRNA sequencing results from flies of the same age (Figure 2.2). *Acetobacter* and *Lactobacillus* colonies dominated the profile on day 12. This changed from day 19 onwards - while *Acetobacter* and *Lactobacillus* reduced in numbers, colonies with distinct morphologies from these two increased in dominance over time. The total CFU number (usually between ~ 10^3 to 10^6 in our other experiments as well) is well within the range of CFU estimates available for the fruit flies in the literature (Blum et al. 2013; Broderick et al. 2014a; Dodge et al. 2023; Gould et al. 2018; Koyle et al. 2016; I. S. Pais et al. 2018; M. Pais 2011; Ren et al. 2007; Storelli et al. 2011).

2.3.3 The sex-specific difference in the number of associated microbes can be partially explained by the body size difference between the two sexes

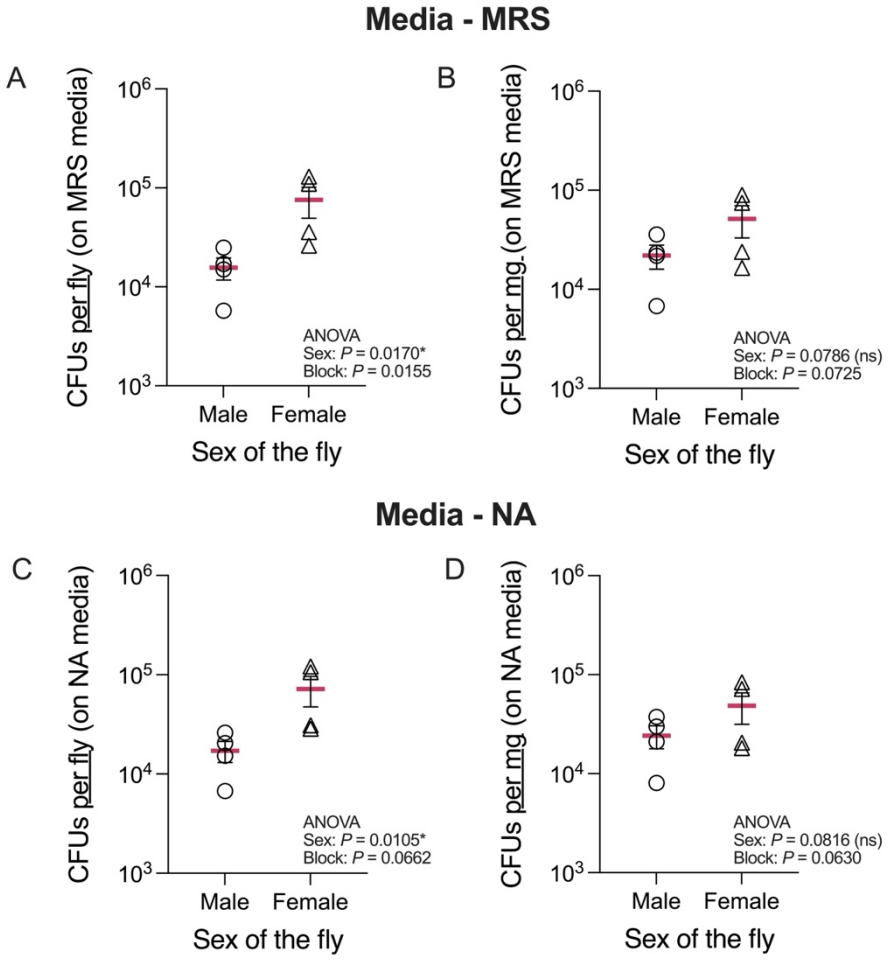

Figure 2.5 Sex-wise difference in total associated bacteria of *Drosophila.*
Total CFUs per fly: (A) on MRS-agar and (C) on NA media. Total CFUs per fly normalized by the body weight of flies before plating: (B) on MRS-agar and (D) on NA media. Each symbol represents the mean CFUs for each of the baseline populations. Four such symbols per treatment correspond to four baseline populations NDB_{1-4}. The red line represents the mean, and the error bars represent SEM.

To investigate if males and females harbor different amounts of bacteria, we plated male and female fly homogenates separately (on day 12 after egg collection). This was done on four baseline populations NDB_{1-4}. On both NA and MRS media, females had more CFUs associated with them than males (Figures 2.5A and 2.5C). The results were analyzed using ANOVA on population means and are reported in Table 2.4. This trend was seen for all four baseline populations. *Drosophila* exhibits sexual dimorphism in multiple traits, including adult body weight. On average, female body weight is twice that of males. We surmised that bacterial load differences could be due to differences in their body weights. To test this, we

normalized the total bacterial load found for each replicate by the body weight of that replicate taken just before plating them. We found that the difference between male and female bacterial loads was not statistically significant with this normalization on both NA and MRS (Figures 2.5B and 2.5D). The results were analyzed using ANOVA on population means and are reported in Table 2.5. This reduction was captured by a concomitant decrease in Cohen's d effect size estimates, as shown in Table 2.6. Even with the normalization by body weight, effect sizes remained large (d > 0.8) on both media.

Table 2.4 Results of Two-way ANOVA on Log CFU per fly

Measurement	Factor	Effect	Test-statistic	P-value	Sample size
CFU per mg on MRS	Sex	Fixed	$F_{1,24} = 23.26$	0.0170	N = 4 per treatment
	Block	Random	$F_{3,24} = 5.16$	0.1055	
CFU per mg on NA	Sex	Fixed	$F_{1,24} = 32.92$	0.0105	N = 4 per treatment
	Block	Random	$F_{3,24} = 7.48$	0.0662	

Table 2.5 Results of Two-way ANOVA on Log CFU per mg

Measurement	Factor	Effect	Test-statistic	P-value	Sample size
CFU per mg on MRS	Sex	Fixed	$F_{1,24} = 6.90$	0.0786	N = 4 per treatment
	Block	Random	$F_{3,24} = 6.97$	0.0725	
CFU per mg on NA	Sex	Fixed	$F_{1,24} = 6.67$	0.0816	N = 4 per treatment
	Block	Random	$F_{3,24} = 7.78$	0.0630	

Table 2.6 Cohen's d effect size estimates for the sex-wise difference in bacterial load

Media:	**MRS**	**NA**
CFU per fly	1.94	1.97
CFU per mg	0.93	0.87

2.3.4 Diet can alter the quantity of bacteria associated with the flies

The host's diet is one of the factors that can potentially influence the associated microbiome (Chandler et al. 2011; Erkosar et al. 2013; Téfit et al. 2018). To investigate the effect of diet on the microbiota of our flies, we used a previously standardized diet regime where protein to carbohydrate ratio (henceforth, P:C ratio) in the fly diet is systematically varied by changing amounts of yeast (protein source) and sugar (carbohydrate source). In this experiment, we

reared one of the baseline populations, NDB_1, in diets with different P:C ratios. See Method section 2.2.9 for the details of this diet. The bacterial load associated with the flies was quantified by plating fly homogenates on NA and MRS media (on day 12 after egg deposition).

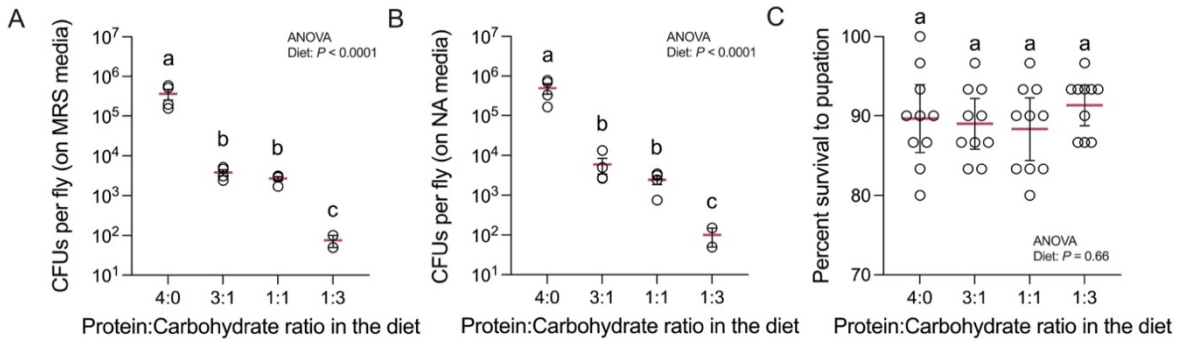

Figure 2.6 Bacterial load and percent survival to pupation when the protein-to-carbohydrate ratio in the diet is changed. Total CFUs per fly on (A) MRS and (B) NA media. On both media, CFUs per fly decreased as the protein-to-carbohydrate ratio reduced. This experiment was performed on the baseline population NDB_1. Each treatment had four biological replicates (except 1:3, which had only two replicates; the other two had bacterial load below the highest dilution assay detection limit). (C) To see if changes in bacterial load in diets with different P:C ratios correlate with survival during early development, we looked at percent survival in these diets till puparium formation. Flies raised in different diets showed similar survival as assessed using one-way ANOVA. For all three graphs, the red line represents the mean, and the error bars represent SEM. The small case letters (a, b, c) indicate status of the statistical differences between different diet types as assessed by Tukey's pairwise multiple comparison test. If the pair under comparison shares the same letter (e.g., "b" and "b"), then all such comparisons are statistically non-significant. If the pair does not share a letter (e.g., "a" and "b") then those comparisons are statistically significant. This assay was done on a cornmeal diet, which is different from the usual banana-jaggery-based diet of these flies. See Method section 2.2.9 for the details of the diet.

We found that the bacterial load associated with the flies decreased as protein in the diet reduced and carbohydrate increased (One-way ANOVA for MRS, $F_{3,10} = 156.69$, $P < 0.0001$, and One-way ANOVA for NA, $F_{3,10} = 69.25$, $P < 0.0001$, Figure 2.6A and B). The Kruskal-Wallis test for equal medians is also significant for both media. We observe that the CFUs per fly do not change significantly when protein is increased from 1:1 to 3:1 (Tukey's pairwise multiple comparison tests, Table 2.7 and 2.8) – it is the extreme 4:0 diet without any sugar where we see a drastic increase of bacterial load from 1:1 (Figure 2.6A and B). The change in the opposite direction, i.e., going from 1:1 to 1:3 is also significant where carbohydrate content is more than the protein content.

These changes in bacterial loads were not accompanied by the host's differential survival to pupation in these diets (one-way ANOVA, $F_{3,36} = 0.54$, P = 0.66, Figure 2.6C). No pairwise comparison was significant either (Tukey's pairwise multiple comparison tests).

Table 2.7. List of all pairwise comparisons on CFUs per fly using Tukey's HSD test on MRS media

Pairwise-comparison	P-Value
4:0 vs. 3:1	0.0002
4:0 vs. 1:1	0.0002
4:0 vs. 1:3	0.0002
3:1 vs. 1:1	0.7645
3:1 vs. 1:3	0.0002
1:1 vs. 1:3	0.0002

Table 2.8. List of all pairwise comparisons on CFUs per fly Tukey's HSD test on NA media

Pairwise-comparison	P-Value
4:0 vs. 3:1	0.0002
4:0 vs. 1:1	0.0002
4:0 vs. 1:3	0.0002
3:1 vs. 1:1	0.4520
3:1 vs. 1:3	0.0006
1:1 vs. 1:3	0.0025

2.4 Discussion

In this chapter, we aim to understand the microbiome composition of the flies in our lab. Several studies across different hosts have shown that the microbiome composition of hosts can vary with age, sex, and host diet (Carmody and Bisanz 2023; Dapa et al. 2022; Erkosar et al. 2018; Han et al. 2017; Kim et al. 2020; Langille et al. 2014; Meng et al. 2022; Muralitharan et al. 2023; Obadia et al. 2018; Trujillo et al. 2022; Turnbaugh et al. 2009). We also needed to understand the impact of these factors on our fly's microbiome before we designed experiments involving manipulation of the microbiome, as every host-microbiome system and its properties can potentially be unique (Angela E. Douglas 2018c).

In addition, the composition of the host-associated microbiota often varies a lot across individuals in a population, or even for the same individual over time and environments. All these variations complicate the design of experiments with appropriate power. We wanted to keep these confounding factors to a minimum by having a handle on how our fly's microbiome changes in response to age, sex, and diet. For example, if the microbiome changes drastically as flies age, then it is important to fix the age of the flies where a particular phenotype is assessed. There is a finite probability that the flies that are not age-matched might produce slightly different results (or results with lots of noise) due to a shift in the microbiome composition.

2.4.1 *Acetobacter* and *Lactobacillus spp.* are the most prominent members of our fly microbiome

We find that *Acetobacter* and *Lactobacillus* dominate the community of bacteria associated with our flies (Figure 2.2). As already discussed above (section 2.1), this observation agrees with other studies on the microbiome composition of *Drosophila* (Angela E. Douglas 2018b; Erkosar et al. 2013). It is fascinating that so many studies show the enrichment of these specific taxa in fly microbiomes. How this ubiquitous association got selected in the laboratory as well as wild populations and is still maintained in many fly populations is an open question. One possibility is that *Drosophila* feeds on rotting fruits in nature, which are a source of acetic acid-producing and lactic acid-producing bacteria (Markow 2015). The symbiosis might have originated in the natural habitat of hosts that continued to benefit the host (I. S. Pais et al. 2018). In response, hosts might have evolved mechanisms to maintain this beneficial association (Storelli et al. 2018). Another possibility of how this association is

maintained in laboratory populations comes from a recent study on *Drosophila* (Sannino and Dobson 2023). Under laboratory conditions, the fly medium often contains antimicrobial preservatives that can have a negative effect on the flies. However, under such preservative-induced toxicity, *Acetobacter* can benefit the host by buffering the Triacyl glyceride (TAG) levels (Sannino and Dobson 2023).

While sequencing has shown that *Acetobacter* is dominant in the fly microbiomes of the lab (Figure 2.2), when we look at the CFU values from fly homogenates on MRS and NA, on average, a ~1:1 ratio of *Acetobacter* to *Lactobacillus* is observed (data not shown). There are two possible reasons behind this mismatch. First, some *Acetobacter* strains may not be culturable and, therefore, were underestimated using the CFU method. Second, it is known that while doing metagenomics, the DNA extraction method, the 16S rRNA PCR primers, or the algorithm used to classify the sequence variants can introduce biases towards detecting a particular taxa (Forry et al. 2024; Gohl et al. 2016; Jovel et al. 2016; Kennedy et al. 2014; Soergel et al. 2012).

Taken together, the recurrent dominance of two taxa over multiple generations raises exciting evolutionary questions about the transmission fidelity of the microbiota and the implications of this fidelity for the local adaption of the host (Guilhot et al. 2023). Is the host actively maintaining the community by "keeping it on a leash" (Foster et al. 2017)? How is similar microbiome composition maintained despite noisy microbiome dynamics? Is our microbiome adapted to the host diet as demonstrated by (M. E. Martino et al. 2018)? What is the mechanism by which the host passes these microbes to the next generation? Is the host receiving fitness benefits from this community? What would happen to the host fitness if this transmission is stopped by microbiome removal for many generations? Several such questions emerge from the observations we made that need further investigation. We will investigate the last two of these questions in the coming chapters.

2.4.2 Microbiome composition changes with age

The microbiome composition of the adult flies in our lab changes as the flies age (Figure 2.4). *Acetobacter* spp. dominate the microbiome of young (3-4 days after eclosion, i.e., day 12 after egg collection) adult flies. However, this dominating *Acetobacter* fraction reduces to undetectable levels in older flies. This is in contrast to a sequencing study where *Acetobacter pomorum* abundance increased in old age (C. N. A. Wong et al. 2011). The changed milieu of

the fly gut towards a more oxic state is an explanation the authors offer for their observation. We see that the abundance of *Lactobacillus* spp. fluctuates around 10-20% of total microbes. While *Acetobacter* and *Lactobacillus* reduce in older flies, bacteria of other types (not yet fully characterized) take over. The total bacterial load fluctuates between ~ 10^4 to 10^5 CFUs per fly on both media (data not shown), showing that the total bacterial load doesn't change drastically with age in our laboratory flies.

Acetobacter pomorum is known to provide fitness benefits to the *Drosophila* hosts by accelerating the development on low yeast diets through insulin signaling (Shin et al. 2011). There is the possibility that *Acetobacter* spp., in our case, also offers some benefits in earlier stages of development, so the association might be needed in early development. However, this association might be costly in later stages, so the host reduces *Acetobacter* spp. in older flies. Recently, antimicrobial peptides (AMPs) and lysozymes have been shown to regulate the microbiome in the gut (Bosch and Zasloff 2021; Marra et al. 2021; Ra and Bang 2024). To see if this is the case, one can look at the correlated changes in the expression levels of these molecules and the *Acetobacter* levels. This correlation can support the hypothesis that the host might be actively regulating *Acetobacter* abundance using AMPs and lysozymes.

The dominance of *Acetobacter* spp. during the early adult life of the flies can also perhaps be explained by a small detail in our fly husbandry. Our fly populations get moist yeast paste a day before eggs are collected to start the new generation. We hypothesize that the presence of this live yeast paste can increase the abundance of *Acetobacter* spp. in the parent flies, which, in turn, might increase the load of *Acetobacter* spp. on the eggs and, hence, the early life of the offspring. This observation is supported by preliminary experiments in our lab where flies raised on high protein diets seem to harbor more proportion of *Acetobacter* spp. as determined by CFU counts on MRS media (data not shown).

2.4.3 Microbial load is different in both sexes, and this can be partially explained by body weight difference

We found that males and females have different total bacterial loads as quantified by CFU counts (Figure 2.5A and C). This difference is not significant when CFU counts are normalized by their average body weights (Figure 2.5B and D), indicating that different body sizes can partially explain the difference. After normalization, effect sizes using Cohen's d remained large, suggesting there still might be interesting overlooked aspects in the trend.

A study showed that there can be differential presence of microbial taxa in males vs. females (Han et al. 2017). Here, we see that the bacterial load of males and females is shown to be very close to each other after normalization. However, without sex-wise microbiome sequencing data, we cannot comment on whether the relative abundance of microbes has changed.

2.4.4. Bacterial load in flies increases with the protein-to-carbohydrate ratio in the host diet over the range studied

We found that over the P:C ratio range we studied, increased protein content in the host diet and concomitant lowering of carbohydrates was associated with greater bacterial abundance in *Drosophila* (Figure 2.6).

The observed trend agrees with the results of a previous long-term study where fly populations were given diets with different yeast percentages (4%, 10%, 27%) for ~ 60 generations without affecting other dietary components (Erkosar et al. 2018). This study showed an exponential increase in microbiota abundance with increasing yeast content. In this study, while microbiome abundance increased, the microbiome diversity reduced. In our case, we would like to conduct 16S rRNA sequencing in future studies to determine if microbiome diversity is affected.

We might be seeing this pattern because the guts of the flies in a protein-rich diet have a higher carrying capacity for microbes as the P:C ratio increases in the diet. Greater carrying capacity could be driven by alternate life histories induced in these populations as a consequence of different diet types (Mudunuri et al. 2024). Consistent with this notion, a study has shown that on low-yeast diets, flies can have smaller body sizes (Y. Henry et al. 2020).

It is possible that a specific taxon of microbes in our flies might be the dominant fraction present on high P:C diets, deriving benefits from the protein-rich diet in the form of molecules that boost bacterial growth and survival, thus contributing to the greater abundance of total microbes in high P:C diets. This possibility can also be checked with the 16S rRNA sequencing in future studies.

To summarize, in this chapter, we studied the microbiome composition of our flies and how it changes with the age, sex, and diet of the flies. The insights obtained in this chapter can be combined to generate more testable hypotheses. For example, we can surmise that older flies

that have lost the dominant portion of the early microbiome (which might have beneficial roles) might be more susceptible to environmental insults, pathogen infection, etc., if we put them on a low-protein diet, as this diet is known to reduce microbiome abundance. It will be interesting to see whether host fitness indeed decreases in experiments designed to test this susceptibility and what interventions, such as a better diet and a young microbiome, can alleviate the state of fitness deficit.

Chapter 3
Standardization of bleaching method and short-term microbiome removal studies

3.1 Introduction

The ubiquitous presence of *Acetobacter* and *Lactobacillus* taxa in fly microbiomes, including ours, as seen in the last chapter, might not be just "co-occurrence." In many studies from different labs, microbes from these taxa are known to affect the host's fitness (Gould et al. 2018; Lesperance and Broderick 2020; Matthews et al. 2021; Walters et al. 2020). Hosts, in turn, provide a stable niche (Dodge et al. 2023; Ludington 2024) and reciprocal modulation of the microbiome (Foster et al. 2017). If we see that the same microbes are maintained or tolerated by the host for many generations, then there is a possibility that hosts might be deriving benefits from these interactions. However, there are host-microbe systems (even with *Drosophila* as hosts) where the effect of microbes on the host is absent or limited (T. J. Hammer et al. 2019; Selkrig et al. 2018). Thus, it seems that there exists a spectrum where, at one end, the host and microbe interact, exchanging goods and services, and at another, there might not be any interaction at all. This tells us that, *a priori*, we cannot expect host-microbe interactions in every system under study, even ours.

In the first half of this chapter, we standardize various assays to study the microbiota's contribution to host biology. Then, in the second half, we use these assays to see what fly phenotypes the microbes modulate and to what extent. Both of these efforts were guided by past studies on *Drosophila* examining how host-associated microbiomes impact them.

Previous studies on fruit flies have shown that microbiota can affect a large number of host traits. For example, two studies in 2011 independently showed that fruit flies in nutrient-poor conditions, without their symbiotic microbes, develop slowly and have lower larval as well as adult body weight (Shin et al. 2011; Storelli et al. 2011). In one case, *Acetobacter pomorum* was sufficient to rescue this phenotype by acting on the insulin pathway (Shin et al. 2011), and in the other case, *Lactobacillus plantarum* (now renamed as *Lactiplantibacillus plantarum*) reverted the malnutrition phenotype through the mTOR pathway (Storelli et al. 2011). These two studies utilized the techniques of rearing gnotobiotic *Drosophila* (i.e., flies with defined microbiome) and genetic manipulation to show that symbiotic microbiome might be more tightly integrated with host physiology than traditionally thought (Macke et al. 2017). These observations also showed that different microbes can modulate similar aspects of host physiology by distinct underlying mechanisms. The most exciting aspect of these studies is that the effect exerted by *L. plantarum* was studied further in detail (Combe et al. 2014; Erkosar et al. 2015; Matos et al. 2017; Storelli et al. 2018), and later, a similar growth-promoting effect by *L. plantarum* was discovered in mice (Schwarzer et al. 2016, 2023). This

line of work demonstrated that *Drosophila* can be a powerful exploratory model to investigate health-related traits in mammals.

Along with the effect on development rate and body weight, microbes are implicated in modulating fecundity. In *Drosophila subobscura,* eliminating microbes through antibiotic treatment resulted in increased fecundity (B. S. Walsh et al. 2017). On the contrary, the removal of microbiota through antibiotic treatment led to a loss of fecundity in olive fruit flies raised on diets lacking essential amino acids, but not when the diets contained those essential amino acids (Ben-Yosef et al. 2010). Thus, the authors concluded that bacteria might play a crucial role in buffering the effects of unbalanced diets. However, in studies that use antibiotics for bacterial removal (e.g. (Ben-Yosef et al. 2010; Ridley et al. 2013)), the observed results could also be an effect of the antibiotic treatment. In the absence of an appropriate control to account for the effects of antibiotics, such studies remain inconclusive.

Another method for microbiota removal involves egg dechorionation, which can lead to reduced female fecundity in *Drosophila*, which can be rescued by *Acetobacter* alone (Elgart et al. 2016). In fruit flies without microbes, mitochondrial activity, and levels of ATP and *Aldehyde dehydrogenase* (*Aldh*) are reduced (Gnainsky et al. 2021). This leads to the reduction of oogenesis, thus implying that gut bacteria can influence distant tissues such as the ovary. Interestingly, supplementation with *Acetobacter* spp. or the addition of riboflavin (vitamin B2) re-instated normal oogenesis (Gnainsky et al. 2021). In another study, *Acetobacter*-mediated beneficial effects on host oogenesis were exerted via hormonal control of germline stem cell increase and egg maturation (Suyama et al. 2023). Such studies highlight that microbes can play a major role in modulating the host's fitness, sometimes through multiple mechanisms.

Along with these life history traits, microbes are also shown to alter the host behavior (Chiang et al. 2022; Cryan and Dinan 2012; Ezenwa et al. 2012; Fischer et al. 2017; Henriques et al. 2020; Jia et al. 2021; Johnson and Foster 2018; Masuzzo et al. 2020; Schretter et al. 2018; Vuong et al. 2017). In *Drosophila*, endosymbionts such as *Wolbachia* were shown to affect mating choice (Markov et al. 2009). However, the contribution of gut symbionts to mating behavior was not well studied till about a decade ago. At that time, a study by Sharon et al. showed that flies raised on the same diet mated within themselves, giving rise to diet-induced mating preference in only one generation (Sharon et al. 2010). However, when antibiotics removed microbes, this preference was not observed. This study showed that re-association with *L. plantarum* could partially restore the mating preference.

Since mating preference can potentially lead to speciation based on sexual isolation, microbes can be a potential driver of this effect (Sharon et al. 2010). Another study was able to reproduce this mating preference in inbred strains (*Canton-S* and *Oregon-R*) but not in outbred strains (Najarro et al. 2015). Another study that tried reproducing these results could not do so (Leftwich et al. 2017). Various explanations were proposed to explain this discrepancy, including differences in experimental conditions, starting microbiomes, and diets (Leftwich, Clarke, et al. 2018; Obadia et al. 2018; Rosenberg et al. 2018). This shows that across-study consistency can only be ensured with standardized protocols and careful experimental design that mitigates as many confounding factors as possible. Nonetheless, these studies implied that the microbiota's potential goes beyond altering life history to affect key behavioral processes, in some cases, with direct implications for the evolution of the host within a few generations (Leftwich, Hutchings, et al. 2018; Sharon et al. 2011).

Although the role of microbiota in affecting mating is relatively unexplored, their effect on locomotor activity and aggression in *Drosophila* has been studied in detail. For example, Schretter et al. (2018) found that flies without symbiotic microbes were hyperactive with increased speed and a different temporal profile of walking. *Lactobacillus brevis* alone (but not *L. plantarum*) was found to be sufficient to restore the conventional walking profile. A single-mutation screen in *E. coli* that targeted amino acid and carbohydrate metabolism revealed that sugar modifying enzyme xylose isomerase was a candidate for modulating fly activity (Schretter et al. 2018). This study also showed that bacterially derived xylose isomerase could reduce trehalose levels, thereby tuning down locomotor activity via negative regulation of octopamine signaling (Angela E. Douglas 2018d; Schretter et al. 2018).

Octopamine signaling is also involved in another host behavior that was shown to be modulated by microbes. In a study by Jia et al., removing the microbiome from the hosts reduced inter-male aggression, while locomotor and courtship behavior was unaffected (Jia et al. 2021). When the microbiome was reconstituted, aggression was restored. *L. plantarum* alone was capable of complete phenotype rescue. The study also showed that without microbes, octopamine production is decreased in a subset of octopaminergic neurons, thus reducing the levels of aggression (Jia et al. 2021). This indicates that aggression levels correlated with octopamine levels and the microbiome's presence. In the case of the study by Schretter et al., the presence of the microbiome negatively regulated the octopamine levels, reducing locomotor activity (Schretter et al. 2018). In contrast, in the case of Jia et al., the presence of the microbiome elevated the Octopamine and thus aggression. These results show

that microbes can potentially tune the host's physiology in different directions. In contrast to these two studies with prominent modulation of aggression and locomotion by microbes (Jia et al. 2021; Schretter et al. 2018), a study by Selkrig et al. found that the Drosophila microbiome had only a minor influence on the host's locomotor activity, sleep, and courtship (Selkrig et al. 2018). This suggests that there might be a lot of context-specificity in how microbiota affects the behavior of their host.

Most studies on microbes' impact on host biology focus on a single trait. Few studies look at multiple related traits, but only a subset of these traits are often found to be affected by microbes (Jia et al. 2021; Ridley et al. 2012a; Selkrig et al. 2018). These studies, often from different labs, involve different (mostly inbred) *Drosophila* strains. In the case of diet-induced mating preference, it was shown that trends observed in inbred strains did not hold for outbred strains (Najarro et al. 2015). This is crucial because outbred strains are often closer to the natural populations as they harbor more genetic variation than inbred strains (Godinho et al. 2020). If results hold in outbred strains, then there is a greater possibility that they are much more relevant in natural populations. In addition, these studies usually investigate either males (Chaston et al. 2016) or females (Schretter et al. 2018), potentially hiding the differential effects of microbes across the sexes.

Taking all these points together, while several different microbiome studies with diverse host-microbiome combinations across different labs have given us potential phenotypes of interest that are modulated by microbes, studies that use the same microbiome-host combination to find out how microbiome manipulation affects multiple types of traits (life-history traits, behavioral traits, stress-related traits) across sexes, are rare. If we can have such an integrated picture of the trait correlations in the host phenotype, one can begin to speculate on the ecological and evolutionary relevance of the links that the host forms with their microbiomes. For example, if the egg-laying capacity of the female hosts gets affected by microbes, that could affect what happens to the longevity of the host, as there can be a tradeoff between these two traits (Gould et al. 2018). If the host lifespan is under strong selection (or late reproduction, for that matter), then reduced early fecundity would make sense as a possible cost incurred by the host to increase its lifespan (or to reproduce later). Alternatively, reduced fecundity could also be explained by the allocation of host resources to stress-related traits such as desiccation, heat, or starvation resistance. Then, one can investigate if stress-related traits have indeed changed due to microbiota removal. These observations can also tell us what kind of selection pressure the host is under and the

ecological and evolutionary constraints that come with it. This study of tradeoffs and evolutionary constraints only makes sense when multiple trait associations are studied together. In other words, *we need to study the interrelation of the microbiome-induced phenotypic changes for the host.*

In this chapter, we report on the method that we adopted and standardized to study the contribution of the microbial community to *Drosophila* life history. Then, using this method, we test if the microbial community present in our flies has the ability to affect the fitness of the host. We test multiple host phenotypes for possible modulation by the native community of microbes in our flies through which overall fitness might be affected. The goal is to understand how trait associations and tradeoffs play out in the absence of the microbes. We find that the method of egg dechorionation using bleach, followed by aseptic rearing, renders flies microbe-free. As an appropriate control for this treatment, control flies with microbes were generated with a reconstituted microbiome. This reconstitution mirrored the native microbiome composition as validated by 16S rRNA sequencing. In addition, the F1 progeny of the bleach-treated flies were used for the phenotypic comparison. We used two types of flies, i.e., microbe-free (M-) and with-microbes (M+), to see the contribution of microbes to host phenotypes. We find that the microbiome can modulate multiple fly phenotypes in males and females, showing sexual dimorphism in its influence on a few traits. Thus, we show that there is ample cross-talk in our lab's host-microbiome system that has important implications for the host's fitness. We show that the patterns of microbial contribution to host phenotypes are robust, holding over many host generations, and possibly reflecting a perpetuated core microbiome dominated by *Acetobacter* and *Lactobacillus.*

3.2 Methods

3.2.1 Choice of the method to generate flies without their microbes

In the literature, there are two major ways of making microbe-free flies: first, using antibiotics in the fly diet, and second, with egg dechorionation, followed by aseptic rearing (Angela E. Douglas 2019; Heys, Lizé, Blow, et al. 2018; Kietz et al. 2018; Koyle et al. 2016; Ridley et al. 2013). Antibiotics are known to have off-target effects (Cain et al. 2021; Heys, Lizé, Blow, et al. 2018; H.-Y. Lee et al. 2019; Ridley et al. 2013). Also, when mixed in the diet, it might be challenging to control the amount of antibiotics that individual flies consume, as the antibiotics can degrade over time. That is why egg dechorionation using bleach might be a preferred technique for studying the *Drosophila* microbiome (Jia et al. 2021; Ridley et al. 2013; J.-H. Ryu et al. 2008; Shin et al. 2011). Moreover, bleach treatment is a transient process; hence, it is easy to reconstitute the microbiome after the bleach treatment to see the potential reversion of the phenotype, and control for any potential effects of the bleach treatment itself. While theoretically, the same can be done for antibiotics, it is impossible to ensure that the entire amount of antibiotics in the host has been degraded, before starting the reconstitution. If the antibiotics are not totally degraded, they can potentially affect some components of the microbiota, which in turn affects the reconstitution process. Therefore, we chose the egg dechorionation method to rear *Drosophila* microbe-free, which is in accordance with the recommendations of a study that performed an extensive comparison between the bleach and the antibiotic treatment (Ridley et al. 2013). Some studies have used either bleach and antibiotics together (Schretter et al. 2018; Storelli et al. 2011; Suyama et al. 2023) or bleach and ethanol together (I. S. Pais et al. 2018), but we thought that an extra layer of complexity may be unnecessary if the bleach treatment works without the antibiotics or the ethanol.

A second ancillary factor that needed standardization was the nature of the medium on which *Drosophila* is raised. Many labs that rear *Drosophila* use autoclaved medium, which automatically suits rearing microbe-free flies. However, our lab uses a fly medium whose preparation does not involve autoclaving. To see if shifting to autoclaved fly food causes differential mortality or major developmental defects, we used both autoclaved and non-autoclaved media for the initial standardization.

3.2.2.1 Bleach treatment standardization: deciding the dose of bleach

To ensure an adequate procedure for microbiome removal, we needed to check two things: (a) the method should not cause unintended side effects, and (b) the method should eliminate the microbiome to the maximum extent possible (Ridley et al. 2013).

In the literature, there is a wide range of bleach concentrations (0.5% to 10%) and the time for which egg-dechorionation is carried out (50 sec to 20 min) (Broderick et al. 2014b; Brummel et al. 2004; R. I. Clark et al. 2015; Fast et al. 2018; Kietz et al. 2018; Koyle et al. 2016; H.-Y. Lee et al. 2022; I. S. Pais et al. 2018; Ridley et al. 2013; Simhadri et al. 2017). After a careful survey, we chose a subset of these values (Figure 3.2 x-axis) representing the wide range of bleach treatments to initiate the standardization process. The detailed protocol for this bleach treatment is illustrated in Figure 3.1. The eggs for this study were collected from the DB_3 fly populations as already described in Chapter 2, method section 2.2.2. Section 2.2.3 has details of the DB_3 population.

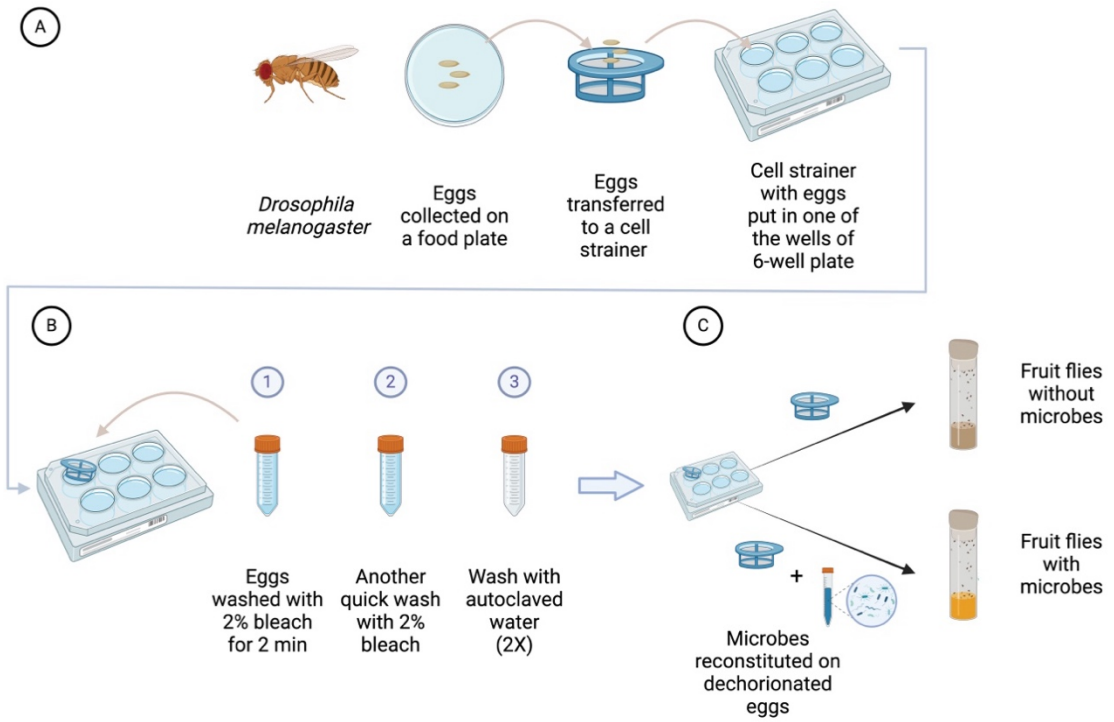

Figure 3.1 Egg dechorionation protocol using bleach. (A) To produce microbe-free adult flies, eggs laid by untreated/conventional mothers (day 12 after egg collection) were collected in a cell strainer using a sterile paintbrush. (B) These eggs were washed in bleach twice (Steps B1 and B2), followed by two washes of autoclaved dH_2O (Step B3) to get rid

of the residual bleach. The procedure was carried out in a biosafety cabinet to avoid environmental contamination. (C) These dechorionated eggs were aseptically transferred to sterile fly bottle/vials with around 6 ml of autoclaved banana-jaggery food. These bottle/vials were plugged with autoclaved cotton plugs. The opening of the bottle/vials (in which cotton plug was inserted) was sealed with double layer of parafilm such that it partially covers the cotton plug as well making sure there the plug doesn't fall off easily while handling. These flies were transferred to a fly incubator maintained at 25^0C till they reached adulthood. Emerging adult flies were tested for the microbe-free state as described in section 3.2.2.2. To make a set of microbiome-reconstituted flies (discussed in detail later in section 3.2.3) microbes were provided to dechorionated eggs after the second water wash. This figure is created with BioRender.com.

As a control to the bleach treatment, we used eggs from untreated flies. An additional treatment of wash-control was set up to see if the physical manipulation of the eggs during the procedure harms the eggs (for example, paintbrush squashing the eggs during transfer). In the wash-control, autoclaved dH_2O replaced bleach, but the rest of the protocol was the same. Any additional mortality in the wash-control compared to the untreated eggs could only come from the physical handling (as bleach is absent in this treatment to cause any mortality).

To understand the effects of this treatment on flies, we looked at two metrics: (a) egg-to-pupa survivorship and (b) egg-to-pupa development time. A drastic change in survivorship or development would indicate that the bleach dose is unsuitable for microbiome manipulation.

We found that the higher bleach concentration (2% vs. 4%) and longer egg dechorionation time at 2% bleach (5 min to 10 min) caused higher mortality (Figure 3.2). The detailed statistical analysis using two-way ANOVA is given in Table 3.1. This observation implied that we needed to reduce the dose of bleach to a minimum. Complete removal of microbiota via bleach treatment is known to induce developmental delay (Ridley et al. 2013; Shin et al. 2011; Storelli et al. 2011), and this was seen in all four treatments and on both types of fly foods (Figure 3.3). The detailed statistical analysis using two-way ANOVA is given in Table 3.2. We also see that out of the four bleach doses that we tested, except for 4% bleach for 10 min treatment, the delay in the time to pupation was less for autoclaved food than the non-autoclaved food (Tukey's post-hoc tests).

Looking at the survival percentage and development time, we further reduced dechorionation time to 2 min from 5 min (at 2% bleach) without compromising the treatment's ability to achieve a microbe-free state in adulthood. Closer observation of the dechorionated eggs under the microscope revealed that reducing bleach concentration further than 2% reduced

the percentage of dechorionated eggs after the treatment (data not shown). A reduced percentage of dechorionated eggs decreases the chance of achieving a microbe-free state, especially when a large number of eggs are processed together. Throughout this study, we used 2% bleach with 2 min egg dechorionation time as a standard treatment to remove microbes. This bleach wash was supplemented by another quick wash of 2% bleach to remove any residual microbes (Figure 3.1).

Additionally, autoclaving the fly food did not cause differential mortality in all six treatments (Table 3.1, Figure 3.2). The wash-control group had similar mortality to the untreated flies, showing that physical manipulation during egg dechorionation did not harm eggs (Figure 3.2).

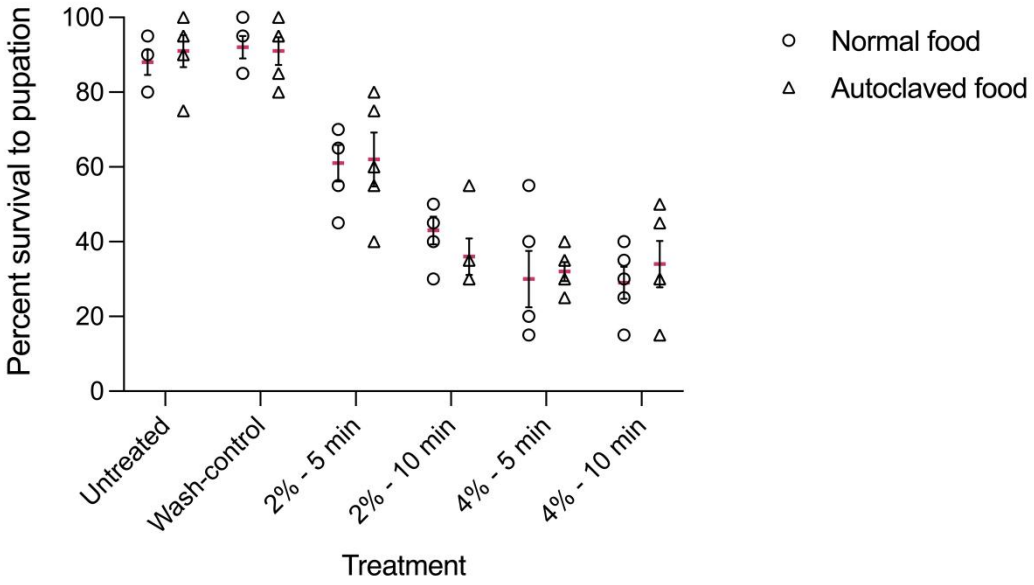

Figure 3.2 Effect of bleach treatment on percent survival to pupation. Circles represent the flies reared on normal banana-jaggery food and triangles represent the flies reared on autoclaved banana-jaggery food. The red horizontal lines are means over the four replicates and the error bars represent the SEM. This experiment was done on DB_3 population. The results were analyzed with two-way ANOVA as given in Table 3.1.

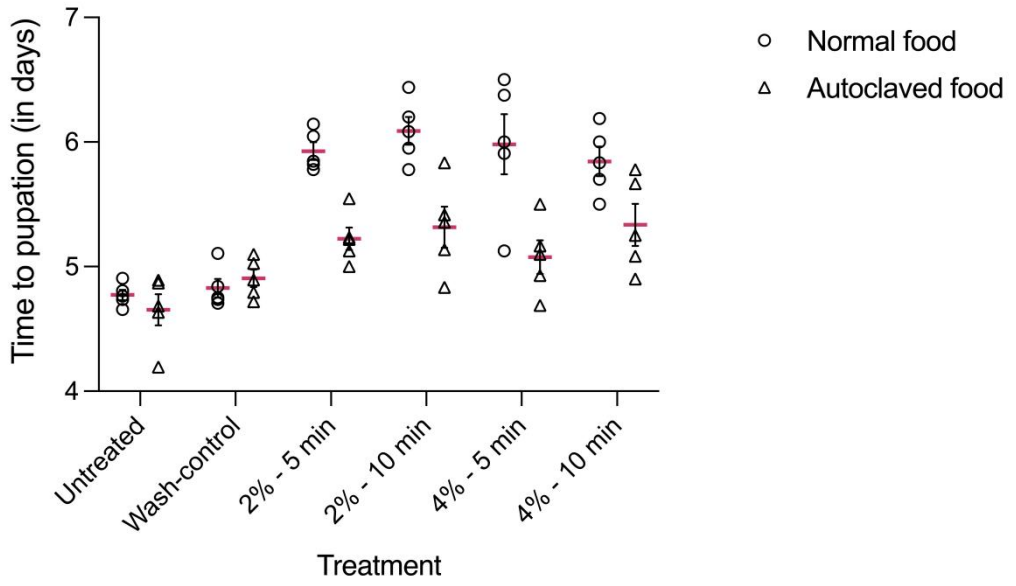

Figure 3.3 Effect of bleach treatment on time to pupation. Circles represent the flies reared on normal banana-jaggery food and triangles represent the flies reared on autoclaved banana-jaggery food. The red horizontal lines are means over the four replicates and the error bars represent the SEM. This experiment was done on DB_3 population. The results were analyzed with two-way ANOVA as given in Table 3.2.

Table 3.1 Results of two-way ANOVA on percent survival to pupation

Factor	Effect	Test-statistic	P-value	Sample size
Type of food	Fixed	$F_{1,48} = 0.17$	0.679	N = 5 (5 vials/treatment, 20 flies/vial)
Treatment	Fixed	$F_{5,48} = 54.68$	P < 0.0001	
Type of food × Treatment interaction	Fixed	$F_{5,48} = 0.36$	0.876	

Table 3.2 Results of two-way ANOVA on time to pupation

Factor	Effect	Test-statistic	P-value	Sample size
Type of food	Fixed	$F_{1,48} = 43.10$	P < 0.0001	N = 5 (5 vials/treatment, 20 flies/vial)
Treatment	Fixed	$F_{5,48} = 21.68$	P < 0.0001	
Type of food × Treatment interaction	Fixed	$F_{5,48} = 4.58$	0.0017	

3.2.2.2 Bleach treatment standardization: making sure that the method works

To ensure that our chosen dose of bleach was able to render the flies microbe-free, we plated fly homogenates from treated flies and did PCR on the gDNA extracted from them using 16S rRNA universal primers to detect bacterial presence. See method section 2.2.4 in Chapter 2 for the detailed methodology.

We assessed the microbe-free state at adulthood (day 12 post egg collection) by comparing colony-forming units (CFUs) from a full-body homogenate of microbe-free flies with untreated counterparts (Figure 3.4). CFUs were compared on NA and MRS. NA is a general-purpose media, and MRS is a fly microbiome-specific media.

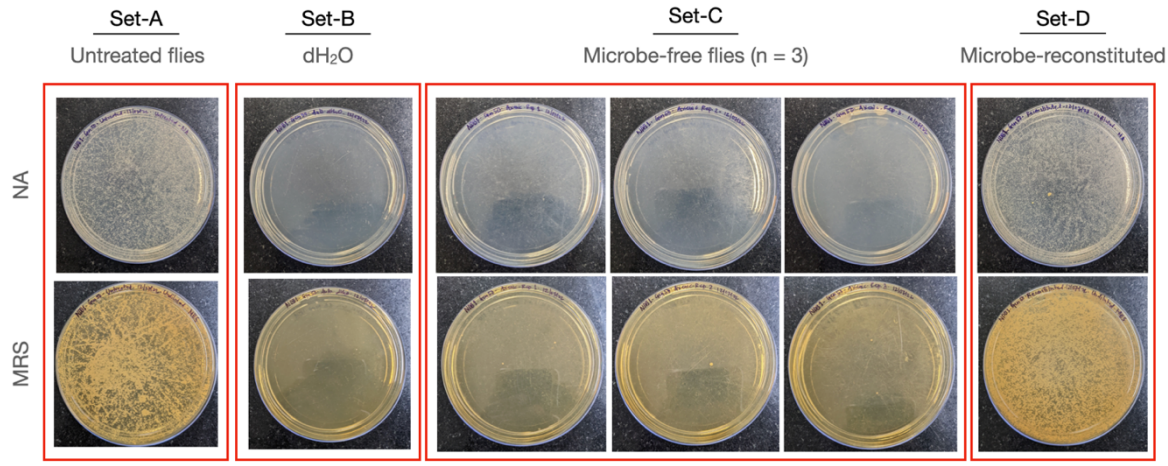

Figure 3.4 Plates showing the absence of microbiota from the adult flies at day 12. To confirm that the technique of egg dechorionation by bleach followed by aseptic rearing works, we plated fly homogenates on NA (upper row) and MRS-agar (bottom row). Set-A shows untreated flies. Set-B shows the plating of autoclaved dH_2O in which flies were homogenized. Set-C shows three replicates of microbe-free flies plated on both media. Set-D shows that microbes can be successfully reconstituted after bleach treatment. For all treatments, bacterial load from ten flies per biological replicate was plated, of which five were male and the other five female.

We see that homogenate from the untreated flies shows a lot of microbes growing on NA as well as MRS (Figure 3.4, Set-A). From various quantitative experiments, we have seen that there are ~ 10^5 CFUs per fly (data not shown here). The autoclaved dH_2O control shows that the water in which flies were homogenized was completely sterile (Figure 3.4, Set-B). Flies that had gone through the bleach treatment at the egg stage and reared aseptically afterward showed no bacterial growth, except ~1-2 colonies on MRS plates (Figure 3.4, Set-C). When we provided the bleached eggs with the microbial community of interest just after the bleach

treatment, we were able to see microbes from this community in day 12 flies. We refer to these flies as *flies with reconstituted microbiomes* (Figure 3.4, Set-D). This treatment is described in depth in the upcoming sections 3.2.3, 3.2.4, and 3.2.5.

It is known that a fraction of any microbiota can consist of unculturable bacteria. Therefore, although we failed to see bacteria in the plates from our microbe-free flies, the presence of unculturable bacteria in these flies cannot be ruled out. To ensure that our treatment for microbiota removal was effective, we used PCR with 16S rRNA universal primers on the DNA extracted from the homogenates of microbe-free and untreated flies. The details about the PCR protocol and the corresponding primers are given in Chapter 2, Method section 2.2.4.2 The absence of any bands for the three biological replicates of the microbe-less flies (Lanes labeled as "–" (minus) in Fig 3.5) confirmed the microbe-free state of the flies.

Here, it is essential to note that DNA sequencing has shown that even if CFU count (via plating) in a microbe-free state (using bleach) goes close to zero, it does not necessarily mean a total 100% absence of microbiome; one could still detect sequencing reads corresponding to the microbiota in the microbe-free state (Youn Henry and Colinet 2018). This is perhaps the reason for which we could see very faint bands in the flies raised aseptically, especially when PCR conditions are saturating (Figure 3.5). These vary faint bands could also be explained by another observation from the plating fly homogenate: while most flies are microbe-free, a tiny fraction of a fly population can have very few colonies associated with them (Figure 3.4, Set-C, MRS plates).

The alternate explanation for the faint PCR bands is that our flies have a few unculturable bacteria.

Taken together, in this section, we reported standardization of the dose of the bleach treatment, which is enough to achieve a microbe-free state for flies with relatively less effect on *Drosophila* development time or survivorship.

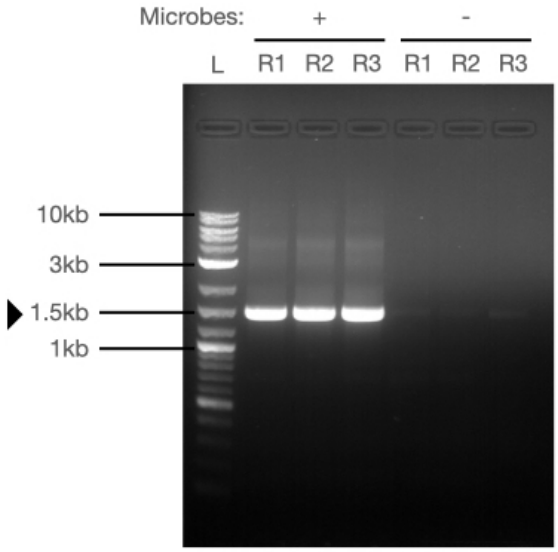

Figure 3.5 PCR using bacterial 16S rRNA universal primers on flies with microbes (+) and flies without them (-)

3.2.3 Method of microbiome reconstitution

The last section outlined a protocol to generate flies without microbes. This section discusses the rationale behind our choice of using flies with reconstituted microbiomes as a control for the phenotypic comparison.

When microbe-free flies are generated using any treatment, such as bleach or antibiotics, there is a possibility that the treatment itself will affect the host (H.-Y. Lee et al. 2019; Ridley et al. 2013). For example, antibiotics can have side effects such as water and food aversion (Cain et al. 2021). Bleach can cause mortality during fly development (Heys, Lizé, Blow, et al. 2018; Ridley et al. 2013). Now, if we create microbe-free flies using an agent such as bleach, then the phenotype of these microbe-free flies can be due to the toxic side effects of bleach or due to the absence of microbes (Figure 3.6A). One cannot decipher which of these factors drive the phenotype change. Thus, directly comparing these two treatments is not a good idea due to the confounding effect of the bleach treatment.

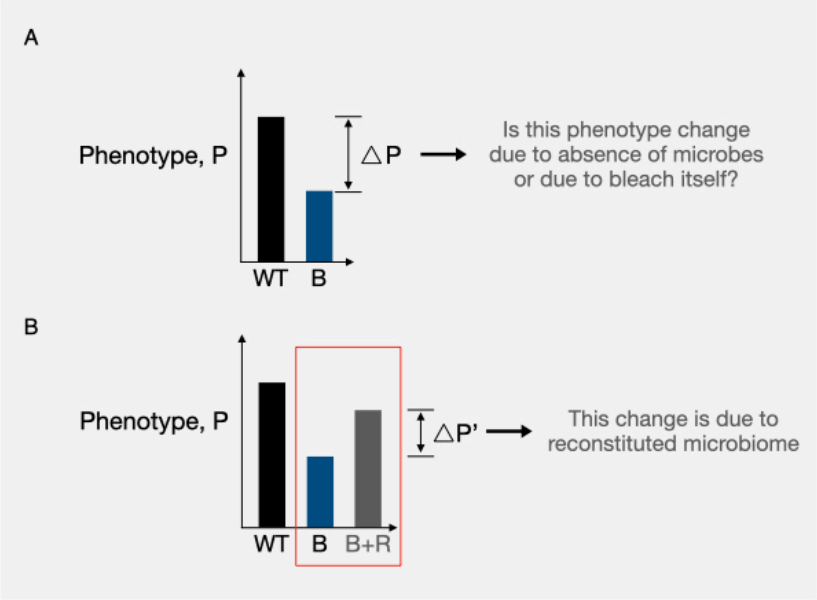

Figure 3.6 A schematic describing the possible confounding effect of bleach on microbe-free flies' phenotype. WT = untreated wild type files, B = bleached flies, B+R = flies with reconstituted microbiome after bleach.

This problem can be alleviated if we treat untreated eggs also with bleach and then reconstitute their native microbiome back just after the bleach treatment (Figure 3.6B). Now, since both treatments have received the bleach, the phenotype change in the flies with the reconstituted microbiome compared to microbe-free flies (B vs. B+R comparison in Figure 3.6B) will just be due to the reconstituted microbiome, effectively giving us the impact of the added microbiome on the host.

In an ideal world, flies with reconstituted microbiomes should be exactly like the untreated flies in every aspect. However, that may not be the case in reality, as multiple studies have shown that microbiome assembly is a complex process involving stochastic dynamics and priority effects (Jones et al. 2022; Obadia et al. 2017; Vega and Gore 2017). Even with the current knowledge of how microbiome assembles, one cannot faithfully reconstitute the complete microbiome to its native state in untreated/wild-type flies. So, the goal here is not to aim for a perfect reconstitution that can exactly phenocopy the untreated flies in all aspects, but to get a robust and reproducible phenotype change upon microbiome reconstitution (Figure 3.6B) that can be probed further.

3.2.4 Nature of microbiome reconstitution

Microbiome reconstitution can consist of a single strain or a community of microbes (Angela E. Douglas 2018b; Gould et al. 2018). We had a choice of doing reconstitution either via some combination of pure culture of microbes with well-defined community composition (actively controlling the microbiota composition) or by passively sampling the microbiome from the flies' environment (similar to fecal microbiome transfer). We chose the latter because of the following reasons.

First, when we isolated single colonies of bacteria from the flies and tried to grow a pure culture to make defined communities, some strains could grow on solid NA/MRS plates but failed to grow in liquid cultures, even when media with different components were tried and tested. This made the defined community approach challenging to implement.

Second, we were interested in studying multiple fly traits, and potentially, these traits could be affected by different microbes. Therefore, we used passive reconstitution as a starting point rather than asking what exact microbe or which community of microbes is responsible for affecting a trait. This strategy, in principle, can later be followed by the focused identification of specific microbes that drove this change.

Furthermore, passive microbiome reconstitution might be a better approach in our case because it has the potential to represent not only diverse strains of a given bacteria but also species from taxa other than bacteria, such as yeasts etc., native to the wild-type microbiome in untreated flies. In addition, it might preserve antagonistic interactions (e.g., due to the presence of pathogenic bacteria that might be part of the normal microbiome), if any. A recent study of the Duckweed microbiome showed that if microbes are taken outside their native communities, then there might be an underestimation of their effect on host fitness (O'Brien et al. 2024). This study shows that a community closer to the host's natural microbiome might be the one that matters most for the host. The authors suggest the culturing or the preservation step can take the community away from its natural form (Panke-Buisse et al. 2017). In this light, simple passive reconstitution also offers an advantage by sampling the source community without culturing or storage that is closer to its native state instead of using a community made up of just a few strains of a particular taxa.

The only flipside is that we should ensure that the community composition we use for microbiome reconstitution doesn't change significantly while we redo the experiments, increasing the noise in the control treatment itself. By doing population-level 16S rRNA

sequencing, we validated the consistency of the bacterial part of the microbiome reconstitution, as described in detail in the next section.

3.2.5 Validation of the microbiome reconstitution

When doing microbiome reconstitution using any method, it is essential to check that the reconstituted microbiome is similar to the native source community. To check this, we compared the 16S rRNA gene profiles of the NDB_1 (source of the microbiome) and reconstituted fly population (Figure 3.8A) using Illumina amplicon sequencing (see Method section 2.2.7 of Chapter 2). We found that almost all sequencing reads aligned with *Acetobacter* and *Lactobacillus* pair, with *Acetobacter* reads being the most abundant (~80-90% relative abundance). This result showed that reconstitution reproduced the microbiome composition of the source NDB_1 flies (shown in Figure 2.2, Chapter 2). For the same experiment, the total CFU counts were also similar to the normal range of ~10^5 CFUs per fly (Figure 3.8B). The detailed protocol used for this reconstitution is illustrated in Figure 3.7.

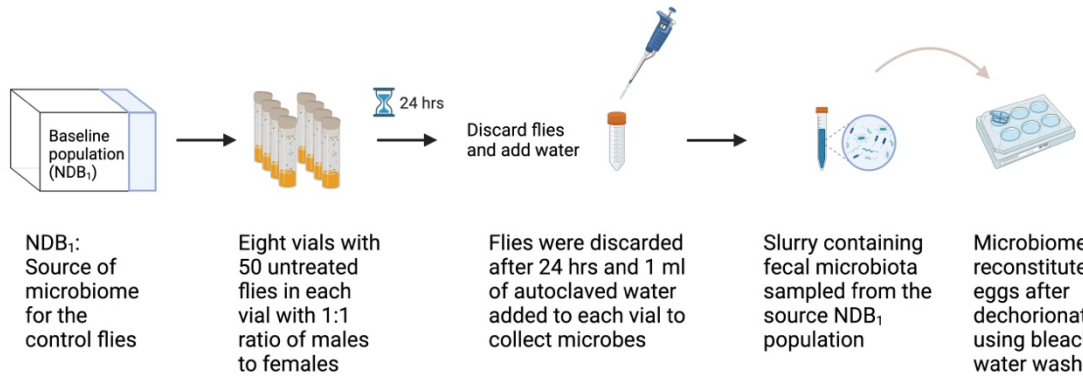

Figure 3.7 Detailed protocol of microbiome reconstitution from NDB_1. The source population for the microbiome reconstitution is NDB_1 unless stated otherwise. Total eight fly vials with non-autoclaved normal banana-jaggery food were setup to keep the flies for 24 hrs. These flies were collected on day 13 after egg collection. In each vial, 50 flies with equal male to female ratio were transferred using mild CO_2 anesthesia. After 24 hrs, flies were discarded and 1ml of autoclaved water was added to each vial to extract their microbiome. Vials were swirled with water till no visible fecal spots were seen on the vial walls. This method of sampling of microbes from the frass of flies housed in vials has been used earlier in the literature (Chandler et al. 2022). The microbe-free eggs after the bleach treatment were washed with autoclaved water twice and then dipped twice into this microbiome slurry to seed the source microbiome on eggs. These eggs were then gently transferred to *Drosophila* vials or bottles with autoclaved food to generate flies with reconstituted microbiome derived from NDB_1. This figure is created with BioRender.com.

To summarize, we created two groups of flies to study the impact of microbes on hosts: (a) microbe-free flies using bleach and (b) flies treated with bleach followed by microbiome reconstitution. We have confirmed these states by plating fly homogenates and PCR on gDNA extracted from adult flies using 16S rRNA universal primers (Figure 3.4 and Figure 3.5). Any phenotypic difference between these two will be attributed to the differential presence of the microbiome (explained in Figure 3.6), thus revealing the contribution of these microbes toward the given host phenotype.

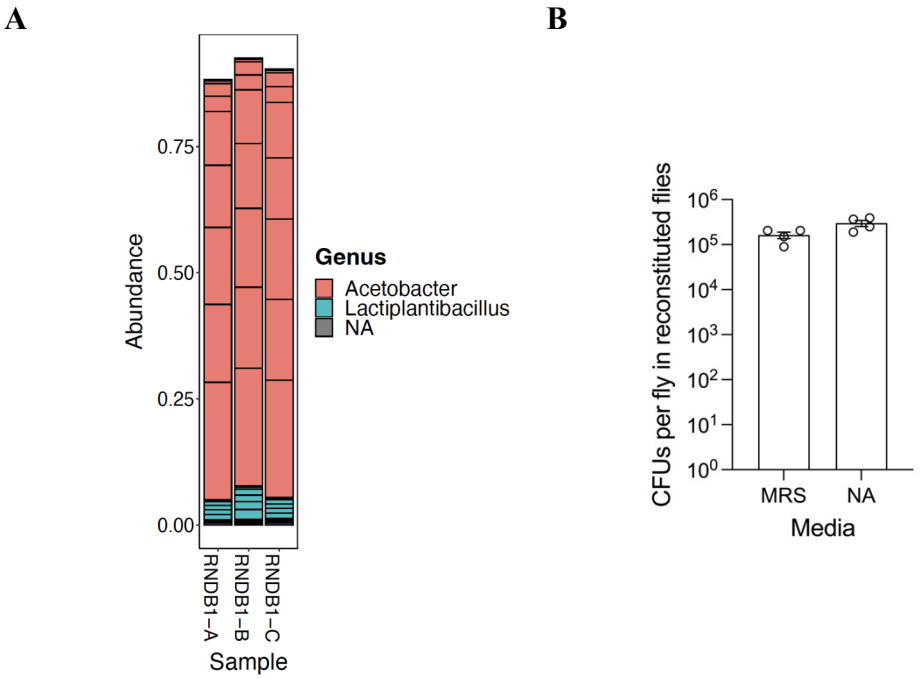

Figure 3.8 Microbiome of flies with reconstituted microbiome. (A) Results of the 16S rRNA amplicon sequencing for the population with the reconstituted microbiome that is used as a control for assays (labelled as R-NDB$_1$ population here). The relative abundance of top 20 most abundant genera is shown. The sequencing data was processed using dada2 pipeline. (B) CFU per fly of the same flies on two different media, MRS and NA. See method section 2.2.4.1 (chapter 2) for the detailed protocol.

3.2.6 Note on the use of F1 flies for the assays

In our flies, we removed microbes via dechorionation at the egg stage. Even if we have taken care of the confounding effect of bleach by using proper control, as explained in section 3.2.3, we wanted to keep the effect of the bleach on flies to a bare minimum (ideally none). To that end, we employed an additional measure of using F1 flies for the assays, as explained below (Figure 3.9).

In this approach, to generate flies without the microbes, we followed a protocol employed by several other studies where F1 flies (progeny of the flies that experienced the bleach at the egg stage) were used in the assays (H.-Y. Lee et al. 2019; I. S. Pais et al. 2018; Selkrig et al. 2018). This ensured that the F1 assay flies of both types, microbiome-reconstituted (M+) and microbe-free (M-), did not experience bleach in their lifetime (their respective parent population did). To generate F1 M- assay flies, F1 eggs were collected from the flies without microbes (i.e., F0 M- flies, Figure 3.9) and were raised in aseptic conditions, ensuring the absence of microbes. To generate control F1 M+ assay flies, F1 eggs were collected without any manipulation from the flies whose microbiome was reconstituted (i.e., F0 M+ flies, Figure 3.9).

In our conventional/untreated baseline lab populations, about 86 percent of the flies survive till adulthood. With the approach of using the F1 flies we described, control F1 assay flies whose microbiome was reconstituted (i.e., M+ flies) showed just an additional ~5 percent mortality (Figure 3.12A), showing that the overall mortality is kept to a minimum.

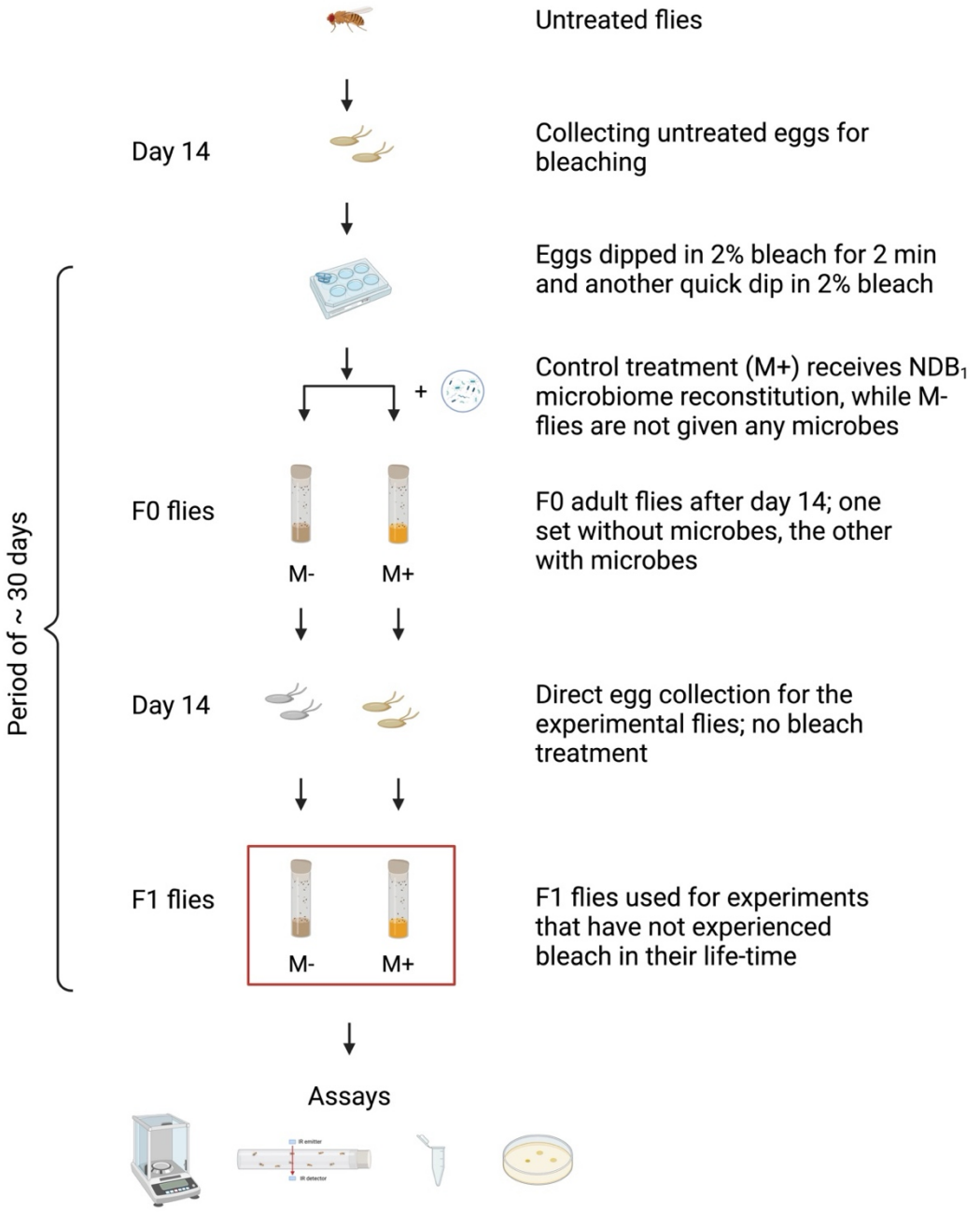

Figure 3.9 Illustration of the full experimental design to study microbiomes' contribution to host phenotypes. To create two types of flies (with the minimal effect of bleach), flies with microbes (M+) and without them (M-), we combined two procedures. First, as discussed in section 3.2.3, we chose microbiome-reconstituted flies as a control instead of using untreated flies. The F0 flies of both (M+ and M-) types were generated using bleach at the egg stage. This protocol is illustrated in detail in Figure 3.1. Second, as discussed in section 3.2.6, assays were conducted on progeny of these F0 flies (i.e., F1 flies) that have not experienced bleach in their lifetime to avoid any recency effect of bleach. The eggs collected for F1 experimental flies either had the microbes (M+) or lacked the microbes (M-) depending on the state of their parent's microbiome. Throughout this thesis, we use this same methodology illustrated here whenever we compare phenotypes of flies with-microbes and without them. This figure is created with BioRender.com.

3.2.7 Details of the phenotypic assays

For all the following assays, eggs were processed for dechorionation using bleach as per the protocol illustrated in Figure 3.1. These dechorionated eggs were transferred to the *Drosophila* vials with ~ 6 ml of food at a specified density (30 eggs per vial unless stated otherwise).

The first two assays (3.2.7.1 and 3.2.7.2) were used to see the impact of different bleach treatments on flies. These six treatments are: **1**. Untreated eggs, **2**: dH_2O wash-control (no bleach), **3**: 2% bleach for 5 min, **4**: 2% bleach for 10 min, **5**: 4% bleach for 5 min, **6**: 4% bleach for 10 min (Figures 3.2 and 3.3). Both the assays happened on two types of food: **1**: Normal banana-jaggery food and **2**: Autoclaved banana-jaggery food.

3.2.7.1 Percentage survival to pupation

For this assay, the eggs put into food vials after the treatments were counted precisely under a microscope using a paintbrush to the specified egg density (here, 30 eggs per vial). In this procedure, treated/untreated eggs were transferred over a thin strip of solidified 1.3% non-nutritive agar with a moist paintbrush. These eggs were spread over the agar strip such that a single egg could be counted and moved into a stack. This egg stack was cut away along with the agar layer using a scalpel that was pre-cleaned with cotton soaked in 70% ethanol. This piece of agar with exactly 30 eggs was transferred to the food vials. The number of total pupae were counted to determine the percent survival to pupation. A total of four vial replicates were used per treatment for this assay.

3.2.7.2 Time-to-pupation

In the experiment where we studied the impact of various bleach doses on fly development, we used time to pupation as a proxy for fly development. The vials used for this assay were the same as those described in the previous section 3.2.7.1. These vials were checked in 12-hour intervals for new pupations. The average pupation time for the whole vial, starting from the egg collection time, was calculated. Four such vial replicates were analyzed for each treatment.

The following set of assays used autoclaved banana-jaggery food. For these assays, egg dechorionation was done with 2% bleach for 2 min with the protocol illustrated in Figure 3.1.

3.2.7.3 Dry body weight

On day 12 after egg deposition, the adult flies were sorted by sex in batches of 20 individuals per sex into 1.5ml micro-centrifuge tubes using CO_2 anesthesia. These flies were then flash-frozen using liquid nitrogen and, if required, stored at -80^{0}C till weighing. The flies were then dried at 60^{0}C for 72 hrs and weighed on an analytical weighing balance (ME104, Mettler Toledo, least count = 0.1mg). First, the total weight of (micro-centrifuge tube + flies) was recorded. Next, flies were removed from the tubes with a dry paintbrush, and the weight of the empty micro-centrifuge tube was recorded. The difference between these two gave us the body weight of 20 flies. This number was divided by 20 to get the average dry body weight per fly. When flies were removed, we counted these flies to double-check if every micro-centrifuge tube had 20 flies. If not, then the total weight was divided by that count. A total of ten micro-centrifuge tubes were weighed per treatment per sex. Thus, 200 flies per treatment per sex were sampled.

3.2.7.4 Egg-to-adult developmental time

This assay was conducted similarly to the time-to-pupation assay (section 3.2.7.2), with the only difference being that instead of pupation, the eclosion of the flies was scored for male and female flies at regular intervals of 2 hours.

3.2.7.5 Percent egg-to-adult survival

This assay was conducted similarly to the percent survival to pupation assay (section 3.2.7.1), with the only difference being that, instead of pupation, adult fly survival was scored on day 12 after egg deposition.

3.2.7.6 Female fecundity

On day 12 after egg deposition, flies were transferred to fresh food vials. On day 13, flies were anesthetized using mild CO_2. A male-female pair was introduced into an aerated 50 ml falcon tube containing a food cup. The food cup provided a surface for egg-laying. The falcon tubes were kept for 12 hrs in an incubator maintained at 25^{0}C. After 12 hours, the total number of eggs laid was counted under a microscope. A total of 50 replicates per treatment were set for this assay.

For the assay on $MB_{1\text{-}4}$ and $MBL_{1\text{-}4}$ in Generations 54-57 (Chapter 4), a male-female pair was introduced in the falcon tube using aspirators, thus completely avoiding CO_2 anesthesia.

3.2.7.7 Desiccation resistance

Ten adult flies of the given sex were introduced into an empty plastic vial (Height: 3.5 inches x Diameter: 1 inch). The flies had no access to food or water. The vials were plugged with fresh cotton plugs. These vials were checked every two hours for any deaths, till all the flies were dead. The datasheet kept track of flies alive (total flies to start with - count of total dead flies) at a given time point. The average time to death due to desiccation was calculated for the vial. A total of ten such vial replicates were set per treatment per sex.

3.2.7.8 Locomotor activity

The flies' locomotor activity was measured using the *Drosophila* Activity Monitoring (DAM) system (Trikinetics Inc). Using an aspirator, a single fly was introduced into a glass DAM tube (Length: 6.5 cm, Diameter: 4 mm). The locomotor activity for a given fly is the average number of times the fly crosses the infrared beam running through the middle of the glass DAM tube per hour, recorded over six hours. The activity was assessed for six hours as prior laboratory tests showed that after six hours, mortality starts due to desiccation stress in the locomotor tubes. Adult flies (day 12 after egg deposition) were used for this assay. A total of about 32 flies were used per treatment per sex. The dead flies during the six hours were excluded from the analysis. The first 15 minutes of activity were excluded from the analysis as this was taken as the time taken for flies to get acclimatized to the glass DAM tubes.

3.2.7.9 Mating latency and mating time

The mating assay was performed on virgin males and females. After the eclosion (typically around day eight after egg deposition), male and female flies were separated under mild CO_2 anesthesia into vials with fly food. The interval of the fly collection was six hours, starting with the very first eclosion, to ensure that the eclosed flies were virgins. Males and females were held separately at the density of ten flies per vial till the mating experiment on day 12 after egg deposition. On the day of the experiment, a female was introduced into a plastic *Drosophila* vial using an aspirator, followed by the introduction of a male. The floor of the vials was covered with solid 1.3% non-nutritive agar to avoid desiccation of the flies. The time at which the male fly was introduced (T1), the time at which mating started (T2), and the time at which mating ended (T3) were noted by live manual observation. The mating latency was calculated as (T2-T1), and the mating time was calculated as (T3-T2). A total of 40 replicates were used for this assay.

3.3 Results

3.3.1 Body weight

For *Drosophila*, body weight is a central life-history trait (Edgar 2006; Nylin and Gotthard 1998; Partridge et al. 1999). We looked at how adult body weight changes when flies are grown without the symbiotic microbes. We found that males and females had a lower dry body weight without microbes (Welch's t-test, $t_{15.55} = 5.38$, $P < 0.0001$ for males and $t_{16.73} = 16.77$, $P < 0.0001$ for females, Figure 3.10). While for males, body weight was reduced by ~11% (Cohen's d = 2.54, large), for females, the effect of microbiome removal was more pronounced with ~26% reduction (Cohen's d = 7.91, large).

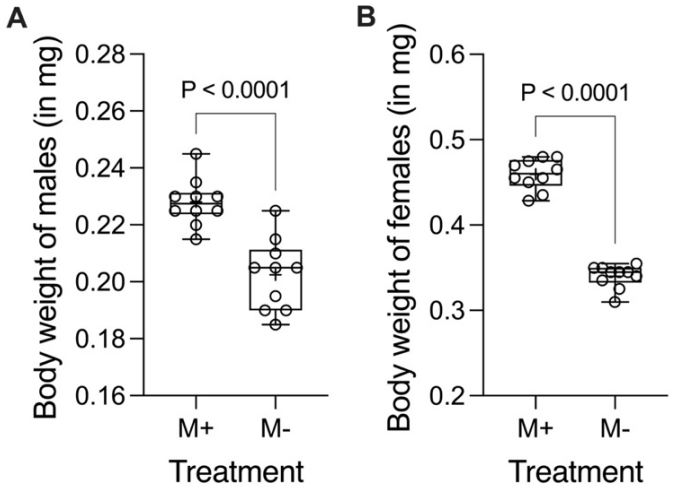

Figure 3.10 Dry body weight of adult *Drosophila* when reared with and without microbes. (A) males (B) females. In box-plots, centre lines show the median, box limits indicate the 25th and 75th percentiles, whiskers extend from the minimum to maximum data points, plus sign within the boxes represents the sample mean. See method section 3.2.7.3 for the assay details. Data were analyzed using two-sample unequal variance t-test (Welch's t-test).

3.3.2 Egg-to-adult developmental time

In flies, the time spent during development can affect the adult body weight/size (Alpatov 1929; Flatt 2020; Robertson 1960) and microbes can modulate these effects (Ridley et al. 2013; Shin et al. 2011; Storelli et al. 2011). In the absence of microbes, while development time does not change for males (Welch's t-test, $t_{13.60} = 1.40$, $P = 0.1855$, Figure 3.11A), females eclosed slightly earlier (Welch's t-test, $t_{12.62} = 4.51$, $P = 0.0006$, Figure 3.11B).

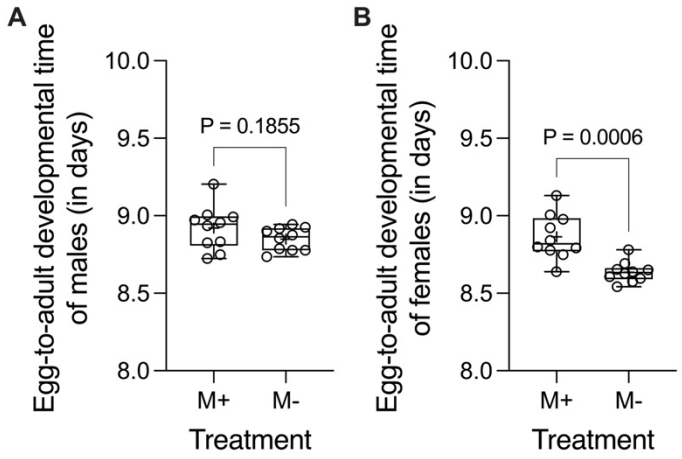

Figure 3.11 Egg-to-adult development time with and without microbes.
(A) males (B) females. In box-plots, centre lines show the medians, box limits indicate the 25th and 75th percentiles, whiskers extend from the minimum to maximum data points, black plus sign represents the sample means. See method section 3.2.7.4 for the assay details. Data were analyzed using two-sample unequal variance t-test (Welch's t-test).

3.3.3 Egg-to-adult survival

To know if microbe-free rearing has changed the overall egg-to-adult survival of the flies, we looked at what percentage of eggs successfully go through the larval and pupal phases to reach adulthood (day 12 after egg collection). We found that egg-to-adult survival was reduced in microbe-free flies (Welch's t-test, $t_{16.60} = 3.25$, P = 0.0048, Cohen's d = 1.53 (large), Figure 3.12A).

3.3.4 Female fecundity

In *Drosophila*, female body weight positively correlates with female fecundity (Lefranc and Bundgaard 2000; Prasad and Joshi 2003; Robertson 1957). Having observed that flies without microbes are smaller, we looked at whether that, in turn, has changed female fecundity as well. Females laid fewer eggs when raised without microbes (Welch's t-test, $t_{85.41} = 8.41$, $P < 0.0001$, Cohen's d = 1.70 (large), Figure 3.12B).

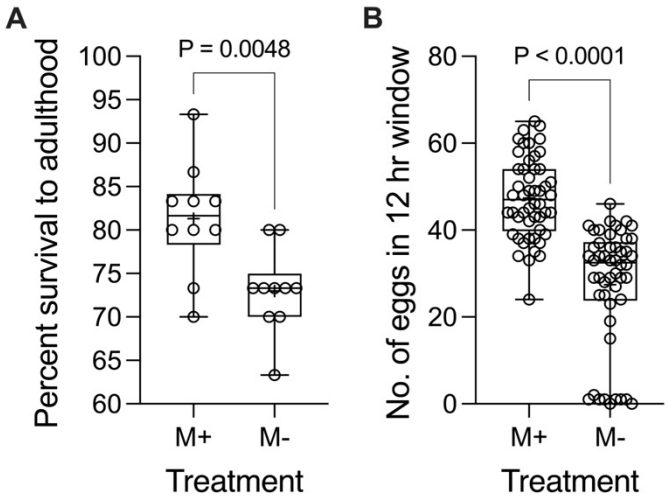

Figure 3.12 Changes in survival to adulthood and female fecundity in microbe-free flies. (A) Percent survival to adulthood (B) Total number of eggs laid by the female in 12 hr window. In box-plots, centre lines show the medians, box limits indicate the 25th and 75th percentiles, whiskers extend from the minimum to maximum data points, black plus sign represents the sample means. See method section 3.2.7.5 and 3.2.7.6 for the assay details. Data were analyzed using two-sample unequal variance t-test (Welch's t-test).

3.3.5 Desiccation resistance

One possible consequence of the change in life-history strategy can be the reallocation of body resources (Stearns and Stearns 1992). This reallocation, in turn, can affect the host's resistance to various stresses (Flatt 2020). To check if the change in life-history traits we observed when *Drosophila* developed without microbes resulted in any alteration in the host's capacity to withstand stresses, we looked at the flies' resistance to desiccation (Figure 3.13). We found that while males slightly reduced their resistance to desiccation (Welch's t-test, $t_{15.21} = 4.00$, P = 0.0011, Cohen's d = 1.88 (large), Figure 3.13A), females' ability to withstand desiccation had increased considerably (Welch's t-test, $t_{15.60} = 11.09$, P < 0.0001, Cohen's d = 5.23 (large), Figure 3.13B).

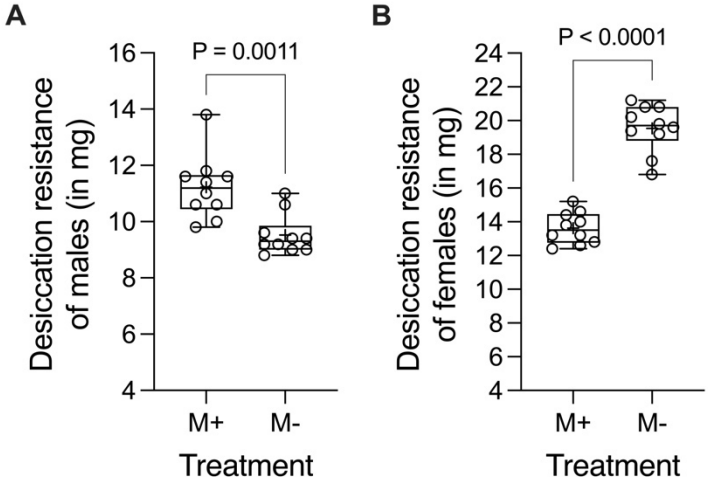

Figure 3.13 Desiccation resistance with microbes and without them. (A) males (B) females. In box-plots, centre lines show the medians, box limits indicate the 25th and 75th percentiles, whiskers extend from the minimum to maximum data points, black plus sign represents the sample means. See method section 3.2.7.7 for the assay details. Data were analyzed using two-sample unequal variance t-test (Welch's t-test).

3.3.6 Locomotor activity

Microbes are shown to modulate behavior in various model systems, including fruit flies (Cryan and Dinan 2012; Masuzzo et al. 2020; Vuong et al. 2017). We looked at the flies' locomotor activity using the *Drosophila* activity monitoring (DAM) system (Trikinetics Inc.). In the absence of the microbes, while males were more active (Welch's t-test, $t_{56.65} = 4.22$, $P < 0.0001$, Cohen's d = 1.1 (large), Figure 3.14A), female activity was reduced (Welch's t-test, $t_{58.86} = 4.00$, $P = 0.0288$, Cohen's d = 0.58 (medium), Figure 3.14B).

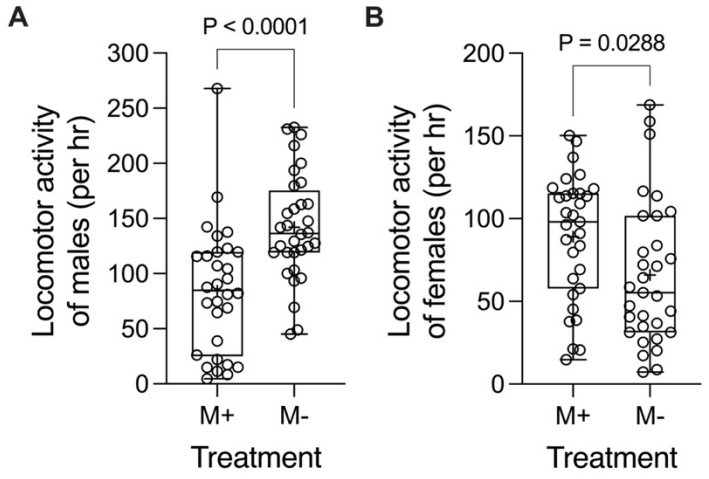

Figure 3.14 Sexual dimorphism in locomotor activity upon microbiome absence. (A) locomotor activity of males, and (B) females. In box-plots, centre lines show the medians,

box limits indicate the 25th and 75th percentiles as determined by GraphPad Prism software, whiskers extend from the minimum to maximum data points, black plus sign represents the sample means. See method section 3.2.7.8 for the assay details. Data were analyzed using two-sample unequal variance t-test (Welch's t-test).

3.3.7 Mating latency and mating time

Mate finding and aspects of the mating process influence the fitness of the organisms. Microbes can provide cues that host use to find mates (Heys, Lizé, Colinet, et al. 2018; Rowe et al. 2020; Sharon et al. 2010; B. S. Walsh et al. 2017). Disruption in these cues due to changes in the microbiome could affect mating-related phenotypes. We looked at two phenotypes related to the mating ritual of hosts – time to start mating (i.e., mating latency) and total time spent mating. Mating latency with microbes was significantly lower than without microbes (Welch's t-test, $t_{51.71} = 2.10$, $P = 0.0403$, Cohen's $d = 0.53$ (medium), Figure 3.15A). Mating time increased for the hosts without microbes (Welch's t-test, $t_{61.38} = 5.29$, $P < 0.0001$, Cohen's $d = 1.43$ (large), Figure 3.15B).

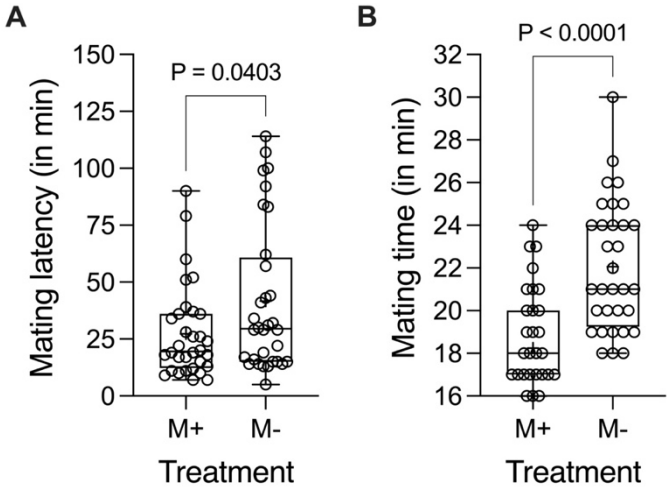

Figure 3.15 Microbiome removal affects the mating in fruit flies.
In box-plots, centre lines show the medians, box limits indicate the 25th and 75th percentiles as determined by GraphPad Prism software, whiskers extend from the minimum to maximum data points, black plus sign represents the sample means. See method section 3.2.7.8 for the assay details. Data were analyzed using two-sample unequal variance t-test (Welch's t-test).

3.3.8 Reproducibility of the body weight results over three independent experiments

To see if the patterns of microbial contribution to the host are robust over multiple host generations, in Figure 3.16, we have plotted results from three independent experiments that had different aims but used flies from the same population of NDB_1 at generations 52, 67, and 71. The generation 52 data is the same as in section 3.3.1 of this chapter. The results are analyzed using the Scheirer-Ray-Hare test (SRH test, a non-parametric equivalent of two-way ANOVA) with Microbiota (two levels: with and without microbes) and Generation (three levels: 52, 67, and 71) as fixed factors. Analyzing the same data using ANOVA led to the same statistical conclusion as the SRH test.

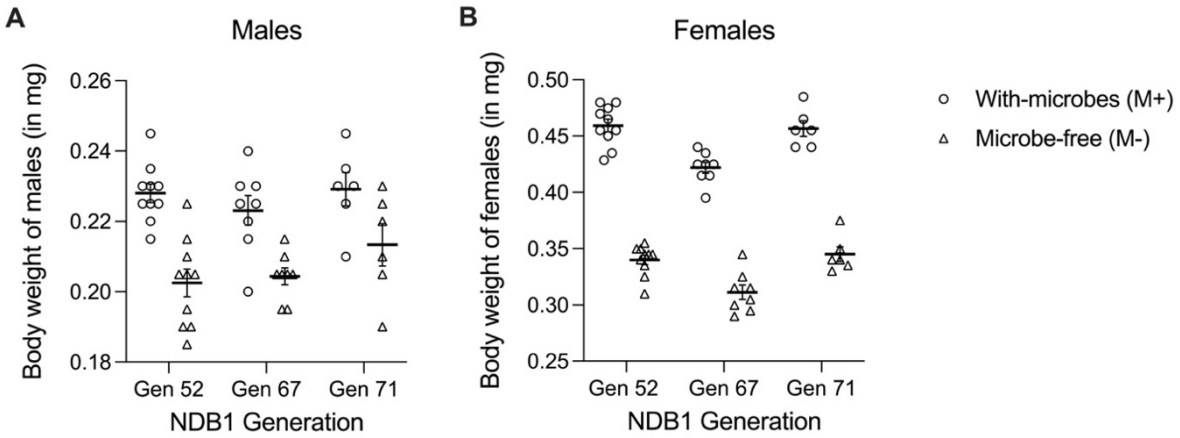

Figure 3.16 Reproducibility of the bodyweight results at different time points in (A) males and (B) females. Circles represent the treatment with microbes (M+) and triangles represent the flies without microbes (M-). The horizontal lines are means over the replicates and the error bars represent the SEM. The data from three generations are shown: Gen 52 data is same as presented earlier in this chapter. Gen 67 and 71 datasets were generated for experiments with different aims. Gen 71 data is also part of the Chapter 5. We plotted these results together to see if our results changed across multiple generations. This can happen in microbiome studies as fly microbiomes in labs are open systems that can get perturbed by environmental factors such as batch effects in their diet components (e.g., banana, jaggery), fly handling etc.

For both males as well as females, the effect of microbiota on host body weight was statistically significant (Males: $H_{1,42} = 23.04$, $P < 0.0001$, Figure 4.7A, and Females: $H_{1,42} = 35.35$, $P < 0.0001$, Figure 4.7B). The effect of generation was statistically not significant (Males: $H_{2,42} = 2.14$, $P = 0.3428$ and Females: $H_{2,42} = 5.86$, $P = 0.0535$). Microbiota × Generation interaction was not significant, indicating that the effect of microbiota on host body weight did not change with host generations (Males: $H_{2,42} = 0.7056$, $P = 0.7027$ and Females: $H_{2,42} = 0.38$, $P = 0.981$).

Table 3.3 Table summarizing statistical analysis details using two-sample unequal variance t-test (i.e., Welch's t-test), percentage change, effect sizes using Cohen's d, and sample sizes. T refers to the mean for the "M-" flies, while C refers to the mean for the "M+" flies.

	Assay	Test statistic	P-value	% change (T - C)/C	Cohen's d	Effect size*	Sample size[$]
1	Dry body weight (Male)	$t_{15.55} = 5.38$	< 0.0001	-11.2	2.54	Large	(10, 10)
2	Dry body weight (Female)	$t_{16.73} = 16.77$	< 0.0001	-25.9	7.91	Large	(10, 10)
3	Development time (Males)	$t_{13.60} = 1.40$	0.1855	-0.8	0.66	Medium	(10, 10)
4	Development time (Females)	$t_{12.62} = 4.51$	0.0006	-2.6	2.12	Large	(10, 10)
5	Survival to adulthood	$t_{16.60} = 3.25$	0.0048[#]	-10.2	1.53	Large	(10, 10)
6	Fecundity (Females)	$t_{85.41} = 8.41$	< 0.0001	-42.1	1.7	Large	(50, 50)
7	Desiccation resistance (Males)	$t_{15.21} = 4.00$	0.0011	-15.1	1.88	Large	(10, 10)
8	Desiccation resistance (Females)	$t_{15.60} = 11.09$	< 0.0001	43.5	5.23	Large	(10, 10)
9	Locomotor activity (Males)	$t_{56.65} = 4.22$	< 0.0001	68.3	1.1	Large	(30, 32)
10	Locomotor activity (Females)	$t_{58.86} = 4.00$	0.0288	-26.1	0.58	Medium	(31, 31)
11	Mating Latency	$t_{51.71} = 2.10$	0.0403	52.6	0.53	Medium	(32, 32)
12	Mating time	$t_{61.38} = 5.29$	< 0.0001	20.9	1.34	Large	(32, 32)

*Interpretation for effect sizes using Cohen's d: d > 0.8 (Large); 0.8 > d > 0.5 (Medium); < 0.5(Small)

[#]Man-Whitney U test is also significant (U = 15.50, P = 0.007).

[$]Note: See methods for detailed information about sample sizes. For example, the sample size of ten per treatment for body weight indicates ten micro-centrifuge tubes with 20 flies each.

3.4 Discussion

The microbiome of the hosts is known to influence the host biology (Knight et al. 2017; McFall-Ngai 2024; McFall-Ngai et al. 2013; Sommer and Bäckhed 2013). Evidence in different vertebrates and invertebrate model organisms shows that host-microbiome cross-talk is ubiquitous (Cornuault et al. 2022; Angela E. Douglas 2019; Hongbing Jiang and David Wang 2018; Kostic et al. 2013; W.-J. Lee and Brey 2013; Margaret J 1999; Motta and Moran 2023; Nguyen et al. 2015; Peixoto et al. 2021). Studies on *Drosophila* have significantly contributed to this development (Angela E. Douglas 2018b; Erkosar et al. 2013; Lesperance and Broderick 2020). These studies have also pointed out that each host-microbiome system can have its own governing principles, and different host-microbiome pairings might produce different insights into the host-microbe crosstalk (Angela E. Douglas 2018c).

We started our inquiry in Chapter 2 by understanding which microbes are closely associated with our flies. The natural course of inquiry after knowing microbiome composition was to see if these microbes can influence host biology. In this chapter, we report the adoption and standardization of methods to establish if microbes can influence the host phenotypes. We used this method on our baseline population NDB_1 to find out what phenotypes get affected when microbes are removed from the hosts over a short period of one host generation. We discuss the results in the following sections.

3.4.1 Life-history traits are modulated by symbiotic bacteria in outbred NDB_1 populations

We found that microbes modulate the host's body weight in our flies (Figure 3.10). This was seen for males and females, and agrees with previous studies (Shin et al. 2011; Storelli et al. 2011). This observation is important because, in *Drosophila*, body weight is correlated with several other traits. For example, female fecundity is often positively associated with the fly's body weight (Lefranc and Bundgaard 2000; Prasad and Joshi 2003; Robertson 1957). This study shows that this correlation between fecundity and body weight holds for our flies – symbiotic bacteria also increase female fecundity (Figure 3.12B). This observation also agrees with the broadly consistent trend of symbiotic bacteria improving the host fecundity reported in *D. melanogaster* (Elgart et al. 2016; Gnainsky et al. 2021; Suyama et al. 2023). However, it does not agree with the one in *D. subobscura,* where its microbiome was implicated in reducing female fecundity (B. S. Walsh et al. 2017).

Besides microbes' positive contribution to body weight and fecundity, our flies survive better till adulthood in the microbe's presence (Fig. 3.12A). Body weight, fecundity, and survival to adulthood are fitness components, i.e., these traits substantially influence the overall fitness of an organism and are often taken as a proxy for fitness (Flatt 2020). The observation that environmental microbes have a say in determining a host's fitness has implications for the microbe's role in host evolution. In contrast to our results, Ridley et al. found that fitness components - adult body weight, survival to adulthood, and female fecundity - do not change when the microbiome is removed (Ridley et al. 2012a).

We measured the time these flies took to develop from the egg to the adult stage. Microbes did not influence the male developmental time but were found to reduce female developmental time only slightly (Figure 3.11). Overall, the percent change for males is 0.8% and for females it is 2.6%. The contribution of the microbes to host development is often realized in unbalanced diets such as diets with low protein content (Shin et al. 2011; Storelli et al. 2011; Yun and Hyun 2023). However, the banana-jaggery diet used in this study is not unbalanced but conducive to fly growth. This might explain why the egg-to-adult development time was little affected by the removal of microbiota. While in the case of previous studies, the lower body weight of microbe-free flies can be explained by the delayed development (Shin et al. 2011; Storelli et al. 2011), we see that the same is not valid for our system. That leads to the question, in our flies, what is the mechanism by which the body weight is reduced without a concomitant effect on the development time? We flag this as an interesting open question for future investigations.

3.4.2 Altered life-history traits could have changed resource allocation patterns influencing stress tolerance phenotype

When life-history traits (i.e., fitness components) of the organism change in response to intrinsic or extrinsic factors, there is a possibility that this has happened through re-purposing of the body resources. Stress-related traits can show correlated changes when life-history traits or underlying resource distribution changes (Flatt 2020). We found that removing the microbes has divergent effects on different sexes - while males die slightly earlier due to desiccation stress, females show a marked increase in desiccation resistance (Figure 3.13). This means the presence of microbes is beneficial for males to survive desiccation, but females are better off without the microbes. We have not found any study that has reported such sexually dimorphic effects on desiccation resistance. It remains to be seen what

physiological changes (e.g., change in protein or triglyceride levels) might be implicated in driving such sexually dimorphic effects of the microbiome on the host.

In the case of males, lost body weight without the microbes might explain the slightly lower resistance to desiccation. However, the same logic does not apply to females. A possible explanation for these results comes from a previous study that found that in the absence of microbes, glycogen levels of female flies were elevated, but that of male flies was reduced (Ridley et al. 2012a). The increased level of glycogen can confer better desiccation tolerance in flies (Chippindale et al. 1998; Graves et al. 1992). This is possible because glycogen metabolism releases water, and glycogen can help retain more water than lipids, thus countering the water loss during desiccation (Wang et al. 2021). The differential allocation of glycogen in our microbe-free flies can potentially explain the pattern: females without microbes could resist desiccation better through increased allocation of glycogen, while males might die earlier due to desiccation as their glycogen is reduced on losing their microbial partners.

This possibility also hints that we need to check how body resource allocation, including glycogen storage, changes in the presence or absence of microbes for males and females. That might provide us further clues on how tradeoffs and constraints on resource allocation affect other phenotypes such as body weight, fecundity, and lifespan. Furthermore, if acquiring or losing a particular microbiome changes stress-related traits differentially for males and females, then they can experience different selection pressures, even exacerbating or improving intersex conflict.

3.4.3 The influence of microbes goes beyond affecting physiology to affect host behavior

Microbes produce several biomolecules that can interact with the host's physiology and nervous system, influencing the host’s behavior (Angela E. Douglas 2019; Henriques et al. 2020; Needham et al. 2022). Through these metabolites, several behaviors across taxa are shown to be modulated by microbes. We looked at the flies' locomotor activity and mating behavior to see if removing microbes changed these behaviors. While males were more active, females showed lower activity without the microbiome (Figure 3.14). Like in the case of desiccation resistance, the effect of microbiota removal was sexually dimorphic. A previous study that observed hyperactivity in the absence of *Lactobacillus brevis* demonstrated that the effect was mediated through sugar metabolism (Schretter et al. 2018).

However, the study was done only on females. Another study reported that the microbiome has limited influence on the fly's locomotor activity (Selkrig et al. 2018). These studies and our results together show that these effects on the host's locomotor activity are specific to the given host-microbiota combination and also depend on the sex of the flies.

Increasing evidence suggests that microbes can influence host mating, possibly via the modulation of pheromones (Engl and Kaltenpoth 2018). We found that the hosts without symbiotic microbes have longer mating latency (Figure 3.15A) and longer mating duration (Figure 3.15B). The increase in mating latency is consistent with results from a previous study, which interestingly found no difference in mating duration (Heys et al. 2020). The mating duration was also unaffected in a study by Walsh et al. on *Drosophila subobscura* (B. S. Walsh et al. 2017).

Overall, our results establish that our lab flies are associated with microbes that influence the host's biology under our lab's standard (routine) nutrition conditions. We do not consider our fly diet unbalanced but one with plenty of nutrition. While many times, the microbes' contribution is highlighted in unbalanced diets, and our results show that microbes associated with our lab flies do so in a diet rich in nutrition.

Lastly, we examined how robust the microbiome composition is and its effect on different phenotypes. To that end, we have sequenced the microbiome of our flies over multiple generations from every set of reconstituted flies we made for various experiments. The microbial community shows small sampling fluctuations but is always dominated by *Acetobacter* and *Lactobacillus* strains (See Figures 2.2 for untreated/conventional flies and Figures 3.8A, 4.26, 4.27, and 5.11 for reconstituted flies). This largely stable community composition also seems to be reflected in the broadly robust patterns in phenotypic assays over multiple host generations in independent experiments (Figure 3.16).

When we started these investigations, we expected there would be only a few traits modulated by microbes. However, almost all the traits we investigated showed that the microbes have the potential to alter them (Table 3.4). This is also exciting because the same community of microbes present in our lab baseline populations affected multiple traits. This observation reinforces the theme that symbiotic microbes can modulate diverse aspects of the host's physiology and affect the host's fitness. Both these aspects are expected to have consequences for the evolution of the host.

Table 3.4 Integrated picture of outbred fly phenotypes to which microbes contribute

No	Type of trait	Phenotype of the flies	Microbes ↑ or ↓ the phenotypic value		
			males	**females**	**males and females**
1	Life-history related	Dry body weight	↑	↑	
2		Development time	~	~↑	
3		Survival to adulthood			↑
4		Female fecundity			↑
5	Stress-tolerance	Desiccation resistance	↑	↓	
6	Behavioral	Locomotor activity	↓	↑	
7		Mating Latency			↓
8		Mating time			↓

Note: The up and down arrows just convey the direction of phenotype change, and they may or may not correlate with the fitness of the organism. For example, an increase in female fecundity due to microbes might very well contribute to the fitness increase of the hosts. However, microbes decreasing mating latency can also contribute to an increase in the host's fitness. This is because initiating mating quickly might offer some evolutionary advantage to the host, for example, in the presence of predators or in stressed conditions. In other words, here, in the case of mating latency, a decrease in the trait value of the host could ultimately increase the host's fitness. The details of microbes' contribution to the host fitness would depend on the environment the organism faces.

Chapter 4
Experimental evolution of *Drosophila* without its microbiome for 54 generations

4.1 Introduction

Host-associated microbes are shown to modulate host biology (Cryan and Dinan 2012; Lynch and Hsiao 2019; McFall-Ngai 2024; McFall-Ngai et al. 2013; Sommer and Bäckhed 2013). These symbiont microbes can enhance the host's nutritional capacity (A. E. Douglas 1998; Ridley et al. 2012b; Sun et al. 2022), offer protection from pathogens through colonization resistance (Caballero-Flores et al. 2023; Spragge et al. 2023), and shape the host's physiology (Grenier and Leulier 2020b; Sommer and Bäckhed 2013), life history (Arias-Rojas and Iatsenko 2022; Gnainsky et al. 2021; Gould et al. 2018; H.-Y. Lee et al. 2019; Shin et al. 2011; Storelli et al. 2018; Suyama et al. 2023), behavior (Cryan and Dinan 2012; Schretter et al. 2018; Vuong et al. 2017; Adam Chun-Nin Wong et al. 2017), and immunity (Ansaldo et al. 2021; Belkaid and Hand 2014; Chervonsky 2013; Jordan and Clarke 2024; K.-A. Lee et al. 2013; Selosse et al. 2014). In hosts where the impact of this symbiont-mediated modulation is substantial, microbes can alter the host's fitness (Ma et al. 2023; Rosenberg and Zilber-Rosenberg 2016). The ability of symbionts to contribute to diverse host phenotypes and, in turn, influence host fitness raises the possibility that the presence of such microbes can influence the host's evolution (Gilbert et al. 2015; L. P. Henry et al. 2021; Kolodny et al. 2020; Macke et al. 2017).

As microbiome acquisition can happen on faster timescales than the host's generation time, the microbiome can confer rapid adaptation to the hosts (Alberdi et al. 2016; Lau and Lennon 2012; Voolstra and Ziegler 2020; Zilber-Rosenberg and Rosenberg 2008). These adaptations can begin with the hosts expanding their niche with the help of microbes (Borges 2017; Hoang et al. 2021; Koide 2023; Kolodny and Schulenburg 2020; Kopac and Klassen 2016). For example, a study showed that stinkbugs can gain insecticide resistance within a single generation by acquiring the bacterial strain of *Burkholderia* that can degrade the insecticide fenitrothion (Kikuchi et al. 2012). This study also showed that along with the insecticidal resistance, the stinkbugs gained a fitness advantage which could help this symbiosis to spread in the population.

Theoretical studies have shown that under certain conditions (strong vertical selection and short generation time), hosts can get a fitness advantage from symbiont microbes, even if the microbes pay costs for such an advantage (Vliet and Doebeli 2019). It has been argued that the microbiome can alter the mean and the variance of the host's phenotypic distribution, which in turn can enable the latter to explore a novel fitness landscape (L. P. Henry et al.

2021). These authors also suggest that the variance contributed by microbes can alter the heritability of the traits, thus affecting the host's response to selection.

Another way in which the microbiota can affect the host's evolutionary potential is through phenotypic plasticity. It is known that symbiotic microbes can be an important source of phenotypic plasticity for their host (Alberdi et al. 2016), which can serve as an initial step in the host's adaptation to novel environments (Hoang et al. 2021).

In light of the above discussion, it comes as no surprise that the potential impacts of microbiota on the evolution of their host has become a major topic of investigation in the field (L. P. Henry et al. 2021; Kolodny et al. 2020; Macke et al. 2017). However, there are more pragmatic anthropocentric reasons for understanding the long-term impact of microbiota on host evolution. In modern human societies, the overuse of antibiotics and a hygienic lifestyle, along with other factors, could lead to the disappearance of beneficial microbes (Blaser and Falkow 2009; Noverr and Huffnagle 2004). The recent COVID-19 pandemic-induced lifestyle changes, such as the overuse of soaps and sanitizers, can add to this selection pressure on certain symbiotic microbes. It has been hypothesized that such loss of ancestral microbes (e.g., *H. Pyroli*) can increase the risk factors for conditions such as gastroesophageal reflux disease (GERD) and childhood asthma (Blaser 2008; Blaser and Falkow 2009). Even the microbiota of many naturally occurring species might have been altered due to increased antibiotic load in the environment (Baquero et al. 2019; Larsson and Flach 2022) and microenvironmental alterations brought by climate change (Williams et al. 2023). Therefore, understanding the evolutionary implications of altering or removing the microbiota on the hosts has emerged as a key question in evolutionary biology (L. P. Henry et al. 2021; Macke et al. 2017).

Here, we used experimental evolution using *D. melanogaster* as a model system to understand how a host can evolve in the absence of its microbiome. For this, we kept four large (~2400 individuals) outbred populations of *D. melanogaster* microbe-free using bleach (labeled 'MBL' populations) for more than 50 generations. This involved bleaching *D. melanogaster* eggs every generation and rearing them in sterile food in a sterile environment. Each of these four selected populations had an ancestry-matched control (labeled 'MB' populations) where the microbiome was reconstituted just after the bleach treatment from a common source pool. For this reconstitution, microbes were sampled from the environment of the flies via fecal microbiome transfer. We compared the selected MBL and control MB populations in two assay environments: (a) in the presence of microbes (labeled "with-

microbes" environment) and (b) in the absence of microbes (labeled "microbe-free" environment). The previous chapter showed that the symbiotic microbiome associated with our outbred baseline lab population affects diverse host traits and host fitness. This observation underscored the important role that the microbiome plays in maintaining the host's homeostasis. Based on these results and the available literature, we hypothesized that the host would face substantial selection pressure in the absence of microbes. Therefore, we expected a rapid evolution of the host to maintain the homeostasis seen in the presence of microbes in our ancestral baseline populations.

We assayed these populations twice during selection: generations 17-20 and generations 54-57. We found that even after 54 generations without microbes, contrary to expectations, there were only modest adaptations in MBL populations. The percent survival from egg to adulthood was higher for MBLs when tested under the microbe-free assay environment. We also found that in the with-microbe assay environment, MBL males had lower desiccation resistance. The increase in survival might be an adaptation, and the reduction of male desiccation resistance might be a cost paid by MBLs. We also saw that the effect sizes over evolutionary differences between MB-MBLs shifted from "small to medium" effects in Gen 17-20 towards "medium to large" effects in Gen 54-57. When looking at the phenotypic plasticity of MB-MBLs across with-microbe and microbe-free environments, we saw that MBLs had lesser plasticity than MBs. This might hint at MBLs reducing their dependence on microbes. To see if there are changes in gene expression patterns, we performed RNA-Seq on one population each of MB and MBL. We found that a cluster of anti-microbial peptides (AMPs) was up-regulated, and another cluster of heat shock proteins (HSPs) was down-regulated in MBL as compared to MBs. These RNA-Seq results also hinted that the peritrophic matrix could be a site of interest for carrying out further investigations related to these populations.

4.2 Methods

4.2.1 Details of the experimental evolution lines

The experimental evolution lines were derived from the four ancestral baseline populations named NDB_{1-4} (at their generation 21 in the lab). The details about the NDB_{1-4} are provided in Chapter 2, section 2.2.1. Each ancestral NDB line gave rise to two populations: MBL ("Microbiota-Less", selected population that is without the microbiome) and MB (corresponding ancestry-matched control population with microbiome) (Figure 4.1). For example, NDB_1 gave rise to MBL_1 and MB_1, NDB_2 gave rise to MBL_2 and MB_2, and so on. Thus, populations with the same numerical index (e.g., index 2 for MBL_2 and MB_2) share a common ancestry and were always assayed together and treated as blocks in statistical analysis. This design means a total of four replicate selected populations (MBL_{1-4}) were compared against four replicate control populations (MB_{1-4}) (Figure 4.1). Each population in this setup is maintained at a population size of ~2400 in the plexiglass cage setup described in the method section 2.2.2 of Chapter 2, with the added implementation of a selection protocol and selection conditions described in the next section. NDB_1 served as the common source of the microbiome for all the MBs (for the rationale of this step, see section 4.2.3).

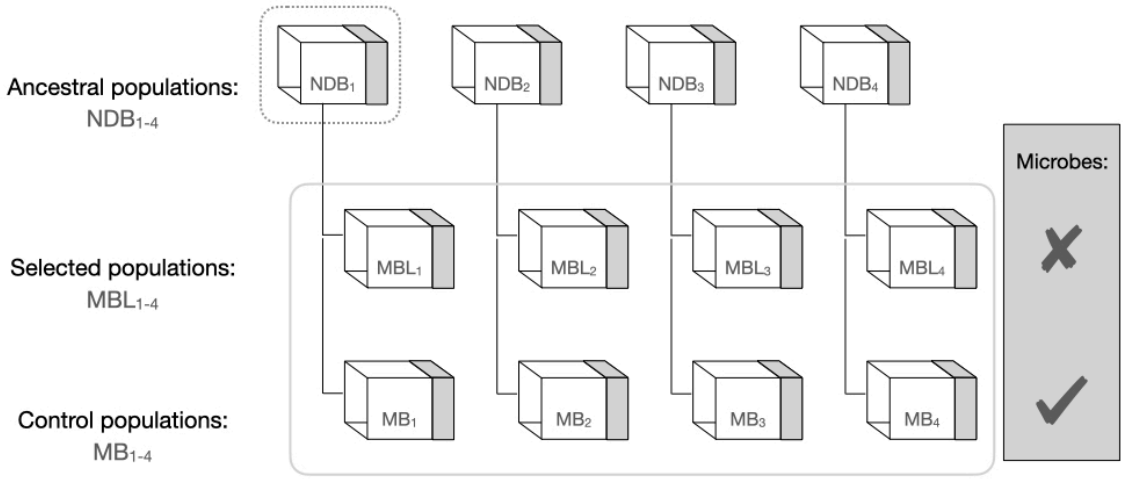

Figure 4.1 Details of the ancestral and experimental evolution populations.

4.2.2 Experimental evolution protocol employed each generation

MBLs were handled aseptically inside a Level II biosafety cabinet (make: Microfilt, India). All things that came in contact with MBLs for their maintenance were either autoclaved, filtered through a 0.2-micron filter, or surface-sterilized with 70% ethanol.

Every generation, on day 12 after egg deposition, each experimental evolution population of MBLs and MBs (total 8 populations) was provided with autoclaved yeast in the form of moist paste to boost their egg output. This paste also contained a few drops of acetic acid so that the flies are attracted to yeast. A fresh food plate with an exposed vertical food surface was provided for 12-16 hrs to these populations at the end of day 13 to collect eggs for the next generation. On day 14, these eggs were collected using a moist paintbrush and transferred to cell strainers (TCP026, HiMedia) for egg dechorionation (Figure 4.2A). The cell strainer was then placed into one of the wells of a 6-well plate. These eggs were washed with 2% bleach for 2 min to remove the chorion layer and any associated microbes (Figure 4.2B). The cell strainer with dechorionated eggs was transferred to another well of the 6-well plate for another quick wash with 2% bleach to kill any residual microbes. Next, the cell strainer with eggs was transferred back-to-back to two fresh wells of the 6-well plate for two successive washes with autoclaved dH_2O to remove bleach. To create MBLs, ~400 eggs were transferred to translucent, plastic *Drosophila* milk bottles (Laxbro Inc., Catalog No. FLBT-20) that had ~50-60ml autoclaved banana-jaggery media (section 2.2.8 of Chapter 2) at the bottom (Figure 4.2C). To make MBs, the native microbiome of NDB_1 was reconstituted (see next section) on the dechorionated eggs before they were transferred to the food bottles (Figure 4.2C).

4.2.3 The procedure of microbiome reconstitution

As discussed in sections 3.2.3 and 3.2.4 (Chapter 3), we chose passive microbiome reconstitution to create flies with microbes for single-generation and for long-term experimental evolution. Figure 4.3 illustrates the reconstitution protocol in detail. The microbiome was reconstituted from the ancestral NDB_1 population for all the MBs (i.e., MB_{1-4}). This ensured that there were no differences across the four MB populations in terms of the microbiota that they were harboring. Several different sources of the microbiome (say, every MB_i getting their microbiome from their ancestral NDB_i) would mean there is a greater chance of differential microbial association.

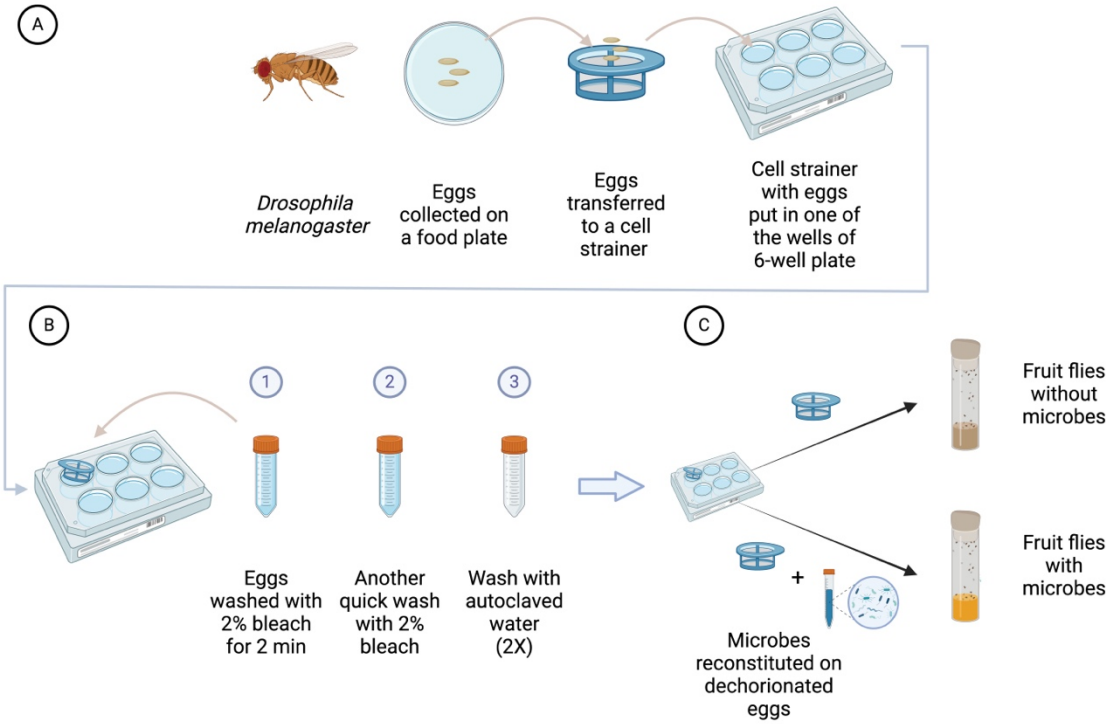

Figure 4.2 Egg dechorionation protocol using bleach to make MBL_{1-4} and MB_{1-4} populations. This figure is created with BioRender.com.

We describe the microbiome reconstitution protocol in detail in Figure 4.3. The source population for the microbiome reconstitution is NDB_1 unless stated otherwise. A total of eight fly vials with non-autoclaved normal banana-jaggery food were set to keep the flies for 24 hrs. These flies were collected on day 13 after egg collection. In each vial, 50 flies with an equal male-to-female ratio were transferred using mild CO_2 anesthesia. After 24 hrs, flies were discarded, and 1 ml of autoclaved water was added to each vial to extract their microbiome. Vials were swirled with water till no visible fecal spots were seen on the vial walls. This method of sampling of microbes from the frass of flies housed in vials has been used earlier in the literature (Chandler et al. 2022). The microbe-free eggs, after the bleach treatment, were washed with autoclaved water twice and then dipped twice into this microbiome slurry to seed the source microbiome on the eggs. These eggs were then gently transferred to *Drosophila* vials or bottles with autoclaved food to generate flies with reconstituted microbiomes derived from NDB_1.

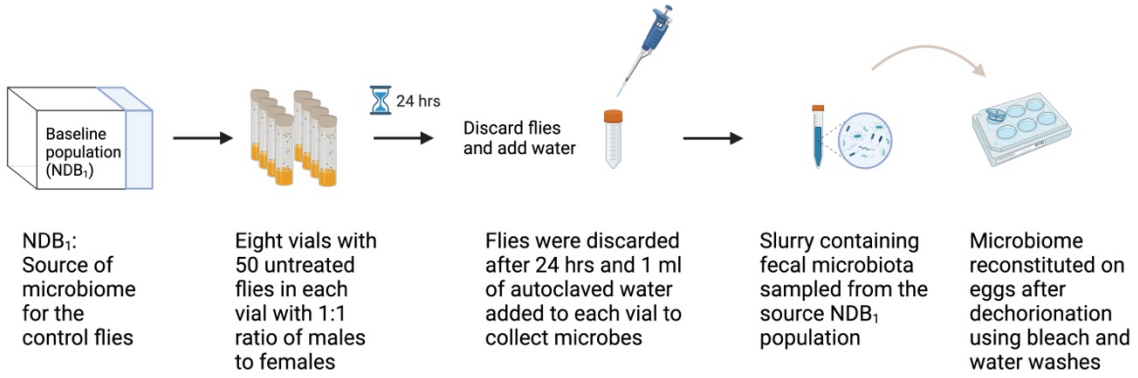

Figure 4.3 Detailed protocol of microbiome reconstitution from NDB_1. This figure is created with BioRender.com.

4.2.4 Assessing the microbiome status of MBs and MBLs during experimental evolution

We plated adult flies from MBL_{1-4} every generation to see if the microbiome is indeed absent in these populations (data not shown here). Except for generations and blocks specified in Table 4.1, in all other generations, MBLs did not show the presence of a microbiome. We sometimes did observe ~1-2 colonies (per 10 flies plated) in some replicates.

Table 4.1 Generations in which MBLs showed transient contamination

Generation	Affected MBL blocks
37	Block 1 and 3
38	Block 1
39	Block 1

MB_{1-4} were plated every 2-3 generations and CFUs were counted to make sure they received the reconstituted microbiome (data not shown here).

In generation 40 of the selection line, we performed PCR with 16S rRNA universal primers (details in section 2.2.4.2) on DNA extracted from adult MB_{1-4} and MBL_{1-4} females (day 15 after egg collection). In Figure 4.4., we see that MB_{1-4} show the presence of the reconstituted microbiome, and the microbiome is absent from MBL_{1-4}. This also confirms that, by

generation 40, the contamination that we saw in MBLs in previous generations was completely gone.

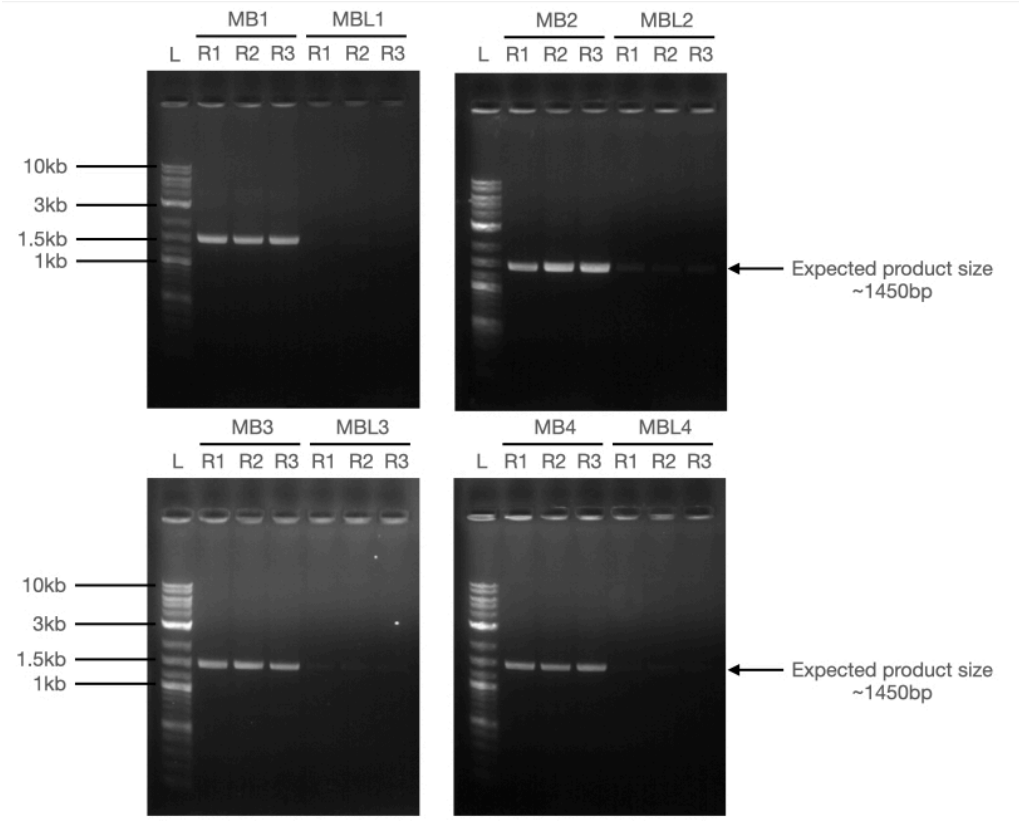

Figure 4.4 16S rRNA PCR to check the status of microbiome in MB_{1-4} and MBL_{1-4} .

4.2.5 Common-garden assay environments for the phenotypic assessment

We explain the environments using the MB_1-MBL_1 block (Figure 4.5). However, the same is true for the rest of the three blocks of MB-MBL as well. MB_1 received microbiome from NDB_1 every generation. MBL_1 is maintained without any microbes (details in section 4.2.1). If we directly compare the phenotypes of MB_1-MBL_1 in their experimental evolution conditions (i.e., MB with microbes and MBL without microbes) (Figure 4.5A), any observed differences between MB/MBL lines can be due to their evolutionary history (with/without microbes) or the assay environment (with/without microbes) or both. Therefore, we needed to provide a common assay condition for the phenotypic comparison of MB_1-MBL_1. We performed a phenotypic comparison between MB_1-MBL_1 in the native environment of MB_1, which is with-microbes environment, and also in the native environment of MBL_1, which is microbe-free (Figure 4.5B). For the with-microbe environment, MB_1 as well as MBL_1 were

maintained in this common-garden environment in the presence of microbes for a generation. F1 flies on which we performed the assays were raised as described in Figure 3.9. For a microbe-free environment, MB_1 and MBL_1 were maintained in this common-garden environment in the absence of microbes for a generation. F1 flies on which we performed the assays were raised as described in Figure 3.9. Matching of environments via common-garden rearing for a generation minimizes the non-genetic effects, giving us the evolutionary effects in which we are primarily interested.

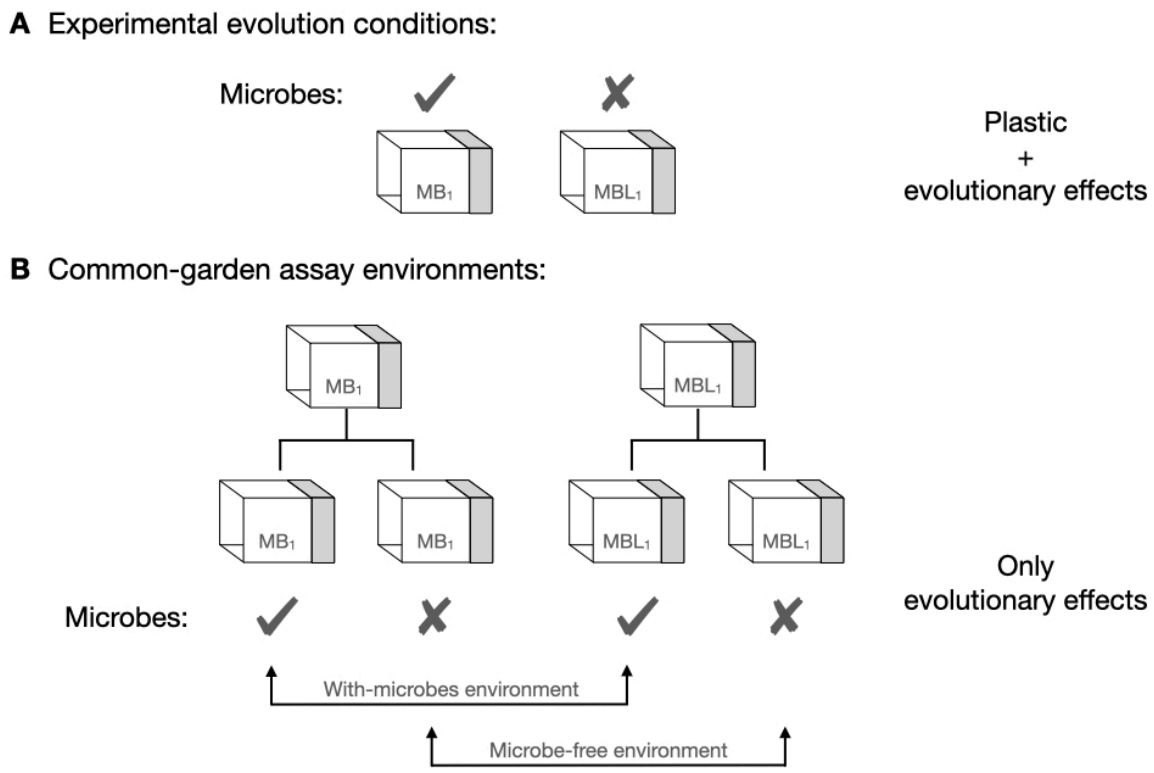

Figure 4.5 Illustration of environments in which experimental evolution was carried out and the environments in which phenotypic assays are carried out.

Table 4.2 Four types of populations generated for comparison in two types of environments

	Comparison 1		Comparison 2	
Experimental evolution population	MB_1	MBL_1	MB_1	MBL_1
Experimental evolution environment	+ Microbes	- Microbes	+ Microbes	- Microbes
Common-garden assay environment	With-microbes (+)		Microbe-free (-)	

4.2.6 First phenotypic assessment of MB_{1-4} and MBL_{1-4} at host generations 17-20

After 17 generations of selection, we looked at various host traits to see if the experimental evolution without the microbes has led to any divergence in MBLs vis-à-vis the MBs. This assessment was done in two assay environments: (1) with microbes and (2) microbe-free. The design of the experiments and details about the phenotypic assays are given in method sections 4.2.5 and 4.2.9, respectively.

In this design, logistically, it was not possible to assay all four blocks of MB and MBL together. Hence, we assayed them in sets of two blocks, namely 1 - 2 and 3 - 4. Table 4.3 gives details of the generations in which the assays took place. MB and MBL populations in each block were always assayed together in the same generation to know the effect of the selection procedure (e.g., MB_1 compared with MBL_1). For all assays done in this set, flies for separate environments (i.e., with microbes and microbe-free) were generated in different host generations (Table 4.3). As two different environments were not assayed in the same host generation, we do not discuss the effect of the environment on these populations. We only discuss the effect of selection in this first set of assays.

Table 4.3 Details of the assay environments and host populations for Gen 17-20 assays

No.	Assay environment	Block	Host generation
1	With-microbes	3 and 4	17
		1 and 2	18
2	Microbe-free	1 and 2	19
		3 and 4	20

The validation of the microbiome states of flies raised in with-microbes and microbe-free assay environments is done in Appendix section 4.5 of this chapter.

4.2.7 Second phenotypic assessment of MB_{1-4} and MBL_{1-4} at host generations 54-57

In the first assessment of MBL_{1-4} vs. MB_{1-4} that happened in generations 17 to 20 (section 4.3.1), we noticed that plastic effects across two assay environments (with-microbes and

microbes-free) on both of these populations were more significant than the effects of the selection (Figures 4.11-4.13). (By plastic effects, we mean differences seen in a phenotype when the environment changes without a change in host genotype). However, as with-microbes and microbe-free flies were generated in separate host generations (see Table 4.3), we couldn't compare these plastic effects quantitatively with this design. We amended our experimental design so that these plastic effects could be compared directly by conducting the assays in the two environments simultaneously. Table 4.4 shows the details of our experimental plan for the set of assays conducted in generations 54 to 57. This amendment meant, due to logistical reasons, we could only assess one block at a time in the same host generation (in contrast to the Gen 17-20 assays where two blocks were assessed in the same host generation).

This experimental design allows us to make two kinds of comparisons: (1) the effect of experimental evolution in a common-garden assay environment obtained by directly comparing MBs vs. MBLs (Result sections – 4.3.2.1 to 4.3.2.3 and section 4.3.3) and (2) the plastic effect of the presence vs. absence of microbes on both MB and MBL populations (Result section – 4.3.2.4)

Table 4.4 Details of the assay environments and host populations for Gen 54-57 assays

No.	Common-garden environment	Block	Host generation
1	With-microbes	1	54
		2	55*
		3	56
		4	57
2	Microbe-free	1	54
		2	55*
		3	56
		4	57

*For block 2, body weight and female fecundity were assessed in Gen 59

4.2.8 RNA isolation, RNA-Seq, and analysis

A subset of flies from the phenotypic assays (at generation 54-57) were flash-frozen in liquid nitrogen and stored at -80^{0}C. These flies from MB_1 and MBL_1 populations were sent to a commercial vendor (miBiome Therapeutics LLP, Mumbai, India) for RNA-Seq, starting with total RNA isolation. RNA-Seq was done only on female flies.

4.2.8.1 RNA isolation

Total RNA was extracted using miRNeasy Mini kit (Qiagen, Catalog No. 1038703) following the manufacturer's protocol. About 30 females were homogenized and lysed in Trizol reagent, followed by chloroform extraction. The aqueous phase was mixed with 1.5x chilled ethanol, and the mix was loaded on the columns provided in the kit. RNA was eluted in 30 µl RNase-free water. The RNA samples were quantitated on a Qubit fluorometer. Appropriate dilutions were loaded on a high-sensitivity RNA screen tape to determine the RNA integrity number (RIN).

4.2.8.2 Library preparation and sequencing

1.5 µg of RNA per sample was depleted of fly rRNA using QIAseq FastSelect - rRNA Fly Kit (Qiagen, Catalog No. 333262). The NEBNext Ultra II directional RNA library prep kit (Illumina, Catalog No. E7760L) was used to construct double-stranded cDNA libraries from the rRNA-depleted RNA. The cleaned libraries were quantitated on a Qubit fluorometer, and appropriate dilutions were loaded on high-sensitivity D1000 screen tape to determine the library size.

These libraries were sequenced Illumina NGS platform with 2x150bp chemistry to generate 10 million paired-end reads (3 Gb data per sample).

4.2.8.3 RNA-seq analysis

The quality of the reads was assessed using *FastQC v0.11.9*. These reads were passed through *fastp* to remove adaptors and low-quality bases. The filtered reads were mapped against the *Drosophila melanogaster* BDGP6.46 top-level chromosome (GCA_000001215.4) retrieved from ENSEMBL using *HISAT2.* The program *featureCounts* (mode = fragment) was used to count mapped reads. The count table from *featureCounts* was given as input to DESeq2 to find differentially expressed genes in the populations. Transcripts with counts less than ten were removed from all samples. The false discovery rate (FDR) cut-off was at 0.05, and the log fold change (LFC) threshold was at 1.5.

4.2.9 Assay details

We assayed the following phenotypes in MB_{1-4} vs. MBL_{1-4}.

Table 4.5 List of traits assessed

No	Type of trait	Phenotype of the flies	Assessment done on		
			males	females	males and females
1	Life-history related	Dry body weight	Yes	Yes	
2		Development time	Yes	Yes	
3		Survival to adulthood			Yes
4		Female fecundity			Yes
5	Stress-tolerance	Desiccation resistance	Yes	Yes	
6	Behavioral	Locomotor activity	Yes	Yes	
7		Mating Latency			Yes
8		Mating time			Yes

The protocols for all the above phenotypic assays are already described in detail in section 3.2.7 of Chapter 3.

4.2.10 Statistical analysis

The selected populations (MBL_{1-4}) were compared with their corresponding controls (MB_{1-4}) in two types of environments (with and without microbiota; details in section 4.2.5). The analysis for these two environments was done separately. In each environment, four block means of MB_{1-4} were compared against four block means of MBL_{1-4} using a paired t-test performed in GraphPad Prism (v10.2.1).

For interpreting effect sizes using Cohen's d, the following criteria were followed: $d > 0.8$ (high); $0.8 > d > 0.5$ (medium); $d < 0.5$ (low) (Cohen 2013). The graphs are plotted in GraphPad Prism (v10.2.1). The figures drawn using BioRender have that specification in their legend description.

In the Results section, along with the trends that are statistically significant (i.e., $P < 0.05$), we have also highlighted trends that are not statistically significant (i.e., $P > 0.05$) but have come close to statistical significance ($P \sim 0.1$) with large effect sizes (using Cohen's d). This is highlighted in blue in Tables 4.6 - 4.9, which provide detailed information about the statistical comparisons for the phenotypic assays.

4.3 Results

4.3.1 First phenotypic assessment of MB_{1-4} and MBL_{1-4} at host generations 17-20

4.3.1.1 Male traits

None of the four phenotypes tested for males – adult body weight, egg-to-adult development time, desiccation resistance, and locomotor activity- showed an effect of selection as assessed by the paired t-test over block means (Figure 4.6A-D). Table 4.6 and Table 4.7 provide details of the statistical comparison for the with-microbe assay environment and microbe-free assay environment, respectively.

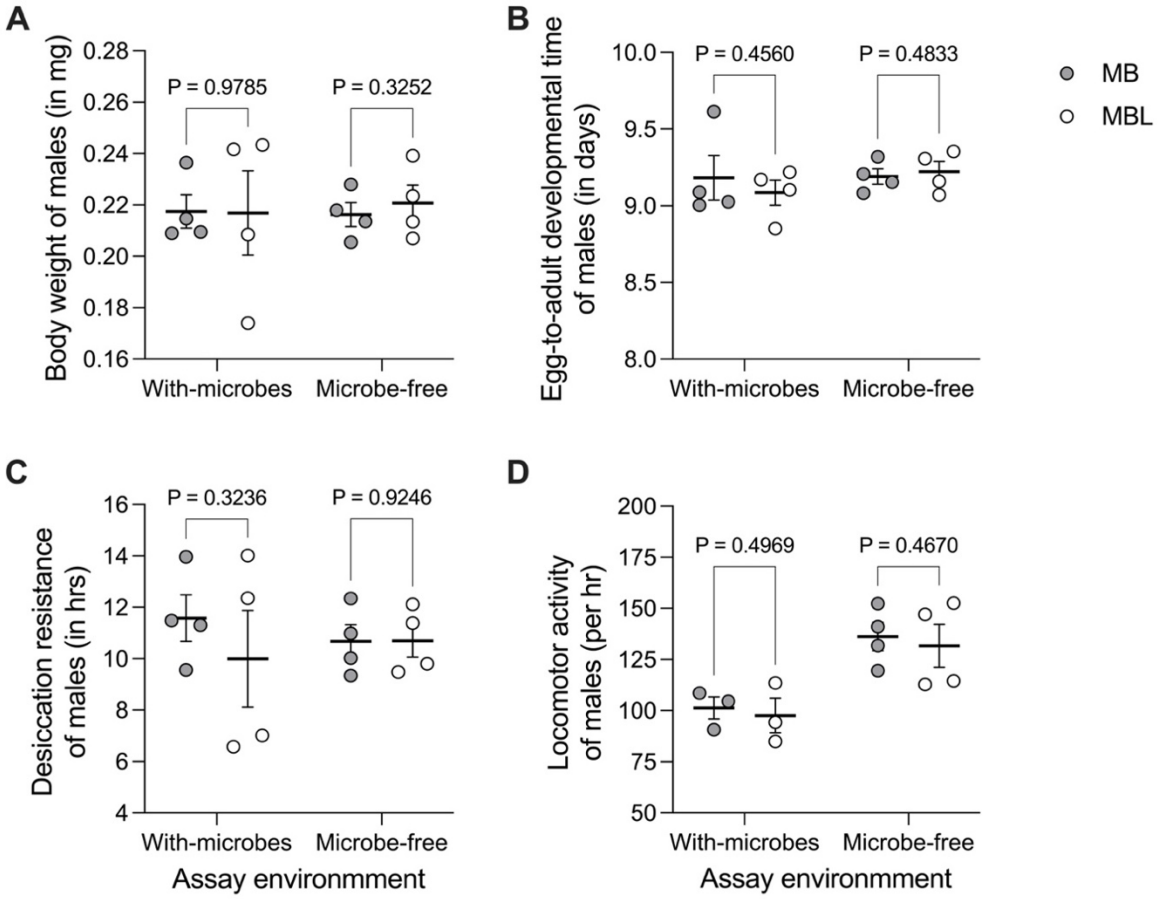

Figure 4.6 Male traits assessed in Gen 17-20 in with-microbe and microbe-free environments. (A) Body weight. (B) Egg-to-adult development time. (C) Desiccation resistance. (D) Locomotor activity using DAM system. In each assay environment, four filled circles are means for each of the four control populations MB_{1-4} and, similarly, four empty circles are means for each of the four selected populations MBL_{1-4}. The black horizontal lines represent the grand means over four population means and the error bars represent the SEM. For each host phenotype, the grand mean over MB_{1-4} was compared against the grand mean over MBL_{1-4} using a paired t-test. DAM data for block 4 in with-microbes environment is not included here in the analysis.

4.3.1.2 Female traits

In the case of females, egg-to-adult development time, desiccation resistance, and locomotor activity didn't differ between MBs and MBLs (Figure 4.7A-D) (Table 4.6 and Table 4.7). The only trait in females that showed some difference between the MBs and the MBLs was female body weight in the microbe-free environment (Paired t-test, $t_3 = 3.05$, P = 0.0554, Cohen's d = 0.81 (large), Figure 4.7A). Here, although the P-value is slightly larger than our pre-determined level of statistical significance (i.e., 0.05), we still interpret this trait as the effect size was found to be large.

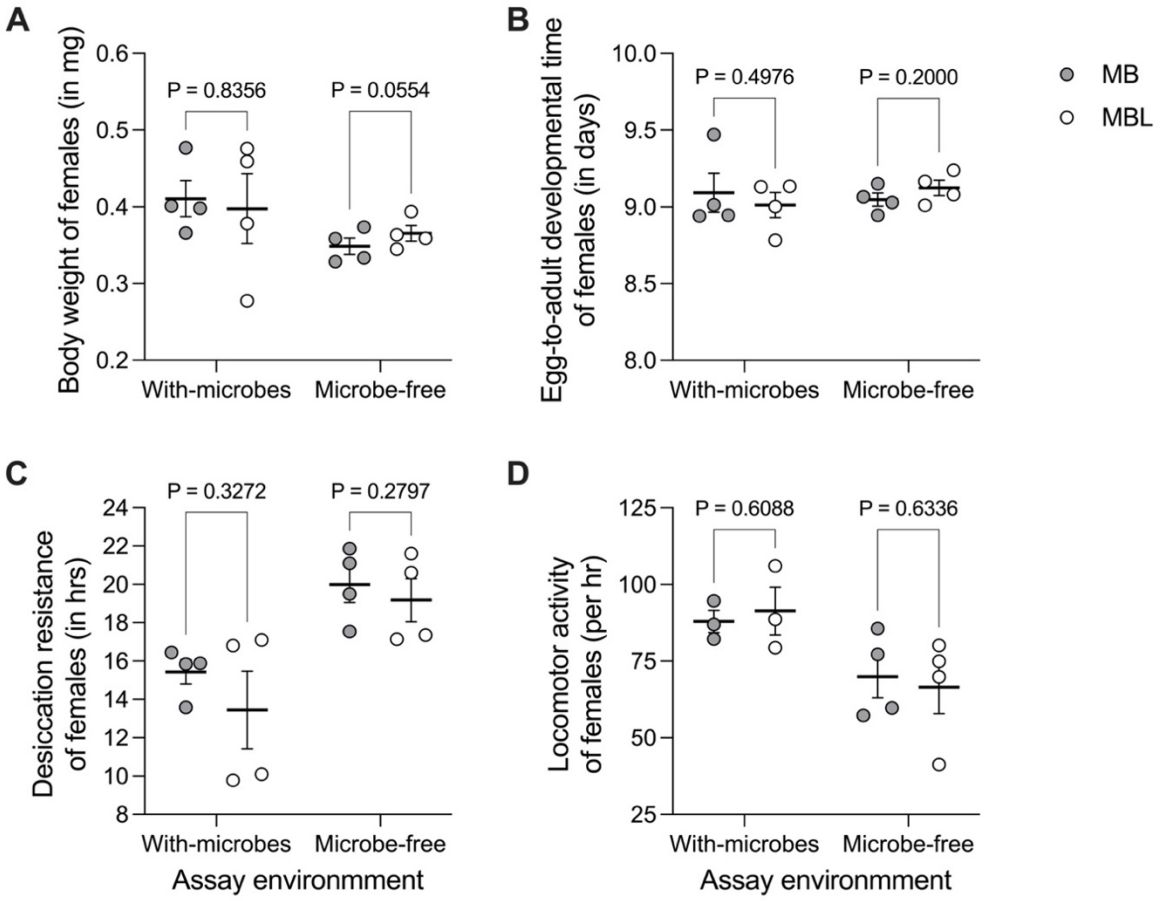

Figure 4.7 Female traits assessed in Gen 17-20 in with-microbe and microbe-free environments. (A) Body weight. (B) Egg-to-adult development time. (C) Desiccation resistance. (D) Locomotor activity using DAM system. In each assay environment, four filled circles are means for each of the four control populations MB_{1-4} and similarly, four empty circles are means for each of the four selected populations MBL_{1-4}. The black horizontal lines represent the grand means over four population means and the error bars represent the SEM. For each host phenotype, the grand mean over MB_{1-4} was compared against the grand mean over MBL_{1-4} using a paired t-test. DAM data for block 4 in with-microbes environment is not included here in the analysis.

4.3.1.3 Pair traits

In traits where both males and females were involved in the assays - percent survival to adulthood, female fecundity over 12 hrs (assayed in the presence of a male), and mating latency - did not show any difference (Figure 4.8A-C, Table 4.6 and Table 4.7). The mating time for MBL_{1-4} was lower than MB_{1-4} in the with-microbes environment (Paired t-test, t_3 = 3.67, P = 0.0349, Cohen's d = 2.35 (large), Figure 4.8D).

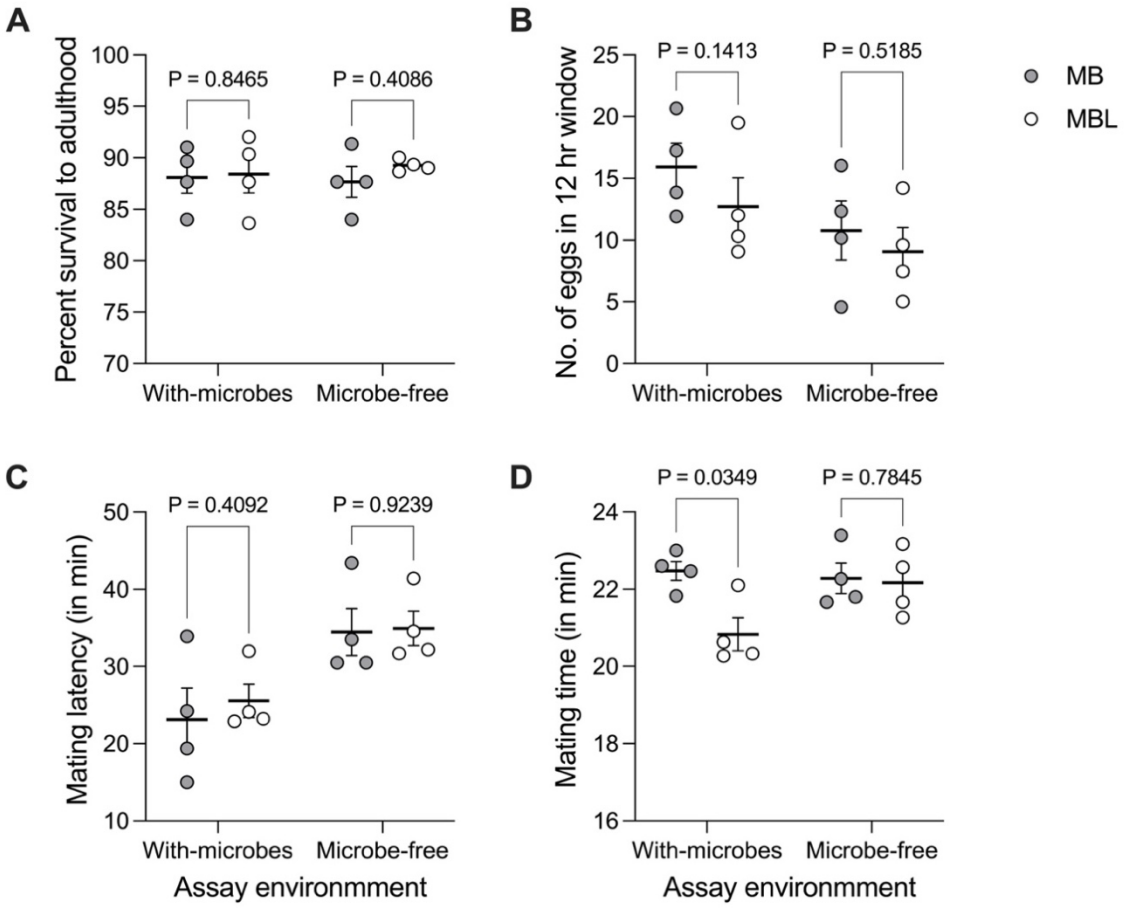

Figure 4.8 Pair traits assessed in Gen 17-20 in with-microbe and microbe-free environments. (A) Survival to adulthood. (B) Female fecundity. (C) Mating latency. (D) Mating time. In each assay environment, four filled circles are means for each of the four control populations MB_{1-4} and similarly, four empty circles are means for each of the four selected populations MBL_{1-4}. The black horizontal lines represent the grand means over four population means and the error bars represent the SEM. For each host phenotype, the grand mean over MB_{1-4} was compared against the grand mean over MBL_{1-4} using a paired t-test.

Table 4.6 Summary of all assays in the with-microbes environment (Gen 17-20). T refers to the grand mean of the MBLs, while C refers to the grand mean of the MBs.

	Assay	Test statistic	P-value	% change (T - C)/C	Cohen's d	Effect size*	Sample size[$]
1	Dry body weight (Male)	$t_3 = 0.03$	0.9785	-0.3	0.02	Small	10
2	Dry body weight (Female)	$t_3 = 0.23$	0.8356	-3.1	0.18	Small	10
3	Development time (Males)	$t_3 = 0.85$	0.4560	-1.1	0.41	Small	10
4	Development time (Females)	$t_3 = 0.77$	0.4976	-0.9	0.37	Small	10
5	Survival to adulthood	$t_3 = 0.21$	0.8465	0.4	0.10	Small	10
6	Fecundity (Females)	$t_3 = 1.96$	0.1413	-20.1	0.75	Medium	50
7	Desiccation resistance (Males)	$t_3 = 1.18$	0.3236	-13.7	0.54	Medium	10
8	Desiccation resistance (Females)	$t_3 = 1.17$	0.3272	-12.9	0.66	Medium	10
9	Locomotor activity (Males)	$t_3 = 0.82$	0.4969	-3.7	0.30	Small	25 to 32
10	Locomotor activity (Females)	$t_3 = 0.60$	0.6088	3.9	0.32	Small	31 to 32
11	Mating Latency	$t_3 = 0.96$	0.4092	10.4	0.37	Small	30
12	Mating time	$t_3 = 3.67$	0.0349	-7.3	2.35	Large	30

*Interpretation for effect sizes using Cohen's d: $d > 0.8$ (Large); $0.8 > d > 0.5$ (Medium); < 0.5 (Small)

[$]Note: See methods for detailed information about sample sizes. For example, the sample size of ten per treatment for body weight indicates ten micro-centrifuge tubes with 20 flies each. Also, this is a sample size per block, i.e., each circle in the plots is an average over this sample size.

Table 4.7 Summary of all assays in the microbe-free environment (Gen 17-20). T refers to the grand mean of the MBLs, while C refers to the grand mean of the MBs.

	Assay	Test statistic	P-value	% change (T - C)/C	Cohen's d	Effect size*	Sample size[$]
1	Dry body weight (Male)	$t_3 = 1.17$	0.3252	2.1	0.38	Small	10
2	Dry body weight (Female)	$t_3 = 3.05$	0.0554	4.8	0.81	Large	10
3	Development time (Males)	$t_3 = 0.80$	0.4833	0.4	0.28	Small	10
4	Development time (Females)	$t_3 = 1.64$	0.2000	0.8	0.82	Large	10
5	Survival to adulthood	$t_3 = 0.96$	0.4086	1.8	0.73	Medium	9 to 10
6	Fecundity (Females)	$t_3 = 0.73$	0.5185	-15.9	0.39	Small	50
7	Desiccation resistance (Males)	$t_3 = 0.10$	0.9246	0.2	0.02	Small	10
8	Desiccation resistance (Females)	$t_3 = 1.32$	0.2797	-4.1	0.39	Small	10
9	Locomotor activity (Males)	$t_3 = 0.83$	0.4670	-3.3	0.25	Small	26 to 32
10	Locomotor activity (Females)	$t_3 = 0.53$	0.6336	-4.8	0.22	Small	30 to 32
11	Mating Latency	$t_3 = 0.10$	0.9239	1.4	0.09	Small	30
12	Mating time	$t_3 = 0.30$	0.7845	-0.5	0.14	Small	30

*Interpretation for effect sizes using Cohen's d: $d > 0.8$ (Large); $0.8 > d > 0.5$ (Medium); < 0.5 (Small)

[$]Note: See methods for detailed information about sample sizes. For example, the sample size of ten per treatment for body weight indicates ten micro-centrifuge tubes with 20 flies each. Also, this is a sample size per block, i.e., each circle in the plots is an average over this sample size.

4.3.2 Second phenotypic assessment of MB_{1-4} and MBL_{1-4} at host generations 54-57

4.3.2.1 Male traits

Table 4.8 and Table 4.9 provide detailed information on the statistical comparison between MB_{1-4} and MBL_{1-4} for the with-microbe assay environment and microbe-free assay environment, respectively. Comparing these tables with the corresponding ones for generation 17 (i.e., Tables 4.6 and 4.7) shows that there were a larger number of cases in the later generation where the effect sizes were medium or large. We interpret this as a signature of evolutionary divergence.

There was no difference between the body weight of the male MBL_{1-4} and MB_{1-4} flies in the with-microbe assay environment (Paired t-test, $t_3 = 0.32$, $P = 0.7723$, Figure 4.9A-left). In the microbe-free environment, the body weight of the male MBL_{1-4} was more than their corresponding controls MB_{1-4} with a large effect size even though the P-value was marginally greater than 0.05 (Paired t-test, $t_3 = 2.89$, $P = 0.0628$, Cohen's d = 1.74 (large), Figure 4.9A-right).

The egg-to-adult development time was largely unaffected by the multi-generation absence of microbes, as seen in both assay environments (Tables 4.8 and 4.9, Figure 4.9B).

MBL_{1-4} had lower desiccation resistance than the MB_{1-4} in the with-microbe environment with a large effect size, although the P-value was marginally greater than 0.05 (Paired t-test, $t_3 = 3.16$, $P = 0.0510$, Cohen's d = 1.62, Figure 4.9C-left). No difference was observed in the microbe-free environment (Paired t-test, $t_3 = 0.40$, $P = 0.\ 7164$, Figure 4.9C-right).

In both environments, MB_{1-4} and MBL_{1-4} showed similar levels of locomotor activity (Tables 4.8 and 4.9, Figure 4.9D).

4.3.2.2 Female traits

The body weights of the MB_{1-4} and MBL_{1-4} females were similar in the with-microbes regime (Paired t-test, $t_3 = 0.82$, $P = 0.4726$, Figure 4.10A-left) but showed a marginal increase for MBLs in the no-microbe regime which was not statistically significant, although with a large effect size (Paired t-test, $t_3 = 2.34$, $P = 0.1015$, Cohen's d = 1.35, Figure 4.10A-right).

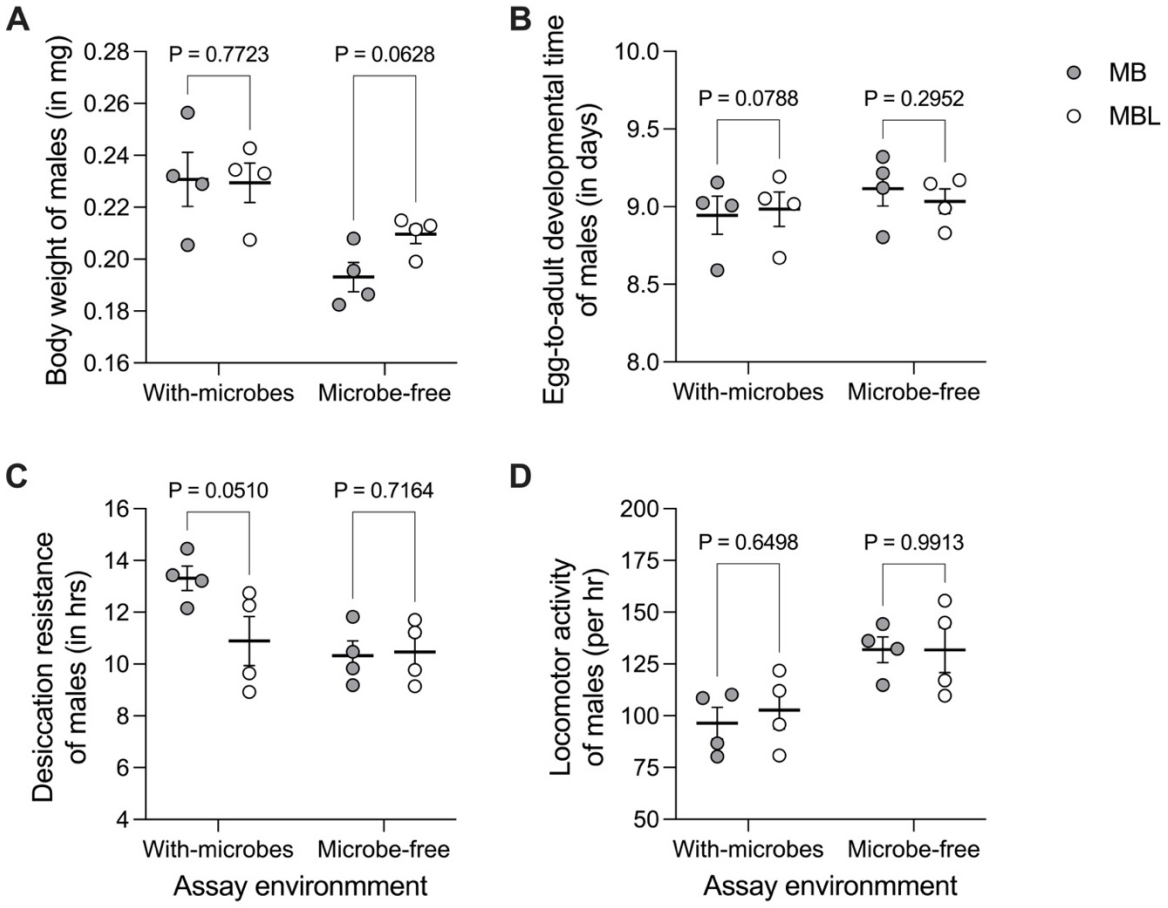

Figure 4.9 Male traits assessed in Gen 54-57 in with-microbe and microbe-free environments. (A) Body weight. (B) Egg-to-adult development time. (C) Desiccation resistance. (D) Locomotor activity using DAM system. In each assay environment, four filled circles are means for each of the four control populations MB_{1-4} and, similarly, four empty circles are means for each of the four selected populations MBL_{1-4}. The black horizontal lines represent the grand mean over four population means and the error bars represent the SEM. For each host phenotype, the grand mean over MB_{1-4} was compared against the grand mean over MBL_{1-4} using a paired t-test.

Female egg-to-adult development time was similar for MB-MBLs after multiple generations without the microbes when assayed in both assay environments (Table 4.8 and 4.9, Figure 4.10B).

MBL_{1-4} females had lower desiccation resistance in the with-microbe regime, which was not statistically significant (Paired t-test, $t_3 = 2.45$, P = 0.0916, Cohen's d = 0.89 (large), Figure 4.10C-left). MBs and MBLs had similar desiccation resistance in the no-microbe regime (Paired t-test, $t_3 = 0.70$, P = 0.5365, Figure 4.10C-right).

MB_{1-4} and MBL_{1-4} females had similar locomotor activity levels in the with-microbes environment (Paired t-test, $t_3 = 0.10$, P = 0.9260, Figure 4.10D-left). In the microbe-free environment, there was a trend of MBL_{1-4} females being more active than the MB_{1-4} that was not statistically significant (Paired t-test, $t_3 = 2.23$, P = 0.1122, Cohen's d = 0.89 (large), Figure 4.10C-left).

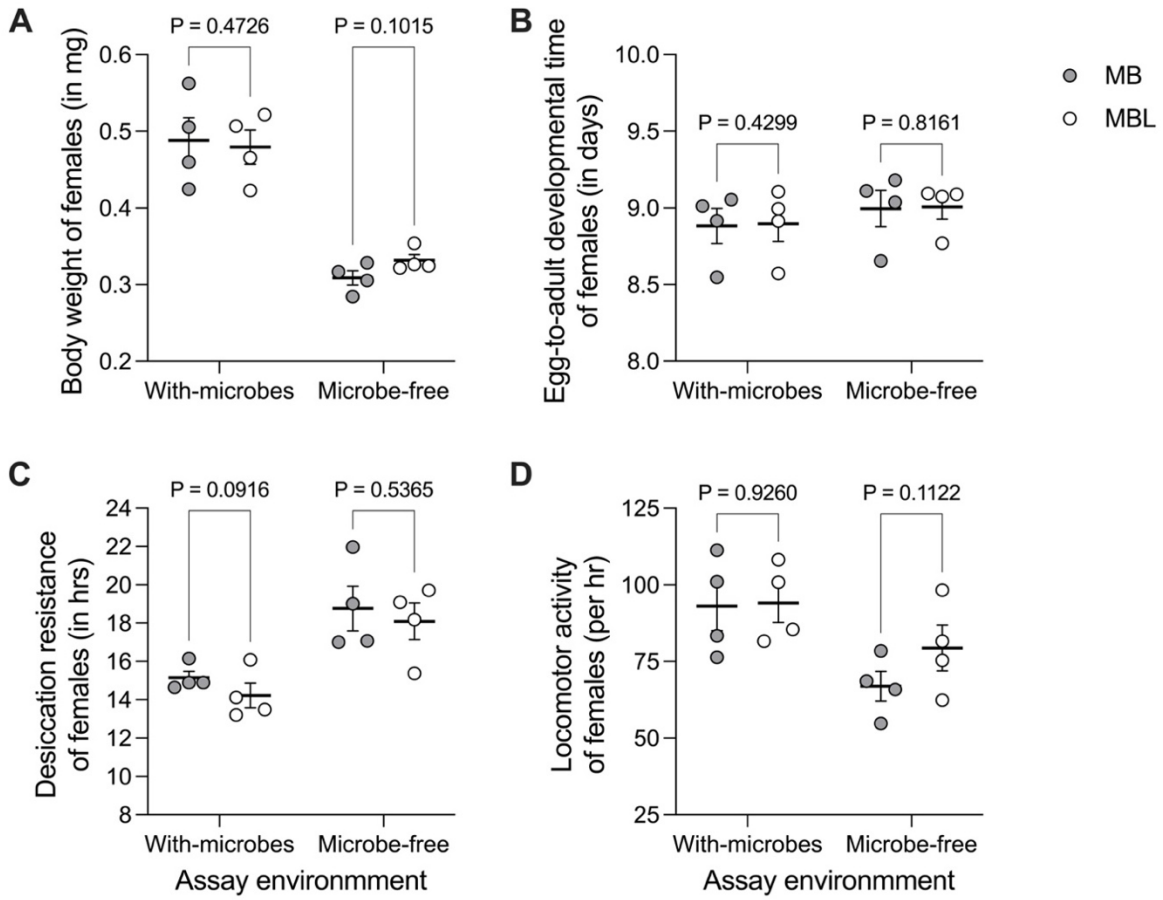

Figure 4.10 Female traits assessed in Gen 54-57 in with-microbe and microbe-free environments. (A) Body weight. (B) Egg-to-adult development time. (C) Desiccation resistance. (D) Locomotor activity using DAM system. In each assay environment, four filled circles are means for each of the four control populations MB_{1-4} and, similarly, four empty circles are means for each of the four selected populations MBL_{1-4}. The black horizontal lines represent the grand mean over four population means and the error bars represent the SEM. For each host phenotype, the grand mean over MB_{1-4} was compared against the grand mean over MBL_{1-4} using a paired t-test.

4.3.2.3 Pair traits

There was a trend of lower survival for $MBL_{1\text{-}4}$ than $MB_{1\text{-}4}$ in the with-microbe regime, but it is not statistically significant (Paired t-test, $t_3 = 2.31$, $P = 0.1044$, Cohen's d = 1.81 (large), Figure 4.11A-left). In the microbe-free environment, $MBL_{1\text{-}4}$ had better survival than $MB_{1\text{-}4}$ (Paired t-test, $t_3 = 3.72$, $P = 0.0388$, Cohen's d = 0.83 (large), Figure 4.11A-right).

There was a trend of $MBL_{1\text{-}4}$ laying more eggs than the $MB_{1\text{-}4}$ in the with-microbe regime, but this difference was not statistically significant (Paired t-test, $t_3 = 1.75$, $P = 0.1791$, Cohen's d = 1.67 (large), Figure 4.11B-left). In the microbe-free environment, $MBL_{1\text{-}4}$ had a trend of slightly higher fecundity, but it was not statistically significant either (Paired t-test, $t_3 = 1.20$, $P = 0.3161$, Cohen's d = 0.58 (medium), Figure 4.11B-right).

$MBL_{1\text{-}4}$ did not differ from the $MB_{1\text{-}4}$ in the latency to start the mating, as assessed in both with-microbe and no-microbe regimes (Table 4.8 and 4.9, Figure 4.11C).

There was no difference in $MB_{1\text{-}4}$ and $MBL_{1\text{-}4}$ mating time in the with-microbe regime (Paired t-test, $t_3 = 0.15$, $P = 0.8928$, Figure 4.11D-left). In the microbe-free environment, there is a trend of shorter mating times for $MBL_{1\text{-}4}$ than $MB_{1\text{-}4}$, but this is not statistically significant (Paired t-test, $t_3 = 2.35$, $P = 0.1007$, Cohen's d = 1.39 (large), Figure 4.11D-right).

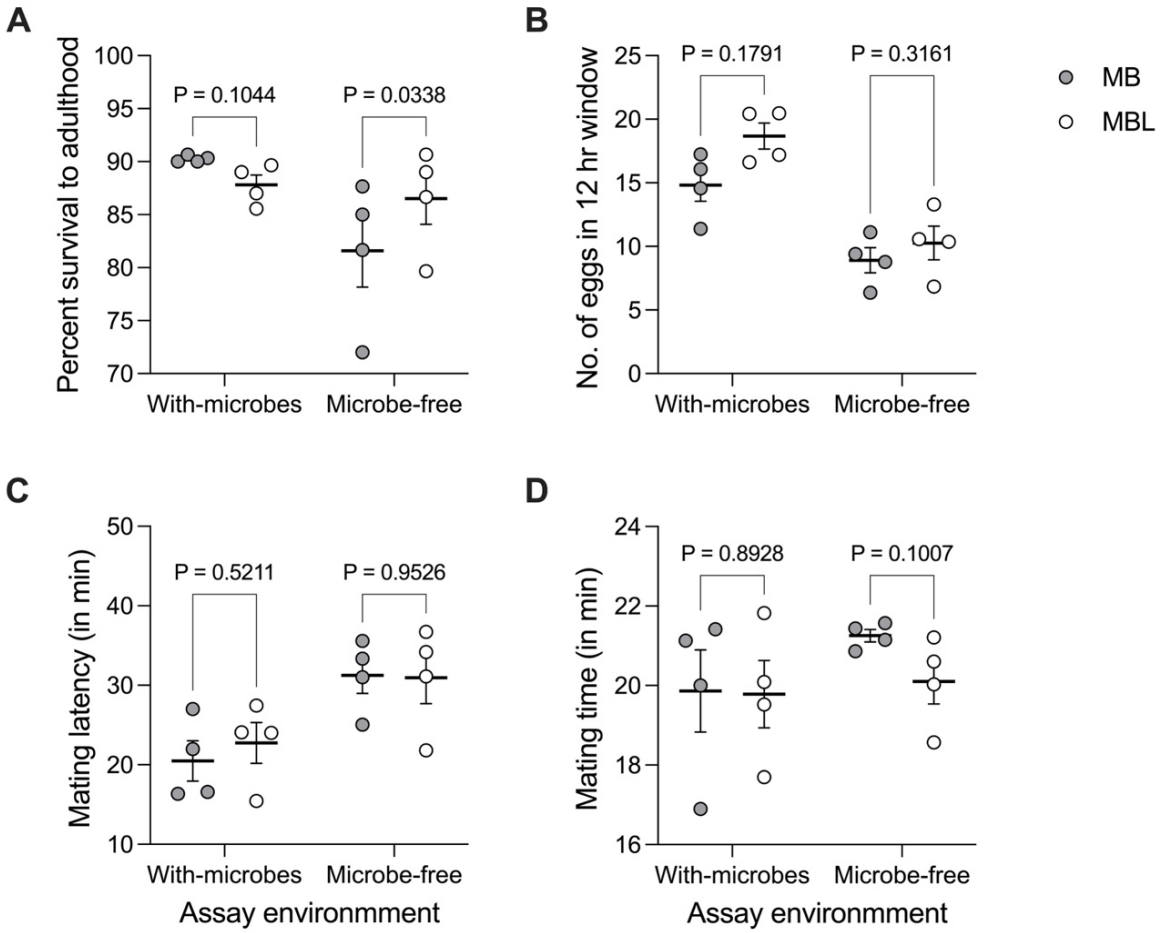

Figure 4.11 Pair traits assessed in Gen 54-57 in with-microbe and microbe-free environments. (A) Survival to adulthood. (B) Female fecundity. (C) Mating latency. (D) Mating time. In each assay environment, four filled circles are means for each of the four control populations MB_{1-4} and similarly, four empty circles are means for each of the four selected populations MBL_{1-4}. The black horizontal lines represent the grand means over four population means and the error bars represent the SEM. For each host phenotype, the grand mean over MB_{1-4} was compared against the grand mean over MBL_{1-4} using a paired t-test.

Table 4.8 Summary of all assays in the with-microbes environment (Gen 54-57). T refers to the grand mean of the MBLs, while C refers to the grand mean of the MBs.

	Assay	Test statistic	P-value	% change (T - C)/C	Cohen's d	Effect size*	Sample size$
1	Dry body weight (Male)	$t_3 = 0.32$	0.7723	-0.6	0.07	Small	10
2	Dry body weight (Female)	$t_3 = 0.82$	0.4726	-1.8	0.17	Small	10
3	Development time (Males)	$t_3 = 2.62$	0.0788	0.4	0.17	Small	9 to 10
4	Development time (Females)	$t_3 = 0.91$	0.4299	0.2	0.06	Small	10
5	Survival to adulthood	$t_3 = 2.31$	0.1044	-2.7	1.81	Large	9 to 10
6	Fecundity (Females)	$t_3 = 1.75$	0.1791	26.0	1.67	Large	50
7	Desiccation resistance (Males)	$t_3 = 3.16$	0.0510	-18.2	1.62	Large	10
8	Desiccation resistance (Females)	$t_3 = 2.45$	0.0916	-6.1	0.89	Large	10
9	Locomotor activity (Males)	$t_3 = 0.50$	0.6498	6.4	0.37	Small	30 to 32
10	Locomotor activity (Females)	$t_3 = 0.10$	0.9260	1.1	0.07	Small	31 to 32
11	Mating Latency	$t_3 = 0.72$	0.5211	11.1	0.44	Small	34 to 40
12	Mating time	$t_3 = 0.15$	0.8928	-0.4	0.04	Small	34 to 40

*Interpretation for effect sizes using Cohen's d: $d > 0.8$ (Large); $0.8 > d > 0.5$ (Medium); < 0.5 (Small)

$Note: See methods for detailed information about sample sizes. For example, the sample size of ten per treatment for body weight indicates ten micro-centrifuge tubes with 20 flies each. Also, this is a sample size per block, i.e., each circle in the plots is an average over this sample size.

Table 4.9 Summary of all assays in the microbe-free environment (Gen 54-57). T refers to the grand mean of the MBLs, while C refers to the grand mean of the MBs.

	Assay	Test statistic	P-value	% change (T - C)/C	Cohen's d	Effect size*	Sample size[$]
1	Dry body weight (Male)	$t_3 = 2.89$	0.0628	8.5	1.74	Large	10
2	Dry body weight (Female)	$t_3 = 2.34$	0.1015	7.4	1.35	Large	10
3	Development time (Males)	$t_3 = 1.27$	0.2952	-0.9	0.42	Small	10
4	Development time (Females)	$t_3 = 0.25$	0.8161	0.1	0.05	Small	10
5	Survival to adulthood	$t_3 = 3.72$	0.0338	6.0	0.83	Large	10
6	Fecundity (Females)	$t_3 = 1.20$	0.3161	15.2	0.58	Medium	50
7	Desiccation resistance (Males)	$t_3 = 0.40$	0.7164	1.3	0.11	Small	10
8	Desiccation resistance (Females)	$t_3 = 0.70$	0.5365	-3.6	0.32	Small	10
9	Locomotor activity (Males)	$t_3 = 0.01$	0.9913	-0.1	0.01	Small	27 to 32
10	Locomotor activity (Females)	$t_3 = 2.23$	0.1122	18.6	0.99	Large	32
11	Mating Latency	$t_3 = 0.06$	0.9526	-1.0	0.05	Small	32 to 38
12	Mating time	$t_3 = 2.35$	0.1007	-5.4	1.39	Large	32 to 38

*Interpretation for effect sizes using Cohen's d: $d > 0.8$ (Large); $0.8 > d > 0.5$ (Medium); < 0.5 (Small)

[$]Note: See methods for detailed information about sample sizes. For example, the sample size of ten per treatment for body weight indicates ten micro-centrifuge tubes with 20 flies each. Also, this is a sample size per block, i.e., each circle in the plots is an average over this sample size.

4.3.2.4 MBLs have reduced phenotypic plasticity than the MBs across with-microbe and microbe-free environments (Gen 54-57 assays)

Fig 4.9-4.11 suggested that, compared to the MBs, the trait values of the MBLs changed relatively less across the two assay environments (i.e., with microbes and microbe-free). In other words, the reaction norms for the various traits across these two environments are expected to be flatter for the MBLs than for the MBs. To quantify this observation, we used effect size (Cohen's d) as a proxy for the measure of phenotypic plasticity shown by MB vs. MBL populations *across two environments*. (Note: These across-environment effect sizes are different than those provided in Tables 4.6-4.9). The Method section 4.2.7 discusses the experimental design used to calculate these plastic effects changes and why such analysis could not be done in Gen 17-20.

We found that the MBLs, on average, had smaller effect sizes than MBs (Paired t-test, t_{11} = 2.14, P = 0.0554, Cohen's d = 0.49 (small), Figure 4.12A), possibly hinting at lower phenotypic plasticity than the MBs across two assay environments. Another way of looking at the trait's reaction norm could be through the percentage change that the trait has undergone across environments. The more the percent change, the more the phenotypic plasticity. We found that for the traits that we assessed, MBLs had a lower percentage of change in their trait values across two environments than MBs (Paired t-test, t_{11} = 2.99, P = 0.0123, Cohen's d = 0.39 (small), Figure 4.12B).

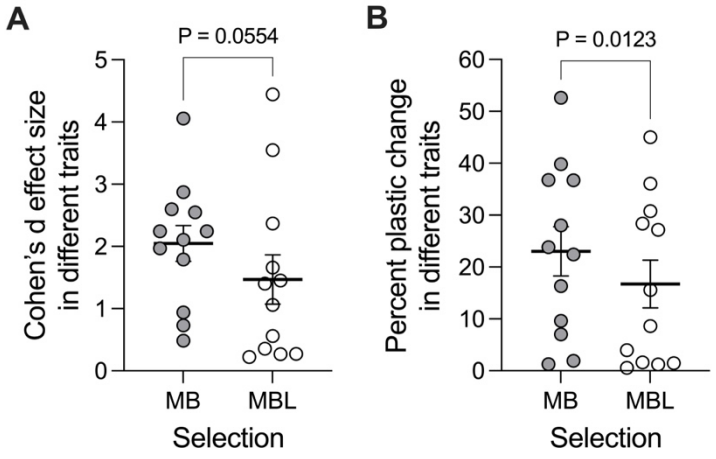

Figure 4.12 (A) Effect sizes and (B) percentage changes as a proxy for plastic changes across two environments. Means are indicated by black horizontal lines. The error bars represent SEM. The data is analyzed using a paired t-test. For percent change, Wilcoxon matched-pairs signed rank test (a non-parametric test) is also significant (W = -64.0, P = 0.0093).

4.3.3 The big picture across both assessments: divergence in MB-MBLs with time?

In the previous section, we used effect sizes on phenotype differences across assay environments for MBs as well as MBLs as a proxy *for the phenotypic plasticity* exhibited by these two populations. In this section, we look at how effect sizes have changed *for the evolutionary difference between MB-MBLs over time* from the first phenotypic assessment in generations 17-20 to the second assessment in generations 54-57 (Figure 4.13). These effect sizes are provided in Tables 4.6-4.9.

The effects sizes (using Cohen's d) for the phenotypic comparison between MB-MBLs in generations 17-20 were mostly small, with some effects being medium or large (Figure 4.13-left). In generations 54-57, it looks like the distribution of effect sizes is shifted in the direction of large effects (Figure 4.13-right). This pattern is seen in both the assay environments: with microbes (circles with a coral fill) and microbe-free (circles without any fill). This might hint that the MBLs are still diverging from MBs even after 54-57 generations of experimental evolution.

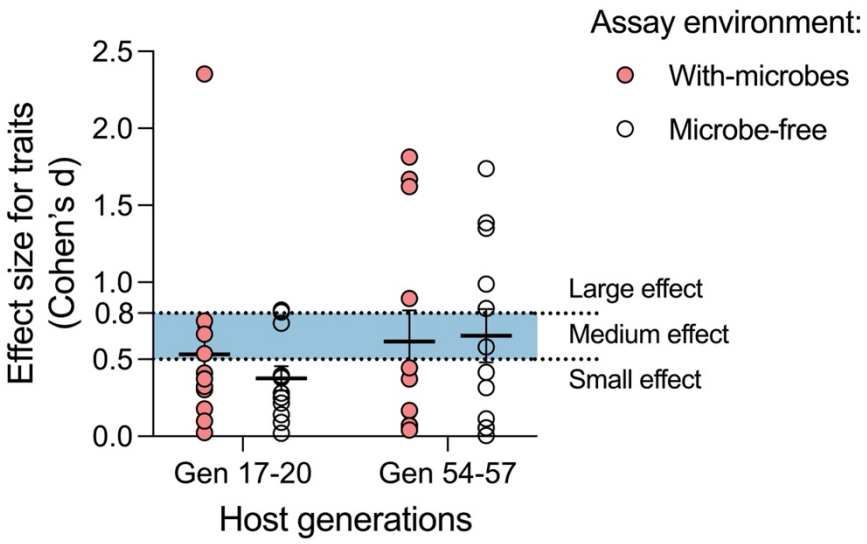

Figure 4.13 Changes in effect sizes (Cohen's d) over all traits with time. Means are indicated by black horizontal lines. The error bars represent SEM. Coral circles indicate with-microbes environment and empty circles indicate the microbe-free environment.

4.3.4 RNA-Seq result – Gen 54-57

After investigating the effects of long-term microbiome absence using broad phenotypic assays, we used RNA-Seq to determine if specific transcripts are differentially expressed in MBL_1 vs. MB_1 as a direct consequence of their different evolutionary history. This comparison was done in two assay environments: (a) microbe-free and (b) with-microbe. The protocol for RNA-seq is described in detail in the method section 4.2.8.

4.3.4.1 No major changes in the gene expression profile of MB_1 vs MBL_1 in the microbe-free environment

In the microbe-free regime, where both selected and control populations were assayed without their microbiome, only a few transcripts showed differential expression when analyzed using DESeq2 (Figure 4.14). Only the significant genes with Log_2(Fold Change) > 1.5 and adjusted P-value < 0.05 are highlighted in Figure 4.14. Except for nAChRbeta2, which is a nicotinic acetylcholine receptor subunit, and CG13617, which codes for cilium assembly protein DZIP1 (zinc finger transcription factor), the functions of the other genes (CG14625, CG12693) are not known in *Drosophila melanogaster*.

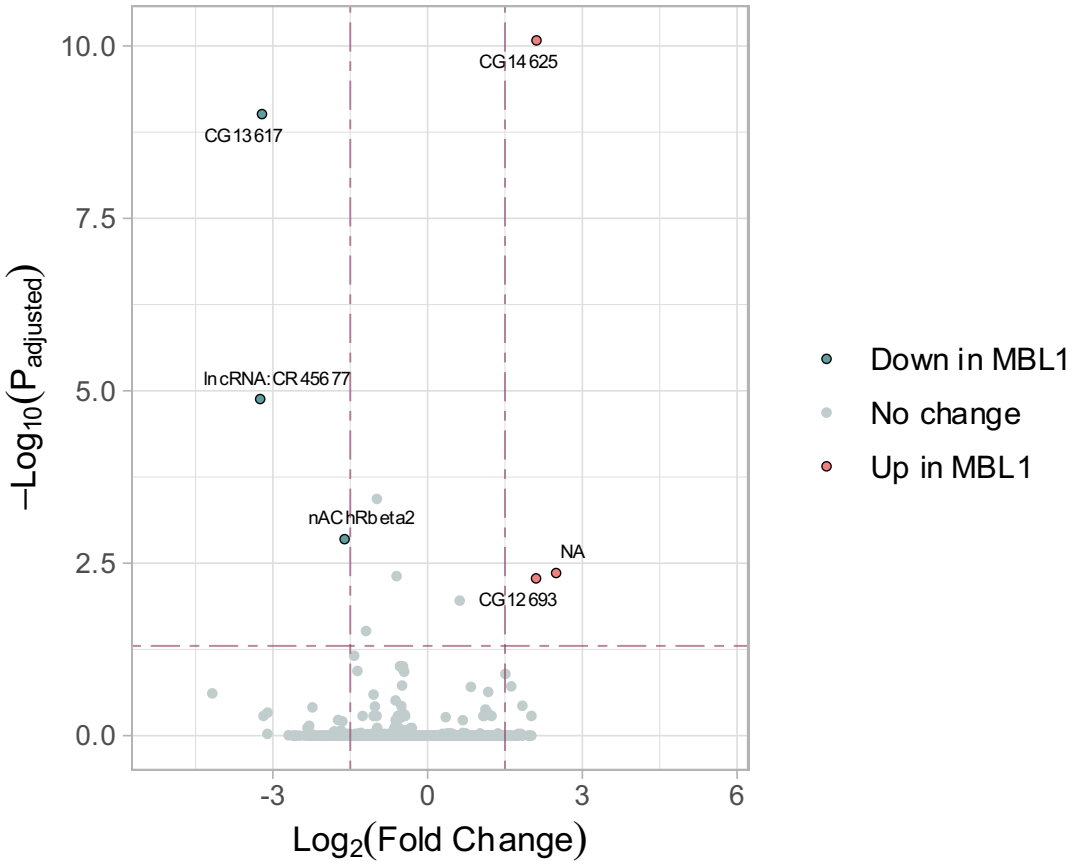

Figure 4.14 Differentially expressed genes in MBL_1 vs MB_1 in the no-microbe environment using DESeq2. The red dots represent the up-regulated genes, while blue dots represent down-regulated genes in MBL_1. The protocol for the RNA-Seq is described in the method section 4.2.8.

4.3.4.2 In MBLs, AMPs are up-regulated while HSPs are down-regulated in the with-microbe environment

In the with-microbe regime, anti-microbial peptide (AMP) expression is up-regulated in MBL_1 compared to their control MB_1 (Figure 4.15). The full list of up-regulated genes in MBL_1 is given in Table 4.10. We also find that a cluster of heat shock proteins (HSPs) is down-regulated along with several other genes in MBL_1. The full list of down-regulated genes in MBL_1 is given in Table 4.11.

Among all the differentially expressed genes, only the significant genes with Log_2(Fold Change) > 1.5 and adjusted P-value < 0.05 are highlighted in Figure 4.15 and given in Tables 4.10-4.11.

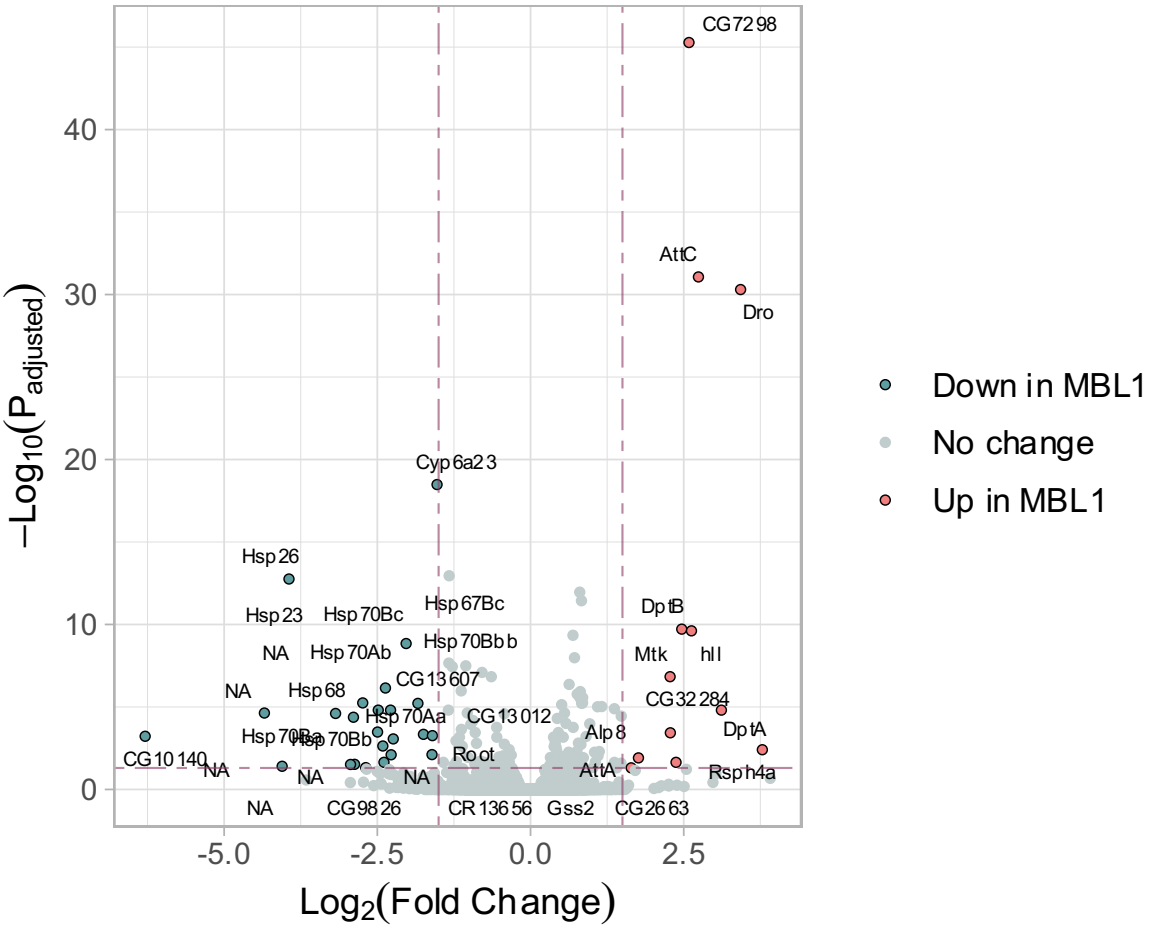

Figure 4.15 Differentially expressed genes in MBL_1 vs MB_1 in the with-microbe environment using DESeq2. The red dots represent the up-regulated genes, while blue dots represent down-regulated genes in MBL_1. The protocol for the RNA-Seq is described in the method section 4.2.8.

Table 4.10 List of up-regulated genes in MBL_1 in RNA-Seq (with-microbes environment)

No.	Gene ID	Log_2(FC)	Adjusted P-value	Symbol	Chr	Description
1	FBgn0036948	2.59	5.29E-46	CG7298	3L	-
2	FBgn0041579	2.74	8.56E-32	AttC	2R	Attacin-C
3	FBgn0010388	3.43	4.99E-31	Dro	2R	Drosocin
4	FBgn0034407	2.47	1.91E-10	DptB	2R	Diptericin B
5	FBgn0286723	2.63	2.44E-10	hll	2L	heimdall
6	FBgn0014865	2.28	1.46E-07	Mtk	2R	Metchnikowin
7	FBgn0004240	3.11	1.55E-05	DptA	2R	Diptericin A
8	FBgn0052284	2.28	0.0004	CG32284	3L	-
9	FBgn0034957	3.78	0.0039	Rsph4a	2R	Radial spoke head protein 4a
10	FBgn0034712	1.76	0.0122	Alp8	2R	Alkaline phosphatase 8
11	FBgn0037323	2.37	0.0227	CG2663	3R	-
12	FBgn0012042	1.64	0.0490	AttA	2R	Attacin-A

Table 4.11 List of down-regulated genes in MBL_1 in RNA-Seq (with-microbes environment)

No.	Gene ID	Log_2(FC)	Adjusted P-value	Symbol	Chr	Description
1	FBgn0033978	-1.53	3.30E-19	Cyp6a23	2R	Cyp6a23
2	FBgn0001225	-3.94	1.75E-13	Hsp26	3L	Heat shock protein 26
3	FBgn0001229	-2.03	1.43E-09	Hsp67Bc	3L	Heat shock gene 67Bc
4	FBgn0013279	-2.37	7.09E-07	Hsp70Bc	3R	Heat-shock-protein-70Bc
5	FBgn0001224	-2.74	5.69E-06	Hsp23	3L	Heat shock protein 23
6	FBgn0039151	-1.84	6.07E-06	CG13607	3R	-
7	FBgn0013276	-2.48	1.55E-05	Hsp70Ab	3R	Heat-shock-protein-70Ab
8	FBgn0051354	-2.28	1.55E-05	Hsp70Bbb	3R	Hsp70Bbb
9	FBti0059721	-4.34	2.33E-05	NA	3R	-
10	FBgn0001230	-3.18	2.48E-05	Hsp68	3R	Heat shock protein 68
11	FBti0019359	-2.89	4.19E-05	NA	3R	-
12	FBgn0013278	-2.50	0.0003	Hsp70Bb	3R	Heat-shock-protein-70Bb
13	FBgn0030769	-1.75	0.0005	CG13012	X	-
14	FBgn0039152	-1.60	0.0005	Root	3R	Rootletin

15	FBgn0036363	-6.29	0.0006	CG10140	3L	-
16	FBgn0013275	-2.24	0.0009	Hsp70Aa	3R	Heat-shock-protein-70Aa
17	FBgn0013277	-2.41	0.0023	Hsp70Ba	3R	Heat-shock-protein-70Ba
18	FBgn0052495	-1.61	0.0078	Gss2	X	Glutathione synthetase 2
19	FBgn0039307	-2.28	0.0079	CR13656	3R	-
20	FBti0020330	-2.39	0.0227	NA	3R	-
21	RR43222_transposable_element	-2.87	0.0300	NA	X	-
22	FBti0059720	-2.94	0.0306	NA	3R	-
23	FBti0019362	-4.05	0.0393	NA	3R	-
24	FBgn0034784	-2.69	0.0476	CG9826	2R	-

To see the biological processes these genes are involved in, we used the ShinyGo tool (version 0.80) (http://bioinformatics.sdstate.edu/go/) for gene ontology enrichment analysis with 'GO biological process' pathway database. We found that the upregulated genes belong to the pathway involved in the fly's humoral immune response (Figure 4.16), while down-regulated genes belong to the stress response pathway (Figure 4.17).

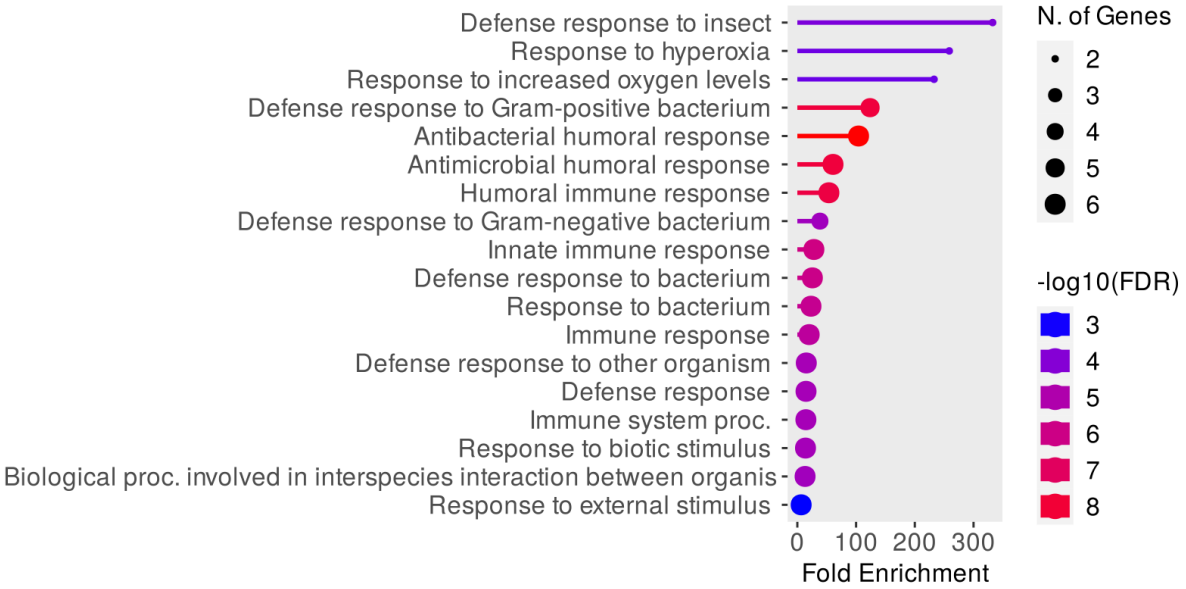

Figure 4.16 Gene ontology (GO) enrichment analysis for 12 Up-regulated genes.

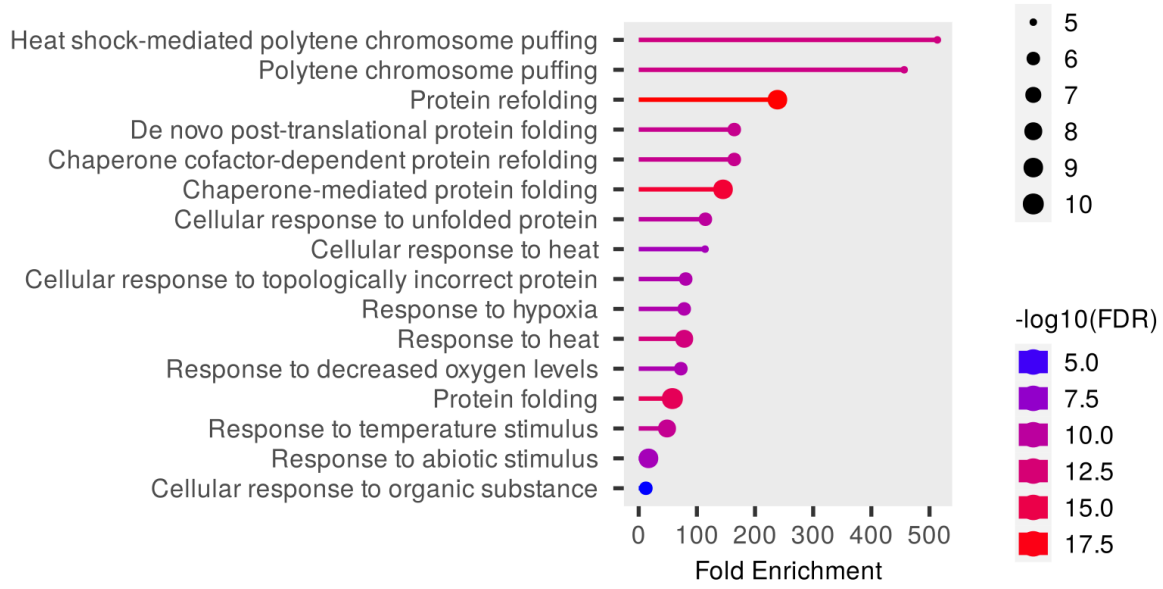

Figure 4.17 Gene ontology (GO) enrichment analysis for 24 down-regulated genes.

4.4 Discussion

4.4.1 MBL_{1-4} show few changes even after 54 generations without microbes

In the single-generation study reported in the last chapter, we saw that almost all the traits we investigated were affected by the removal of the microbiome. This led us to infer that microbiota is crucial for the realization of a large number of traits in *Drosophila melanogaster*, an observation that corroborates the results of countless studies from the literature (Broderick and Lemaitre 2012; Angela E. Douglas 2018b; Erkosar et al. 2013; Lesperance and Broderick 2020; Ludington and Ja 2020). This led to the hypothesis that the removal of microbiota is likely to be extremely stressful for the flies, as many traits would be affected at once. Hence, we expected the microbiota-less environment to exert very strong selection pressure on the MBL_{1-4} flies, leading to changes in multiple life-history traits, maybe including some trade-offs, within relatively few generations. However, the results proved to be otherwise.

In the first set of phenotypic assays done in host generations 17-20 (Figures 4.6 to 4.8), we saw that the mating time of MBLs was shorter than the MBs (Figure 4.8D, with-microbes environment) and female body weight was slightly elevated for MBLs (Figure 4.7A, microbe-free environment). Apart from these two changes, we did not see any evidence of evolutionary divergence between MB_{1-4}-MBL_{1-4} in either of the two assay environments.

In the second set of phenotypic assays done in host generations 54-57 (Figures 4.9 to 4.11), we saw that two traits showed significant change. In the microbe-free environment, we found that MBL_{1-4} had better egg-to-adult survival than MB_{1-4} (Figure 4.11A). In the with-microbes environment, MBL_{1-4} males had lower desiccation resistance (Figure 4.9C). It is possible that this lower desiccation resistance might be a cost incurred by MBLs in this environment for improving their egg-to-adult survival in the selection environment (i.e., microbe-free environment).

When we looked at the effect sizes over all the traits assessed in Gen 17-20 vs. Gen 54-57 (Figure 4.13), we saw that the effect size distribution shifted from mostly “small and medium” effects to “small, medium, and large” effects. This suggests that even after 54 generations, the selected flies are still diverging from the control flies.

We assayed the life-history traits both in the presence and the absence of microbes. Thus, any improvement the fitness of the MBLs (compared to the MBs) in the microbe-free environment can be construed as the effect of adaptation in the selection environment. On the

other hand, any reduction in the fitness of the MBLs (again compared to the MBs) in either assay environment would signify a cost of adaptation under microbe-free conditions. Our results showed that there were very few adaptations or trade-offs.

There are several potential ways to interpret these observations, some of which may not be mutually exclusive.

One possibility is that the selection pressure on the MBLs was so strong that they responded extremely fast, and therefore, by the time we assayed them first (i.e., by 17-20 generation), they had already responded maximally to the selection pressure and were back to the MB levels across all traits. However, if this were to be the case, in the microbe-free assay environments, we would have expected the MBLs to have generally greater fitness than the MBs. This is because the microbe-free assay environment is the "selection" environment for the MBLs but a novel environment for the MBs. Since the MBLs did not do better than the MBs in this assay environment, therefore we conclude that the former had not adapted too well in the selection environment.

Another possibility is that even though the selection pressure is strong, the flies did not possess sufficient genetic variability to respond to the selection pressure. This might have been a valid concern if, like many studies on *Drosophila*-microbiota systems, the experiments were performed on highly inbred or iso-female strains (e.g., *Canton-S, Oregon-R,* and w^{1118}). However, our fly populations are outbred and were reinvigorated through genetic mixing of four outbred populations only one year prior to the initiation of this study (see Chapter 2 Methods section 2.2.3). Our regular observation of these flies indicates that they are vigorous in the cage (in terms of activity) and have high fecundity and egg-to-adult survivorship (A Malwade, personal observations). Therefore, we do not have any reason to believe that these flies did not have sufficient genetic variations. More importantly, the fact that egg-to-adult survivorship did change over time indicates that the populations were indeed evolving, which would not have been possible in the absence of genetic variation.

A third possibility is that while some divergence has indeed happened, they have not been picked up by our assays. For example, some previous studies have shown that the role of microbiota in affecting host physiology becomes prominent only in nutritionally unbalanced diets (Shin et al. 2011; Storelli et al. 2011). It is entirely possible that we will see more differences between the MBs and the MBLs if we assay them on a different diet. However, we have already shown (Chapter 3) that removing the microbiota leads to changes in almost

every trait, even under the standard food medium used in our lab. Therefore, it is unlikely that the lack of differences between MBs and MBLs is due to the food on which they were assayed or selected.

This brings us to the fourth possibility that although the removal of microbiota can lead to large-scale changes in a number of life-history and behavioral traits in a single generation (Chapter 3), this does not translate into strong selection pressure on an evolutionary timescale. Our results would make sense when we consider the possibility that if the selection pressure on the host to evolve in the absence of microbes is low, then the evolutionary response can be slow and mild, as we observed in this study. We hypothesize that the results of our short-term single-generation study and long-term experimental evolution can be explained by the so-called "evolutionary addiction hypothesis" (Angela E. Douglas 2018a; T. J. Hammer 2023; Moran et al. 2019). This hypothesis suggests that when the microbiome is perturbed or culled entirely, hosts experience "withdrawal symptoms" as the host and microbes have lived together for an extended period (T. J. Hammer 2023). Stated differently, the host can take care of itself in the absence of the microbes, it is just that there is an inertia, borne out of very-long coexistence, towards the same. Thus, when we removed the microbiome for the first time (i.e., in our single-generation study), the host was in an altered state of physiology as the host had evolved some dependence on the microbes that were always present many generations prior to the manipulation. But, the host still had the capacity to perform its function without the microbiome. Once several generations passed without the microbes, the host adjusted itself to the absence of a microbiome without any major evolutionary changes. This idea of evolutionary addiction might explain why this experimental evolution happened under low selection pressure and why we haven't seen major evolutionary changes in our experimental evolution so far.

4.4.2 $MBL_{1\text{-}4}$ might be reducing their dependence on the microbes as inferred by the overall lesser phenotypic plasticity than the MBs

For the phenotypic assays done in generations 54-57, the differential response of MBs and MBLs in two different environments (with microbes vs. microbe-free) represents the phenotypic plasticity of these two populations. We wanted to find out if losing microbial partners for multiple generations has made the MBLs more independent of them. If MBLs have indeed started becoming less dependent on their microbial partners, then we might see

that, across the two environments, MBLs might be phenotypically less plastic, as the presence or absence of microbes would affect MBLs less than the MBs.

If there is only one trait under consideration, the phenotypic plasticity of two populations can be compared using the slopes of the reaction norms for each population across the environments: the greater the slope, the more the phenotypic plasticity. What we were looking at was phenotypic plasticity *across multiple traits*. To calculate average phenotypic plasticity across different traits, one needs a dimensionless (or a normalized) measure. This is needed because slopes for different traits (e.g., mg of bodyweight, no. eggs laid, percent survival) cannot be added as they have different units. We used two normalized/dimensionless proxies of phenotypic plasticity that fit the above criteria and then compared the mean phenotypic plasticity for the MB-MBL populations. These proxies were (a) effect size using Cohen's d (Figure 4.12A) and (b) percent change (Figure 4.12B). The results suggest that MBLs had lower phenotypic plasticity across with-microbes and microbe-free environments. This observation, in turn, might hint that MBLs might be less dependent on the microbes.

4.4.3 MBL_1 and MB_1 have at least three sets of differentially expressed gene clusters

To see if MBLs have started showing differences in gene expression compared to MBs, we performed an RNA-seq on one block of the populations, namely MBL_1 and MB_1.

A cluster of heat shock proteins (HSPs) is downregulated in MBL_1

Heat shock proteins are the proteins that are involved when the organism experiences stress (Feder and Hofmann 1999). Even though, as their name suggests, they play an important part primarily in the heat shock response (Lindquist 1986), some of these proteins can be expressed in response to various other stresses such as cold, crowding, anoxia, desiccation, etc., where these proteins can act as chaperones (King and MacRae 2015). We find that several HSPs, such as HSP23, HSP26, HSP67, and HSP68, and subunits from HSPs, such as HSP67 and HSP70, are down-regulated in flies that were maintained without the microbiome for multiple generations (Figure 4.15).

RNA-Seq results on MBL_1 agree with the desiccation resistance data over all four replicate MBLs

Lower expression of HSPs in MBL_1 might hint at a muted stress response of the MBLs due to the lower availability of HSPs to counter the stress. This correlation of stress resistance with the expression of HSPs is well documented (Feder and Hofmann 1999). When we looked at the desiccation resistance of MBL_{1-4} vs. MB_{1-4} in the with-microbes regime, we found that MBL_{1-4} indeed had a lower desiccation resistance than MB_{1-4} for males (Figure 4.9C). For females, it comes close to statistical significance as well (Figure 4.10C). Both these effects are large (Table 4.8). While we haven't established a causal link in this trend of lower desiccation resistance, this observation correlates the lower expression of HSPs to a phenotypic readout of lower desiccation resistance.

Looking at this desiccation-HSP expression correlation, one can speculate that the thermal stress tolerance of MBL_{1-4} will be lower than that of MB_{1-4} due to lower expression and, hence, lower levels of HSPs. But that need not be necessarily true. A study in land snails has shown that the desiccation-sensitive species maintain more HSPs than the desiccation-resistant species (Mizrahi et al. 2010). If the same logic holds true for thermal stress tolerance, then the lower expression of HSP might hint at a better ability of MBLs to resist heat stress.

Antimicrobial peptides (AMPs) upregulated in MBL_1

Antimicrobial peptides (AMPs) are small cationic molecules that interact with bacterial membranes to damage and kill the bacteria (Brogden 2005). They are widespread across the tree of life, with animals and plants harboring various types of AMPs to deal with pathogens (Mookherjee et al. 2020). In *Drosophila* melanogaster, there are seven types of AMPs whose function is known, and a detailed discussion on them is available in (Hanson and Lemaitre 2020; Lemaitre and Hoffmann 2007). In addition to their classical role of defense against pathogens, these AMPs are also shown to control the host microbiome (Franzenburg et al. 2013; Maritan et al. 2024; Marra et al. 2021; Mergaert 2018).

A study that looked at the gene expression changes in the *Drosophila* gut using transcriptomics when the host is colonized with the microbes vs. it is kept without microbes found that the presence of a microbiome leads to upregulated expression of several immune response genes, including AMPs such as Attacin, Drosomycin and stress response genes such

as HSP23, HSP67Bb, and HSP70Bc (Broderick et al. 2014a). While their observation of the up-regulation of the immune response matches our results in MBL_1, the HSP response is down-regulated in MBL_1, in contrast to the observation by Broderick et al., which was done over the host's lifetime (Broderick et al. 2014a). The up-regulation of the immune system upon microbiome colonization is also reported in other subsequent single-generational studies that are done over the host's lifetime (Chandler et al. 2022; Elya et al. 2016).

These gene expression studies discussed above on *Drosophila* within a host's single generation show that the presence of a microbiome leads to AMP up-regulation compared to when a microbiome is absent. In our case, both MBL_1 and MB_1 were given the same native microbiome for a single generation after 54 generations of microbiome absence for MBLs. As both MB and MBL received the same native microbiome from a common pool (shown in the Appendix section of this chapter, Figure 4.22), their different evolutionary history might be the main driving force in the up-regulation of AMPs, not the differential presence of microbiome.

The hygiene hypothesis-like phenomenon can explain the overactive humoral immune response in MBLs

MBLs have been kept away from any microbes for more than 50 generations. Several studies have shown that microbes are indispensable for the proper calibration and modulation of the immune system, especially early in life (Abt et al. 2012; Donald and Finlay 2023; Gensollen et al. 2016; Henrick et al. 2021; Hoang and King 2022; Knoop et al. 2017; Mazmanian et al. 2005; Metcalf et al. 2022; Selosse et al. 2014). This calibration includes proper sorting of self vs. non-self (W.-J. Lee 2008; Lhocine et al. 2008). There is a possibility that MBLs might have lost the delicate balance that maintains this calibration and, as a result, are over-expressing AMPs compared to MBs to actively regulate the microbiome.

In humans, this phenomenon is often discussed in the context of the hygiene hypothesis (or a refined version of the same, called the old friend hypothesis) (Bach 2018; Blackwell 2022; Wills-Karp et al. 2001). This hypothesis states that exposure to certain microorganisms, particularly in early life, can reduce the frequency of atopic diseases and immune-mediated disorders in organisms (Stiemsma et al. 2015). It is possible that the observed increased levels of AMPs signify immune overactivation via immune miscalibration over many generations. It is already known that *Drosophila* can suffer from autoimmune disorders (Mortimer et al. 2021). Therefore, if our conjecture about the hygiene hypothesis is true then one can predict

that the MBLs would be suffering from higher instances of auto-immune disorders, something that we would like to investigate in detail in a future study.

A tradeoff between resources allocated for AMP and HSP production is possible

In honeybees, induction of HSPs in response to heat shock repressed multiple AMP genes, and wounding the cuticle of the abdomen resulted in lower heat shock response gene expression in tissues proximal and distal to the injury site (McKinstry et al. 2017). Similarly, it is also possible that, in our case, the lowered expression of HSPs is a consequence of the trade-off with the over-expression of AMPs.

Another set of differently expressed genes hints at the peritrophic matrix as a potential region of interest in the MBLs

Along with HSPs and AMPs, another set of genes that have an altered expression are chitin-interacting genes. CG7298 and CG32284 are up-regulated chitin-binding enzymes in MBL_1, and down-regulated CG10140 is a probable chitinase (Figure 4.15). As the RNA-seq is done on the full body of flies, it is difficult to predict what this means for the fly. But, the annotation of CG7298 in FlyBase offers a clue. CG7298 is expressed in the proventriculus, which is part of the fly digestive system. A recent study supports the notion that proventriculus is the site where gut bacteria stably interact with flies, possibly through interactions with *N*-acetyl glucosamine, which is a building block of chitin (Dodge et al. 2023). Moreover, *Drosophila melanogaster* CG7298 has an ortholog called peritrophin-48 in related *Drosophila* species, which is a component of the peritrophic matrix. The peritrophic matrix is a chitinous layer that separates food bolus from the gut epithelia. This is also implicated as a physical barrier that keeps gut bacteria away from the gut tissue (Charroux and Royet 2012; Erlandson et al. 2019; Kuraishi et al. 2011; Rodgers et al. 2017). Taking this all together, we think this is the region where chitin-interacting proteins are carrying out various processes that might be of interest. However, the limited available information about these hits prevents us from drawing any further conclusions at this point in time. In future, we plan to confirm all the RNA-seq hits and do additional experiments that can give us more insights.

In a recent study, Kathedar et al. showed that the nicotinic acetylcholine receptor (nAchR) regulates gut barrier function in *Drosophila* through the remodeling of the peritrophic matrix

(Katheder et al. 2023). We have seen that in our no-microbe assay regime, nAchRbeta2 gene expression showed down-regulation in MBL_1. This raises the possibility that the processes of interest happening at the proventriculus that involve the peritrophic matrix might be mediated through nAchR signaling.

Overall, it is also possible that the single-generation response when the microbiome is removed is pronounced, but that is not true for the long term as the host and microbiome are not too integrated with one another. If they had a good enough level of integration, then the loss of microbiome would have elicited a better selection response in the given time frame. This implication might also hint at the idea that a holobiont as a “tightly integrated entity” is perhaps not strongly supported by our data.

4.5 Appendix

4.5.1 Validation of microbiome status in the flies raised in two different environments by plating fly homogenates and PCR

4.5.1.1 By plating fly homogenates

In the with-microbe environment, on both MRS and NA media, we see the presence of microbes (Figure 4.18A, Set-B and Set-C). Set-A is the autoclaved water that is used to homogenize the flies. The serial dilutions with ~10-100 colonies were counted to get the CFUs/fly. While Figure 4.18A shows the representative picture from block 1, CFUs/fly for all four blocks of MBs and MBLs are compared using paired t-test on block means in Figure 4.19.

In the microbe-free environment, both MB_1 (Figure 4.18B) and MBL_1 (Figure 4.18C) show the absence of the microbiome. While block 1 plates do not have any microbes, other blocks show a few colonies in some replicates (data not shown here).

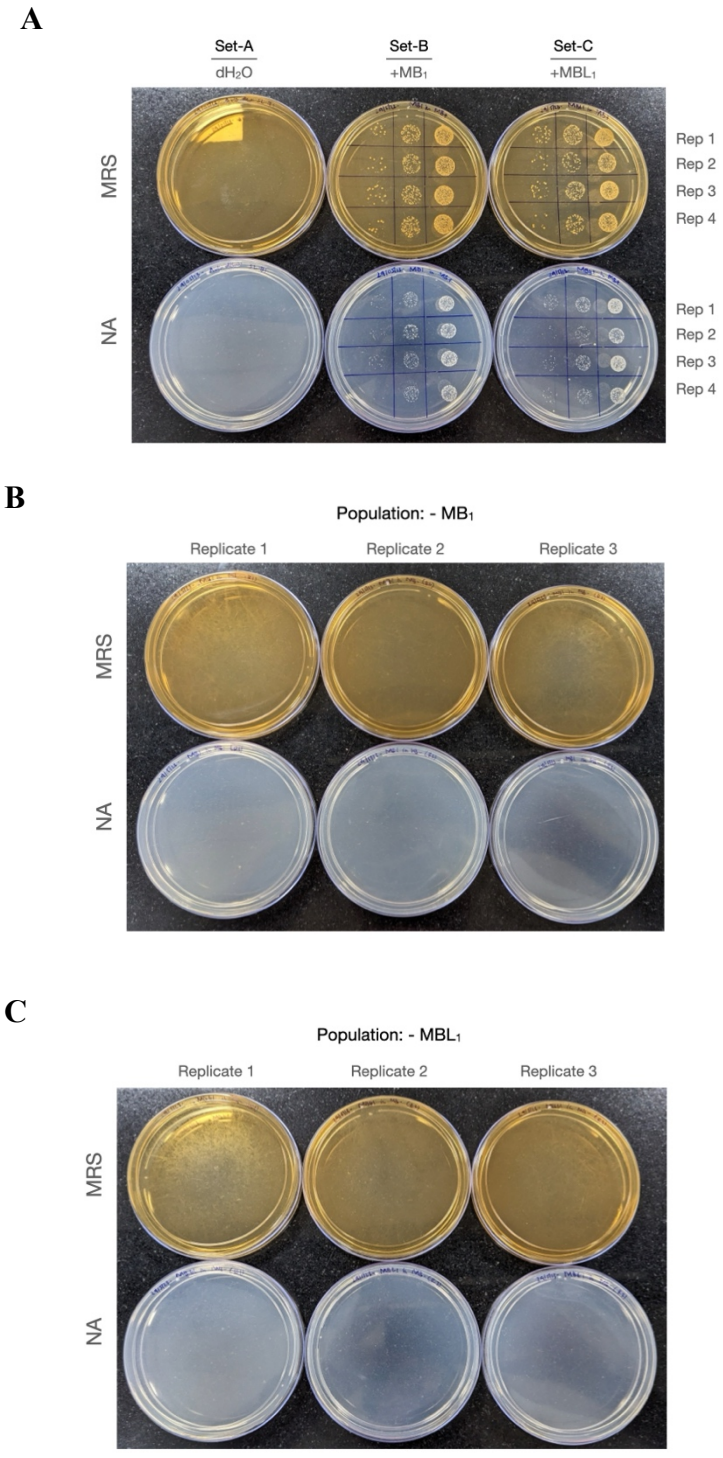

Figure 4.18 MRS and NA plates showing presence of microbes in with-microbe environment and their absence in microbe-free environment. (A) MB_1 and MBL_1 in with-microbe environment (n = 4). The spots show different serial dilutions used to calculate CFUs/fly. These CFU/fly for all the replicate populations are shown in Fig. 4.7. (B) MB_1 microbe-free environment (n = 3). (C) MBL_1 microbe-free environment (n = 3).

4.5.1.2 MBs and MBLs received similar microbial loads in the with-microbe environment (Gen 54-57 assays)

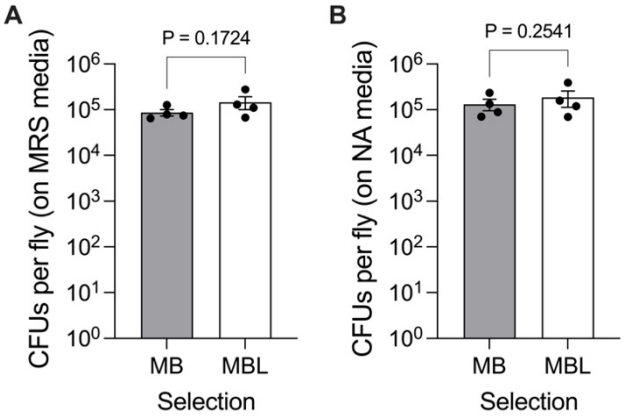

Figure 4.19 Bacterial load in the MB_{1-4}-MBL_{1-4} flies (with-microbe environment) as determined by plating fly homogenates. CFUs/fly on (A) MRS-agar (B) NA-agar. The bacterial load in MBs vs. MBLs is compared using paired t-test on block means. Block means are indicated by black filled circles. The error bar represents SEM. We see that the process of microbiome reconstitution (described in 4.2.3) leads to MBs and MBLs carrying similar bacterial loads. This value is close to the average of ~10^5 CFUs/fly that we see in our earlier single-generation study that involved reconstituted NDB_1 microbiome (Figure 3.8 in Chapter 3).

4.5.1.3 PCR showing the presence of microbiome in the with-microbiome environment and depletion of the microbiome in microbe-free environment

To validate the status of the microbiome in both environments with a culture-independent method, we extracted DNA from the four types of populations (Table 4.2). We performed PCR with 16S rRNA universal primers (details in section 2.2.4.2). For PCR, a subset of flies used for the assay on day 12 were flash-frozen in liquid nitrogen and kept at -80^0C for further use. For the with-microbe environment, 12 female flies from only MB_1 and MBL_1 (block 1) were used for the DNA extraction. For the microbe-free environment, total DNA was extracted from 12 female flies (3 flies per block, four such blocks). The PCR was done with 40ng of input DNA with 30 cycles.

In the with-microbe environment, MB_1 and MBL_1 showed the presence of microbes as expected (Figure 4.20). In the microbe-free environment, while two replicates have no band, one replicate each from pooled MB_{1-4} and MBL_{1-4} contains a faint band. This faint band might be indicative of a few colonies that show up on NA and MRS-agar plates, even after aseptic handling (Chapter 3, section 3.2.2.2). This signal might get picked up due to saturating PCR conditions. As we are not bleaching assay flies or using any antibiotics to

avoid their toxic effects, we are essentially maintaining flies without any chemicals for about two consecutive generations, i.e., for ~30 days (Figure 3.9). So, there might be a tradeoff between the efficiency of keeping all the microbes away without any chemicals and how long that efficiency can be sustained without any chemical agent.

Another possibility is that the signal is from very few non-culturable bacteria (discussed in Chapter 3, section 3.2.2.2).

These results match the observations in the previous sub-section 4.5.1.1 obtained after plating flies on NA and MRS. Together, the evidence showed that both MB-MBL populations in a with-microbe environment harbor microbiome and in microbe-free environments do not have microbes with them.

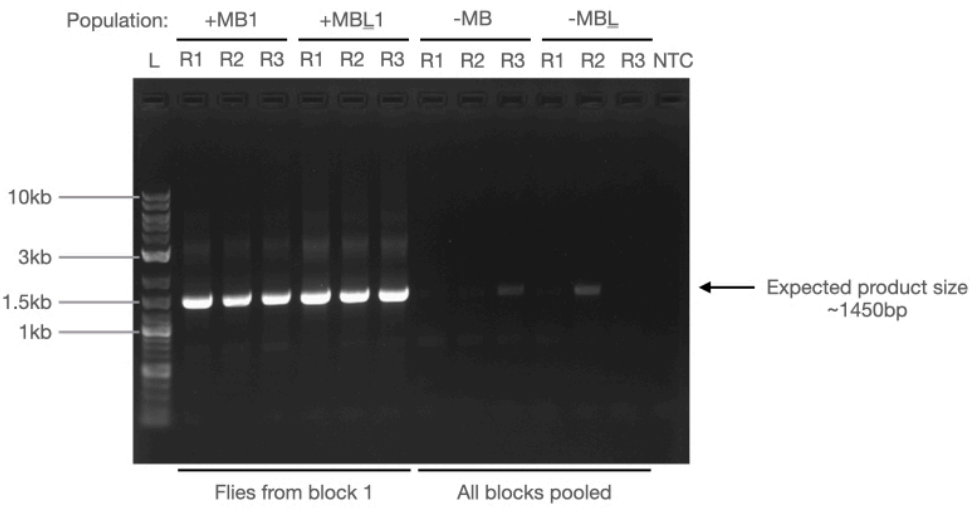

Figure 4.20 PCR on MB_1 and MBL_{1-4} to confirm the status of microbiome.

4.5.2 Validation of microbiome composition of flies raised in with-microbes assay environment

4.5.2.1 MB-MBL generation 17-20 assays

In the previous section, through plating fly homogenates and PCR, we showed that flies raised in the with-microbes environment indeed have the microbes with them. However, the PCR only indicates that microbes are present - it does not tell us the composition of the reconstituted microbiome. For the single-generation experiment, we showed that the reconstituted microbiome mirrors the source microbiome composition (Figure 3.8A), and the number of microbes received is also similar to that seen in untreated flies (Figure 3.8B). In Figure 4.19, we have established that the number of microbes received by MB and MBL

populations is similar. In this section, we test if the reconstituted microbiome composition is similar for MB-MBLs, and this, in turn, matches the source microbiome composition of NDB_1.

To validate that the composition of the reconstituted microbiome of all four blocks (MB_{1-4} and MBL_{1-4}) reflects the native NDB_1 composition, we performed amplicon sequencing on the V3-V4 region of the 16S rRNA gene (Figure 4.21). Three biological replicates were processed per population, giving us 24 samples (8 populations: 4 MBs + 4 MBLs, 3 replicates each). The native source pool of NDB_1 (at Gen 56) was also sequenced. Thus, Figure 4.21 shows a total of 27 samples (24 from MB_{1-4}-MBL_{1-4} and 3 from NDB_1).

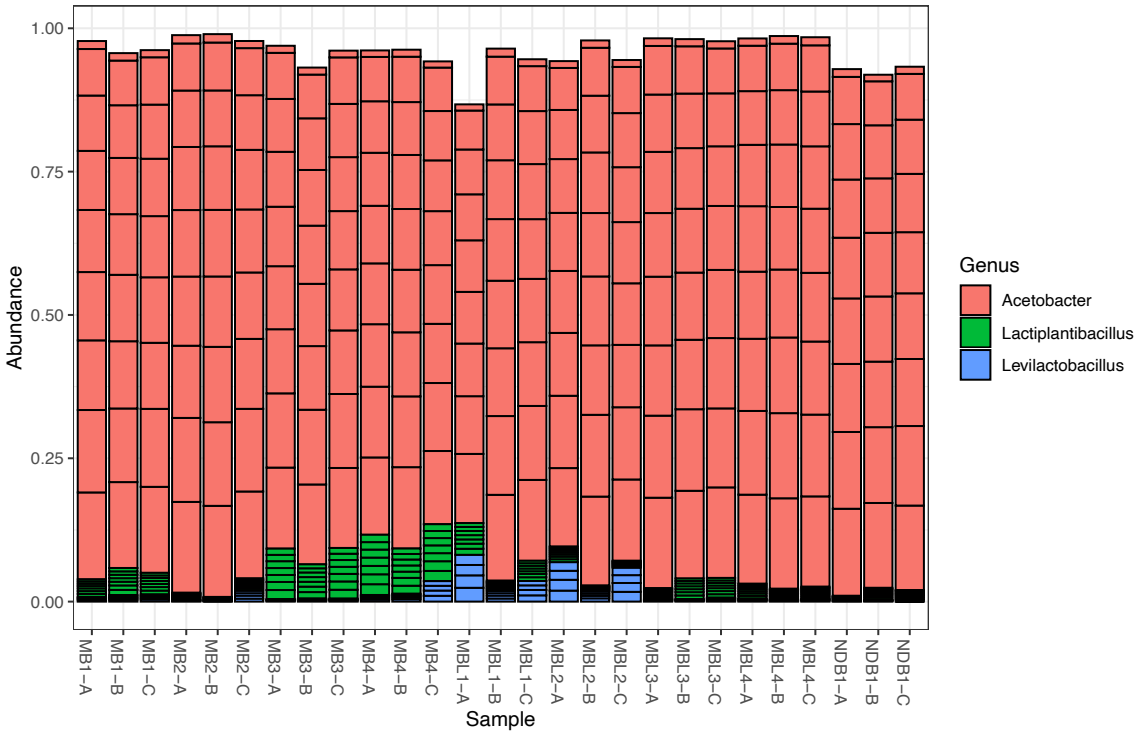

Figure 4.21 Validation of microbiome composition after reconstitution in Gen 17-20 assays. The result of population-level 16S rRNA amplicon sequencing across four replicate populations of MB_{1-4} and four replicate populations of MBL_{1-4}. Native microbiome composition of source population NDB_1 is also given for the reference (last three columns). Most abundant 20 taxa are shown for each population. For each population, three independent replicates where processed (labelled "A", "B", "C"). Each replicate consisted of pooled DNA extracted from surface sterilized full body homogenates of 8-10 females. The DNA extraction method and sequencing data analysis are described in Chapter 2, method sections 2.2.4.2 and 2.2.7 respectively.

We see that the native microbiome composition of NDB_1 is very close to the previous sequencing result (Figure 2.2). The MB_{1-4}-MBL_{1-4} populations also mirror this broad composition with the dominance of *Acetobacter* spp. but with small fluctuations across MB-

MBLs in *Acetobacter* and *Lactobacillus* fractions. In 2020, the old genus *Lactobacillus* was reclassified into 25 new different genera. *Lactiplantibacilus* and *Levilactobacilus* both belong to the old composite *Lactobacillus* genus.

4.5.2.2 MB-MBL generation 54-57 assays

In generation 54-57 assays, we sequenced microbiomes from MB_1 and MBL_1 that were raised in the with-microbes environments (Figure 4.22). We see that MB_1 and MBL_1 have received microbiomes that are very close in composition. *Acetobacter* spp. dominates the microbiomes of both populations.

Together, these sequencing results for generations 17-20 assay and for generations 54-57 assay show that microbiome composition received by MBs and MBLs is broadly similar to each other and to the source microbiome of NDB_1 (Figure 2.2 and Figure 4.21-last three lanes).

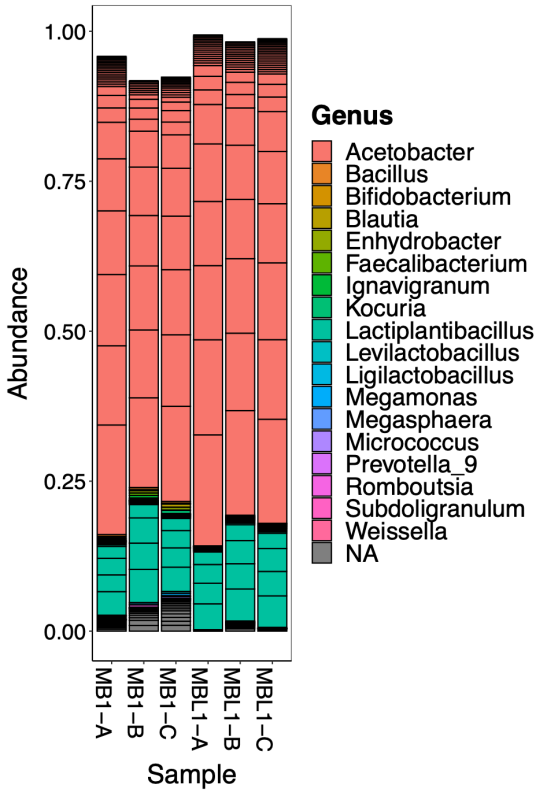

Figure 4.22 Validation of microbiome composition after reconstitution in Gen 54-57 assays. Most abundant 100 taxa are shown for each population. For each population, three independent replicates where processed (labelled "A", "B", "C"). Each replicate consisted of pooled DNA extracted from surface sterilized full body homogenates of 8-10 females. The DNA extraction method and sequencing data analysis are described in Chapter 2, method sections 2.2.4.2 and 2.2.7 respectively.

Chapter 5
Contribution of microbiome to outbred vs. inbred *Drosophila* host

5.1 Introduction

Host-associated microbes can be an important source of genetic variation for the hosts. This expanded genomic repertoire can help the host in their adaptation (Baldassarre et al. 2022) and niche expansion (Kikuchi et al. 2012; Lemoine et al. 2020) as microbes can have capabilities (e.g., metabolic (A. E. Douglas 1998), immune regulatory (Belkaid and Hand 2014), etc.) that the host alone might lack. Thus, hosts associated with the right kinds of microbes can form "a host + microbes unit" that many studies have referred to as a "holobiont" (Bordenstein and Theis 2015; Rosenberg and Zilber-Rosenberg 2018; Zilber-Rosenberg and Rosenberg 2008). Considerable debate surrounds the idea of holobionts (discussed in detail in Chapter 1 as well as (Angela E. Douglas and Werren 2016; Moran and Sloan 2015). However, regardless of the philosophical issues, studying the "host + microbes" system together can be insightful to understand the role of microbes in influencing host fitness and evolution (Fontaine and Kohl 2020; Morris 2018; Nyholm et al. 2020; van de Guchte et al. 2018).

In previous chapters, we have only manipulated the microbes part in this "host + microbes" unit. In this chapter, we attempt to tinker with the host part as well to study how that can potentially affect the unit. When studying the host + microbes unit, one cannot entirely remove the host from the picture – but we can manipulate it. One can study the effect of the same microbiome community on different host backgrounds and understand if the host genetics plays an important role in driving the host-microbe interactions (Chong and Moran 2016). Another way to manipulate a host is by reducing the overall genetic diversity of the host through inbreeding.

It is known that the host's genetic variation can influence its gut microbiome composition (Blekhman et al. 2015; Bonder et al. 2016; Early et al. 2017; Goodrich et al. 2014; Jehrke et al. 2018; E. P. Ryu and Davenport 2022). For example, two groups of flies that are given two different kinds of diets develop a preference for mating within the same group (Sharon et al. 2010). This preference was shown to be mediated by the microbiome of the hosts. This study was done on inbred Oregon R flies. When Najarro et al. tried reproducing the mating preference imparted by different diets, inbred strains (Canton-S) showed a similar preference, but the outbred strain (allRAL) did not (Najarro et al. 2015). So, what was the causal mechanism? Is it possible that outbred flies had different microbiome than the inbred strains? Or did the difference in the amount of genetic variation available between the inbred and the outbred populations cause the difference in results? While it is not possible to answer this

question in the context of these two studies (Najarro et al. 2015; Sharon et al. 2010), there seems to be evidence for both phenomena in the literature.

In multiple vertebrate taxa like fishes (Bolnick et al. 2014), birds (Leclaire et al. 2019), and mammals like mice (Khan et al. 2019) and minks (Leeuwen et al. 2024), it is known that hosts that possess greater variation in major histocompatibility complex (MHC) motifs have less diverse microbiota. More interestingly, it has been demonstrated in the mouse model that MHC heterozygosity positively affects a number of microbial functions that enhance the fitness of the host (Khan et al. 2019). Inbreeding, due to its negative effects on heterozygosity (Templeton 2021), is thus likely to reduce the fitness of an organism also through its effect on the microbiota.

Although MHC genes are not present in *Drosophila*, it is known that the composition of the microbiota is affected by components of the immune system like antimicrobial peptides (AMPs) (Marra et al. 2021), immune-related genes like Pdm1/nub (Dantoft et al. 2016), and 4E-BP (Bahuguna et al. 2022). In addition, the developmental "master control" gene *Caudal* is shown to regulate AMPs expression that maintains a normal gut community (J.-H. Ryu et al. 2008). Interestingly, the genetic variation in *Drosophila* antimicrobial peptides is known to be maintained by balancing selection (Chapman et al. 2019). Since selection can have very complex interactions with inbreeding (B. Walsh and Lynch 2018), it is conceivable that the latter can alter the genetic variability of the flies, which in turn can have an effect on the microbiota. Indeed, it is known that harsher population bottlenecks (that reduce the genetic variation of the host and increase inbreeding) reduce the diversity of the microbiome and make the microbial community less robust (Ørsted et al. 2022). While the Ørsted et al. (2022) study looked at the microbiome composition change, the total contribution of the microbiome to different life history, stress-tolerance-related, and behavioral phenotypes was not checked via microbiome removal or microbiome manipulation. Thus, it is not entirely clear whether microbiota removal would have a greater effect on the fitness of inbred or outbred populations. To the best of our knowledge, this aspect of host-microbiota interaction has not been investigated to date.

Here, we used four replicate populations of outbred *Drosophila melanogaster* (labeled Outbred-NDB$_{1\text{-}4}$, population size ~ 2400) to generate corresponding four inbred replicate populations (labeled Inbred-NDB$_{1\text{-}4}$) via full-sib mating of virgin collected flies at the population size of two individuals. This inbreeding treatment was done for ten consecutive host generations and led to populations with reduced fitness, as assessed by percent survival

to adulthood and female fecundity. We then investigated if the contribution of the microbiome to the fitness of the inbred populations is altered as compared to the outbred flies. For this, in both of these populations, we created two types of flies – flies with their microbiome removed and flies with the microbiome reconstituted (this is explained in detail in Figure 3.9 of Chapter 3). The difference between the phenotypes of these two types of flies will give us the microbiome's contribution in both populations. We found that the overall modulation of the host by the microbiome was less pronounced in the inbred flies compared to their outbred counterparts for life history and stress (desiccation) resistance traits, while behavioral traits remained unchanged. Inbred populations had more inter-replicate variation than the outbred populations. Closer inspection revealed that the traits with higher standard deviation in outbred flies further increased their standard deviation in inbred flies. Also, while both males and females showed microbiome modulation in outbred flies, only traits specific to female inbred flies showed modulation by microbiome. Taken together, these results reveal that outbred strains of *Drosophila* might differ in their response to microbiome than the inbred strains. While most of the results in the *Drosophila* microbiome field are obtained in inbred strains, the microbiome's contribution in modulating the host biology might be more pronounced in outbred strains.

5.2 Methods

5.2.1. Generation of inbred populations starting with outbred NDB_{1-4}

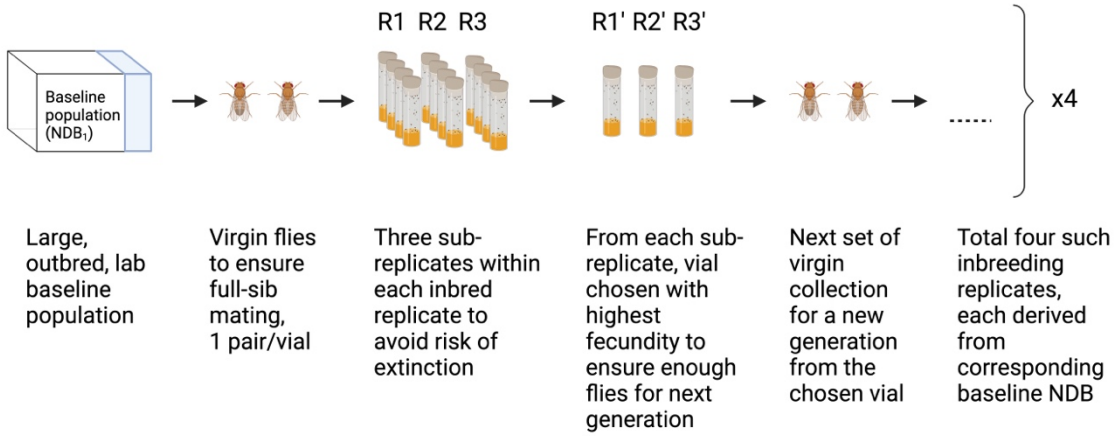

Figure 5.1 Illustration of the methodology followed to generate four inbred lines. This figure is created with BioRender.com.

Four inbred lines (Inbred-NDB_{1-4}) were derived from the corresponding four outbred NDB populations (Outbred-NDB_{1-4}) that trace their ancestry to wild-caught IV lines (Ives 1970) via the JB_{1-4} populations (Sheeba et al. 1998). The details of outbred-NDB_{1-4} populations are given in section 2.2.1 of Chapter 2. To set up single mating pairs of full-sibs, virgin flies were collected every generation (Figure 5.1). The first virgin collection happened in generation 56 of Outbred-NDB_{1-4}. For each inbred population, three sub-replicates were set up (R1, R2, R3) within each inbred line. Each sub-replicate had four vial replicates nested within (Figure 5.1). For every inbred population, every generation, 4 x 3 = 12 mating pairs in 12 different vials (1 mating pair per vial) were allowed to lay eggs for the next generation. Inbred lines tend to go extinct; this sub-replication and keeping four vials per sub-replicate ensured that even if one or two sub-replicates went extinct, we would still have at least one replicate per block to work with at the end of the inbreeding process. As every vial only had a single virgin mating pair laying eggs, all the emerging adults were full-sibs. Out of the four replicate vials nested within a sub-replicate, a vial with the highest fecundity was chosen to provide the virgin full-sib pairs for the next generation. This inbreeding procedure was carried out for ten consecutive generations. At the end of the inbreeding process, out of the three sub-replicates (R1, R2, R3), only one sub-replicate was chosen for final assays, giving rise to a total of four inbred populations (Inbred-NDB_{1-4}).

This inbreeding procedure led to the expected inbreeding coefficient of ~0.88 (Falconer 1996). After ten generations of inbreeding, these populations were assessed for their egg-to-adult survival and female fecundity as a proxy for inbreeding depression. These results are in section 5.5.1 (Appendix) of this chapter.

The inbred population derived and the outbred population from which it was derived (e.g., Inbred-NDB$_1$ and Outbred-NDB$_1$) were always assayed together and treated as blocks in statistical analysis.

5.2.2. Comparing the contribution of microbes to host phenotypes in outbred and inbred populations

To compare the contribution of the microbiome to host traits in outbred vs. inbred populations, we followed the earlier protocol of egg-dechorionation using bleach, which is described in detail in sections 3.2.1-3.2.6 and illustrated in Figure 3.9 of Chapter 3. Using this protocol, Outbred populations either received the microbes ("with-microbes flies") or were raised without them ("microbe-free flies") (Figure 5.2-left). The difference in phenotypic values of these two types of flies gave us the microbiome's contribution to that phenotype. A similar process was also done on inbred populations (Figure 5.2-right).

This procedure gives rise to four types of populations for a single block. For example, for block 1, the four types of populations are: with-microbes Outbred-NDB$_1$, microbe-free Outbred-NDB$_1$, with-microbes Inbred-NDB$_1$, microbe-free Inbred-NDB$_1$ (Figure 5.2).

In this set of assays, for with-microbes flies, outbred flies after egg dechorionation received the native microbiome from unmanipulated outbred flies (i.e., reconstituted Outbred-NDB$_1$ received microbiome from Outbred-NDB$_1$). The other three blocks also received the microbes from their corresponding unmanipulated counterparts. The same procedure was followed for inbred flies as well, i.e., reconstituted Inbred-NDB$_1$ received the microbiome from unmanipulated Inbred-NDB$_1$ and so on for the other three blocks.

The presence of microbes in with-microbe flies and their absence in microbe-free counterparts was checked by plating fly homogenates and by PCR on gDNA extracted from the flies with 16S rRNA universal bacterial primers (see Appendix section 5.5.2 of this chapter for the details). The composition of with-microbes flies in outbred and inbred populations was also compared using population-level 16S rRNA gene amplicon sequencing to see that the microbiomes of these two populations were similar in composition (see Appendix section 5.5.2 of this chapter for the details).

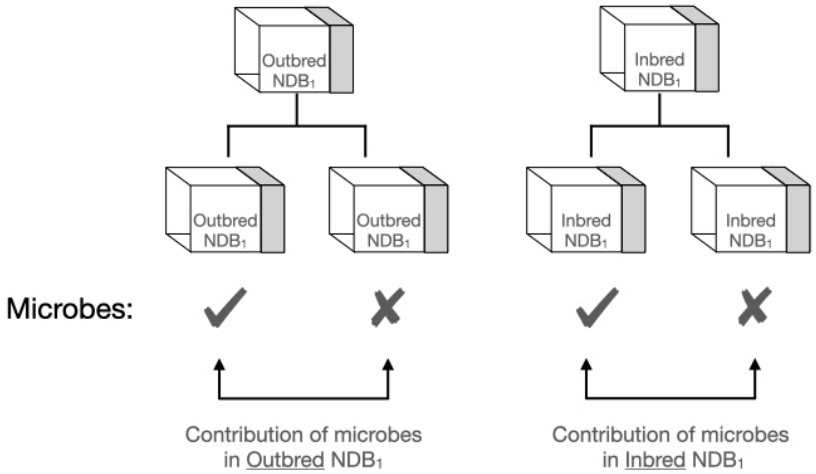

Figure 5.2 Generation of populations with microbes and without them. We illustrate the procedure for block 1 (i.e., Outbred-NDB_1 on the left and Inbred-NDB_1 on right), but the same applies for all four blocks/replicates. All four populations in the same block (e.g., with-microbes Outbred-NDB_1, microbe-free Outbred-NDB_1, with-microbes Inbred-NDB_1, microbe-free Inbred-NDB_1) were always assessed together.

5.2.3 Assay details

We compared the following traits in the outbred vs. inbred flies:

Table 5.1 List of traits assessed

No.	Type of trait	Phenotype of the flies	Assessment done on		
			males	females	males and females
1	Life-history related	Dry body weight	Yes	Yes	
2		Survival to adulthood			Yes
3		Female fecundity			Yes
4	Stress-tolerance	Desiccation resistance	Yes	Yes	
5	Behavioral	Locomotor activity	Yes	Yes	
6		Mating latency			Yes
7		Mating time			Yes

The protocols for all the above assays are described in detail in section 3.2.7 of Chapter 3.

The egg-dechorionation procedure to make flies with microbes and without them is described in detail in sections 3.2.1-3.2.6 and illustrated in Figure 3.9 of Chapter 3.

5.2.4 Statistical analysis

Two types of populations were compared with each other: (a) four replicate outbred populations "Outbred-NDB_{1-4}" (control), and (b) four replicate inbred populations "Inbred-NDB_{1-4}" (treatment) derived from NDB_{1-4} through virgin full-sib matings (population size = 2) as explained in method section 5.2.1. Both of these populations either received microbiome after egg-dechorionation ("with-microbes" treatment) or were kept without any microbes after egg-dechorionation ("microbe-free" treatment). This makes it a 2 x 2 design – i.e., two populations (outbred and inbred) assessed in two environments, i.e., the presence and absence of microbes.

For outbred populations, four block means of with-microbes Outbred-NDB_{1-4} were compared against four block means of microbe-free Outbred-NDB_{1-4} using a paired t-test performed in GraphPad Prism (v10.2.1). A similar procedure was done for Inbred-NDB_{1-4}. The effect sizes for both of these comparisons in two populations were calculated using Cohen's d. For interpreting effect sizes using Cohen's d, the following criteria were followed: $d > 0.8$ (high); $0.8 > d > 0.5$ (medium); $d < 0.5$ (low) (Cohen 2013). The graphs are plotted in GraphPad Prism (v10.2.1). The figures drawn using BioRender have that specification in their legend description.

In the result section, along with the statistically significant trends (i.e., $P < 0.05$), we have also highlighted trends that are not statistically significant (i.e., $P > 0.05$) but have come close to statistical significance ($P \sim 0.1$) with large effect sizes (using Cohen's d). This is highlighted with the blue ink in Tables 5.2 and 5.3, which provide detailed information about the statistical comparisons for the phenotypic assays.

5.3 Results

5.3.1 Body weight

For both outbred and inbred populations, male body weight was unaffected by the presence of microbes (Figure 5.3A, see Tables 5.2-5.3 for the details of the statistical analysis).

For outbred females, the body weight of flies without microbes was lower but not statistically significant (Paired t-test, $t_3 = 2.50$, P = 0.0875, Cohen's d = 1.72 (large), Figure 5.3B). For inbred females, the body weight of flies without the microbe was lower and statistically significant (Paired t-test, $t_3 = 4.15$, P = 0.0274, Cohen's d = 1.50 (large), Figure 5.3B).

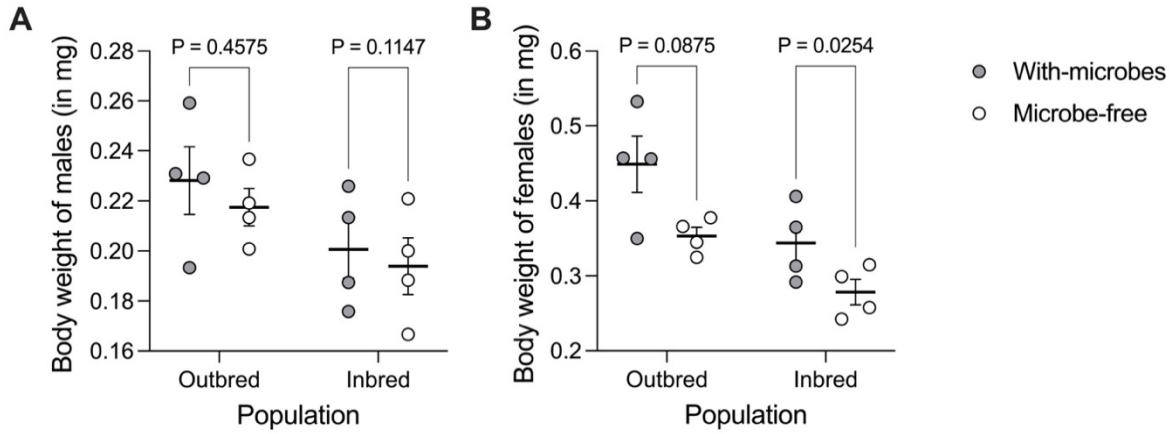

Figure 5.3 Body weight of outbred and inbred flies, in the presence of microbes and their absence (A) Males (B) Females. For each population (outbred and inbred), four filled circles are means for each of the four populations *with-microbes* and similarly, four empty circles are means for each of the *microbe-free* four populations. The black horizontal lines represent the grand means over four population means. For each population (outbred and inbred), the grand mean over populations *with-microbes* was compared against the grand mean over *microbe-free* populations using a paired t-test. Absence of microbes reduced the body weight of the flies in females (but not in males) in both inbred and outbred populations.

5.3.2 Egg-to-adult survival

Outbred flies showed a trend of lower egg-to-adult survival that was marginally non-significant (Paired t-test, $t_3 = 2.87$, P = 0.0640, Cohen's d = 2.55 (large), Figure 5.4A). For inbred flies, egg-to-adult survival was not affected by the absence of microbiomes (Paired t-test, $t_3 = 0.70$, P = 0.5340, Cohen's d = 0.63 (medium), Figure 5.4A).

5.3.3 Female fecundity

In both outbred and inbred populations, female fecundity was reduced without the microbes (For outbred flies: Paired t-test, $t_3 = 4.08$, P = 0.0266, Cohen's d = 2.31 (large), Figure 5.4B, and for inbred flies: Paired t-test, $t_3 = 5.96$, P = 0.0094, Cohen's d = 2.70 (large), Figure 5.4B).

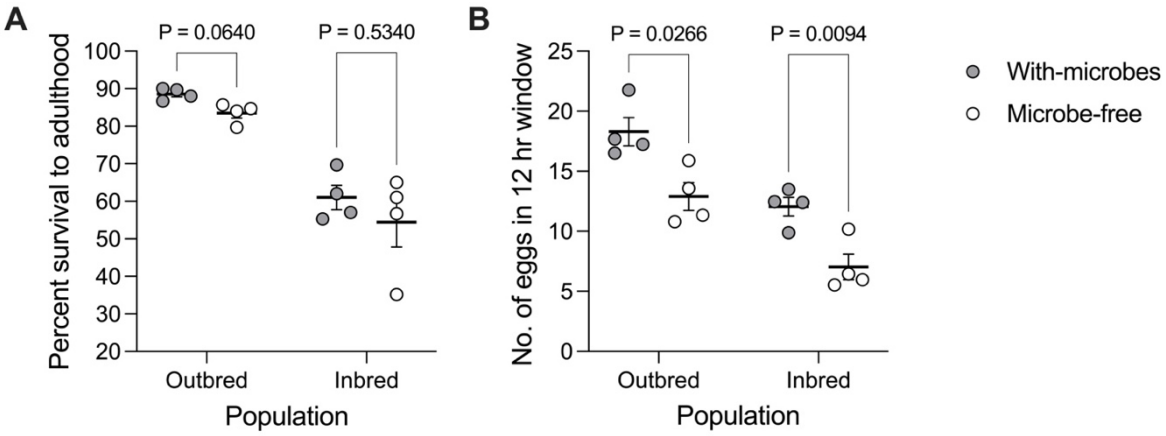

Figure 5.4 (A) Percentage survival to adulthood and (B) female fecundity of outbred and inbred flies, in the presence and in the absence of microbiome. For each population (outbred and inbred), four filled circles are means for each of the four populations *with-microbes* and similarly, four empty circles are means for each of the *microbe-free* four populations. The black horizontal lines represent the grand means over four population means. For each population (outbred and inbred), the grand mean over populations *with-microbes* was compared against the grand mean over *microbe-free* populations using a paired t-test.

5.3.4 Desiccation resistance

For the outbred males, desiccation resistance was lower without microbes, and this difference was marginally non-significant (Paired t-test, $t_3 = 2.88$, P = 0.0636, Cohen's d = 2.09 (large), Figure 5.5A). For the inbred males, desiccation resistance wasn't affected by the microbiome removal (Paired t-test, $t_3 = 0.69$, P = 0.5412, Cohen's d = 0.50 (small), Figure 5.5A).

For outbred and inbred females, desiccation resistance was increased in the absence of microbes (For outbred flies: Paired t-test, $t_3 = 7.51$, P = 0.0049, Cohen's d = 3.95 (large), Figure 5.5B, and for inbred flies: Paired t-test, $t_3 = 4.28$, P = 0.0235, Cohen's d = 1.98 (large), Figure 5.5B).

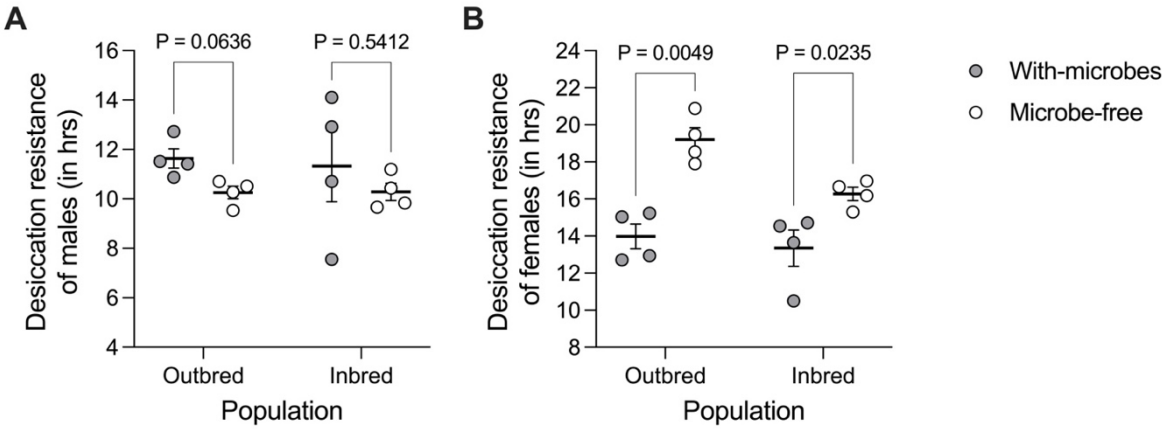

Figure 5.5 Desiccation resistance of outbred and inbred flies, in the presence and in the absence of microbiome for (A) Males and (B) Females. For each population (outbred and inbred), four filled circles are means for each of the four populations *with-microbes* and similarly, four empty circles are means for each of the *microbe-free* four populations. The black horizontal lines represent the grand means over four population means. For each population (outbred and inbred), the grand mean over populations *with-microbes* was compared against the grand mean over *microbe-free* populations using a paired t-test.

5.3.5 Locomotor activity

Locomotor activity of outbred males without microbes was higher than the outbred males with microbes (Paired t-test, $t_3 = 15.65$, P = 0.0006, Cohen's d = 2.51 (large), Figure 5.6A). In inbred males, microbe removal did not change the locomotor activity of the flies (Paired t-test, $t_3 = 0.68$, P = 0.5464, Cohen's d = 0.54 (medium), Figure 5.6A).

The locomotor activity of both outbred and inbred females reduced when microbes were removed (For outbred flies: Paired t-test, $t_3 = 13.14$, P = 0.0010, Cohen's d = 1.17 (large), Figure 5.6B, and for inbred flies: Paired t-test, $t_3 = 28.42$, P < 0.0001, Cohen's d = 1.63 (large), Figure 5.6B).

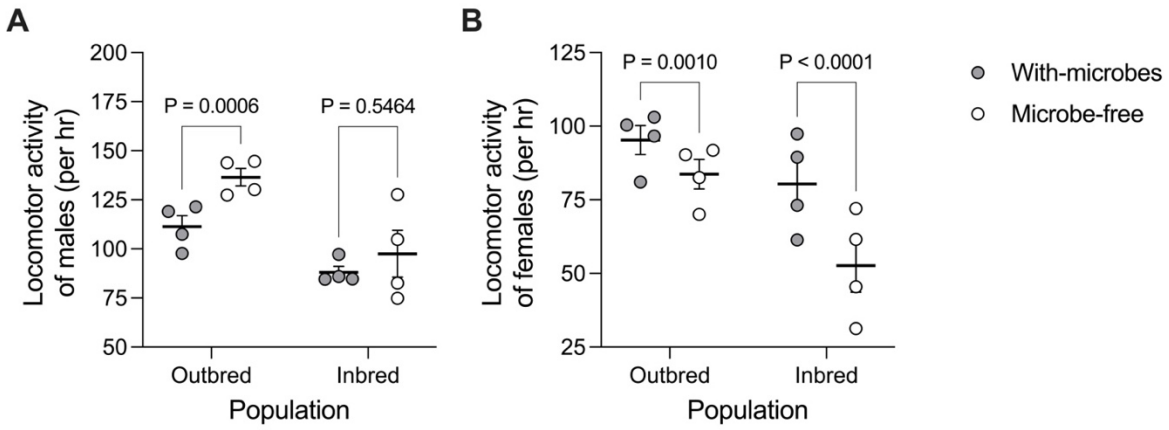

Figure 5.6 Locomotor activity of outbred and inbred flies, in the presence and in the absence of microbiome for (A) Males and (B) Females. For each population (outbred and inbred), four filled circles are means for each of the four populations *with-microbes* and similarly, four empty circles are means for each of the *microbe-free* four populations. The black horizontal lines represent the grand means over four population means. For each population (outbred and inbred), the grand mean over populations *with-microbes* was compared against the grand mean over *microbe-free* populations using a paired t-test.

5.3.6 Mating latency and mating time

The mating-related traits – mating latency and mating time – were not affected by the microbe removal in both outbred and inbred flies (Figure 5.7; see Tables 5.2-5.3 for the details of the statistical analysis).

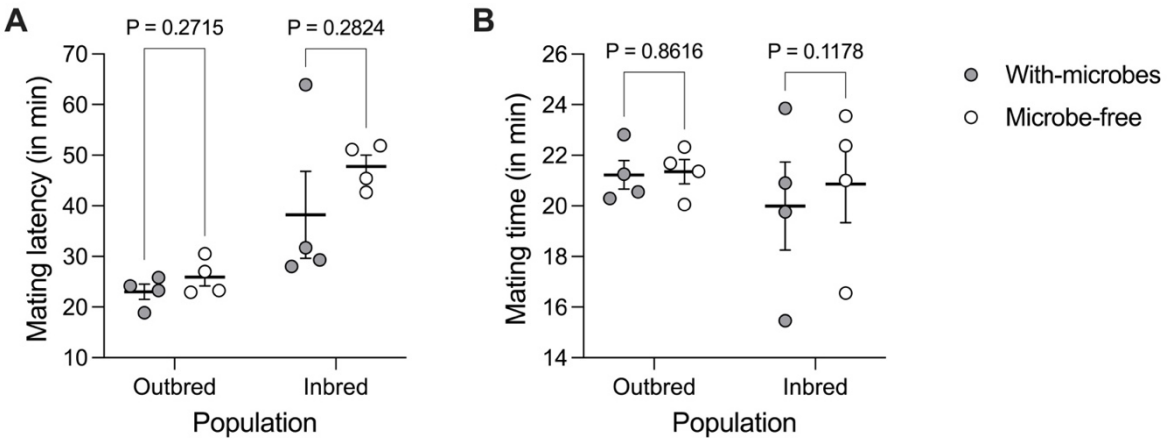

Figure 5.7 (A) Latency to mating and (B) Mating time of outbred and inbred flies, in the presence and in the absence of microbiome. For each population (outbred and inbred), four filled circles are means for each of the four populations *with-microbes* and similarly, four empty circles are means for each of the *microbe-free* four populations. The black horizontal lines represent the grand means over four population means. For each population (outbred and inbred), the grand mean over populations *with-microbes* was compared against the grand mean over *microbe-free* populations using a paired t-test.

Table 5.2 Summary of all assays for the outbred populations. T refers to the grand mean of the microbe-free flies, while C refers to the grand mean of the with-microbe flies.

	Assay	Test statistic	P-value	% change (T - C)/C	Cohen's d	Effect size*	Sample size[$]
1	Dry body weight (Male)	$t_3 = 0.85$	0.4575	-4.7	0.49	Small	06
2	Dry body weight (Female)	$t_3 = 2.50$	0.0875	-21.3	1.72	Large	06
3	Survival to adulthood	$t_3 = 2.87$	0.0640	-5.7	2.55	Large	9 to 10
4	Fecundity (Females)	$t_3 = 4.08$	0.0266	-29.5	2.31	Large	49 to 50
5	Desiccation resistance (Males)	$t_3 = 2.88$	0.0636	-11.8	2.09	Large	10
6	Desiccation resistance (Females)	$t_3 = 7.51$	0.0049	37.3	3.95	Large	10
7	Locomotor activity (Males)	$t_3 = 15.65$	0.0006	22.6	2.51	Large	26 to 32
8	Locomotor activity (Females)	$t_3 = 13.14$	0.0010	-12.1	1.17	Large	31 to 32
9	Mating Latency	$t_3 = 1.34$	0.2715	12.6	0.88	Large	31 to 38
10	Mating time	$t_3 = 0.30$	0.8616	0.6	0.12	Small	31 to 38

*Interpretation for effect sizes using Cohen's d: $d > 0.8$ (Large); $0.8 > d > 0.5$ (Medium); < 0.5 (Small)

[$]Note: See methods for detailed information about sample sizes. For example, the sample size of ten per treatment for body weight indicates ten micro-centrifuge tubes with 20 flies each. Also, this is a sample size per block, i.e., each circle in the plots is an average over this sample size.

Table 5.3 Summary of all assays for the inbred populations. T refers to the grand mean of the microbe-free flies, while C refers to the grand mean of the with-microbe flies.

	Assay	Test statistic	P-value	% change (T - C)/C	Cohen's d	Effect size*	Sample size[$]
1	Dry body weight (Male)	$t_3 = 2.21$	0.1147	-3.3	0.29	Small	06
2	Dry body weight (Female)	$t_3 = 4.15$	0.0254	-19.0	1.50	Large	06
3	Survival to adulthood	$t_3 = 0.70$	0.5340	-10.7	0.63	Medium	9 to 10
4	Fecundity (Females)	$t_3 = 5.96$	0.0094	-41.7	2.70	Large	49 to 50
5	Desiccation resistance (Males)	$t_3 = 0.69$	0.5412	-9.2	0.50	Small	10
6	Desiccation resistance (Females)	$t_3 = 4.28$	0.0235	21.9	1.98	Large	10
7	Locomotor activity (Males)	$t_3 = 0.68$	0.5464	10.7	0.54	Medium	15 to 32
8	Locomotor activity (Females)	$t_3 = 28.42$	<0.0001	-34.5	1.63	Large	28 to 32
9	Mating Latency	$t_3 = 0.19$	0.2824	24.9	0.76	Medium	15 to 35
10	Mating time	$t_3 = 2.18$	0.1178	4.4	0.27	Small	15 to 35

*Interpretation for effect sizes using Cohen's d: $d > 0.8$ (Large); $0.8 > d > 0.5$ (Medium); < 0.5 (Small)

[$]Note: See methods for detailed information about sample sizes. For example, the sample size of ten per treatment for body weight indicates ten micro-centrifuge tubes with 20 flies each. Also, this is a sample size per block, i.e., each circle in the plots is an average over this sample size.

5.3.7 Effect size estimate of microbe's contribution to host traits in outbred and inbred flies

To compare the overall effect of microbe removal in outbred vs. inbred populations, we plotted effect sizes (using Cohen's d) of the differences between the with- and without-microbe treatments for both these populations (Figure 5.8A) across all the traits that we studied. We find that the effect sizes were relatively lower for the inbred populations than the outbred ones, but this trend was marginally non-significant (Paired t-test, $t_9 = 2.15$, P = 0.0606, Cohen's d = 0.7 (medium), Figure 5.8A). When we plotted non-behavioral (life-history and desiccation resistance) traits and behavioral traits separately, we see that, (although statistically not significant), there was a greater reduction in the effect sizes of the life-history and desiccation resistance traits (Paired t-test, $t_5 = 2.18$, P = 0.0808, Figure 5.8B, Cohen's d = 0.88 (large), Figure 5.8B), compared to the behavioral traits (locomotor activity in both sexes, mating latency, and mating time) (Paired t-test, $t_3 = 2.15$, P = 0.5436, Cohen's d = 0.46 (small), Figure 5.8C).

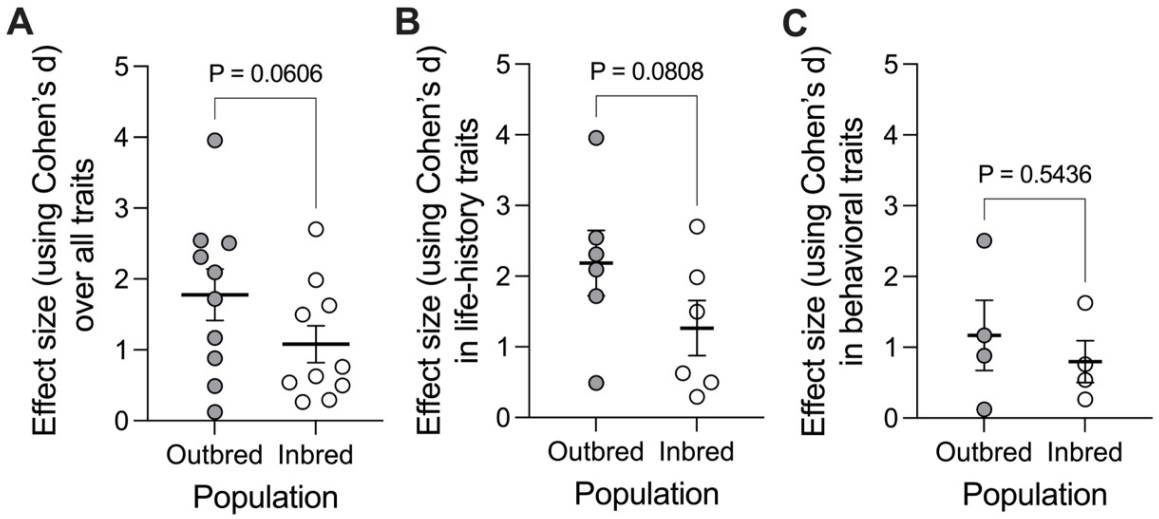

Figure 5.8 Comparison of effect sizes (using Cohen's d) of microbiome's contribution to host traits in inbred vs. outbred *Drosophila.* Effect sizes over (A) all traits (B) life-history traits (C) behavioral traits. Means are indicated by black horizontal lines. The error bar represents SEM. The data is analyzed using a paired t-test.

5.3.8 Pooled standard deviations for outbred and inbred populations

We conjectured that the smaller effect sizes in inbred populations (Fig 5.6) might be a result of increased inter-replicate variation in inbred populations as compared to the outbred ones. To quantify this variation, we used the proxy of pooled standard deviation over four replicate population means for inbred as well as outbred populations.

$$\text{Pooled standard deviation} = \sqrt{\frac{S^2_{With\text{-}micorbes} + S^2_{Microbes\text{-}free}}{2}}$$

This was done for all the ten traits we studied. We see that, indeed, the pooled standard deviation (over with-microbe and microbe-free flies) for inbred lines was more than the outbred lines (Paired t-test, $t_9 = 2.82$, P = 0.0201, Cohen's d = 0.63 (medium), Figure 5.9A). Wilcoxon matched-pairs signed rank test (a non-parametric test) is also significant (W = 43.0, P = 0.0273, Figure 5.9B).

We also found that the traits with higher levels of standard deviation in outbred flies (locomotor activity of males and females, percent survival to adulthood, mating latency) further increased their standard deviation in inbred lines derived from them (Figure 5.9B).

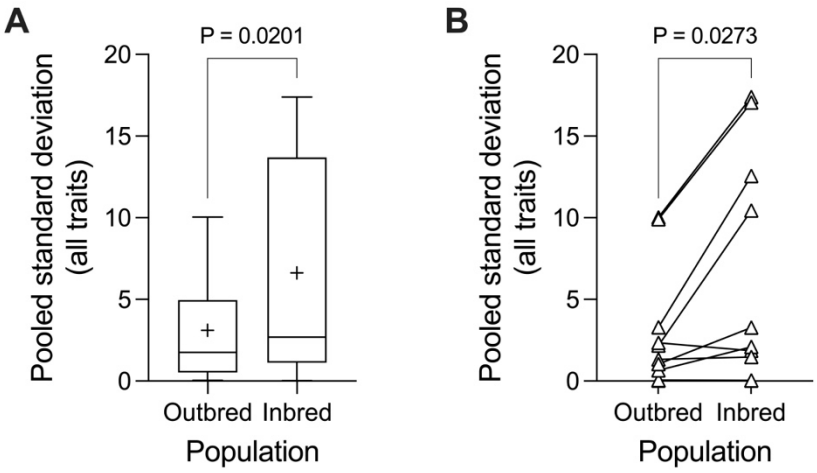

Figure 5.9 Higher pooled standard deviations for the inbred populations. The pooling is over "with-microbe" and "microbe-free" treatments. Both (A) and (B) show the same data. (A) In box-plots, centre lines show the median, box limits indicate the 25th and 75th percentiles, whiskers extend from the minimum to maximum data points, plus sign within the boxes represents the sample mean. The data is analyzed using a paired t-test. (B) Shows the pairings for different phenotypes. The data is analyzed using Wilcoxon matched-pairs signed rank test.

5.4 Discussion

The genetic background of the host used in microbiome studies can influence the outcomes (Benga et al. 2024; E. P. Ryu and Davenport 2022). We checked a diverse set of host phenotypes, both in males and females, to see if the microbiome's contribution to host phenotypes changes when the host is inbred. For this, we generated four replicate inbred lines (Inbred-NDB_{1-4}) from four large (population size ~ 2400), outbred populations (Outbred-NDB_{1-4}). Both of these populations were either given microbiomes via reconstitution or they were kept without microbes for a single generation. By comparing the phenotypes of the with-microbes flies and the flies without them, we assessed the microbe's contribution to outbred and inbred populations.

5.4.1 Low effect sizes and high inter-replicate variation in inbred populations

In mice, changes in the heterozygosity of the host in terms of the MHC loci affect the functioning of the microbiota, which in turn can affect the fitness of the host (Khan et al. 2019). In *Drosophila*, which do not have MHCs, antimicrobial peptides (Marra et al. 2021), and other immunity-related genes (Bahuguna et al. 2022; Dantoft et al. 2016) can alter the diversity of the microbiota. Interestingly, inbreeding can have complex effects on immunity in *Drosophila*. While some studies have reported that inbreeding leads to an enhanced immune response in cold-sensitive flies (Vermeulen et al. 2013), others have shown that the ability to resist a pathogen generally decreases with increasing levels of inbreeding (Spielman et al. 2004). Thus, it is very difficult to predict in what way the interaction between the host and its microbiota would change due to inbreeding.

If the holobiont is indeed a tightly integrated unit made of host and microbes, we expect that the microbiome can elevate the host fitness with its added genomic variation in a state where host fitness falls drastically due to inbreeding. Thus, our working hypothesis was that in the presence of reduced fitness due to severe inbreeding, the host would be less able to resist the effects of microbiota removal. This meant that we expected the effects of microbiota removal, as evidenced by the effect size of the difference between the phenotypic value of the populations with and without the microbiota, would be greater for the inbred populations, than the outbred ones. However, we found that the effects were actually lower in the inbred populations, although this was not statistically significant (Figure 5.8A). This effect was driven by lower effect sizes in life history and desiccation resistance traits in inbred lines (Figure 5.8B), while effect sizes for behavioral traits didn't differ in outbred vs. inbred

populations (Figure 5.8C). We definitely do not see the microbiome's contribution increasing in inbred populations (Figure 5.8A), thus, our working hypothesis that the microbiome's contribution in inbred flies will be greater doesn't hold.

Looking at the spread in data in outbred vs. inbred populations, we wondered if lower effect sizes are the results of the higher spread (i.e., variation) over the block means. To quantify this, we looked at the standard deviation over different traits in outbred vs. inbred populations. As each of these populations had two treatments (with microbes and microbe-free), we took pooled standard deviation over these two treatments (section 5.3.8). We found that variation over block means of inbred populations was more than the outbred populations (Figure 5.9). This might contribute to the observation that the effect sizes of the microbiome's contribution in inbred populations are lower than that of the outbred populations (Figure 5.8A).

Together, larger effect sizes and lower variation in the microbiome's contribution in outbred populations compared to inbred populations might make the former better suited to reveal the microbiome's contribution to host-microbiome studies.

When we looked at which traits contributed to more variation in inbred populations, we saw a pattern (Figure 5.9B). We observed that the traits that had higher standard deviations in outbred flies, increased it further upon inbreeding. We also noted that out of the four high variation phenotypes (both in outbred and inbred lines), three were behavioral (locomotor activity of males and females, mating latency).

This pattern might also hint at what traits will be likely to show more inconsistencies across outbred vs. inbred fly genetic backgrounds in microbiome manipulation studies – these would be ones with more variability in them.

When we look at the overall results in Tables 5.2-5.3, another pattern is apparent. The traits that are statistically different (in red) and marginally insignificant (blue) in outbred populations – both with large effect sizes – are a mix of those assessed in males as well as in females (Table 5.2). But, in inbred populations, the traits that show statistical differences (with large effect sizes) are all exclusively in females (Table 5.3). It appears that only in females, microbes were able to contribute to host phenotypes in outbred and inbred genetic backgrounds. In the other traits tested, we did not see the microbiome's contribution in inbred flies. This might also be another contributing factor for the overall low effect sizes in microbe's contribution to inbred host phenotypes.

5.4.2 Implications

The inbreeding and fitness reduction that follows with it can have implications for conservation efforts (Hedrick and Kalinowski 2000). Microbiomes of species under conservation could impact the fitness of these species (Bahrndorff et al. 2016; Mueller et al. 2020). Depending on the identity of the microbes, microbiome perturbation can either improve host fitness or reduce it. If we look at the proxies for fitness in our inbred populations, while survival from egg to adult stage didn't change upon total microbiome removal, female fecundity reduced by ~42% (Figure 5.4B). This percentage is higher than for the same change in outbred flies (~30%). This shows that contrary to our general interpretation that the overall effect sizes are lower in inbred populations upon microbiome removal, the microbiome is important to sustain certain fitness components, such as female fecundity in inbred flies. This also means that, in certain inbred populations, reconstituting a native beneficial microbiome might help rescue the host from inbreeding depression by increasing its fitness.

If we take a closer look at the phenotypic changes upon microbiome modulation, we see that the inbred females do worse in terms of desiccation resistance when they have their native microbiome than without it (Figure 5.5B). So, in a particular environment, if desiccation resistance is crucial for host fitness (e.g., in an arid climate), then the inbred host might be better off without the microbiome supplement as this supplement will elevate the fecundity, but might reduce the crucial desiccation resistance. Another interesting observation is while this is true for females, inbred male's desiccation resistance is unaffected by the microbiome presence (Figure 5.5A). So, the males and females can respond differently to the same native microbiome transplant.

These observations tell us that while the microbiome can augment conservation efforts, one needs to carefully study its overall implications for the inbred hosts, especially in natural settings where the environment can change, also changing what traits matter for overall host fitness.

5.5 Appendix

5.5.1 Inbred flies generated after 10 generations of full-sib matings have lower fitness

To check if sufficient inbreeding has taken place, we looked at the two metrics – (a) egg-to-adult survival and (b) female fecundity. We found that both of these fitness components had reduced drastically in inbred flies after ten generations of virgin full-sib matings (Egg-to-adult survival, paired t-test, $t_3 = 7.36$, $P = 0.0052$, Figure 5.10A, and female fecundity, paired t-test, $t_3 = 5.36$, $P = 0.0127$, Figure 5.10B).

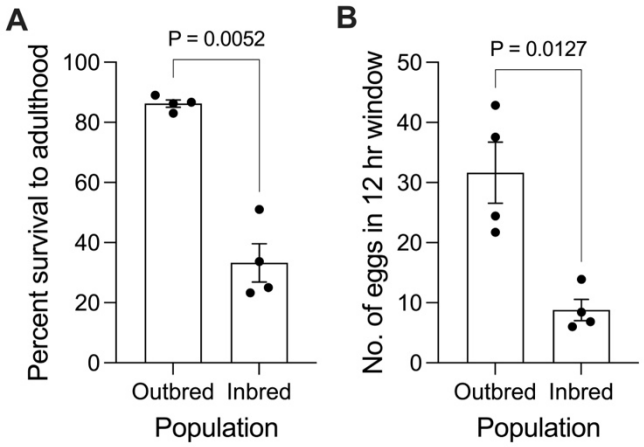

Figure 5.10 Reduction in fitness components in inbred flies (A) Percent survival to adulthood. (B) Female fecundity. Block means are indicated by black filled circles. The error bar represents SEM. The results were compared using paired t-test on block means. This assessment was done prior to the microbiome manipulation experiments to see adequate inbreeding has been achieved through the full-sib matings of population size of two for ten successive generations.

5.5.2 Validation of microbiome presence and absence

5.5.2.1 Plating fly homogenates confirms the status of microbiome in with-microbe and microbe-free flies

To check the status of microbiome in with-microbes and microbe-free flies, we plated these treatments for inbred and outbred flies on two media – MRS and NA (Figure 5.11). The protocol to plate the fly homogenates is already described in section 2.2.4.1 of Chapter 2.

Set-A in Figure 5.11A is the autoclaved water that is used to homogenize the flies (Top row: MRS, bottom row: NA). Set-B and Set-C show the serial dilutions made to count CFUs/fly for Outbred-NDB_1 and Inbred-NDB_1, respectively. The serial dilutions with ~10-100 colonies were counted to get the CFUs/fly. While Figure 5.11A shows the representative picture from

block 1, CFUs/fly for all four blocks of MBs and MBLs are compared using paired t-test on block means in Figure 5.12.

Figures 5.11B and 5.11C show plates for microbe-free Outbred-NDB$_1$ and microbe-free Inbred-NDB$_1$. All three replicates for both of these populations show the absence of a microbiome. While block 1 plates do not have any colonies, other blocks show a few colonies in some replicates (data not shown here).

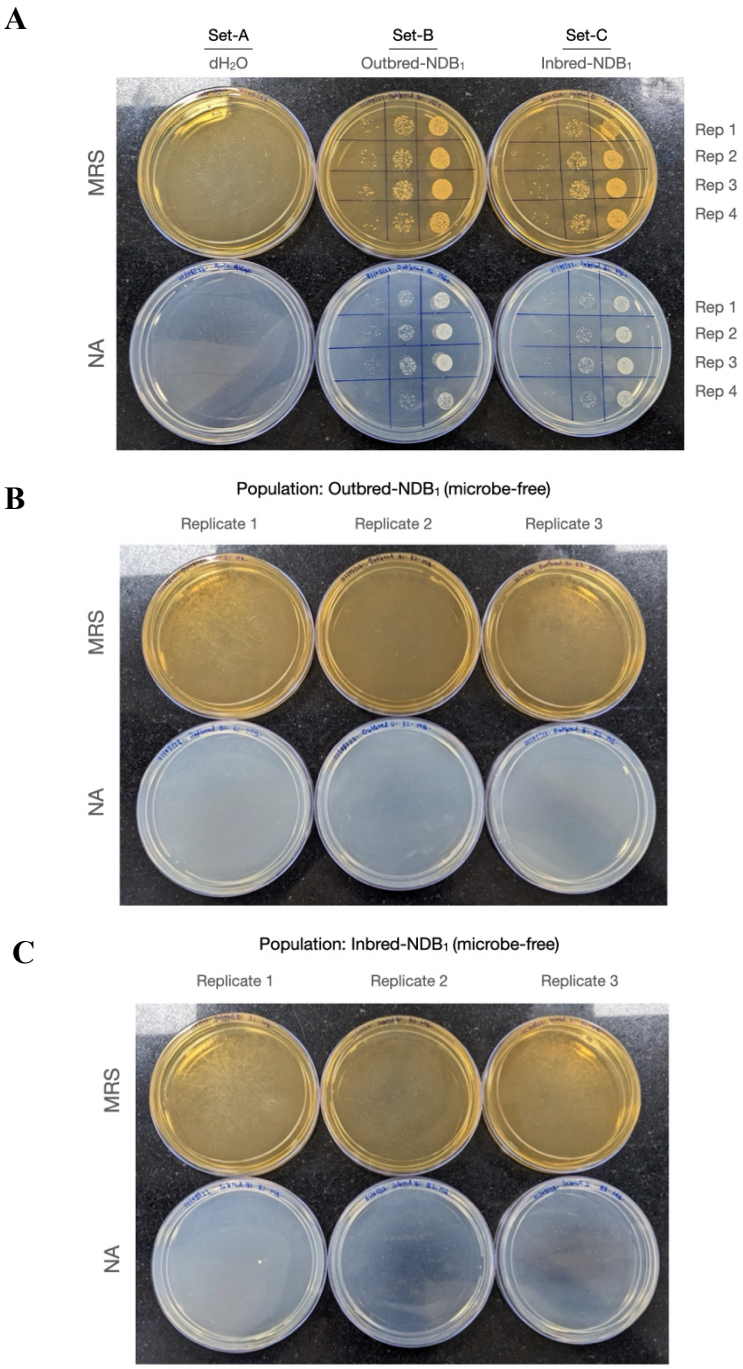

Figure 5.11 MRS and NA plates showing presence of microbes in with-microbe treatment and their absence in microbe-free treatment. Top row: MRS media, bottom row: NA media. (A) Set A: autoclaved dH$_2$O, Set B: Outbred-NDB$_1$ with-microbes (n = 4),

Set C: Inbred-NDB$_1$ with-microbes (n = 4). The spots show different serial dilutions used to calculate CFUs/fly. These CFU/fly for all the replicate populations are shown in Figure 5.12. (B) microbe-free Outbred-NDB$_1$ (n = 3). (C) microbe-free Inbred-NDB$_1$ (n = 3). All the data in this figure is for block 1 (i.e., Outbred-NDB$_1$ and Inbred-NDB$_1$), but similar protocol was followed for all four blocks (i.e., Outbred-NDB$_{1-4}$ and Inbred-NDB$_{1-4}$).

5.5.2.2 Inbred and outbred flies with microbes received similar bacterial load upon microbiome reconstitution

We see that the process of microbiome reconstitution (described in Figure 3.7 of Chapter 3) leads to Inbred-NDB$_{1-4}$ and Outbred-NDB$_{1-4}$ carrying similar bacterial loads on both the media (For MRS, paired t-test, $t_3 = 0.69$, P = 0.5424, Figure 5.12A, and for NA, paired t-test, $t_3 = 0.49$, P = 0.6552, Figure 5.12B). This bacterial load is close to the average of ~10^5 CFUs/fly that we see in our two earlier chapters - (a) single-generation study (Figure 3.8B in Chapter 3) and (b) multi-generational study (Figure 4.19 in Chapter 4) involving reconstituted microbiomes.

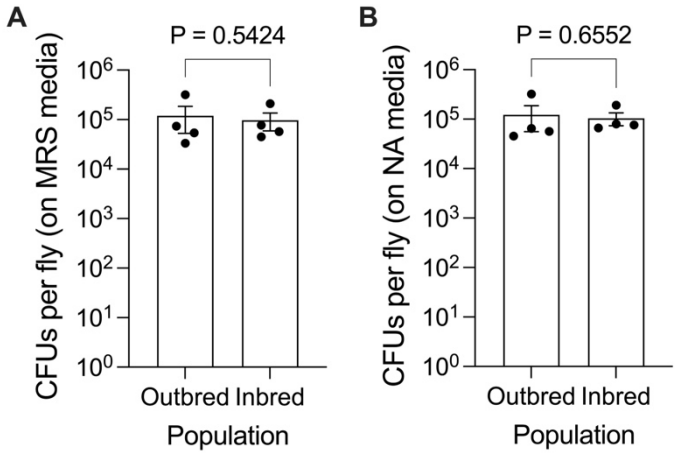

Figure 5.12 Bacterial load in outbred and inbred populations (with reconstituted microbiome) on the day of the assays on (A) MRS media (B) MRS media. The bacterial load in four outbred populations vs. four inbred populations were compared using paired t-test on block means. Block means are indicated by black filled circles. The error bar represents SEM.

5.5.2.3 PCR on 16S rRNA showed microbial presence in with-microbes outbred and inbred flies, while microbe-free outbred and inbred flies showed no microbial presence

Even though in the previous section we validated the status of the microbiome in with-microbes and microbe-free flies, it was with a culture-dependent method. There could be unculturable bacterial isolates present in microbe-free flies. To rule out that possibility, we

extracted gDNA from the flies and subjected it to 16S rRNA PCR using universal bacterial primers (Figure 5.13). The detailed method is described in section 2.2.4.2 of Chapter 2.

The with-microbe control consisted of 12 female block 1 flies (for both Outbred-NDB$_1$ and Inbred-NDB$_1$ populations). The female flies over all four blocks were pooled for the microbe-free set (3 flies per block, total 4 × 3 = 12 such flies per replicate per population). The PCR was done with 40ng of input DNA with 30 cycles.

The results from the PCR-based method agree with the fly homogenate plating, showing that the "with-microbes" flies harbor microbes, and "microbe-free" flies are indeed devoid of any microbe. This is shown for outbred and inbred populations (Figure 5.13).

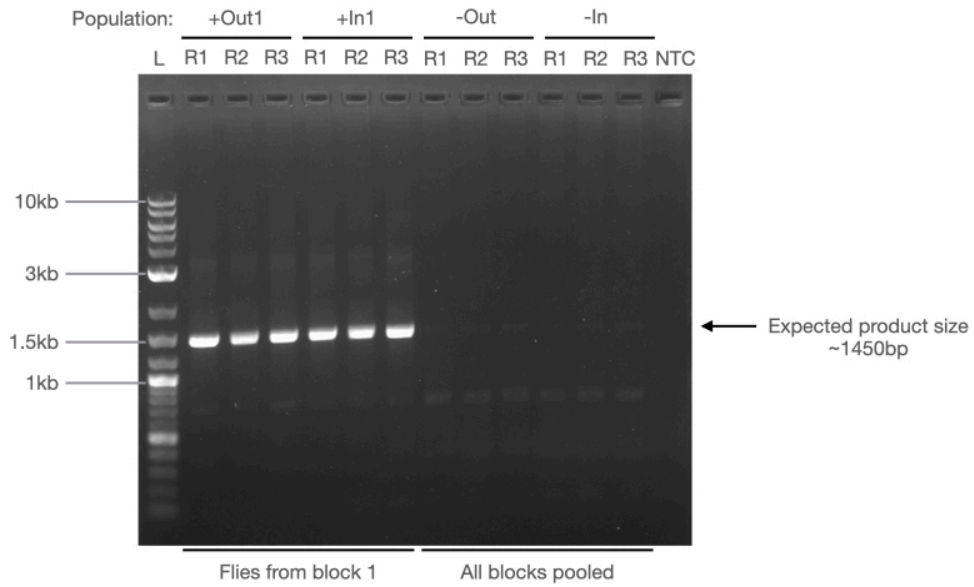

Figure 5.13 PCR using 16S rRNA gene universal primers to check microbiome status. **+Out1**: outbred flies from block 1 with-microbes, **+In1**: inbred flies from block 1 with-microbes, **-Out**: outbred microbe-free flies pooled from all four blocks, **-In**: inbred microbe-free flies pooled from all four blocks, **NTC**: no-template control. All the treatments had three biological replicates labeled R1, R2, and R3. The detailed PCR protocol is described in section 2.2.4.2 of Chapter 2.

5.5.2.4 Population-level amplicon sequencing of inbred-NDB$_1$ and outbred-NDB$_1$ assay flies shows similar bacterial composition

The 16S rRNA PCR using universal bacterial primers shows that both Inbred-NDB$_1$ and Outbred-NDB$_1$ "with-microbes" flies have microbes with them – but the composition of these flies cannot be deciphered using the PCR approach. We needed to make sure that both these populations have similar microbiome compositions that are reconstituted from their respective native stocks that are not manipulated in any way (discussed in section 5.2.2). To

check this, we did population-level 16S rRNA gene sequencing on gDNA extracted from 8-10 female flies per replicate per treatment.

We found that both the outbred and inbred flies whose microbiome was reconstituted from their respective native unmanipulated stocks showed similar microbiome profiles (Figure 5.14). This profile shows that both types of flies are dominated by *Acetobacter* spp. followed by the *Lactobacillus* spp. This observation also agrees with the results of our previous population-level sequencing efforts on reconstituted flies (Figure 3.8A in Chapter 3 and Figures 4.21 and 4.22 in Chapter 4) and on native NDB_{1-4} flies (Figure 2.2 in Chapter 2).

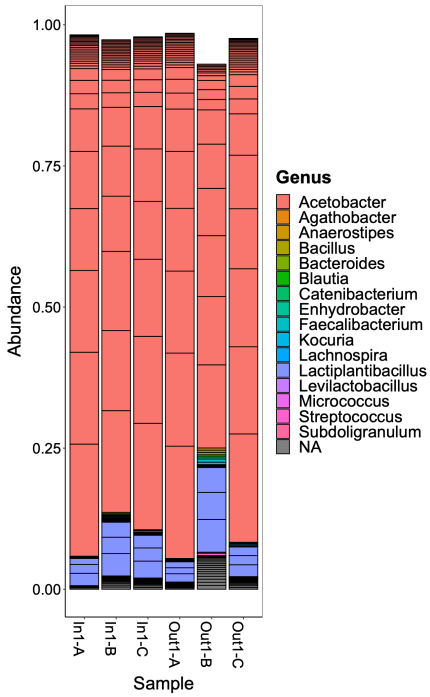

Figure 5.14 Validation of microbiome composition after reconstitution in inbred vs. outbred assay flies. The result of population-level 16S rRNA amplicon sequencing for Inbred-NDB_1 and for outbred-NDB_1 flies with-microbes. Most abundant 100 taxa are shown for each population. For each population, three independent replicates where processed (labelled "A", "B", "C"). Each replicate consisted of pooled DNA extracted from surface sterilized full body homogenates of 8-10 females. The DNA extraction method and sequencing data analysis are described in Chapter 2, method sections 2.2.4.2 and 2.2.7 respectively. In 2020, the old genus *Lactobacillus* was reclassified into 25 new different genera. *Lactiplantibacilus* and *Levilactobacilus* both belong to the old composite *Lactobacillus* genus.

Chapter 6
Conclusion, future perspective, and implications

6. Conclusion

Two decades of studies on the microbiota have led to the realization that there is hardly any biological process of the host that is not affected by the microbiota. This implies that, among many other consequences, our understanding of diseases and disorders of the hosts needs to be updated, as depending on the microbiome one has, the disease progression or even the effect of drugs treating these conditions might be different (Hou et al. 2022; Zimmermann et al. 2019). Also, as microbes emerge to be modulators of host physiology, they can influence the evolution of the hosts. Such possibilities are already discussed in Chapter 4. There is a growing awareness that interactions between host and microbiome genomes can be complex and ultimately influence host evolutionary trajectories (L. P. Henry et al. 2021; Macke et al. 2017). There are not many empirical studies investigating such possibilities.

In this context, testing various hypotheses related to host-microbiome interactions in suitable model systems becomes important. *Drosophila* has been a model of choice in these studies for the reasons we have already outlined in Chapter 1. The extant studies are concentrated on short timescales of a single host generation, most of these studies focus on a single trait in isolation (though there are a few studies on multiple traits, these are very few, e.g. (Gould et al. 2018)), and the strains used for these studies are mostly inbred. In this thesis, we have tried to address these lacunae in the literature.

We were primarily driven by the questions: if the microbiome is crucial for host function, then how will the host cope with the condition where the microbiome is taken away from the host? Will the short-term response over a single-host generation and the long-term response over multiple host generations be similar? What happens when we take the microbes away from the inbred host vs. the outbred host – will the difference in the available genetic variation of the host matter for the extent of the microbiome modulation of the host?

As discussed in the last section of the introduction of this thesis, these questions allow us to study the "host + microbe" unit carefully. Before we started the microbiome removal experiments to understand the "host + microbe" system, we first needed to understand the microbiome composition of our outbred flies and what factors affect this composition. This is needed because each host-microbiome system is unique and can have its own governing principles driven by specific strains present that are part of this system. Also, most microbiome studies are on inbred strains of *Drosophila* (Bost, Martinson, et al. 2018) – so there was little to go by when looking at the microbiome of laboratory-bred outbred flies.

In **Chapter 2**, using 16S rRNA sequencing, we characterized the microbiome of our outbred flies, and by plating fly homogenates, we looked at how it varies with age, sex, and diet. We found that the microbiome of outbred populations (NDB_{1-4}) that trace their ancestry to IV lines (Ives 1970) is simple and dominated by *Acetobacter* and *Lactobacillus* spp. (Figure 2.2). This is similar to the well-studied microbiome composition of inbred *Drosophila* strains (Erkosar et al. 2013). The microbiome composition of our lab flies isn't static, and the *Acetobacter* genus that is dominant in adult flies (day 12 after egg deposition) is almost undetectable in older flies (day 26 onwards) (Figure 2.4). While we have provided some clues on why such a composition shift might happen (section 2.4.2), in the future, we can investigate what are the consequences of such microbiome change - does the loss of *Acetobacter* help/harm the host, or is there no effect? Another question that can be asked is: Does *Acetobacter* fraction go down via active host control, or is it a consequence of a change in environmental factors beyond the host's control? In future studies, it will also be interesting to see if 16S rRNA sequencing across the host's lifespan validates this composition change via culture-independent techniques.

When we looked at the difference in total bacterial load in males vs. females via CFU counts, we observed that females had higher bacterial load than males on both MRS and NA media (Figure 2.5A and C). We thought this pattern could be explained by the higher body weight of females than the males. When we normalized the CFU counts by body weight, there was no statistical difference between the CFU counts (Figure 2.5B and D). Still, the effect sizes using Cohen's d remained large ($d > 0.8$) for the difference (Table 2.6), indicating that there might be interesting yet unknown aspects related to this trend.

Diet can be a major driver of host-associated microbiome compositions (Chandler et al. 2011; Erkosar et al. 2013; Téfit et al. 2018). To see if the microbiome of our outbred flies responds to changes in the host diet, we used a previously standardized host diet regime in our lab with a systematically varied Protein-to-carbohydrate (P:C) ratio (Mishra et al., unpublished results). The yeast formed the main protein source, while sugar was the major carbohydrate source in these diets. When we looked at the bacterial load in the form of CFU counts on these diets with the different P:C ratios, we saw that as yeast content in the diet reduced along with the concomitant increase in sugar, the total CFU count per fly reduced on both MRS and NA media (Figure 2.6A and B). This pattern agrees with the study by (Erkosar et al. 2018). In future studies, we would like to see whether the composition of the microbiome differs with

sex and with dietary manipulation of the P:C ratio via culture-independent techniques using 16S rRNA sequencing.

In the first half of **Chapter 3**, we adopted and standardized a method to look at the microbiome's contribution to the host phenotypes (Figures 3.1-3.3). We used egg dechorionation at 2% bleach for 2 min, followed by aseptic rearing to make flies without the microbiome (Figure 3.1). The corresponding control was also bleached to remove the egg microbiome, and the microbiome from a source population was reconstituted on the bleached eggs (Figures 3.6 and 3.7). Thus, the control consisted of "microbiome-reconstituted" flies. We ensured that the bleach used in making flies microbe-free did not become a confounding factor in two ways: 1. The control also received the bleach along with treatment, and 2. The experimental flies did not see the bleach in their lifetime as they were directly collected from the parents that either had the microbes (thus generating the control flies) or did not have any microbes (thus generating the treatment flies). See section 3.2.6 and Figure 3.9 for details of this experimental protocol. The status of the microbiome in control flies (labeled: "M+" flies, i.e., with-microbes flies) with the reconstituted microbiome and the microbe-free flies (labeled: "M-" flies) was checked using culture-dependent (Figure 3.4) and culture-independent techniques (Figure 3.5). Using 16S rRNA sequencing, we also showed that the microbiome composition of microbiome-reconstituted flies (Figure 3.8A) is similar to that of the native source microbiome of NDB_1 flies (Figure 2.2). The bacterial load in these flies was also similar (Figure 3.8B).

In the second half of **Chapter 3**, by comparing the phenotypes of "M+" and "M-" flies, we found that all the traits that we assessed (except the developmental time of males) were modulated by microbes (Figure 3.10 to 3.15 and Table 3.3). As many current studies are available on the inbred strains of *Drosophila*, we didn't know to what extent removal of the microbiome would change host phenotypes in our outbred flies. We only had a modest expectation of seeing only some traits being influenced by the microbes. However, the absence of a microbiome for over a single generation of the host changed multiple host traits, affecting host fitness. The major components of host fitness – the so-called life-history traits (body weight, egg-to-adult survival, and female fecundity) had taken a hit, demonstrating that the overall host fitness is reduced in the absence of a microbiome.

The developmental time hadn't changed a lot; for future studies, we can plan the same assay in poor nutrition diet as there are studies showing that the effect of the microbiome on host

development is substantial where the host is subjected to unbalanced diet conditions (Shin et al. 2011; Storelli et al. 2011; Yun and Hyun 2023).

We could also see that certain traits had sexually dimorphic effects after microbiome removal (desiccation resistance and locomotor activity) (Figures 3.13 and 3.14). We have speculated that altered life-history traits might have changed resource allocation patterns (e.g., protein and triglyceride levels) differentially in males and females. This could explain the sexual dimorphism in desiccation resistance. Thus, in future studies, we need to assay the macromolecule allocation in different sexes, with and without the microbes.

We saw that the reduced body weight of females was correlated with reduced female fecundity after microbiome removal, but the desiccation resistance of females was higher in spite of reduced body weight. It is possible that resources used for female fecundity (egg output in 12 hrs) in the presence of microbes are diverted to other body functions (such as desiccation resistance) in the absence of microbes. It'll also be interesting to see if reduced early-life female fecundity leads to a greater lifespan in females.

These findings showed that the microbiome is important to maintain host homeostasis and host fitness in outbred flies, and trait associations might change differentially for males and females depending on whether the microbes are present or absent. To the best of our knowledge, such studies using a microbiome reconstitution in the community form (discussed in detail in section 3.2.4) as a control and providing an integrated picture of trait associations that change in males and females of *Drosophila* upon microbiome removal are rare. In the future, we would like to complement organismal-level phenotype changes with RNA-Seq data to see what kind of gene expression alterations accompany these changes. We speculate that on such a short timescale, epigenetic changes might be involved in phenotype changes seen after microbiome removal (Pepke et al. 2024).

As multiple host traits were affected by microbiota in both sexes and host fitness was reduced without the microbiome, we expected that the absence of microbiota over multiple host generations would result in strong selection pressure on the host to increase its fitness, and the evolutionary signature might be seen in multiple different traits. This expectation was also based on past studies on host microbiomes in different taxa that have underscored the important role of microbiomes for the host (Rosenberg and Zilber-Rosenberg 2016, 2018).

In **Chapter 4,** we experimentally evolved *Drosophila* populations without their microbiome for 54 host generations (section 4.2.1). This was done using the bleaching protocol similar to

that used in Chapter 3 (section 4.2.2). This treatment had corresponding control where the host microbiome was reconstituted in every generation (section 4.2.3). The treatment flies were called MBL_{1-4} ("Microbiome-Less," selected population without microbes), and control flies were called MB_{1-4} (flies with reconstituted microbiome). MBL_{1-4} vs. MB_{1-4} phenotypes were assessed in two common environments: (a) with microbes and (b) microbe-free (section 4.2.5 and Figure 4.5). These populations were assessed at two time points: 1. Generations 17-20 and 2. Generations 54-57.

Although removing the microbes had far-reaching effects on the short timescale of a single-host generation, in the evolutionary experiment, the microbiota-less flies did survive for a long period of time (Chapter 3 results). At the time of writing this thesis, these populations have survived 90 generations without the microbes. This shows that the relationship that the microbiome has with the host isn't obligate when observed on the evolutionary timescale.

Contrary to our expectation, we saw very few and very small changes in the phenotypes of MBLs vs. MBs even after 54 generations of experimental evolution in both assay environments (Table 4.8 and 4.9). The only statistically significant change was increased egg-to-adult survival of MBLs in the microbe-free environment (Figure 4.11A). Typically, we have seen fast evolutionary responses (within 5-10 host generations) in our other experimental evolution lines dealing with other selection pressures. In this context, this observation of a slow and mild evolutionary response to the selection pressure of the absence of a microbiome when we clearly expected a stronger response was interesting. To understand these results, we have discussed multiple, potentially non-mutually-exclusive ways (section 4.4.1). We speculate that, among all these possibilities, the scenario of low functional integration between the host and microbes in our system is the most plausible. In such a case, the results of our short-term and long-term studies of microbiome removal can be reconciled by the evolutionary addiction hypothesis (Angela E. Douglas 2018a; T. J. Hammer 2023; Moran et al. 2019).

This hypothesis states that hosts might evolve partial dependence on microbes as the latter are ubiquitously present in the host's environment throughout the evolution of the host (Angela E. Douglas 2018a) (Moran et al. 2019). That is why, when the microbes are taken away from the host suddenly, the host might seem to be in an altered state. But, over time, there can be a new equilibrium without the microbes and without major changes due to long-term microbiome absence. We surmise that this is what we might have observed for our host-microbiome system – the initial sudden microbiome removal for over a single generation

altered the phenotypic state of the host, giving rise to "withdrawal symptoms" (T. J. Hammer 2023). But, over a longish timescale, there were no major costs or benefits observed without the microbiome, as seen in both assay environments.

If true, then the low functional integration between host and microbiome and the unfolding of a scenario like evolutionary addiction might indicate that this host-microbiome system may not be the tightly integrated one as usually expected from the "holobiont" perspective (Bordenstein and Theis 2015; Rosenberg and Zilber-Rosenberg 2018).

When we looked at how effect sizes for the evolutionary differences between phenotypes of MBLs and MBs have changed over time, we see that the effect size distribution is shifting from mostly "small and medium" effects in generations 17-20 to "small, medium, and large" effects in generations 54-57 (Figure 4.13). This shows that MBLs are still evolving. Therefore, it would be interesting to revisit the life history and behavioral differences between the MBs and the MBLs at a future time point.

When we quantified the phenotypic plasticity of MBLs vs. MBs over all the traits across two assay environments using two metrics: 1. Cohen's d and 2. percent change, we saw that MBLs, overall, had lower phenotypic plasticity (Figure 4.12). This might hint at MBLs getting less affected by the microbiome's presence/absence. In future studies, we can investigate if this lower phenotypic plasticity is limited to the presence/absence of the microbiomes or can be attributed to a general consequence of experimental evolution. For this, we can check this phenotypic plasticity in various environments, such as different temperatures, larval densities, diet regimes, etc. The lower phenotypic plasticity of MBLs was in line with expectation as the microbiome environment of MBLs is very stable as compared to MBs whose microbiome could change with factors such as host age or due to stochastic effects on community composition. The stable microbiome environment would be less conducive to high phenotypic plasticity (Berrigan and Scheiner 2004).

To see if the experimental evolution of *Drosophila* without the microbes has resulted in the differential expression of genes, we performed RNA-Seq on one population of MBLs (MBL_1) vs. one population of MB (MB_1) with three biological replicates each. This was done on female flies. We observed that there weren't many changes in the microbe-free environment (Figure 4.14), but there were several differentially regulated genes in the with-microbes environment (Figure 4.15 and Tables 4.10 and 4.11). A group of anti-microbial peptides (AMPs) was up-regulated in MBL_1, and a group of heat shock proteins (HSPs) was

downregulated. We also found a set of three differentially expressed genes that can interact with chitin, but their functions aren't known. One of these genes, CG7298, is annotated on FlyBase to be expressed in the proventriculus. This could be a site of interest as proventriculus could be a potential niche in the gut where bacteria stably interact with *Drosophila* (Dodge et al. 2023). Proventriculus has a chitinous layer called a peritrophic matrix (see section 4.4.3 for the detailed discussion) where our chitin-binding genes could be active. In the future, we want to investigate if this organ plays any role in the difference between MBs and MBLs. Also, we would like to verify all the RNA-Seq hits with RT-PCR.

We have a hypothesis on why flies selected without the microbes for multiple generations have an up-regulated immunity in the form of AMPs. As these flies have not encountered any microbes for 54 generations, it is possible that their ability to perceive gut microbes as native symbionts (and hence non-harmful) might be reduced. So, when the same native microbiome was provided to the MBLs and MBs, MBLs reacted to it as if it was a non-native microbiome, up-regulating the AMP response that can control the gut microbiome. We think the immunity up-regulation is similar to the immune response discussed broadly under the umbrella of the hygiene hypothesis/old friend hypothesis, where no exposure to microbes can result in miseducated immunity, further leading to allergic, inflammatory, or autoimmune reactions (Blackwell 2022). This possibility is discussed in detail in section 4.4.3. In future studies, we can give back the microbes to MBLs for multiple generations to see if that rescues the AMP up-regulation phenotype.

Another hypothesis that can explain the up-regulated AMP response and the chitin-interacting genes expressed in the peritrophic matrix is the leaky gut of MBLs. If the MBL's protective peritrophic matrix layer is thinned over the years owing to no exposure to the microbes, then that can leak the microbes on the non-gut side of the matrix (endoperitrophic space). This leak of microbes can also result in the up-regulated AMP response.

We also think that as heat shock proteins affect the stress response (Feder and Hofmann 1999; Sørensen et al. 2003) and longevity (Morimoto and Cuervo 2009; Tower 2011), MBLs might be affected due to the down-regulation of HSPs. In the future, we can check this by assessing different stress-related traits (such as heat and cold tolerance, the concentration of reactive oxygen species (ROS)), and the longevity of the MBLs.

In **Chapter 5**, we asked what happens to the microbiome's contribution to host phenotype when the inbred host has reduced genetic variation compared to their outbred counterparts.

The host's genetic variation is known to affect the microbiome composition (Blekhman et al. 2015; Bonder et al. 2016; Early et al. 2017; Goodrich et al. 2014; Jehrke et al. 2018; E. P. Ryu and Davenport 2022). Moreover. there are several candidate host genes that are involved in controlling the microbiome composition (Bahuguna et al. 2022; Dantoft et al. 2016; J.-H. Ryu et al. 2008), which can, in turn, affect the microbiome's contribution to various host phenotypes. Thus, in principle, inbreeding-induced loss of genetic variation can affect both these aspects. While in *Drosophila*, a study has shown that inbreeding reduces microbiome diversity (Ørsted et al. 2022), what happens to diverse traits of inbred hosts when the microbiome is completely removed is not known. The difference between the phenotypic values when the microbiome is present vs. absent is taken as the microbiome's contribution to that phenotype of the host (similar to what we did in Chapters 3 and 4).

For this, we generated four inbred populations from four baseline outbred populations via virgin full-sib mating for ten consecutive generations (Census size, N = 2), giving us the expected inbreeding coefficient of 0.88 (Falconer 1996). Then, for both of these populations (inbred vs. outbred), we created microbe-free flies and flies with reconstituted microbiomes (using a protocol discussed in Chapter 3, Figure 3.9).

Our working hypothesis was that the microbiome will play a greater role in determining the host traits when the host has a lower genetic variation to work with. We found that the effect sizes after microbiome removal were lower for inbred populations but not statistically significant (Figure 5.8A). Thus, the data we obtained here does not support our working hypothesis. When we partitioned the effect sizes on the overall traits as non-behavioral vs. behavioral, we found that while non-behavioral traits (life history and desiccation resistance) showed a difference in microbiome contribution in inbred vs. outbred populations (with large effect size) (Figure 5.8B), behavioral traits didn't change much (small effect size) (Figure 5.8C). The trend of overall lower effect sizes could be explained by higher variation in block means of inbred lines (Figure 5.9). When male traits, female traits, and pair-traits (where both sexes were assayed together) showed modulation by the microbiome in outbred lines, in inbred lines, only the female traits showed evidence of modulation by the microbiome (Table 5.2 and 5.3).

We have discussed in detail (section 5.4.2 of Chapter 5) what these results mean in the context of conservation efforts of inbred populations. We note here that as the effects of the microbiome on inbred hosts are context-specific, one should be cautious in deploying a microbiome community as a conservation measure.

Limitations of the model system

There has been a lot of enthusiasm related to the *Drosophila* model of microbiome research, particularly in terms of its potential to inform issues related to human-microbiota interactions. However, such enthusiasm needs to be tempered, as several limitations exist. First, flies lack adaptive immunity. Hence, studies done on flies might not assess adaptive immunity's role in host-microbe interactions, making direct comparison with the human condition difficult. Second, mammalian gut flora is complex, and many microbes need aerobic conditions to grow as opposed to fly gut flora that can be easily grown in normal oxic lab conditions. While this is good news for those who want to culture fly microbiota, how this fundamental difference influences applied studies, such as those involving microbiome-mediated drug metabolism, is unclear. Third, the *Drosophila* gut is a dynamic organ that can respond very differently to dietary and microbial challenges than the mammalian gut. Such considerations mean rules that govern host-microbe interactions in humans can be entirely new and contrary to what is expected from the fly studies (Angela E. Douglas 2018b).

Limitations of this study

While we used a community of microbes for our reconstitution that is closer to the native microbiome community of our flies, similar experiments can be done with a single microbiome species (e.g., Only *Acetobacter* spp. or only *Lactobacillus* spp.). Once we know what phenotypes are getting altered, one can look at the underlying mechanism by precise genetic manipulation of the bacteria (e.g., through mutant libraries) to see what genes or gene products of these bacteria are driving the host phenotype changes. We haven’t looked at any such underlying mechanism in this thesis.

In this study, we used only egg-dechorionation-based methods to generate microbe-free flies using bleach. It is possible to create the microbe-free flies using antibiotics. We haven’t confirmed our single-generation results with bleach-independent methods like antibiotics. We actively avoided antibiotics in our experimental evolution study, as the host would likely have responded to them, which would have introduced a confounding effect.

The RNA-Seq data on experimental evolution lines is based on only one block of MB-MBL out of a total of four blocks that we have. We would like to get RNA-Seq done on the rest of the three blocks. We also haven’t validated the RNA-Seq hits with RT-PCR.

It is possible to sequence the genomes of MB-MBLs to see if there is any divergence in their genomes with time. We haven't performed any sequencing on the fly hosts.

While immune-related phenotypes could be the interesting targets to show a response after microbiome manipulation, we were not able to see what happened to these phenotypes (e.g., survival after pathogen infection, the population of different immune cells in hemolymph) in the current study. Some of these assays are currently happening in the lab.

Final thoughts

In a nutshell, in this thesis, we studied the effects of microbiome removal on the short ecological timescale of over a single-host generation and on the evolutionary timescale of multiple-host generations. While the short-timescale removal of the native microbiome resulted in broad changes in host biology, contrary to our expectation, removing microbes for the extended period of 54 host generations didn't result in prominent evolutionary changes in the host. This might be due to weaker evolutionary pressure on the host and can be explained using the "evolutionary addiction hypothesis." This might also mean that, in the case of outbred *Drosophila* as the host, the "host + microbe" unit (as a whole) may not be well-integrated on evolutionary timescales as proposed by the hologenome theory of evolution.

This has implications for how host-microbe interactions are viewed and modeled. While in a given host, certain traits can be more affected by the microbes (e.g., egg-to-adult survival in our case), certain other traits may not show this functional integration with microbes (e.g., developmental time in our case) and, hence, remain unaffected after microbiome removal. So, instead of focusing on the entire "holobiont" as an independent entity, we think looking at the functional integration between host and microbes *trait-by-trait* might be of greater value to appreciate the microbiome's role in host evolution.

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