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GRADUATE SCHOOL OF ARTS AND SCIENCES

Dissertation

ROLE OF *WOLBACHIA* IN SHAPING THE MICROBIOME OF *DROSOPHILA MELANOGASTER*

by

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B. Tech., Indian Institute of Technology, Madras, 2010

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Doctor of Philosophy

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ROLE OF *WOLBACHIA* IN SHAPING THE MICROBIOME OF *DROSOPHILA MELANOGASTER*

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Boston University Graduate School of Arts and Sciences, 2018

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ABSTRACT

The endosymbiotic bacteria *Wolbachia* and the gut microbiome have independently been shown to affect several aspects of insect biology, including reproduction, development, lifespan, stem cell activity and resistance to human pathogens in insect vectors. This research shows that *Wolbachia,* which reside mainly in the fly germline, affect the microbial species present in the gut of a lab reared strain of *Drosophila melanogaster*. Fruit flies host two main genera of commensal bacteria – *Acetobacter* and *Lactobacillus*. *Wolbachia*-infected flies have significantly reduced titers of *Acetobacter*. Analysis of the microbiome of axenic flies fed with equal proportions of both bacteria shows that *Wolbachia*'s presence is a determinant in the microbiome composition throughout fly development. This effect of *Wolbachia* on the *Drosophila* microbiome is host genotype-dependent. To investigate the mechanism of microbiome modulation, the effect

of *Wolbachia* on Imd and ROS pathways, the main regulators of immune response in the fly gut was measured. *Wolbachia*'s presence did not cause significant gene expression changes of the effector molecules in either pathway. It was also found that *Wolbachia* slightly reduce the relative length of the acidic region of the gut. However, this observation lacks the robustness necessary to provide a mechanism for the significantly reduced *Acetobacter* levels. Furthermore, microbiome modulation is not due to direct interaction between *Wolbachia* and the gut microbes, as confocal microscopy shows that *Wolbachia* is absent from the gut lumen. These results indicate that the mechanistic basis of the modulation of microbiome composition by *Wolbachia* is more complex than direct bacteria interaction or *Wolbachia*'s effect on fly immunity. The findings reported here highlight the importance of considering the gut microbiome composition and host genetic background during *Wolbachia*-induced phenotypic studies and microbial based-disease vector control strategies.

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LIST OF ABBREVIATIONS

AMPs – Anti-Microbial Peptides

bp – base pair

BCAA – branched-chain amino acid

C – Celsius

Cad99c – Cadherin 99c

d – day

DI – de-ionized

DNA – deoxyribonucleic acid

Duox – Dual oxidase

FBS – fetal bovine serum

FISH – fluorescence in situ hybridization

Flag – Flag tag

h – hour

HCl – hydrochloric acid

Hh – Hedgehog signalling pathway

HOCl – hypochlorite

hPABP – human poly-A binding protein

Imd – Immune deficiency pathway

imd – Immune deficiency protein

kDa – kilo daltons

min – minutes

NaOH – sodium hydroxide

PBANG – PBT + 0.2% BSA + 5% normal goat serum + 0.005% sodium azide

PBS – phosphate buffered saline

PBT – PBS + 0.2% Triton X-100

PCR – polymerase chain reaction

PFA – paraformaldehyde

PM – peritrophic matrix

PQQ-ADH – pyrroloquinoline quinone-dependent alcohol dehydrogenase

qPCR – quantitative PCR

qRT-PCR – quantitative reverse transcription PCR

Rel – Relish

RNA – ribonucleic acid

RNAi – RNA interference

ROS – reactive oxygen species

RPKM – reads per kilo base of gene per million reads

rRNA – ribosomal RNA

SDS – Sodium Dodecyl Sulfate

SSC – Saline-Sodium Citrate

TOR – Target Of Rapamycin

UAS – upstream activating sequence

VNTR – variable number tandem repeats

wMel – *Wolbachia* strain present in *Drosophila melanogaster*

yeast – *Saccharomyces cerevisiae*

CHAPTER 1

Introduction

Animal-microbe interactions are ubiquitous and microbes play a pivotal role in host development, physiology, immunity, reproduction and evolution (McFall-Ngai et al., 2013, Dale and Moran, 2006, Gilbert et al., 2012). Interactions between animals and microbes can range from beneficial to detrimental. While mutually beneficial interactions are based on communication between the host the microbe, the detrimental interactions arise when the pathogens subvert this communication for their own benefit (Hughes and Sperandio, 2008, Fischbach and Segre, 2016). Communication between microbes within a host is an equally important component in understanding host-microbe interactions (Coyte et al., 2015), (Marx, 2014).

1.1 Insects and their microbiota

Insects are the most abundant and diverse clade of organisms in the animal kingdom (Engel and Moran, 2013, Basset et al., 2012). A wide assortment of environmental habitats, diet, developmental stage and physiological characteristics of insects make their guts a unique microenvironment that can support a tremendous variety of microbes (Engel and Moran, 2013, Yun et al.,

2014, Hooper and Gordon, 2001). The variety of interactions between insects and microbes contributed to their evolutionary success (Yun et al., 2014, Engel and Moran, 2013). Numerous examples of the beneficial nature of microbial-host interactions exist. Many plant sap feeding Hemipterans have developed obligate symbiosis with bacteria. The plant sap is sugar rich, but a poor nitrogen source, and the obligate symbionts provide the host with essential amino acids in return for a safe residence (Douglas, 2006). Insects that feed on plant and woody material harbor cellulose-degrading microbial communities (Anand et al., 2010, Warnecke et al., 2007). Microbes also play protective roles against pathogens of the host (Koch and Schmid-Hempel, 2011, Koch and Schmid-Hempel, 2012, Ayres and Schneider, 2012).

In extreme cases, beneficial microbes become intracellular, and are maternally transmitted. These heritable symbionts can be obligate or facultative. Obligate endosymbionts live in the cytosol of specialized host cells such as bacteriocytes and provide the host with essential nutrients (Baumann, 2005). The bacteria and host coevolve for extended periods of time and tend to lose large portions of their genome that are not essential for provisioning the host with nutrients (Baumann, 2005). On the other hand, facultative endosymbionts are not required by the hosts. They are maternally transmitted, but can undergo

horizontal transmission events which forces the symbionts to retain a larger and more dynamic genome (Werren et al., 2008, Degnan et al., 2009).

One of the most commonly found endosymbiotic bacteria of insects are *Wolbachia*. Depending on the insect host, *Wolbachia* range from being obligate to facultative endosymbionts (Werren et al., 2008).

1.2 *Wolbachia*, a global pandemic in invertebrates

Wolbachia infect about 40%-70% of all arthropods making them the most abundant intracellular symbionts on earth (Hilgenboecker et al., 2008, Zug and Hammerstein, 2012). Since *Wolbachia* are transmitted both vertically and horizontally, they are capable of coevolving with a single host species for millions of years forming obligate mutualistic relationships, as well as forming pathogenic short-term relationships in relatively newer hosts (Werren et al., 2008). However, horizontal transmission events are rare and have been documented only in a couple of instances in a lab setting (Huigens et al., 2004, Frydman et al., 2006), and the primary mode of transmission is from the mother to the offspring. The vertical transmission of *Wolbachia* stems from their ability to robustly colonize the germline of their host species. Several studies show tropism to certain niches in and around the reproductive organs that facilitate *Wolbachia*'s entry into the germline (Frydman et al., 2006, Fast et al., 2011, Toomey et al.,

2013, Toomey and Frydman, 2014, Riparbelli et al., 2007, Clark et al., 2002, Clark et al., 2003, Hosokawa et al., 2010, Genty et al., 2014, Zouache et al., 2009, Sacchi et al., 2010, Taylor et al., 2005).

A key feature of *Wolbachia* is their ability to manipulate reproductive processes in their host. Depending on the host genetic structure, cytoplasmic incompatibility, parthenogenesis induction, feminization, and male killing are the four most common reproductive phenotypes induced by *Wolbachia* in their host (Werren et al., 2008). Apart from these reproductive manipulations, *Wolbachia* can also alter several aspects of the host biology, including lifespan (Chrostek and Teixeira, 2015, Min and Benzer, 1997), fecundity (Fast et al., 2011, Zhao et al., 2013, Caragata et al., 2014), immunity (Wong et al., 2011b, Moreira et al., 2009, Kambris et al., 2009, Bian et al., 2010b, Kambris et al., 2010b), metabolism (Molloy et al., 2016, Kremer et al., 2009, Nikoh et al., 2014, Moriyama et al., 2015, Dobson et al., 2015), and stem cell activity (Fast et al., 2011).

Wolbachia provide the host with fitness advantages such as resistance to certain positive strand RNA viruses (Hedges et al., 2008, Teixeira et al., 2008, Osborne et al., 2012, Martinez et al., 2014). This phenotype has been utilized in controlling vector transmitted diseases such as dengue, chikungunya, and malaria (Hoffmann et al., 2011, Walker et al., 2011, Bourtzis et al., 2014, Kambris

et al., 2009, Moreira et al., 2009, Bian et al., 2010b, Glaser and Meola, 2010, Kambris et al., 2010b, Hughes et al., 2011a, Pan et al., 2012, Wong et al., 2011b, Rances et al., 2012, Chrostek et al., 2013). Another area of disease control is filarial diseases such as river blindness and filariasis are caused by parasitic nematodes. These disease-causing agents are in an obligate symbiotic relationship with a strain of *Wolbachia*. Instead of treating the disease with the current regimen of a year-long anti-worm medication, a much shorter dose of tetracycline class of compounds to eliminate *Wolbachia* from these nematodes is very effective (Hoerauf et al., 2000, Taylor et al., 2005, Slatko et al., 2010).

Many of the phenotypic studies discussed here have been performed in a wide range of insects. However, owing to ease of maintenance, availability of a tremendous range of genetic tools, and experimental tractability, most of the current knowledge of the molecular and cellular interactions of *Wolbachia* and their hosts comes from the *Drosophila* model system.

1.3 *Wolbachia* in *Drosophila*

Wolbachia are facultative endosymbionts in *Drosophila* species. They are vertically transmitted and infect the germline robustly (Werren et al., 2008). *Wolbachia* grow to high densities in the germline. Depending on the fly species, a large fraction of the *Wolbachia* present in the germline localizes to the oocyte by

mid-oogenesis (Veneti et al., 2004, Ferree et al., 2005). Further, the fifteen nurse cells which support the development of the oocyte also support *Wolbachia* to a high density. Post mid-oogenesis, the nurse cells dump their cytoplasm into the oocyte to provide essential nutrients required for the nourishment of the embryo. In the cases where *Wolbachia* does not localize to the oocyte by this stage, the dumping process ensures that *Wolbachia* stream in to the oocyte and colonize it heavily (Veneti et al., 2004). In a fully mature oocyte, *Wolbachia* are uniformly distributed throughout its entire volume.

Upon fertilization of the oocyte, pole cells are the first set of cells to form that eventually give rise to the germline stem cells in the developing embryo. In several *Drosophila* species, *Wolbachia* reliably colonize pole cells to ensure that the developing offspring faithfully pass on *Wolbachia* to successive generations (Hadfield and Axton, 1999, Serbus and Sullivan, 2007, Veneti et al., 2004). Since *Wolbachia* are also present throughout the developing embryo, they colonize several somatic tissues such as the gut, brain, fat bodies, and malpighian tubules as the host develops (Fig. 1.1) (Pietri et al., 2016). Figure 1.1 illustrates *Wolbachia* infection in the gut cells of a larva.

Another fail safe mechanism to ensure *Wolbachia*'s transmission to the offspring is their tropism to the stem cell niches. *Wolbachia* colonize the somatic

and germline stem cell niches in the ovaries to very high densities. Since these cell types are in direct contact with the germline and are crucial for the development of the oocyte, this tissue tropism has been implicated as a contributor to the near perfect infection of all offspring (Toomey and Frydman, 2014, Toomey et al., 2013, Fast et al., 2011, Frydman et al., 2006).

Wolbachia infection in fruit flies has been shown to affect a wide range of phenotypes mentioned in the section above, including protection to the host from positive strand RNA viruses, lifespan, fecundity, stem cell activity, apoptosis, insulin signaling, and metabolism. Each of these phenotypes are described in detail below.

1. Protection from positive-strand RNA viruses

Wolbachia-infected *Drosophila melanogaster* flies are resistant to several natural pathogenic positive-strand RNA viruses such as *Drosophila* C virus, Nora virus, and Flock House virus. *Wolbachia* confer higher survival rates compared to their *Wolbachia*-free counterparts (Teixeira et al., 2008, Hedges et al., 2008). Several human pathogenic viruses that are carried by mosquitoes such as West Nile virus, Chikungunya virus, and La Crosse virus can also be harbored by *Drosophila melanogaster*. *Wolbachia*-infected flies can efficiently eliminate viral infection compared to the *Wolbachia*-free flies (Glaser and Meola, 2010). Further,

this *Wolbachia*-mediated anti-viral protection extends to other *Drosophila* species such as *D. simulans* (Osborne et al., 2009).

2. Fecundity, stem cell activity and apoptosis

In *Drosophila mauritiana* flies, *Wolbachia* infection results in a 4-fold higher egg production. The increased fecundity was linked to lowered programmed cell death and increased stem cell division during oogenesis (Fast et al., 2011). The germline stem cells are housed by the germline stem cell niche, and this tissue is heavily infected by *Wolbachia*. Developmentally, *Wolbachia* accumulate in the niche and can potentially coordinate the replication and differentiation of the stem cells (Fast et al., 2011). Further, there are checkpoints in oogenesis where the developing egg chambers can undergo programmed cell death under unfavorable environmental conditions. *Wolbachia*-infection reduces the programmed cell death in the germarium by half. Taken together, the increased stem cell activity, and the reduced programmed cell death results in higher fecundity of *Wolbachia*-infected flies (Fast et al., 2011). However, this increased fecundity is subject to variation due to factors such as the host genotypes and the microbiome (Frydman lab, unpublished).

3. Insulin signaling and metabolism

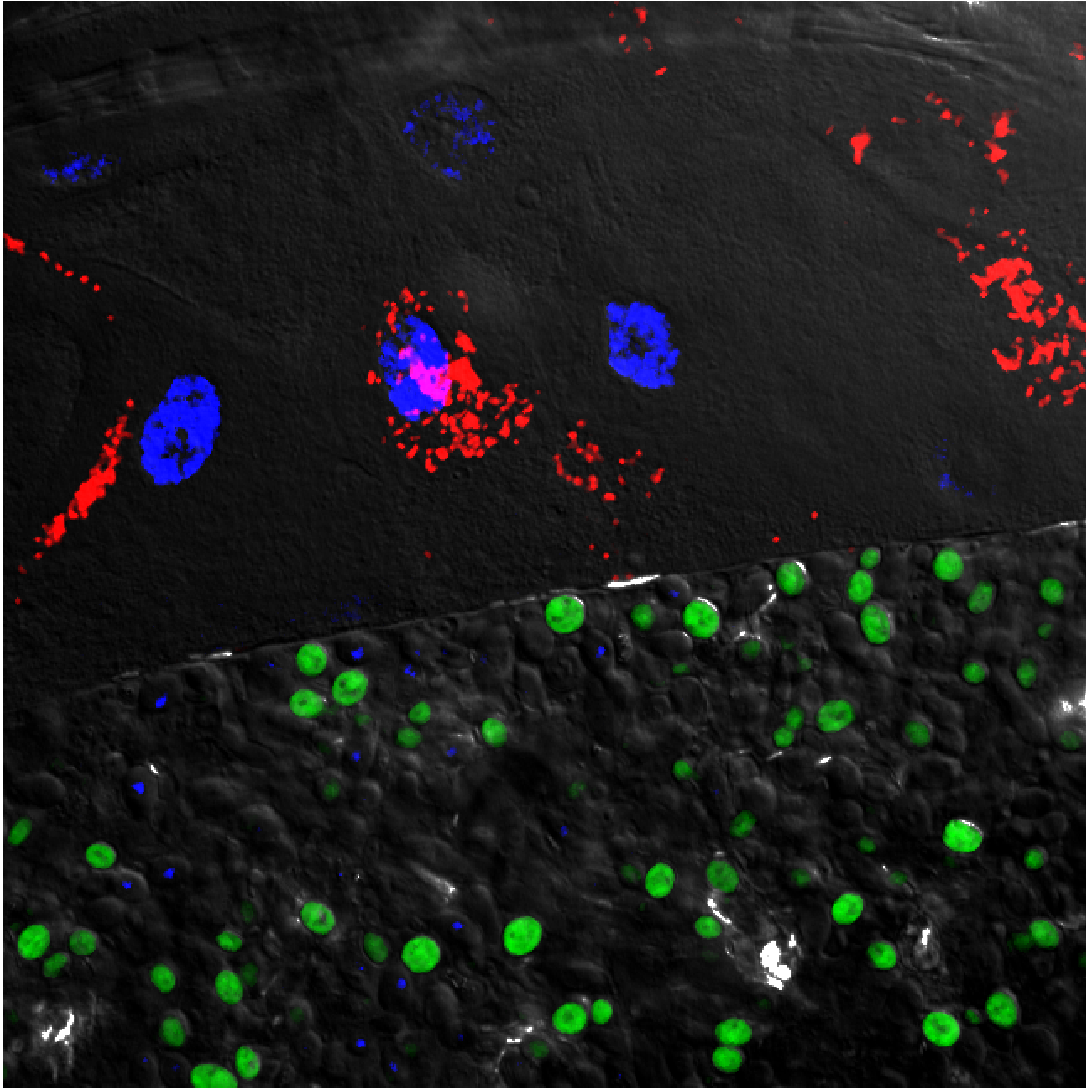
Insulin signaling in flies has pleiotropic effects on several host processes such as growth, development, metabolic homeostasis, lifespan, resistance to stress, and fecundity (Ikeya et al., 2009). Insulin signaling is regulated by a variety of factors to match the energy demands of the host. Insulin mutants are characterized by reduced growth and fecundity, and increased triacyl glycerides (Ikeya et al., 2009). However, *Wolbachia* infection in insulin mutants significantly rescues the mutant phenotype. While the *Wolbachia*-free mutant flies laid about 2 eggs/female/day, *Wolbachia*-infected flies laid 28 eggs/female/day. *Wolbachia*-infected mutant female flies also weighed more and lived longer compared to their uninfected counterparts (Ikeya et al., 2009).

However, this study does not consider the contribution of the gut microbiota that might have been closely associated with the presence of *Wolbachia* in the flies. Several reports suggest that the gut microbiota such as *Acetobacter* (Shin et al., 2011) and *Lactobacillus* (Storelli et al., 2011) species can affect insulin signaling and alter several phenotypes measured in the above study.

There is mounting evidence suggesting that the gut microbiota of *Drosophila* can alter some of the phenotypes mentioned above, it's important to understand the relative contributions of both *Wolbachia* and the gut microbiota

towards these phenotypic alterations. It is also possible that *Wolbachia* affect the functional composition of the microbiota to give rise to some of these phenotypes. To untangle these two variables, we need to first understand the microbiome of the flies and the anatomy of the fly gut.

Figure 1.1: *Wolbachia* in somatic tissues of *Drosophila melanogaster*



Wolbachia shown in red is completely intracellular in the gut epithelial cells (Top, nuclei shown in blue). The epithelium is juxtaposed to the gut lumen containing green auto fluorescent food particles (bottom). Image is an overlay of several confocal sections of midgut stained by FISH probes against *Wolbachia* and DAPI against nuclei.

1.4 Microbiome of *Drosophila*

The gut microbiome of *Drosophila* is well characterized and consists of about 5-20 bacterial species. Flies collected in the wild have a higher diversity of microbiome compared to lab reared flies. The gut bacteria are constituted primarily by bacteria in *Acetobacteraceae*, *Lactobacillaceae*, *Enterococcaceae*, and *Enterobacteriaceae* families (Chandler et al., 2011, Corby-Harris et al., 2007, Broderick and Lemaitre, 2012, Cox and Gilmore, 2007, Ren et al., 2007b). However, *Acetobacter* and *Lactobacillus* are the most commonly found genera irrespective of the growth conditions of the flies (Shin et al., 2011, Lee and Brey, 2013). Bacteria from both the genera are culturable in vitro. Gnotobiotic flies can be generated easily by associating a predetermined composition of the gut bacteria from culture with axenic embryos generated by surface sterilization (Ridley et al., 2013, Bakula, 1969). These gnotobiotic flies greatly facilitate studies that aim at uncovering the functional role of microbiota on host biology.

Gut microbiota have been shown to affect several phenotypes in the fly. A few examples are listed below.

1.4.1 Development

Lactobacillus and *Acetobacter* species have a profound role in the development of flies (Chaston et al., 2014, Storelli et al., 2011, Shin et al., 2011).

The contribution of the microbes in aiding host development is amplified under poor diet conditions (Shin et al., 2011). Axenic larvae fail to develop in a low protein diet. However, association of the larvae with *Acetobacter pomorum* or *Lactobacillus plantarum* was enough to restore their development into adults (Storelli et al., 2011, Shin et al., 2011). The mode of action of developmental rescue by both *A. pomorum* and *L. plantarum* relies on provisioning limiting nutrients and promoting the nutrient sensing and allocation pathways in the host. In *A. pomorum*, a genome wide screen was performed to show that the periplasmic oxidative respiratory chain initiated by pyrroloquinoline quinone-dependent alcohol dehydrogenase (PQQ-ADH), and secondary metabolites of this pathway, such as acetic acid are essential for host development (Shin et al., 2011). *L. plantarum* on the other hand promote protein assimilation from diet by optimizing diet-derived branched-chain amino acids (BCAA) in the hemolymph. Target of Rapamycin (TOR) is activated in the fat bodies in response to BCAAs, and promotes systemic insulin-signaling activation (Storelli et al., 2011). These two studies have been validated by a metagenome-wide association study of various bacteria and their effects on the host (Chaston et al., 2014).

1.4.2 Immunity

Gut epithelial immune response must tolerate the presence of commensal bacteria, or else risk chronically active immune activation and inflammation which are deleterious to the host. Therefore, a very tight regulation of the pathway that provides tolerance to the beneficial microbes has evolved (Lemaitre and Hoffmann, 2007, Davis and Engstrom, 2012, Royet, 2011). *Drosophila* lack an adaptive immune system; hence the innate immune system must be able to identify and differentiate between pathogen and commensal species.

1.4.2.1 Primer on *Drosophila* gut immunity

There are three major components of the innate immune system in the gut: (1) Physical barriers such as the peritrophic matrix (PM) (Kuraishi et al., 2011, Lehane, 1997, Hegedus et al., 2009), mucus (Syed et al., 2008, Buchon et al., 2009b), and epithelial integrity (Bonnay et al., 2013, Hegan et al., 2007), (2) Imd signaling and Anti-Microbial Peptide (AMP) production (Tzou et al., 2000), and (3) Reactive Oxygen Species (ROS) production by Duox (Ha et al., 2005).

1. Physical barriers

The peritrophic matrix is a grid-like structure primarily constituted by chitin polymers and peritrophins (Lehane, 1997, Hegedus et al., 2009). PM forms

the first line of defense by preventing direct contact of bacteria and their toxins (Lehane, 1997, Kuraishi et al., 2011) with the epithelium (Fig. 1.2).

Below the PM is the mucus layer, which lines the entire midgut (Vodovar et al., 2005). Although the genes encoding mucin-like proteins have been identified, the functional role of the proteins is unknown (Syed et al., 2008). However, it is known that during infection, the genes encoding for PM and mucus proteins are differentially regulated suggesting their role in fighting pathogen invasions (Buchon et al., 2009b). Finally, the enterocytes form a barrier that prevents the pathogen entry into the circulatory system of the fly as the last line of physical defense. Mutants that have defective septate junctions between the enterocytes (Bonnay et al., 2013) or the brush border (Hegan et al., 2007) have a higher susceptibility to bacterial infections.

2. Imd pathway

Two major pathways involved in the production of AMPs in the body cavity of flies are the Imd and Toll pathways (Lemaitre and Hoffmann, 2007). Of these, the Imd pathway is responsible for controlling AMPs in the midgut region (Fig. 1.3) (Buchon et al., 2009b, Tzou et al., 2000, Ryu et al., 2006).

The Imd pathway is activated by the peptidoglycans released by gut bacteria. PGRP-LC and PGRP-LE are the receptors that bind to DAP-type

peptidoglycan found in gram-negative and some gram-positive bacteria, and activate the Imd pathway (Neyen et al., 2012, Leulier et al., 2003, Bosco-Drayon et al., 2012, Takehana et al., 2002, Kaneko et al., 2004). Both *Acetobacter* and *Lactobacillus* species have the DAP-type peptidoglycan (Buchon et al., 2009a, Ryu et al., 2008, Lhocine et al., 2008). Imd is the adaptor protein that transduces the signal from the receptors to the effector molecules in the cytoplasm (Lemaitre et al., 1995, Corbo and Levine, 1996). The subsequent activation of Dredd, a caspase that cleaves the inhibitory ANK domain of the inactive transcription factor Rel leads to its activation and nuclear translocation (Leulier et al., 2000, Stoven et al., 2000). The active Rel then transcribes multiple AMPs which are then released into the luminal space containing the gut microbiota. *Drosophila* AMPs are small cationic proteins that exhibit a broad range of activities against bacteria and fungi, while those activated by the Imd pathway are highly effective against gram-negative bacteria (Imler and Bulet, 2005). To make sure that the Imd pathway derived AMP response is not chronic, there are several negative regulators of the pathway. PGRP-LB and PGRP-SC are efficient amidases that convert peptidoglycans into non-immunostimulatory fragments (Mellroth et al., 2003, Zaidman-Remy et al., 2006, Mellroth and Steiner, 2006, Bischoff et al., 2006). PGRP-LF (Maillet et al., 2008) and Pirk (Lhocine et al., 2008, Kleino et al., 2008,

Aggarwal et al., 2008) bind to the receptors PGRP-LC and PRGP-LE respectively, to reduce their peptidoglycan sensing ability. Another layer of regulation is provided by *Caudal* which controls the transcriptional activity of Rel by competitively binding to the promoter regions of the Rel responsive genes (Ryu et al., 2008). Together, the Imd pathway provides a robust response to pathogenic invasion, but has regulatory mechanisms to provide tolerance to commensal gut microbiota.

3. Reactive Oxygen Species

ROS response to pathogens in the gut is primarily mediated by the Dual oxidase (Duox) pathway (Ha et al., 2005, Bae et al., 2010, Lee et al., 2015, Geiszt et al., 2003, El Hassani et al., 2005). The NADPH oxidase domain of Duox produces extracellular H_2O_2 that is then converted into hypochlorite (HOCl) by the extracellular peroxidase homology domain in the gut mucosa (Ha et al., 2005). Pathogen derived uracil induces Hedgehog (Hh) signaling, which is required for intestinal expression of Cadherin 99c and formation of Cad99C-dependent formation of endosomes. These endosomes act as signaling platforms for PLCβ/PKC/Ca2+-dependent DUOX activation (Lee et al., 2015). The ROS produced by Duox are effective against gut pathogens, but are also cytotoxic to

the host cells. Immune Regulated Catalase (IRC) is expressed in flies in response to infection-induced ROS to protect the host (Ha et al., 2005).

1.4.2.2 Effect of microbiome on Immunity

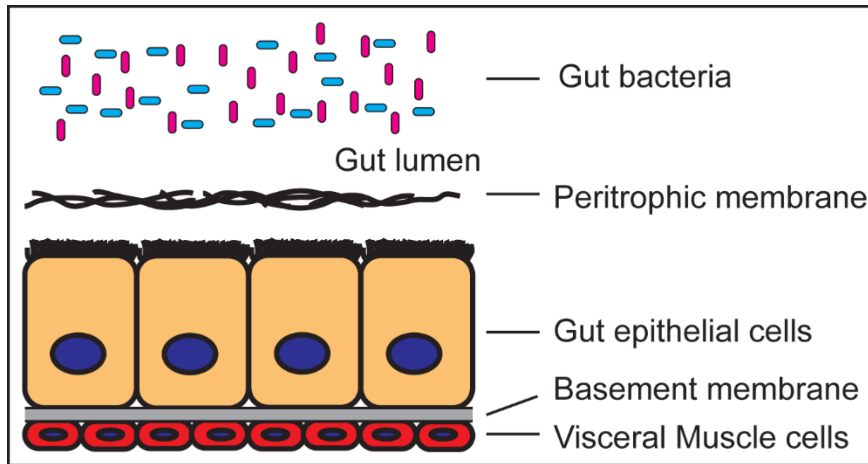
Bacteria are constantly ingested along with food, and in the gut, they are not in direct contact with the gut epithelium, but are contained within a chitinous membrane called the peritrophic matrix (PM) (Ren et al., 2007b, Ryu et al., 2008, Buchon et al., 2009a, Paredes et al., 2011). Bacterial derived molecules such as free peptidoglycans can diffuse past the PM and act as a substrate to activate the Imd pathway. However, the Imd pathway is not perpetually active as the presence of the microbiota also activates the negative regulators of the pathway such as Pirk (Lhocine et al., 2008, Kleino et al., 2008, Aggarwal et al., 2008), PGRP-LF (Maillet et al., 2008), PGRP-SC (Paredes et al., 2011), and PGRP-LB (Zaidman-Remy et al., 2006). Further, the presence of microbes also activates Cad, a repressor of transcription of the AMPs (Ryu et al., 2008). The basal level of AMP expression in axenic flies is lower than in conventionally reared flies with gut microbiota, but not enough to cause prolonged inflammation.

The ROS responses are critical in eliminating harmful pathogens in the gut (De Deken et al., 2000, Bae et al., 2010). Interestingly, peptidoglycans are unable to activate the DUOX pathway to generate ROS (Bae et al., 2010). This ensures

that ROS are not produces chronically in response to commensal microbes that are part of the regular diet. Instead, the pathway is activated by the presence of free uracil, a hallmark of many pathogenic bacteria (Lee and Brey, 2013, Ha et al., 2009).

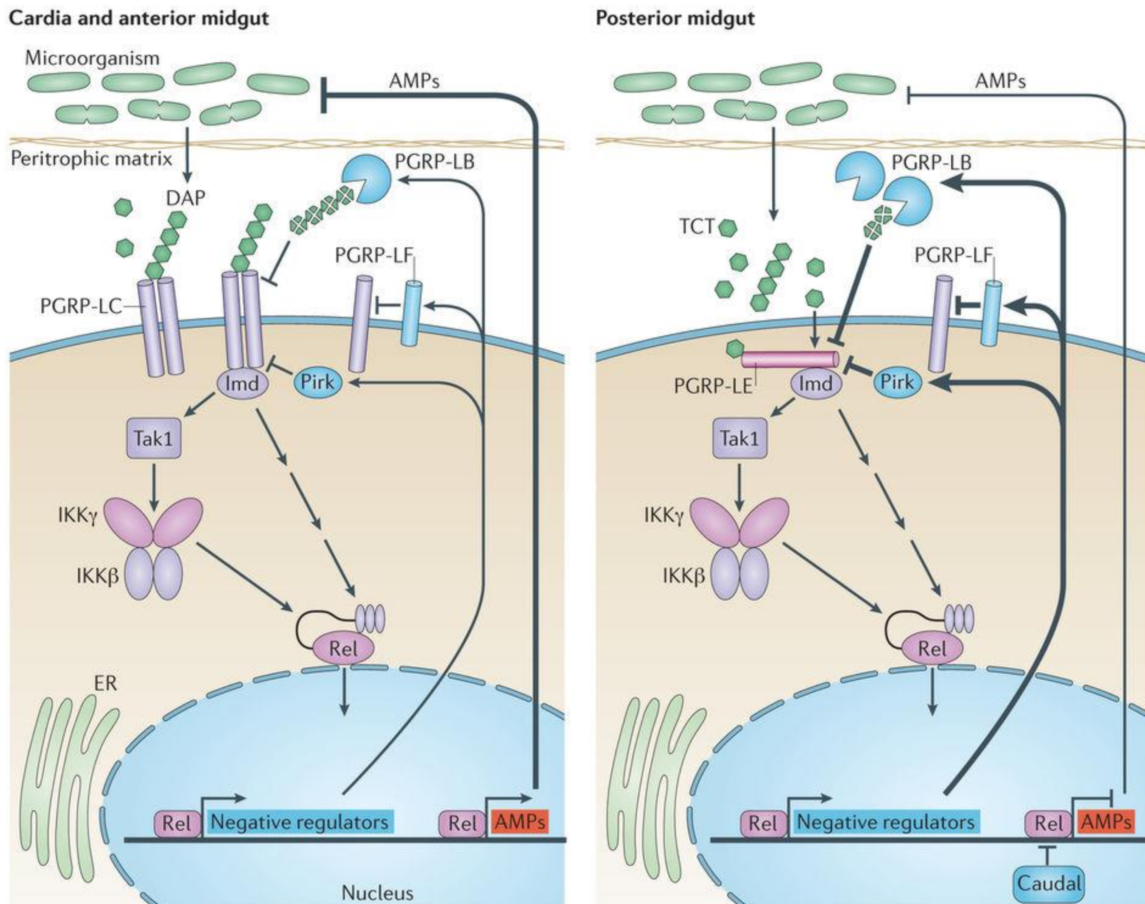
Gut commensal bacteria also play a protective role against certain pathogens like *C. albicans*, and promote the survival of larvae. This protective effect is independent of the Imd pathway, and could be a direct consequence of competition between the bacteria for nutrients and space (Glittenberg et al., 2011).

Figure 1.2: Cellular outlay of the *Drosophila melanogaster* gut



The cross section of the gut showing a layer of epithelial cells supported by the basement membrane and visceral muscle cells. The epithelium is protected from the contents of the gut lumen by a very thin porous chitinous layer called the peritrophic matrix. The commensal bacteria that the fly ingests do not come into direct contact with the epithelium as they cannot penetrate the peritrophic matrix.

Figure 1.3: Imd pathway in *Drosophila melanogaster*



Immune deficiency pathway is a key component of the response to infection in the fly gut. This pathway is basally activated by the gut microbiota and ingested microorganisms, and strongly induced by microbial infection. In addition, it has a demonstrated regional specificity depending on the tissue responding to infection (cardia and anterior midgut (left) versus posterior midgut (right)).

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1.5 *Drosophila* gut physiology

The *Drosophila* gut is a tubular organ that consists of a layer of epithelial cells, lined by visceral muscles, nerves and trachea (Fig. 1.2). There are three major cell types in the epithelial layer – Intestinal stem cells (ISCs), enterocytes (ECs), and enteroendocrine cells (EEs) (Ohlstein and Spradling, 2006, Micchelli and Perrimon, 2006). The ISCs are largely quiescent, but efficiently renews the midgut epithelium over two-three weeks. ISC activity is influenced by environmental factors such as pathogenic invasion and dietary conditions (Broderick et al., 2014, Buchon et al., 2013a, Buchon et al., 2009a, Buchon et al., 2009b). ECs constitute most of the gut epithelium. They are large polyploid cells that absorb nutrients and release digestive enzymes. EEs are relatively smaller than ECs and are less abundant. The precise role of EEs is unknown, but they broadly function in regulating peristalsis and fluid retention along with the enteric neurons (Lemaitre and Miguel-Aliaga, 2013).

The gut can be broadly divided into three regions – foregut, midgut, and hindgut based on their developmental origins. The midgut can be further divided into six regions based on their distinct metabolic and digestive functions. These six regions are separated by narrow epithelial boundaries and are controlled by anatomically distinct set of muscles. In addition, adult foregut also

consists of the crop which acts as a storage organ (Marianes and Spradling, 2013, Buchon et al., 2013b).

The digestive capabilities of the gut are compartmentalized and the pH of the region of the gut plays a major role in its function. The neutral pH of the anterior midgut is followed by an acidic zone that corresponds to the copper cells. The middle midgut is neutral and the posterior midgut is more alkaline. Like the mammalian stomach, the acidic region of the midgut aids in denaturing and degrading proteins and facilitates absorption of lipids and metals. The posterior regions of the gut aid in absorption of carbohydrates that have been previously broken down by digestive enzymes (Buchon et al., 2013b, Marianes and Spradling, 2013).

1.6 Dissertation rationale

The microbiota of *Drosophila* plays an important role in its development and homeostasis. The microbiota mainly consists of commensal gut bacteria such as *Acetobacter* and *Lactobacillus* species, and endosymbiotic bacteria *Wolbachia*. Growing evidence shows that both *Wolbachia* and gut microbiota affect several physiological functions in flies. However, there are few studies on the interaction of *Wolbachia* and the microbiome, and whether this interaction has functional consequences on host health.

The first reported interaction between *Wolbachia* and the microbiome was observed in *Anopheles* mosquitoes(Hughes et al., 2014). *Wolbachia* do not infect these mosquito species naturally, and *Wolbachia* injections into these mosquitoes to create stably infected lines that carry *Wolbachia* were unsuccessful. Hughes *et. al* have shown that *Asaia*, acetic acid bacteria found in these mosquitoes impede the successful colonization of *Wolbachia*. After removing the gut microbiota including *Asaia*, *Wolbachia* could infect the germline to be stably passed on to the offspring.

Here, whether *Wolbachia* can affect the composition of the gut microbiome is investigated (Chapter 3). Further, mechanistic bases for any interaction between the components of the fly microbiome is explored (Chapter 4). Finally, the phenotypic contributions of *Wolbachia*, and of each of the components of the microbiome on the developmental rate of flies is studied (Chapter 5).

CHAPTER 2

Materials and Methods

2.1 Fly husbandry

All fly stocks used were maintained on a cornmeal-molasses-yeast based diet (66g/l of cornmeal, 66ml/l of molasses, 27g/l of baker's yeast, 10g/l agar, 7ml/l of propionic acid, and 20ml/l of tegosept). All gnotobiotic experiments were performed on the same fly food without propionic acid and tegosept. All flies were maintained in a 25°C and 60% humidity incubator.

2.2 Fly stocks used

Drosophila mauritiana fly stocks used were as described in Fast et. al. 2011 (Fast et al., 2011). *Drosophila melanogaster* fly stocks used in this study were *Upd*-gal4; CyO/Sco; P(UAS-hPF)B (*upd*>hPABP-Flag in short), and a *white* mutant (w^{1118}). *Upd*>hPABP-Flag fly stocks with and without *Wolbachia* share a similar genetic background through backcrossing as previously described (according to Toomey et al., 2013, Toomey and Frydman, 2014), and *white* mutant (*w*) flies with and without *Wolbachia* were a generously gift of Luis Teixeira (Chrostek et al., 2013). gstD1-GFP (;gstD1-GFP) flies were a gift of Dirk Bohmann (Sykiotis and Bohmann, 2008). rel^{E20} (;;rel^{E20}) flies were obtained from the Bloomington *Drosophila* Stock Center, stock# BL55714.

2.3 Generation and rearing of axenic and gnotobiotic organisms

Gnotobiotic flies were generated by exposing sterile embryos placed on sterile food to 100 CFUs/ml of fly food with the desired bacteria. Embryos were collected on apple juice plates (12.5g/l of sucrose, 22.5g/l of agar, 750ml/l of water, 250ml/l of apple juice, and 15ml/l of Tegosept solution) coated with yeast paste for six hours. Embryos were sterilized by three washes in 50% bleach (Clorox, diluted in sterile water, final concentration approximately 4% sodium hypochlorite) for two minutes per wash. Subsequently the dechorionated sterile embryos were washed three times with sterile Triton salt solution (4g/l of NaCl, 300ul/l of Triton-X 100 (Fisher Scientific, Cat# BP151)) to prevent sticking to surfaces. After removal of all the Triton salt solution, an equal volume of fresh Triton salt solution was added to the embryos. 10ul of this mix was added to autoclave-sterilized fly food. Sterilized fly food was prepared like the regular fly food without preservatives after autoclaving. A correlation of OD600-CFU for each species was generated, *L. plantarum* was 1.11x10^9 CFUs/ml/OD and *A. pasteurianus* was 2.6x10^8 CFUs/ml/OD. Using appropriate dilutions of log phase cultures whose ODs were measured, 1000 CFUs of each of *A. pasteurianus* and *L. plantarum* were added to the sterile hatching L1 larvae. L3 larvae and adult flies

were collected under sterile conditions, and their genomic DNA was extracted after surface sterilization with 50% bleach.

2.4 Bacterial cultures and propagation

All bacterial cultures were grown in De Man, Rogosa, and Sharpe (MRS) liquid medium or agar (Cat# 288130, BD). *Lactobacillus plantarum*, *Lactobacillus casei*, and *Lactobacillus brevis* cultures were grown at 37°C and *Acetobacter pasteurianus* cultures were grown at 30°C.

2.5 DNA extraction from flies, l3 larvae, and fly food

Genomic DNA was isolated using a modified protocol from Qiagen DNeasy Blood & Tissue Kit (Cat# 69506). Flies or l3 larvae were surface sterilized by vortexing with 50% household bleach solution (4% sodium hypochlorite) for 5 min and washing three times with sterile water. Effective removal of external bacteria was confirmed as previously described with modifications (Ridley et al., 2012, Chandler et al., 2011). The efficiency of this procedure was confirmed by PCR of the water from the final wash using universal 16S rRNA gene primers. Flies were then homogenized in 200ul of lysis buffer (20mM Tris pH8.0, 2mM EDTA, 1.2% Triton X-100) with 20mg/ml of lysozyme (MP Biomedicals, Cat# 210083401) and incubated for 90 min at 37°C. 200ul of AL buffer (Qiagen, Blood and Tissue Kit) and 20ul of proteinase-K was then added to the mix and

incubated further for 90 min at 56°C. Subsequent extraction using the columns was performed as recommended by the kit. To isolate DNA from the food, 50-100mg of food was collected from the bottles, and the same protocol as above without bleach treatment was followed

2.6 Sequencing the fly microbiome

The 16S rRNA gene sequences were utilized for characterizing the microbial composition. Specifically, the hypervariable regions V1 and V2 were sequenced and compared to green genes library to generate a list of OTUs present in the flies. The results were then confirmed by culture-based methods.

2.6.1 Sample preparation by elimination of *Wolbachia* 16S rRNA gene

Total genomic DNA was extracted and digested with NEB's BstZ17I restriction enzyme (Cat# R0594S) for 1hr at 37°C followed by 10min at 65°C to prevent amplification of *Wolbachia*'s V1 and V2 regions of the 16S rRNA gene prior to high throughput sequencing.

500ng of BstZ17I-digested total genomic DNA was utilized per sample for each library. All PCRs were performed with Platinum® *Taq* DNA Polymerase High Fidelity polymerase from Life Technologies (Cat# 10790-020) using appropriate primers (listed below) in triplicate. 16S rRNA gene amplicons from the triplicate PCRs were pooled and separated by agarose gel electrophoresis and

subsequently extracted using the Qiagen QIAquick Gel Extraction Kit (Cat# 28706). Kappa Biosystems DNA standards (Cat# KK4903) were used for calibration of DNA concentration used for sequencing by qPCR with Illumina adaptors. qPCRs were performed with SYBR® GreenER™ qPCR SuperMix Universal from Life Technologies (Cat# 11790-01k). All samples were pooled at equal concentrations and submitted for sequencing at the Harvard FAS Bauer core.

2.6.2 Primers used for amplification and sequencing

The different regions of the primers are Illumina adaptor (red), indexing barcode (black), primer pad and linker (green), and 16S annealing primer sequence (blue)

Table 2.1: List of primers for 16S rRNA gene sequencing

Primer name	Sequence
Bacterial 16S 27F	AGAGTTTGATCMTGGCTCAG
Bacterial 16S 338R	TGCTGCCTCCCGTAGGAGT
Bacterial 16S 27F with Illumina adaptor	AATGATACGGCGACCACCGAGATCTAC ACGGCTACTATGGTAATTCTAGAGTTTG ATCMTGGCTCAG
Bacterial 16S 338R with Illumina adaptor for W- female	CAAGCAGAAGACGGCATACGAGATTAG CTTAGTCAGTCAGATTGCTGCCTCCCGT AGGAGT
Bacterial 16S 338R with Illumina adaptor for W- male	CAAGCAGAAGACGGCATACGAGATCAG ATCAGTCAGTCAGATTGCTGCCTCCCGT AGGAGT
Bacterial 16S 338R with Illumina adaptor for *w*Mel-infected female	CAAGCAGAAGACGGCATACGAGATGGC TACAGTCAGTCAGATTGCTGCCTCCCGT AGGAGT
Bacterial 16S 338R with Illumina adaptor for *w*Mel-infected male	CAAGCAGAAGACGGCATACGAGATACT TGAAGTCAGTCAGATTGCTGCCTCCCGT AGGAGT
P5 adaptor primer for quantification of amplified DNA	AATGATACGGCGACCACCGAGAT
P7 adaptor primer for quantification of amplified DNA	CAAGCAGAAGACGGCATACGA

2.6.3 Data acquisition and analysis of sequencing data

Sequencing of the 16S rRNA gene amplicons was performed on Illumina's MiSeq platform using 250bp paired end reads (at the FAS Bauer core, Harvard University). Analysis of the reads was performed using the QIIME 1.8.0 package. Default parameters were used for the analysis and Greengenes database was used to assign taxonomy. The accession number of the BioProject containing the raw reads on NCBI is PRJNA381361.

2.6.4 Isolation of fly microbiota

20 flies were homogenized in 1ml of MRS medium (Cat# 288130, BD), the debris was centrifuged down on a table top centrifuge for 10s, and 200ul of the supernatant was plated on MRS agar plates. The plates were incubated at 37°C or 30°C. Well separated colonies with varying morphology were restreaked, and their 16S rRNA gene's V1 and V2 regions were PCR amplified, gel extracted and sequenced.

2.6.5 Bacterial whole-genome sequencing

DNA from overnight cultures of *L. plantarum* and *A. pasteurianus* was extracted using modified protocol for Qiagen blood and tissue kit as described above. DNA was sheered using Covaris spin tubes (Cat# 520079). Genome libraries were prepared according to the PACBIO Template Preparation and

Sequencing Guide selecting for approximately 10 kb genome fragments. DNA quality and size was confirmed on Bioanalyzer followed by sequencing with PACBIO RS II sequencer (at New England Biolabs). Raw reads were assembled de novo by SMRT analysis software. Manual curation and closing of the genome was done by NCBI alignment. The *A. pasteurianus* chromosome (3.12 Mb) and plasmid (140 kb) were annotated using a database of closed *Acetobacter* strains (CP012111 and NC_013209). The *L. plantarum* (3.32 Mb) genome was annotated using the rapid annotation using subsystem technology (RAST) (Aziz et al., 2008, Overbeek et al., 2014). The accession ID of the complete genomes on NCBI is PRJNA384998.

2.7 PCR-based methods

Since the microbiome of *D. melanogaster* is quite simple, qPCR-based assays are cost effective and efficient compared to sequencing-based assays to determine the shifts in microbiome composition.

2.7.1 Quantitative PCR for determining bacteria composition

qPCRs were performed with SYBR® GreenER™ qPCR SuperMix Universal from Life Technologies (Cat# 11790-01k). BstZ17I was used to digest 200ng of total genomic DNA for 1hr at 37°C followed by 10min at 65°C. 5ng of the digested DNA was used to amplify with species-specific primers (listed

below), and universal 16S rRNA gene primers were used for normalization. When the normalization was performed to the host 14-3-3 gene instead of 16S rRNA gene, BstZ17I digestion was not performed.

2.7.2 Species-specific primers for qPCR

Table 2.2: List of species-specific *glmS* primers

Species	Forward primer	Reverse primer
Acetobacter pasteurianus	GCACCCTCATGGTACCGAGC	ACCAGCAGGGCGATGGTTTC
Lactobacillus plantarum	ACGTTAGGGCTACTCGGCCA	GCCTTCGCCGACCCCAATTA
Lactobacillus casei	GGCCCAGAAATTGCGGTTGC	CCTCGCCGACTGCTTTCGAT
Lactobacillus brevis	ACGATGCGGGTTACCCAAGC	ATTTGGGCCGTGTACGCCTT

2.7.3 qRT-PCR for transcriptional profiling

Ten guts of the axenic or gnotobiotic L3 larvae were dissected in Grace's Insect Medium from Lonza (Cat# 04-457F), and the RNA was immediately extracted using the Qiagen miRNeasy Mini Kit (Cat# 217004) as per the manufacturer's protocol. For quantification of the mRNA levels of the genes, EXPRESS One-Step SYBR® GreenER™ Kit, with premixed ROX from Life Technologies was used (Cat# 1179001K). 20ng of RNA were used as input for each reaction and the conditions used were as recommended by the kit.

2.7.4 Primers for qRT-PCR of fly genes

Table 2.3: List of primers used for qRT-PCR of fly genes

Gene Symbol	Forward primer	Reverse primer	Product size (bp)
Rpl32	ATGCTAAGCTGTCGCAC AAATG	GTTCGATCCGTAACCG ATGT	107
AttA	AACACAAGCATCCTAAT CGTGG	GTCAGATCCAAACGA GCATCAG	152
AttB	GACCCATTCGGGTCCGT CG	GACCAGCATTGTTGTA GGCCA	198
AttC	TGCCCGATTGGACCTAA GC	GCGTATGGGTTTTGGT CAGTTC	173
AttD	AAGGGAGTTTATGGAGC GGTC	GCTCTGGAAGAGATTG GCTTG	117
CecA1	CGCTCAGACCTCACTGC AATA	CGCGTTCGATTTTCTTG CCAAT	173
CecA2	CGATCACTTTCCATTGC AACAGC	TGCTGACCAACACGTT CGATTTT	191
CecB	TCAGCCTGGGAAACTCA GAG	GAGGACCTGGATTGA GGCA	98
CecC	AGTCGCTCAGTTTCCAC AGC	GCCAATGCGCTCGATT CTCT	200
Dpt	GACGCCACGAGATTGG ACTG	CAGCTCGGTTCTGAGT TGCC	76
DptB	CTATTCATTGGACTGGC TTGTGC	ATCGAATCCTTGCTTT GGGCT	189
Dro	TTTTCCTGCTGCTTGCTT GC	GGCAGCTTGAGTCAGG TGAT	149
Dredd	GACATGAACTTTGCCCA AAAGG	CGGGAAGTCTGATCGT GTCA	114
imd	TGCATGGCAATATTAGA	GGGACTCCCACAGCA	111

	GAGGTGAT	GTGTA	
Rel	TTGAACCAGGTGCGGCTCT	TTGTGCCGACTTGCGGTTAT	114
PGRP-LE	AGCACTATGACACTAGGCACT	GTCTGAATGCTGTTGATCGAGT	98
PGRP-LC	GCATTTCTACGAGGGACCCG	GTGTTTGGAGCCCGGCG	151
PGRP-LF	GAAGGATGCGAACAAGAGGATG	AGGCTATGCTAACGGAGATGG	196
PGRP-LB	CCGGCAGGGGATTCAATGTC	GTTCTCCAATCTCCGATCAGC	88
PGRP-SC1a	TGGGCAACTACAACTGGGAC	GTACAGGATGTAGCCGGAGC	110
PGRP-SC2	ACCCTCACCTCTGCTCAGAT	GTGCGGATCTCGTTCCAGAT	152
PGRP-LA	TGGCGCAAAATCAGACTAAGGT	CTATTGCTTGGCGTCCCACG	158
Def	TGTCCTGGTGCATGAGGATG	AGTTCCACTTGGAGAGTAGGTC	89
Drs	CTGGGACAACGAGACCTGTC	ATCCTTCGCACCAGCACTTC	92
Mtk	ATGCAACTTAATCTTGGAGCGA	GACGGCCTCGTATCGAAAATG	116
Mcr	CGAGGTTTCCTCGTTGACCG	CCTGAACCTTGATGGTGCCC	140
Anp	AAATACTTTGTGGTCCTTGTCGT	TGCGTTTTCCACTTTGTCAAGAA	102
cact	GCGACACACCTTTGCACCT	ATGTTGAGCAGGCACGGATG	94
Dif	ACATCGATCCCTTCAATGCCAAA	ATACGATGGGATCCAGGGGC	132
spz	GACACCTGGCAGTTAATTGTCA	CGAAGTCACAGGGTTGATCCG	91
Toll-7	ATCCATCGCAACCCAGTGG	GCTGTCGCTCAATGAGACG	96

Myd88	GTGGGCCAAAGTGTGCA GAT	CGCAGGAATAGCCTG AGATTGT	173
dl	ATCCGTGTGGATCCGTT TAA	AATCGCACCGAATTCA GATC	77
PGRP-SA	ACGGGCATAGCCTTTAT CGG	TAATCCTCGCTCAGCT CACC	116
PGRP-SD	ACTTGGATCGGTTTGCT CATC	AGGGAGTTTCCATGCT GTCTAT	127
Drsl2	TCCGGCAAATACAAGG GTCC	GGCCACTGATATGTCC CTCC	85
Drsl3	TCCTTGCTGTAATGACC ATTGTC	GGACCTCCGAAAGTTC CAGATAG	82
Duox	ATGGCTGGTACAATAAC CTGGC	AACCCCATCCGAATAG GAGGG	92
NOX	CATCGCGGTTCAGTGTC GT	ACTGCTGGTTGATGGG TTGC	133
cat	GATGCGGCTTCCAATCA GTTG	GCAGCAGGATAGGTC CTCG	139
irc	GCGTTGGACTCAGTAAA TAGGC	GCTGCCGTTCTTAACA GTGAT	78

2.8 High-throughput sequencing of RNA from JW18 cell lines

JW18 cell lines and their *Wolbachia*-free counterparts, JW18DOX were a gift of William Sullivan at the University of California, Santa Cruz (Serbus et al., 2012). Cell lines were maintained in M3 Shields and Sangs medium from Sigma (M3 medium) (Cat# S3652) with 10% Fetal Bovine Serum (FBS) from Fisher Scientific (Cat# SH3007003I) in a 27°C incubator with water pans. JW18 cells were passaged by splitting 1:2 and JW18DOX cells are were passaged by splitting 1:4 or 1:5 every week. Frozen stocks were generated for future use by taking a confluent 25Cm^2 flask of cells, adding culture medium with 10% DMSO, and storing this suspension at -80°C. Frozen stocks can be revived by washing the thawed cells with culture medium 3 times to reduce the presence of DMSO and growing at 27°C.

2.8.1 Generating *Wolbachia* re-infected JW18DOX cells

To minimize the genetic differences between the JW18 and JW18DOX cells as a result of the doxycycline treatment to remove *Wolbachia*, *Wolbachia* from JW18 cells was extracted and was used to re-infect the JW18DOX cells. Confluent flask of JW18 cells were resuspended in 10ml of culture medium, 2ml of glass beads were added, and vortexed for 5 min to lyse the insect cells. The lysate was centrifuged for 5 min at 2500g at 4°C, and the supernatant was passed through a

5µm filter. The flow through was spun down at 13,000g for 5min to pellet the *Wolbachia*-containing fraction. This pellet was resuspended in 5ml of culture medium, and this suspension was to culture JW18DOX cells. These cells were re-infected with *Wolbachia* within a week and these were called JW18R (JW18 Re-infected).

2.8.2 Sample preparation and data acquisition

Three replicates of JW18, JW18DOX, and JW18R cells in their exponential growth phase were used for RNA extraction using the Qiagen miRNeasy Mini Kit (Cat# 217004). The RNA quality was checked on a Bioanalyzer using an RNA nano chip (Cat# 5067-1511) (at FAS Bauer core, Harvard University). The cDNA libraries were prepared using Illumina's TruSeq RNA Library Prep Kit V2 (Cat# RS-122-2001). The library quality was checked on a bioanalyzer using the HS DNA chip (Cat# 5067-1504). Kappa Biosystems DNA standards (Cat# KK4903) were used for calibration of DNA concentration used for sequencing by qPCR with Illumina adaptors. The libraries were pooled at equal concentrations and sequenced on the Hi-Seq 2000 with 50b standard run to generate about 200M reads (at the FAS Bauer core, Harvard university).

2.8.3 Analysis of sequencing data

The fastq files of sequence data were analyzed using the TopHat and Cufflinks packages. TopHat was used for the alignment of reads to the *Drosophila melanogaster* genome, and Cufflinks was used to perform the differential gene expression analysis. DAVID was used for determining pathways that were enriched from the differentially expressed genes. MATLAB scripts were used to filter out genes of interest based on pathways and gene expression.

2.9 Confocal microscopy of cells and tissues

2.9.1 Imaging *Wolbachia* and gut bacteria by FISH

Wolbachia localization in the gut was determined by FISH as previously described (Toomey et al., 2013). *A. pasteurianus* probes were designed and tested for specificity by performing BLAST against other species in the *Drosophila* gut microbiome. The probe used in this study is 5' 6-FAM/AGAGTGCCCAGCCCAACCTGA from IDT DNA. Both *Wolbachia* and *A. pasteurianus* probes were used at 1ng/ul. For performing FISH on the gut contents, the fly food was modified by substituting yeast with yeast extract and sugars to eliminate autofluorescence of yeast. Modified fly food composition - Dextrose 50g/L, Sucrose 25g/L, Yeast extract 15g/L, Cornmeal 60g/L, Agar 6.5g/L, Tryptone 30g/L, and Molasses 65g/L. L3 larval guts were dissected, fixed in 4%

paraformaldehyde in PBS for 1 h, and then in 50% ethanol with PBS for 30min at -20°C. The hybridization was performed as previously described (Toomey et al., 2013). Image acquisition was performed with an Olympus FluoView 1000 Confocal microscope.

Images of full guts were assembled using Microsoft Image Composite Editor and MosaicJ package in FIJI from individual images collected at 40X (for Fig. 3.11) or 60X (for Fig. 3.12) magnification. Images were processed with Photoshop to eliminate pixels outside the gut and equalize channels intensity within the same composite image.

2.9.2 Imaging Reactive oxygen species (ROS) in the larval gut

GstD-GFP reporter flies display green fluorescence in response to oxidative stress in a tissue-specific manner. The reporter flies were crossed to the flies of interest, the appropriate tissue at the required life stages are dissected, fixed and mounted for visualization of green fluorescence.

2.9.3 Immunohistochemistry of cells and tissues

Poly-L-lysine coated 12mm cover slips (MP Biomedicals, Cat #150176) were prepared by incubating clean cover slips in 0.01% poly-L-lysine solution for one h, washing thrice with DI water, and then air drying. Confluent cells were resuspended in M3 medium without FBS, 250ul of the suspension was added on

the cover slips and incubated for two h at room temperature. The cells were then fixed for 20 min using 4% PFA in M3 medium, along with 0.2% triton for permeabilization, followed by three washes with PBT (PBS + 0.2% Triton X-100). The fixed cells were prepared for antibody staining by incubating in PBT for 30 min, followed by PBT for 30 min, and finally in PBANG (PBT + 0.2% BSA + 5% normal goat serum + 0.005% sodium azide) blocking solution for 30 min. To stain for the target protein, cells were incubated in PBANG containing appropriate amount of primary antibody for two-three h, followed by three washes with PBT. To remove excess and free primary antibody, cells were incubated in PBT with two changes over a course of two h. The primary antibody stained cells were then blocked again using PBANG for 30 min, and then with PBANG with the secondary antibody for one-two h to stain the primary antibody. Excess and free secondary antibody was washed away with three washes of PBT, followed by incubation with two changes over a course of two h with PBT. To stain for DNA, the cells were incubated in 10 ug/ml of Hoecsht (Life Technologies Cat# H3570) in PBT, and excess was washed away with three washes with PBT. The coverslips were then mounted on slides with prolong gold (Life Technologies, Cat# P36930) for visualization.

2.9.4 Counting *Wolbachia* cells in host cells by imaging

JW18 cells were split into fourths, and each portion was used separately for the analysis as replicates. The cells were plated into 24-well plates and three wells were used for each day for counting the number of *Wolbachia* per host cell. FISH was performed as described above in section 2.9.1 on three wells of JW18 cells. After mounting, 10 confocal stacks chosen at random from the slide from each replicate slide were taken. This process was repeated for seven days. The images were then processed in a custom MATLAB script to count the number of individual *Wolbachia* per each host nucleus. The script essentially detects the edges of individual bacteria and count the number of bacteria. In case of clumping, the local maxima of intensities at the centers of each bacteria in the clump were counted to estimate the number of bacteria. The script and its usage can be found at https://github.com/RamaSimhadri/Bacteria_counting.

2.10 pH analysis of larval gut

Drosophila gut pH can be measured by feeding the organisms with a pH indicator in the food and subsequently imaging dissected gut to analyze the hues the dye assumes in the gut.

2.10.1 Sample preparation

Gnotobiotic organisms were generated with equal proportions of *A. pasteurianus* and *L. plantarum* on sterile fly food. L3 larvae were then transferred to apple juice plates containing saturating concentrations of four pH indicators – 0.1% thymol blue (Honeywell Fluka, Cat# 32728), 0.1% bromophenol blue (Fisher Chemical, Cat#B392-5), 2% bromocresol purple (Fisher Chemical, Cat# B393-5), and 0.07% phenol red (ACROS Organics, Cat# AC15143-0050). After allowing the l3 larvae to feed for three h, the guts were dissected in 0.9% saline solution, and imaged under Olympus SZX9 dissection scope (oblique 0%, maximum bottom light intensity, 9.4 magnification) with Canon S5 powershot camera (ISO 80, daylight white balance, 2s self-timer, 3.5 f stop, 1/20s shutter speed, maximum zoom).

2.10.2 Image analysis

The images were analyzed on a custom MATLAB script (https://github.com/RamaSimhadri/gut_pH). The script measures the length of the midgut region, the hue profile of the midgut, and the fraction of the gut at each of the pH ranges for every pH dye used. The hue-pH correlation was obtained by generating a calibration curve of every pH dye used. 200ul of the saturated solution of the dye in a clear 96-well plate at various pHs was imaged

using the same imaging parameters as the guts. A custom MATLAB script was used to generate a hue-pH correlation. Hue-pH correlation was generated by first producing saturated solutions of each pH indicator in distilled water, adjusting the pH of the solution using HCl or NaOH and reading out using a pH probe, aliquoting 200ul of the known pH solution into a well of a clear 96-well plate, and then taking an image of the well using the settings of the camera and microscope as described in 2.10.1. The hue of the center of each well was determine and a correlation curve of hue-pH was generated.

2.11 *Drosophila* development assays

Wolbachia-free and infected embryos were collected for six h on apple juice plates and axenic organisms were generated by bleaching the embryos. Gnotobiotic organisms with only *A. pasteurianus*, or with only *L. plantarum*, or with both organisms in equal proportions were generated to measure for their development rate in comparison to axenic organisms. Roughly 9 days after generating the gnotobiotic organisms, the number of flies eclosing from each bottle was counted at eight h intervals for four days. Survival curves were then compared between every condition to assess differences in development rates due to *Wolbachia* and the microbiota.

CHAPTER 3

Wolbachia modulate the *Drosophila* microbiome

(Portions of this chapter previously published in Simhadri et al., 2017)

A previous study in our lab showed that *Wolbachia* affect the fecundity of *D. mauritiana* flies (Fast et al., 2011). In a parallel study, we observed that the presence of *Wolbachia* in *D. mauritiana* changes the color of the grape juice plates over time. The plates used for egg collections from *Wolbachia*-free flies were pink, but displayed a yellowish tinge when exposed to *Wolbachia* infected flies (Fig. 3.1). Adding an acid or a base to the grape juice plates indicated that the color change is pH dependent. (Fig. 3.1). Since flies defecate on the agar medium, we suspected that fecal microbiota-derived metabolites contribute towards determining the final pH of the agar. In addition, if there is a difference in the microbial composition between *Wolbachia*-free and infected flies, this might contribute to the differential coloration of the grape juice agar plates. Since the *Drosophila mauritiana* system does not have powerful genetic tools to further analyze this phenotype, we investigated whether *Wolbachia* affect the microbiome in *Drosophila melanogaster* flies. Moreover, results in *D. melanogaster* will be of broader interest than in *D. mauritiana*.

For this analysis, we examined the strain of *D. melanogaster, upd*-Gal4, UAS-hPABP-Flag (*upd*>hPABP-Flag). The hPABP-Flag strain facilitates the isolation of mRNA from specific cell types for transcriptional profiling (Yang et al., 2005). We used the *upd* promoter, which is active in the stem cell niche (hub) of the testis, to drive the expression of hPABP-Flag. Since *Wolbachia* show tropism to the hubs in males (Toomey and Frydman, 2014), this construct allows for the isolation of RNA for studying gene expression patterns that are altered due to *Wolbachia* infection. The *upd*>hPABP-Flag strain was made isogenic and was utilized for molecular characterization of the effects of *Wolbachia* on stem cells and their niches (see Materials and methods). Since the microbiome has been shown to affect host phenotypes such as fecundity (Elgart et al., 2016, Coon et al., 2016), which is a direct result of stem cell activity (Fast et al., 2011), we considered testing the effects of *Wolbachia* on the microbiome to be especially important in this specific genotype.

Figure 3.1: ***D. mauritiana*** **flies change grape juice agar plate color depending on** ***Wolbachia*** **status.**

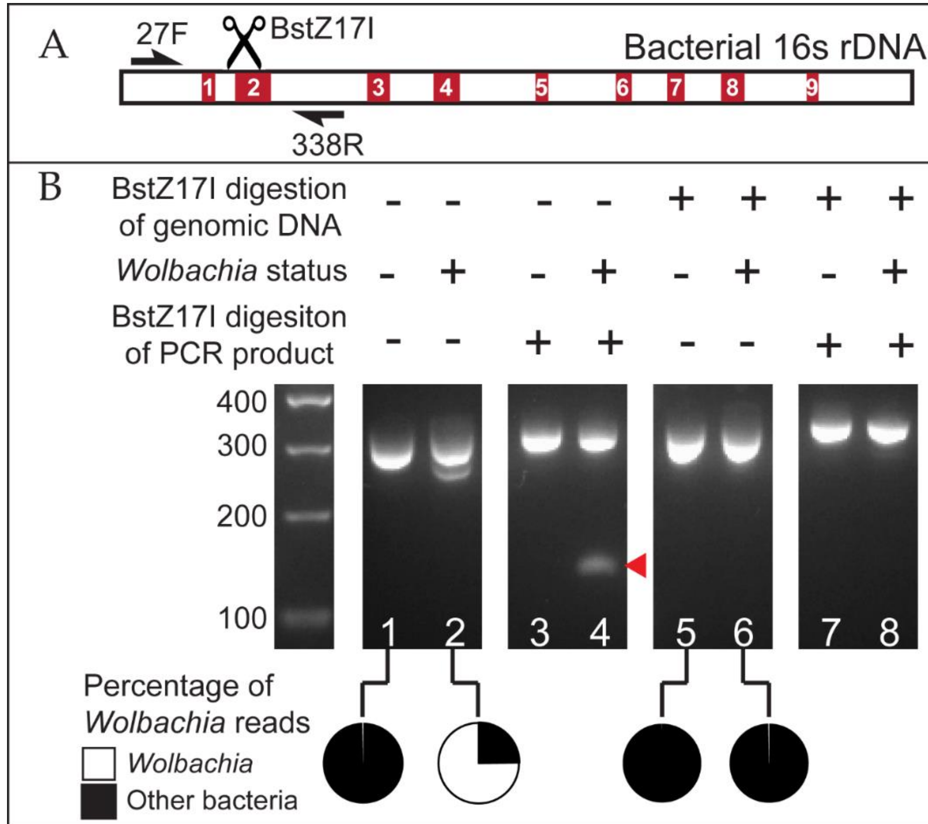
Grape juice agar plates used for *D. mauritiana* egg collection. Grape juice acts as a pH indicator, turning pink in acidic conditions and yellow in basic conditions. Over time, the microbial composition in the feces of *Wolbachia*-infected flies turns plates more yellowish compared to plates utilized by *Wolbachia*-free flies.

3.1 *Wolbachia* pose problems for sequencing 16S rDNA, but their 16S rDNA can be eliminated

To determine the microbial content of the *upd*>hPABP-Flag flies, we employed high-throughput sequencing of the 16S rRNA gene amplicons from total genomic DNA isolated from flies. A major hurdle in sequencing the 16S rRNA gene sequences from flies infected with *Wolbachia* is the overrepresentation of *Wolbachia* sequences (Chandler et al., 2011). BstZ17I digestion of the total genomic DNA specifically prevents the amplification of *Wolbachia* 16S rRNA gene as the restriction enzyme cleaves between the V1 and V2 regions. Microbes normally found in *Drosophila* do not contain the BstZ17I restriction sites. Other bacteria containing this restriction site are present in the orders *Rhizobiales*, *Myxococcales*, and non-*Wolbachia Rickettsiales* that have been reported to be absent or occur at low numbers in *Drosophila* (Staubach et al., 2013). We found that BstZ17I digestion prior to the PCR of the V1 and V2 regions of the 16S rRNA gene effectively removed most of the *Wolbachia* amplicons (Fig. 3.2). While more than 70% of the reads originated from *Wolbachia* in the case of undigested genomic DNA, the BstZ7I-digested genomic DNA produced less than 1% *Wolbachia* reads (Fig. 3.1B, Lane 6, Table 3.1). We also confirmed the strain of

Wolbachia present to be *w*Mel by Variable Number Tandem Repeat (VNTR) analysis (Fig. 3.3)

Figure 3.2: BstZ17I digestion eliminates *Wolbachia* 16S rRNA genes



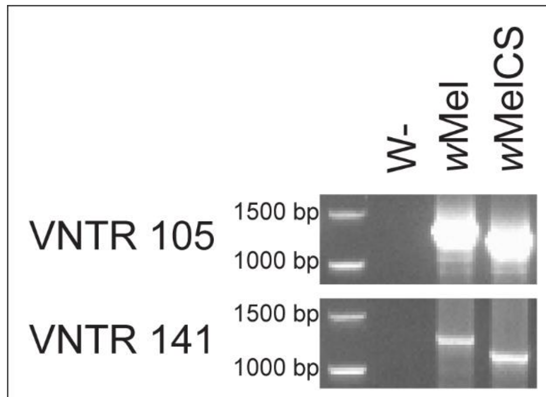
(A) Schematic of bacterial 16S rRNA gene. Hypervariable regions (V1-V9) are shown in red. Primers (arrows, 27F and 338R) amplify V1 and V2 regions. BstZ17I restriction *dig*estion of the total 16S rRNA gene pool selectively digests only *Wolbachia* ribosomal DNA between V1 and V2. (B) Agarose gel image showing the 16S rRNA gene PCR products from the microbiome of *D. melanogaster* and efficient digestion of *Wolbachia* 16S rRNA gene amplicon using BstZ17I restriction enzyme. The red arrowhead indicates the digested *Wolbachia* product. The pie charts indicate the percentage of *Wolbachia* reads before and after BstZ17I digestion

Table 3.1: Number of reads for each sample and the effectiveness of BstZ17I digestion

Sequencing the 16S rRNA gene PCR products from either BstZ17I digested or undigested total genomic DNA from flies shows that the BstZ17I enzyme can eliminate the amplification of *Wolbachia* 16S rRNA gene.

Wolbachia status and sex	BstZ17I digested prior to PCR	Number of reads	Fraction of *Wolbachia* reads	Number of *Wolbachia* reads
W- Female	Yes	114263	0.000255493101686	29
W- Male	Yes	38980	0.00164928708236	64
*w*Mel Female	Yes	98656	0.00304534321666	300
*w*Mel Male	Yes	34940	0.00341541575308	119
W- Female	No	42049	0.000252003	11
W- Male	No	45406	0.0004679	21
*w*Mel Female	No	53757	0.697802742	35712
*w*Mel Male	No	30173	0.910362365	27468

Figure 3.3: Variable Number Tandem Repeats (VNTR) analysis to determine *Wolbachia* strain.

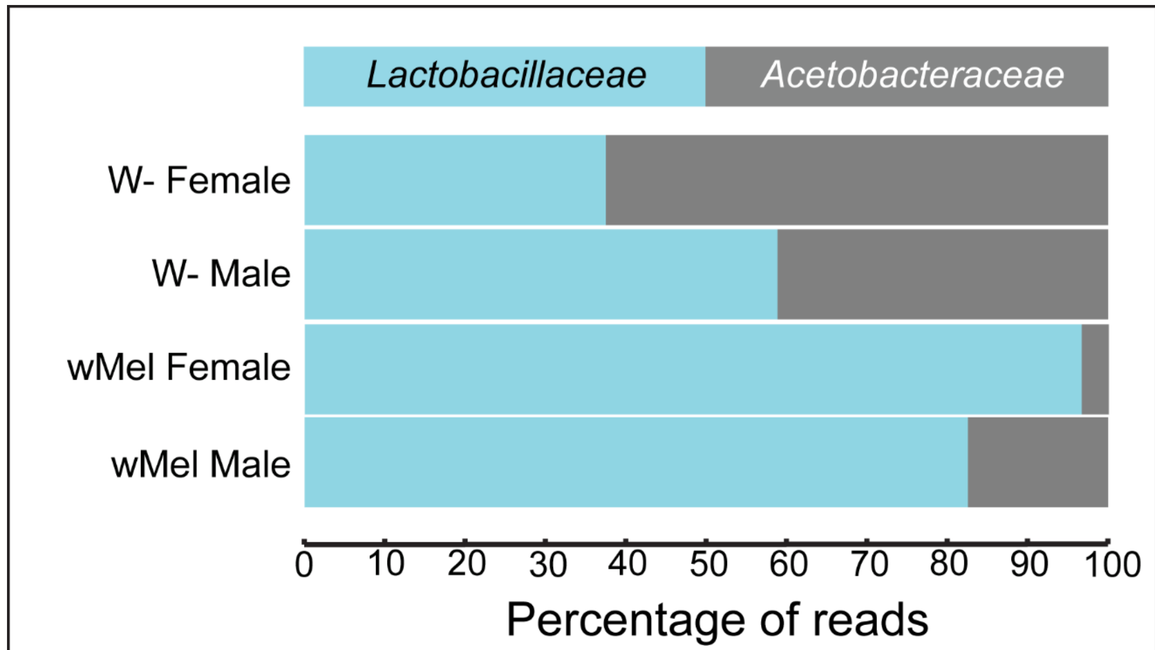


Different *Wolbachia* strains have different numbers of repeats in some VNTRs, specifically, VNTR 105 and VNTR 141. The expected sizes for *w*Mel are 1347bp for VNTR 105 and 1330bp for VNTR 141, and for *w*MelCS are 1241bp for VNTR 105 and 1189bp for VNTR 141 (Riegler et al., 2012). Lanes 1 in both gels are *Wolbachia*-free flies, lanes 2 are *w*Mel-infected flies used in this study. Lanes 3 are flies infected with *w*MelCS (not used for study) for comparison.

3.2 16S rRNA gene sequencing suggests that *Wolbachia* reduce *Acetobacteraceae* in adults

To assess the effect of *Wolbachia* on bacterial composition associated with these flies, we sequenced the non-*Wolbachia* 16S rRNA gene from 0-2-week old adult male and female flies separately (N= 5 flies per sample, 20 flies total). We then grouped the 16S rRNA gene sequences into 97% identity operational taxonomic units (OTUs). We found that the vast majority (>99.8%) of the microbiome of both *Wolbachia*-free and infected flies is restricted to only two families - *Acetobacteraceae* and *Lactobacillaceae* (Fig. 3.4, Table 3.2). Intriguingly, there was a striking contrast between the proportions of the two families of bacteria between *Wolbachia*-free and infected flies. *Acetobacteraceae* make up less than 20% (17% in males and 3% in females) of the microbes in *w*Mel-infected flies and more than 40% (41% in males and 62% in females) in *Wolbachia*-free flies (Fig. 3.4).

Figure 3.4: 16S rRNA gene profiling of *Drosophila melanogaster* shows reduction of *Acetobacteraceae* levels in *Wolbachia*-infected adult flies



16S rRNA gene profiles of male and female *w*Mel-free (W-) or infected strains of *D. melanogaster*. The proportions of *Acetobacteraceae* are significantly different between every pair of conditions (p<0.0001, chi-square test with Yate's correction).

Table 3.2: Percentages of bacterial taxa (non-*Wolbachia*) found in each of the samples by 16S rRNA gene sequencing

The two most abundant families of bacteria are *Acetobacteraceae* and *Lactobacillaceae*, which constitute more than 99.8% of reads in any sample.

Family	Female W-	Male W-	wMel Female	wMel Male
Acetobacteraceae	62.4371752	40.99654	3.2548224	17.33446
Lactobacillaceae	37.5351393	58.87557	96.7308792	82.64438
Unassigned	0.0170372	0.015987	0	0
Propionibacteriaceae	0.00426	0	0	0.00423
Erysipelotrichaceae	0.00213	0.00533	0	0
Pseudomonadaceae	0.00213	0.026645	0.0013	0.00423
Xanthomonadaceae	0.00213	0.010658	0.0026	0.00423
Corynebacteriaceae	0	0.010658	0	0
Bacillaceae	0	0.010658	0.0026	0.00423
OP11-4*	0	0	0.0026	0
Methylobacteriaceae	0	0.031974	0.0039	0
Sphingomonadaceae	0	0.00533	0.0013	0.00423

Burkholderiaceae	0	0.010658	0	0
Acetobacteraceae **+** **Lactobacillaceae**	99.9723145	99.8721	99.9857016	99.97884
Total number of reads	114234	38916	98356	34821
Number of *Acetobacteraceae* **+** **Lactobacillaceae reads**	114202	38866	98342	34814

* Name of the order. Family level identity was unavailable

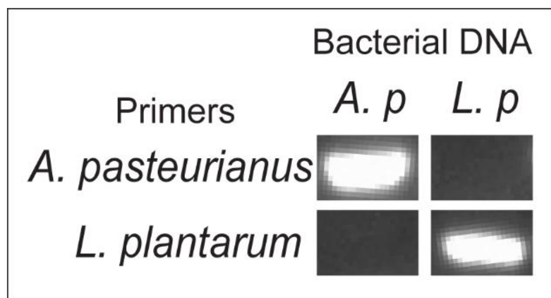
3.3 *Acetobacter pasteurianus* and *Lactobacillus plantarum* are the two species of bacteria found by culture-based methods

To determine the specific bacterial species present in these flies, we employed culture-based techniques followed by Sanger sequencing the 16S rRNA gene of the bacterial isolates. Consistent with our 16S sequencing data, we found that *Acetobacter pasteurianus* and *Lactobacillus plantarum* are the only two species residing in this fly strain. We also confirmed the identity of the bacteria using specific primers against their respective published *glmS* gene (Table 3.3 and Fig. 3.5).

Table 3.3: Species-specific primers against the *glmS* gene for the bacterial species isolated and their PCR amplicons

Species	Forward primer	Reverse primer
Acetobacter pasteurianus	GCACCCTCATGGTACC GAGC	ACCAGCAGGGCGATGG TTTC
Lactobacillus plantarum	ACGTTAGGGCTACTCG GCCA	GCCTTCGCCGACCCCA ATTA

Figure 3.5: Species-specific primers used for quantitative PCR.



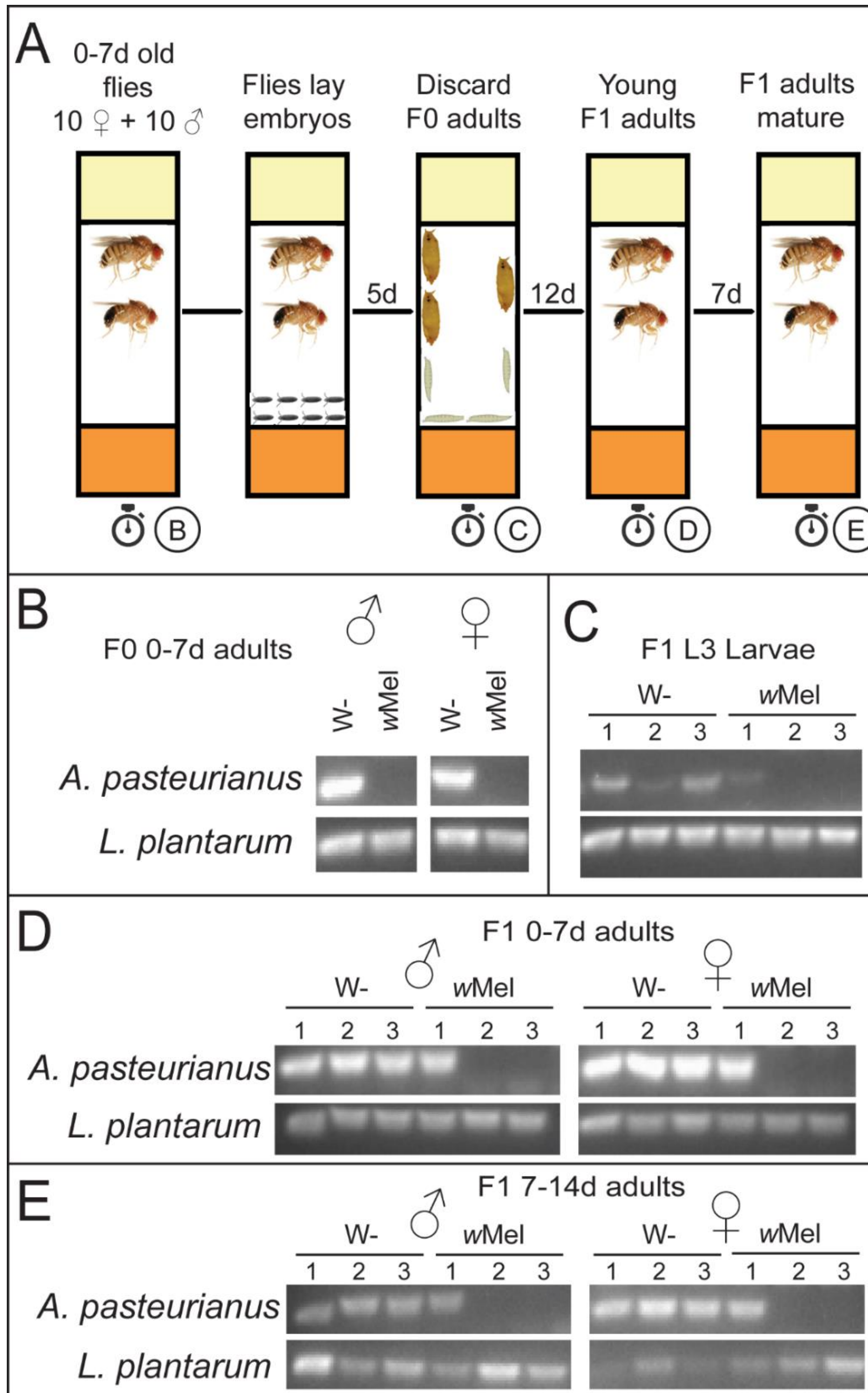
Primers were designed against the *glmS* gene of both the species. The PCR results show that the primers are specific, having no cross-species targets. *A. p* is *Acetobacter pasteurianus* and *L. p* is *Lactobacillus plantarum*.

3.4 *Acetobacter pasteurianus* levels are reduced in all developmental stages

To further corroborate differences in the microbiome composition shown by the deep sequencing results, we performed PCR on the DNA from *Wolbachia*-free and infected *Drosophila* stocks using species-specific primers. We also analyzed the effect of *Wolbachia* on the microbiome during development (see diagram of experiment in Fig 3.6 A): 35 adult males and females were used as the parental generation for the experiment. To analyze the composition of the parental microbiota, a sample of five males and females each were surface sterilized, and DNA extracted. The remaining 30 males and females were each split into three replicates of ten male and ten female parents for both the *w*Mel-free and infected flies. After 7 days of egg laying, these F0 adults were removed from the vials. Five individuals each from the next generation (F1) were collected in triplicate at multiple life stages: L3 larvae, 0-7 day old adults and 7-14 day old adults. DNA was extracted from surface sterilized organisms and digested with BstZ17I to exclude *Wolbachia* 16S rRNA gene amplification. On performing PCR with species-specific primers against the two isolated species, we found that *A. pasteurianus* was not detected in the majority of the *Wolbachia*-infected samples. There was no detectable *A. pasteurianus* in the parent flies, in 2 out of 3 replicates of the F1 L3 larvae, and in both the adult stages. That is, in 12 out of 17 total

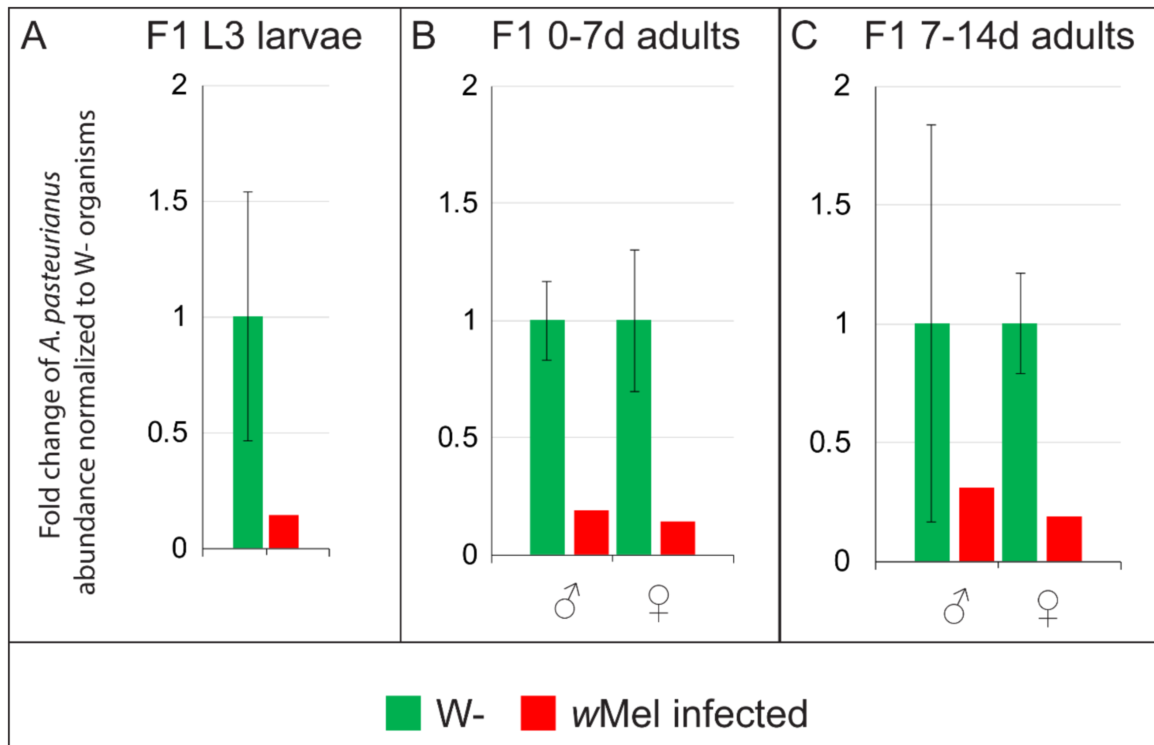
Wolbachia-infected samples, *A. pasteurianus* was absent, compared to its detection in 17 out of 17 *Wolbachia*-free flies. The lack of *A. pasteurianus* was observed in all life stages sampled (Fig. 3.6 B-E). From the samples that did have *A. pasteurianus*, qPCR showed that the levels of the bacteria are about 10-fold lower in the *Wolbachia*-infected organisms than in their *Wolbachia*-free counterparts (Fig. 3.7). These results confirm that the presence of *Wolbachia* is consistently correlated with reduced levels of *A. pasteurianus*.

Figure 3.6: *Wolbachia* suppress *A. pasteurianus* across various life stages of *D. melanogaster*.



(A) Schematic of the experimental setup. Stop clocks indicate sample collection times. (B-E) PCR products using species-specific primers of *A. pasteurianus* and *L. plantarum* on BstZ17I digested total genomic DNA from *w*Mel-free or infected *D. melanogaster*. (B) The parental flies are F0 0-7d old males and females. (C-E) Experiments were done in triplicate, each sample with 5 adults (B, D, E), or 5 larvae each (C). Vial number is indicated on top each of gel image. F1 un-sexed L3 larvae (N=3) (C), F1 0-7d old male and female flies (N=3) (D), and F1 7-14d old male and female flies (N=3) (E).

Figure 3.7: Quantification of the effects of *Wolbachia* on *A. pasteurianus* levels in flies during development.



(A-C) Relative levels of *A. pasteurianus* and *L. plantarum* in *w*Mel-infected flies compared to *Wolbachia*-free flies. (A) F1 un-sexed L3 larvae, (B) F1 0-7d old male and female flies, and (C) F1 7-14d old male and female flies. (A-C) *A. pasteurianus* was absent in 2 out of the 3 replicates of the *Wolbachia*-infected vials tested, hence the absence of the error bars for the *A. pasteurianus* bars. Bar graphs show means (N=3 when the qPCR produced amplicons) and error bars are standard deviations

3.5 *Wolbachia* reduce *Acetobacter pasteurianus* in gnotobiotic flies as well, with L3 larvae showing the greatest effect

Besides the presence of *Wolbachia*, another factor that could influence the microbiome is the difference in the relative abundances of each bacterium passed on by the parents. The general absence of *A. pasteurianus* in the F1 generation (Fig. 3.6) could have been due to the low abundance of this bacteria in the parent flies and not necessarily caused by *Wolbachia* infection. To eliminate the differences in the microbiome that is imparted by the parent flies, we produced gnotobiotic organisms that were infected with equal quantities of *A. pasteurianus* and *L. plantarum* (Fig. 3.8 A). 10ul of bleach-sterilized eggs of *w*Mel-free and infected flies were seeded on sterile fly food in triplicate. To minimize differences of growth rate and survival of each bacterium in the fly food, 1000 CFUs of each bacteria was added to the food after majority of the eggs hatched (see Materials and Methods). Five individuals each from various life stages - L3 larvae, 0-7 day old adults and 7-14 day old adults, were then collected from each biological replicate. Upon performing qPCR for each of the bacteria, we found that the levels of *A. pasteurianus* were indeed higher, about 10-fold ($P < 0.05$), in the *Wolbachia*-free larvae compared to the *w*Mel-infected larvae (Fig. 3.8 B). However, the 0-7day old adults, either *Wolbachia*-free or infected, had no

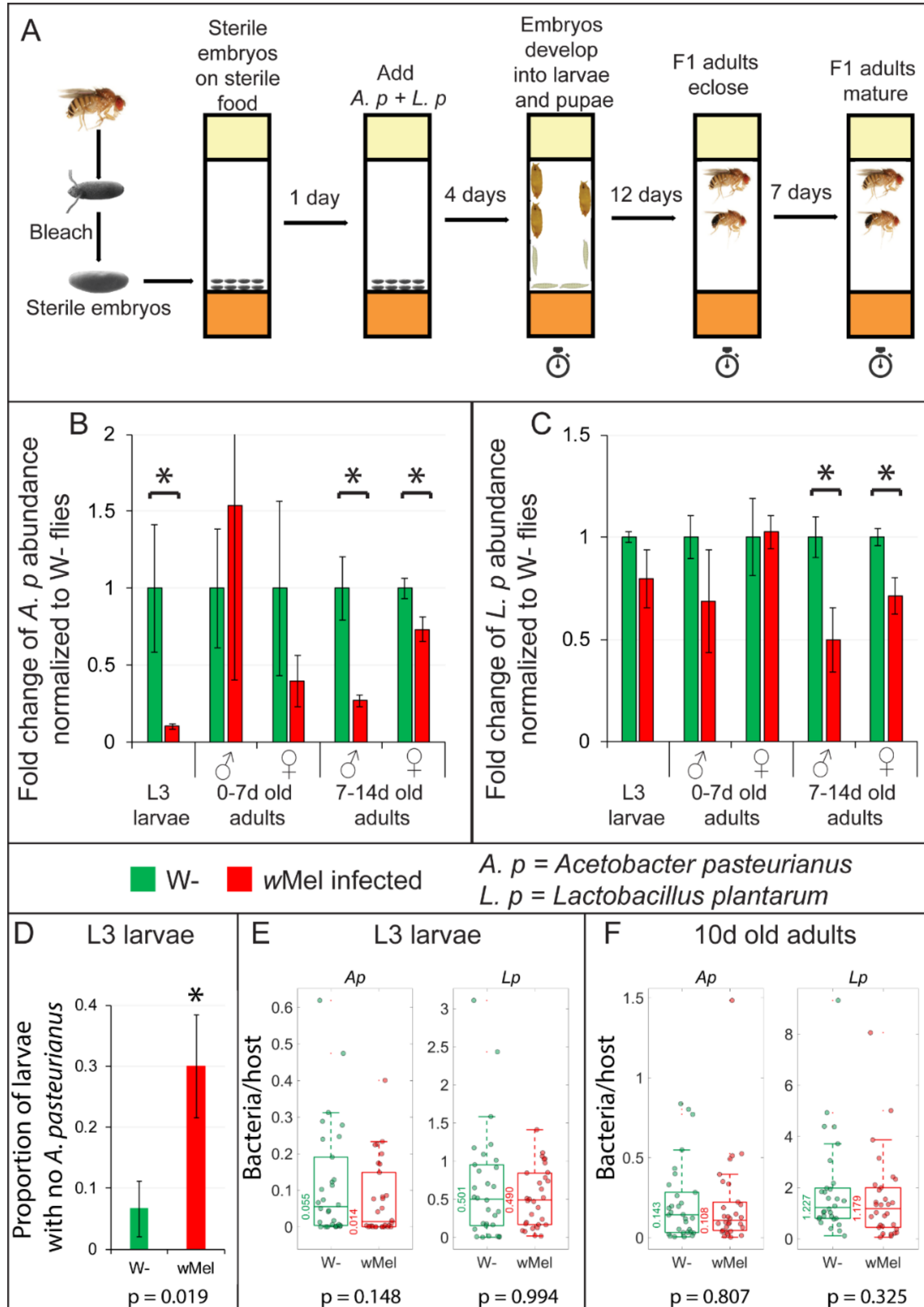
statistically significant difference between the levels of *A. pasteurianus* (Fig. 3.8 B). Finally, the 7-14day old *w*Mel-infected adults had reduced levels of both *A. pasteurianus* and *L. plantarum* compared to the *Wolbachia*-free flies (Fig. 3.8 B, C).

To determine if this reduction of *A. pasteurianus* levels was due to overall reduction of the bacteria in all *Wolbachia*-infected flies or due to the complete lack of *A. pasteurianus* in large fractions of *Wolbachia*-infected flies, we sampled individual organisms to assay the levels of each gut bacteria relative to the host. Gnotobiotic organisms were produced as described above. Three separate experiments were performed, and 10 individuals (at L3 larval stage and 10 day old adults) were sampled from each experiment. On performing qPCR with species-specific primers and comparing to the host DNA, we found that about 30% of the *Wolbachia*-infected L3 larvae did not harbor any *A. pasteurianus*, compared to 6% in *Wolbachia*-free flies ($p = 0.019$) (Fig. 3.8 D). Further, the median levels of *A. pasteurianus* in *Wolbachia*-infected flies were four-fold lower than in *Wolbachia*-free flies ($p = 0.148$), while the levels of *L. plantarum* were unaffected due to *Wolbachia* (Fig. 3.8 E). In the 10day old adult flies, neither *A. pasteurianus* nor *L. plantarum* levels relative to the host were affected due to the presence of *Wolbachia* (Fig. 3.8 F). To ensure that this was not an artifact of lack of *A. pasteurianus* in the food, we sampled the bacterial levels in the food (see

Materials and Methods), and the levels of both the bacteria were comparable between *Wolbachia*-free and infected bottles at both L3 larval and 10day old adult stages (Fig. 3.9).

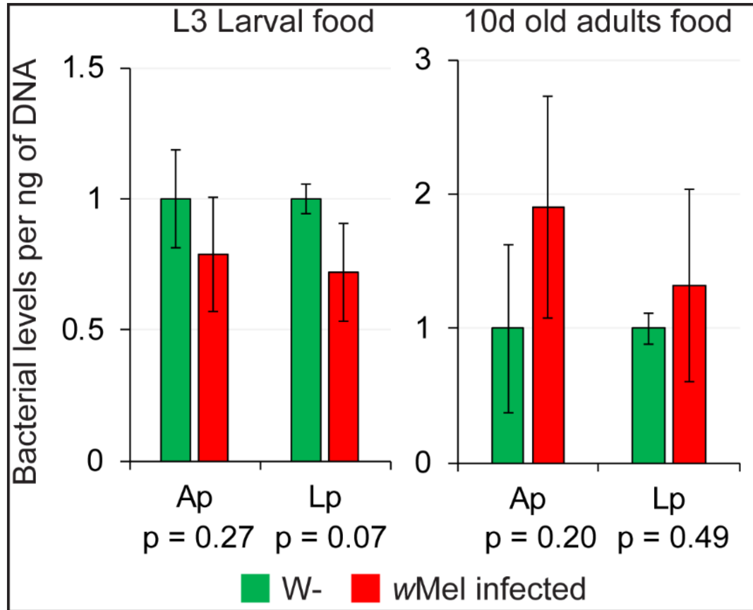
From these results, the greatest species-specific effect of *Wolbachia* on the microbiome appears to act at an earlier developmental stage, but importantly, is independent of the transmission of a specific microbial composition from the parents.

Figure 3.8: *Wolbachia* infection in gnotobiotic flies reduces *A. pasteurianus* in L3 larvae.



(A) Schematic of the experimental setup. Stop clocks indicate sample collection time points. (B, C) qPCR of *A. pasteurianus* (B) and *L. plantarum* (C) titers in *w*Mel-infected gnotobiotic flies compared to *Wolbachia*-free gnotobiotic flies, normalized to 16S rRNA gene levels, at L3 larval stage, 0-7d old adults, and 7-14 d old adults. Bar graph shows means (N=3) and error bars are standard deviations. Asterisk shows statistical significance of $P<0.05$ (student t-test). (D) Proportion of individual L3 larvae that had no *A. pasteurianus* in the gut (N = 30, Chi-squared test, error bars are confidence intervals). (E, F) Box-Whisker plot of levels of each bacterium in L3 larval (E) and 10d adult stages (F) (N = 30, median values are shown next to the boxes. p values of two-sided Wilcoxon rank sum test are reported).

Figure 3.9: Bacterial levels in the food of *upd*>PABP-Flag flies.

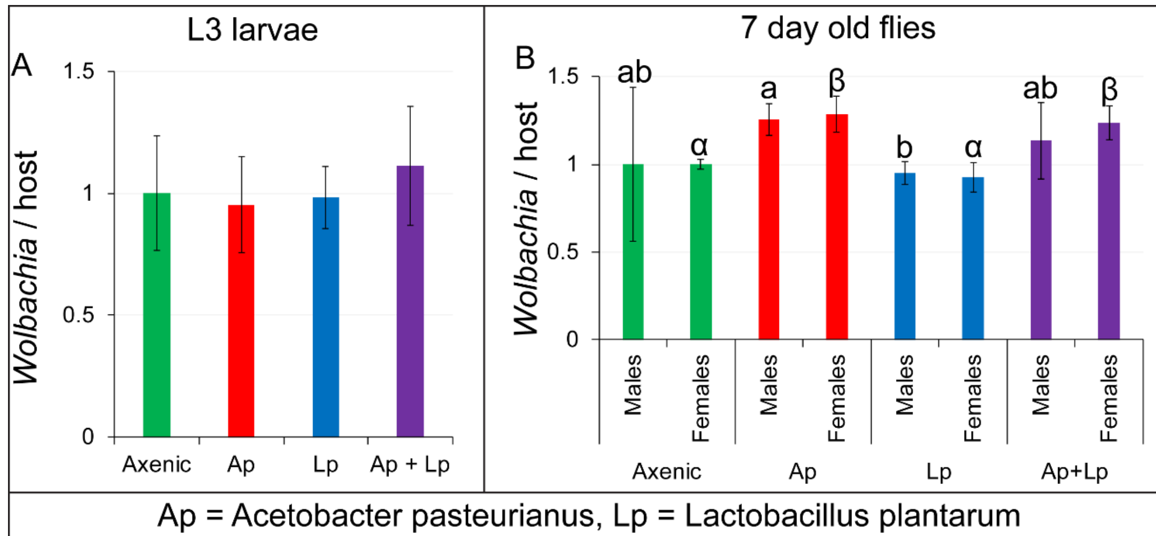


Relative levels of each bacterial species per nanogram of DNA obtained from the food, normalized to the levels in food inhabited by *Wolbachia*-free flies. Bar graphs represent the means of 3 experiments, error bars are standard deviations. p values are from student t-tests.

3.6 The gut microbiome does not alter the levels of *Wolbachia* in whole flies

Previous studies have shown that the presence of certain gut-associated acetic acid bacteria can affect the colonization and stability of *Wolbachia* infection in mosquitoes (Hughes et al., 2014). To determine whether the microbial species in this study alter the levels of *Wolbachia* in flies, we first generated axenic organisms, organisms that were mono-associated with either *A. pasteurianus* or *L. planatarum*, and organisms with both bacterial species, in both *Wolbachia*-infected and free conditions. We then sampled the DNA from three replicates of pools of 5 organisms from every condition at L3 larval stage and in one week old adults, and compared the levels of *Wolbachia* (*wsp*) relative to the host (*14-3-3* gene) by qPCR. We observed no statistically significant change in the levels of *Wolbachia* between any conditions at both life stages (Fig. 3.10), showing that the microbiome does not significantly affect *Wolbachia* levels in these flies.

Figure 3.10: Gut microbiota does not affect *Wolbachia* densities in flies



Levels of *Wolbachia* relative to the host in L3 larvae (A) and 0-7d old adults (B) were assayed by qPCR. Bar graphs represent the means of 4 experiments, error bars are standard deviations. p values are from student t-tests. The characters on bars represent statistical significance at $p<0.05$

3.7 *Wolbachia* are absent in the gut lumen, ruling out direct competition between *Wolbachia* and the gut microbiota

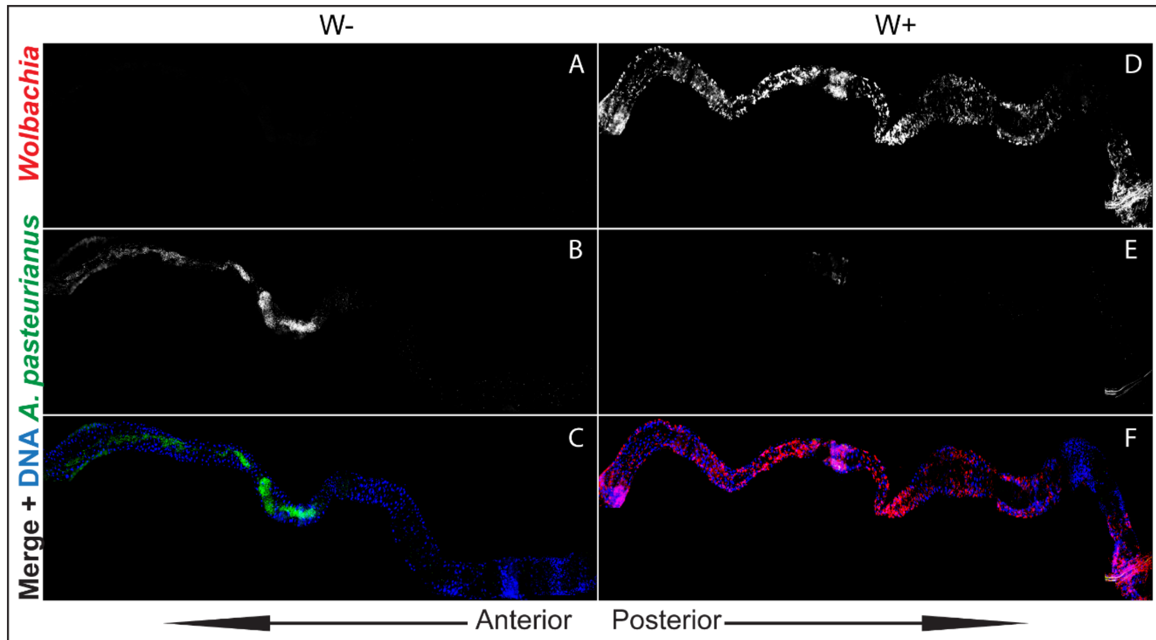
We investigated the stock (*upd*>hPABP-Flag) where *Wolbachia* reduced the levels of *A. pasteurianus* for possible causes of this phenotype. Towards determining the mechanistic basis for this *Wolbachia*-induced microbiome differences, we characterized the distribution of *Wolbachia* and *A. pasteurianus* in the gut by FISH (see Material and Methods).

In five of six *Wolbachia*-free guts that had *A. pasteurianus*, we observed that the bacteria were predominantly present only in the anterior midgut, but not in the posterior regions or the hindgut (Fig. 3.11 B, C). We then looked at the *Wolbachia*-infected guts to determine whether *Wolbachia* and *A. pasteurianus* were spatially exclusive. However, this was not possible as the majority of the guts analyzed (three of four) did not have any *A. pasteurianus* (Fig. 3.11 E). This trend of absence of *A. pasteurianus* ($p=0.0325$, one-tailed Chi-squared test) is in agreement with the previous result, where *A. pasteurianus* was absent in a higher fraction of *Wolbachia*-infected guts than in *Wolbachia*-free guts (Fig. 3.8 D).

Even though *Wolbachia* are primarily a reproductive symbiont found in the gonads, we found them in a majority of the gut cells. *Wolbachia* did not show any preference for a specific region of the gut (Fig. 3.12). We also assessed the

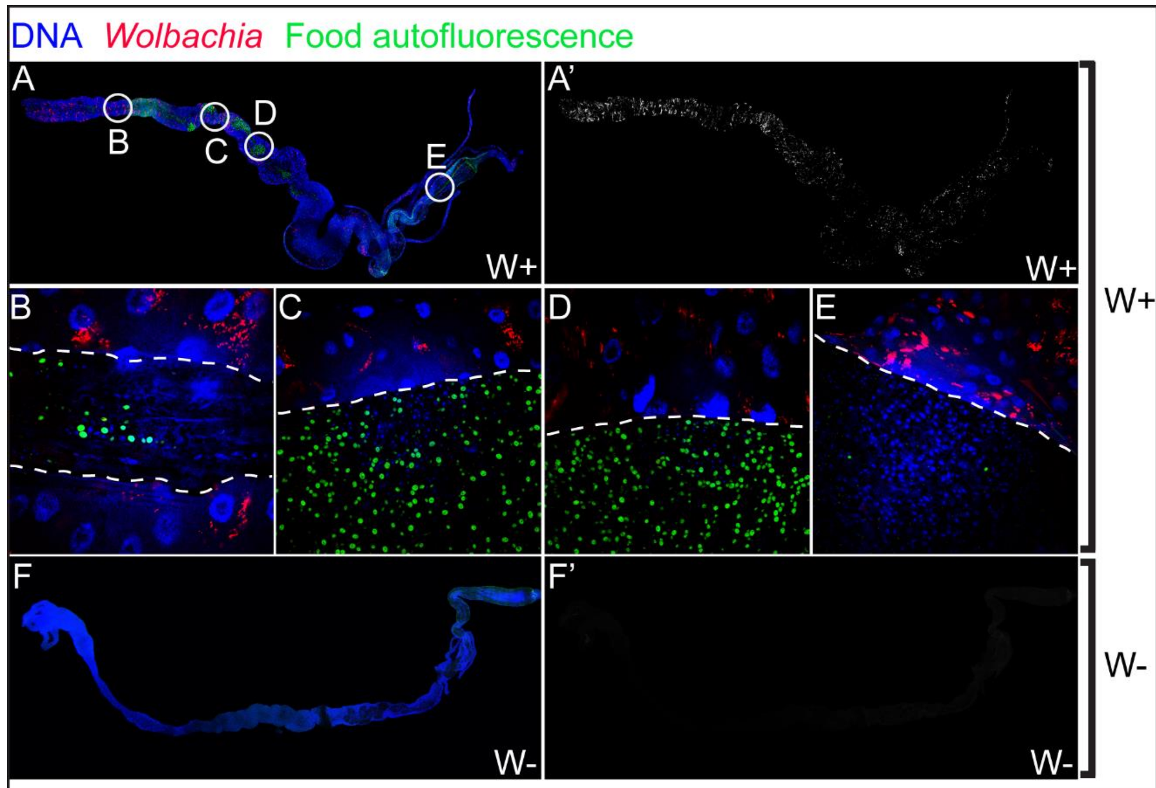
possibility of *Wolbachia* in the lumen directly affecting the gut commensal bacteria. Higher magnification confocal images (Fig. 3.12 B-E) showed that *Wolbachia* were present only in the gut epithelia and absent from the lumen. We found that *Wolbachia* do not occupy the same niche as the gut microbiome, in agreement with previously reported absence of *Wolbachia* from the fecal matter (Fink et al., 2013).

Figure 3.11: *A. pasteurianus* is absent in *Wolbachia* infected L3 larval guts.



(A-F) Composites Z-stack projections of confocal images of gnotobiotic L3 larval midguts stained using FISH probes against *Wolbachia* and *A. pasteurianus*, and Hoecsht against DNA. (A-C) *Wolbachia*-free, and (D-F) *Wolbachia*-infected guts. (A, D) *Wolbachia* channel, (B, E) *A. pasteurianus* channel, and (C, F) merge of *Wolbachia* (red), *A. pasteurianus* (green), and DNA (blue).

Figure 3.12: *Wolbachia* are present in the gut cells but absent from the lumen.



Composite confocal images of the whole midgut and hindgut of *Wolbachia* infected flies (A) and *Wolbachia*-free flies (F), and their respective *Wolbachia* channels (A′ and F′). 60X magnification of midgut (B-D) and hindgut regions (E) illustrates that *Wolbachia* are present intracellularly in the gut cells, but are absent from the lumen of the gut (lumen marked by green autofluorescence).

3.8 Discussion

The intracellular bacteria *Wolbachia* infect a wide range of insect species including fruit flies both in the lab and in the wild. It has been estimated that about 30% of all *Drosophila* lab stocks in the Bloomington stock center are infected with *Wolbachia* (Clark et al., 2005). Since *Wolbachia* alters many aspects of the host biology, this has a significant impact on many phenotypic and genetic studies.

The microbiome of the flies is also an important determinant of many aspects of host health, including development, immunity, metabolism, and fecundity. Given that both *Wolbachia* and the microbiome have independently been shown to affect host phenotypes, it is important to elucidate the relative contributions of each of the components towards host phenotypes. Phenotypes that have previously been thought to be a result of *Wolbachia* infection alone could be due to the changes in microbiome composition due to *Wolbachia* infection. However, little evidence exists to show that *Wolbachia* and the microbiome interact and that *Wolbachia* infection can alter the microbiome composition.

Here we show that *Wolbachia* infection can indeed alter the microbiome composition. *Wolbachia*-free flies harbor both *Acetobacter* and *Lactobacillus* species,

whereas *Wolbachia*-infected flies harbor mostly *Lactobacillus*. In flies, the offspring derive their gut microbiota from the environment. In the case of lab-reared stocks, the feces of the parents populate the microbiome of the offspring. We asked whether the *Wolbachia*-induced changes in the microbiome are due to faulty transmission of the microbiome from the parents to the offspring. To answer this, we generated both *Wolbachia*-free and infected gnotobiotic offspring that have both *A. pasteurianus* and *L. plantarum*. On testing the microbiome composition of both larvae and adults, we found that a significant portion of *Wolbachia*-infected L3 larvae have no *A. pasteurianus* compared to *Wolbachia*-free larvae.

To test whether *Wolbachia* is spatially excluding *A. pasteurianus* in the gut, we performed FISH of both *Wolbachia* and *A. pasteurianus*. We found that *Wolbachia* is present only in the gut epithelia and is absent from the lumen, eliminating the possibility of direct competition between the two species. Further, we found that *A. pasteurianus* was completely absent in all regions of *Wolbachia*-infected guts. This is in concordance with the results from gnotobiotic larvae.

CHAPTER 4

Effects of *Wolbachia* and microbiota on gut immunity and physiology

(Portions of this chapter previously published in Simhadri et al., 2017)

Wolbachia can alter the composition of resident microbes in an insect host. Recent work in *Anopheles stephensi* mosquito also shows variation of *Wolbachia*-induced differences in the mosquito microbiome. Directly after a blood meal, *Wolbachia*-infected mosquitoes had reduced levels of gammaproteobacteria compared to *Wolbachia*-cured animals (see Fig. 2 in (Chen et al., 2016)). A week post blood meal, *Wolbachia*-infected mosquitoes have a significantly more diverse microbiota compared to the *Wolbachia*-free insects. However, when fed with just sugar meal or immediately post blood meal, there are no differences between *Wolbachia*-free and infected mosquitoes (see Table 1 in (Chen et al., 2016)). Another study in *D. melanogaster* showed that the presence of *Wolbachia* reduced the diversity of the gut microbiome (See Fig. 2B in (Ye et al., 2017)), and also reduced the abundance of bacteria in the *Acetobacter* genus (see Table 2 in (Ye et al., 2017)).

Findings presented here are in accordance with mounting evidence showing a complex interaction between *Wolbachia* and commensal microbes, which is influenced by several variables. (Ye et al., 2017, Dittmer et al., 2014,

Rossi et al., 2015, Chen et al., 2016, Hughes et al., 2014). We show here for the first time that the genotype of the host is highly relevant in discerning these interactions. Therefore, we investigated both in vitro and whole animal systems to address the mechanisms by which *Wolbachia* play a role in the determination of the microbial composition.

4.1 *Wolbachia* affects host immunity in a cell line system

We analyzed the effects of *Wolbachia* on a *D.* melanogaster cell line system, JW18. JW18 cell lines were derived from the embryonic cell suspension of a *Wolbachia*-infected transgenic fly strain carrying the Jupiter^{GFP} transgene (Serbus et al., 2012). A *Wolbachia*-free version, JW18DOX was created by treating the JW18 cell line with doxycycline (Fig. 4.1). We reasoned that transcriptional profiling of the two cell lines would provide insights into the processes that *Wolbachia* alter in the host. However, doxycycline treatment can affect many physiological processes such as fitness (O'Shea and Singh, 2015), fecundity, metabolism (Ridley et al., 2013) and mitochondrial function (Moullan et al., 2015) in flies. To eliminate the confounding variable of antibiotic treatment on the cell lines, JW18DOX cells were stably re-infected with *Wolbachia* purified from the JW18 cells to create a new cell line, JW18R (for Re-infected). JW18R is *Wolbachia*-infected and has the same genetic background as JW18DOX cells. RNAseq

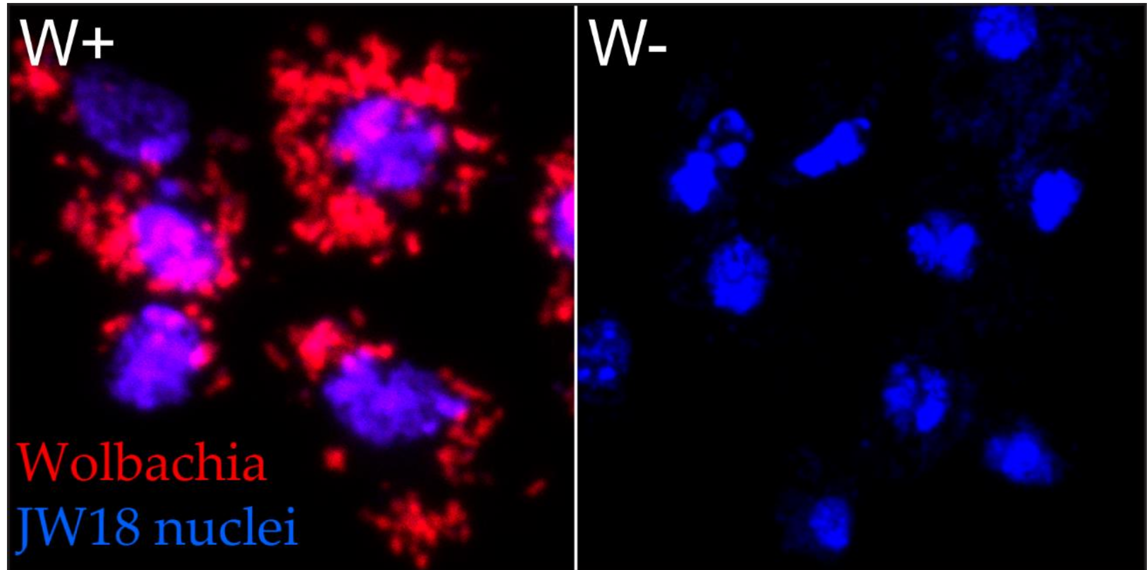
analysis of all three cell lines was then performed in triplicate (see Materials and Methods). TopHat was used for alignment to the fly genome and Cufflinks was used for differential analysis (Trapnell et al., 2012). Differentially expressed genes (p adjusted <0.05) between the JW18 and JW18DOX, or JW18R and JW18DOX were identified and visualized using a custom MATLAB script. DAVID was then used to identify biological pathways that were significantly enriched from the differentially expressed genes between *Wolbachia*-free and infected cells (Huang da et al., 2009). Host defense and immunity-related genes, followed by stress response, were the most significantly enriched when comparing either JW18 or JW18R against JW18DOX (Table 4.1). Fly molecular immunity comprises of two innate immune pathways, Imd and Toll (see section 1.4.2.1). The main effectors of the pathway are the anti-microbial peptides (AMPs), which are secreted by various tissues to eliminate bacterial infections. We inspected the expression of the AMPs in both the cell lines and we found that the AMPs of the Imd pathway, but not the Toll pathway were highly upregulated in the *Wolbachia*-infected cells (Fig. 4.2 A). Some AMPs of the IMD pathway that were expressed in the *Wolbachia*-infected cells were not expressed in the *Wolbachia*-free cells. Among the AMPs that were expressed in both the cell types, the *Wolbachia*-infected cells had 10-1000 fold more expression than the *Wolbachia*-free cells. Further, *Relish* (*Rel)*,

encoding the transcription factor of the Imd pathway that controls the expression of the AMPs, was also elevated in the *Wolbachia*-infected cells (Fig. 4.2 B).

Since AMPs have been routinely used as a readout for the activity of the Imd pathway, the upregulation of *Rel* transcripts might indicate higher activity of the transcription factor or higher levels of its active form. Rel protein is synthesized in an inactive form with the ANK domain in its C-terminal half acting as a negative regulator. However, on activation of the Imd pathway, the ANK domain is removed by cleavage and the active transcription factor translocates to the nucleus to enhance the expression of downstream targets (Fig. 1.3) (Stoven et al., 2000). To probe for the levels of active Rel, we performed western blotting on four separate cell lysates using an antibody against the C-terminus of Rel. The full-length inactive form of Rel is 110kDa, and on activation, the C-terminal ANK domain is 49kDa (Fig. 4.3) (Stoven et al., 2000). The *Wolbachia*-infected JW18 cell lysates predominantly have only the active form of Rel, while the *Wolbachia*-free cell lysates have both forms of the protein. On quantifying the ratio of the active to inactive form of the protein, we found that the *Wolbachia*-infected cells have about 2.75 times more active form of the protein compared to the *Wolbachia*-free cells (p=0.002, student t-test) (Fig. 4.3).

These results together show that the presence of *Wolbachia* in the JW18 cell line system is sufficient to upregulate the immune responses of the host cell, specifically via the Imd pathway.

Figure 4.1: JW18 cells stained for *Wolbachia* using FISH.



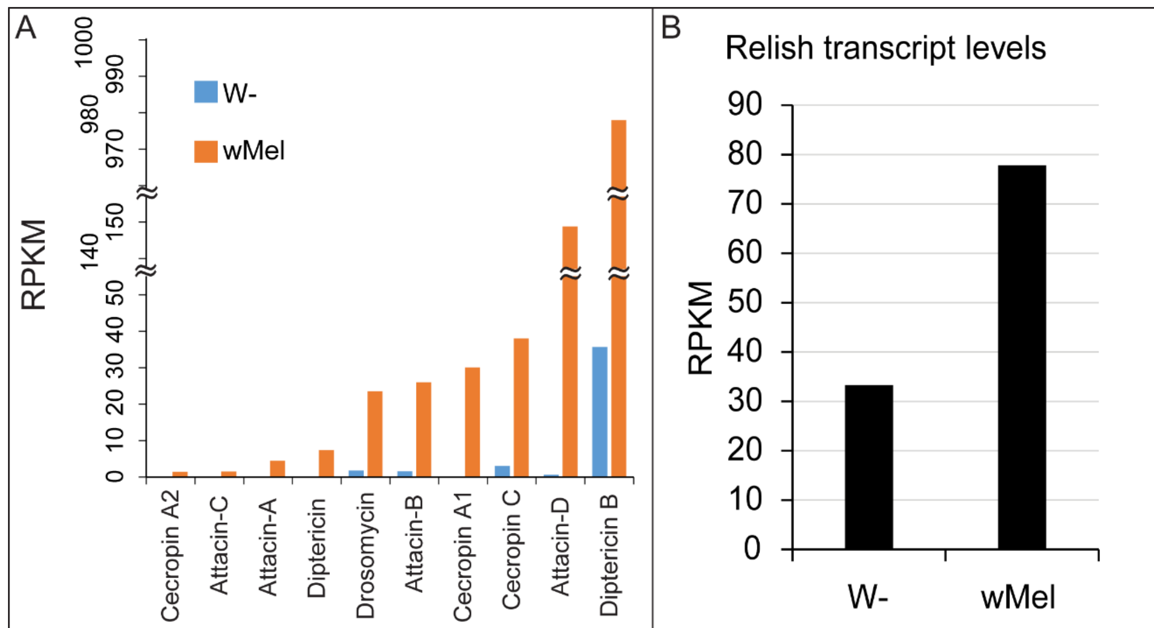
The left panel shows *Wolbachia*-infected JW18 cells with *Wolbachia* in red and host cell nuclei in blue. The right panel shows *Wolbachia*-free JW18DOX cells. Images were obtained by z-stack projection of confocal microscopy of cells stained by FISH probes against *Wolbachia* and Hoecsht against DNA.

Table 4.1: Pathway enrichment analysis of the differentially expressed genes between JW18 and JW18DOX cell lines using DAVID

DAVID was used to identify clusters of biological functions that were significantly altered due to *Wolbachia*-infection based on the list of differentially expressed genes

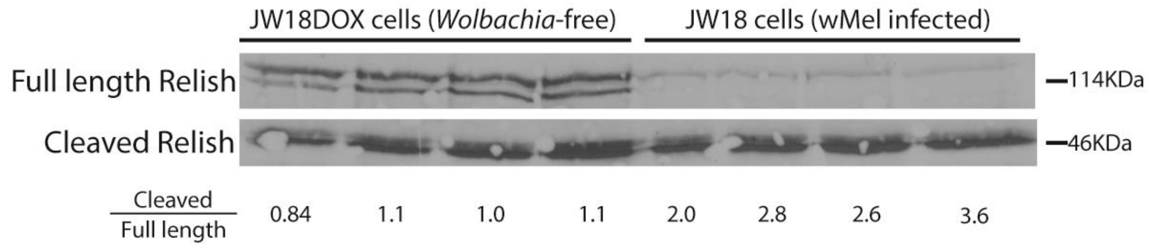
Annotation Cluster 1	Enrichment Score: 4.10	
Term	Count	PValue
Antimicrobial	5	0.00275
innate immunity	7	0.00316
immune response	7	0.00381
antibiotic	4	0.00477
Annotation Cluster 2	Enrichment Score: 4.09	
Term	Count	PValue
stress response	11	1.77E-10
heat shock	7	1.09E-08
stress-induced protein	7	1.09E-08
molecular chaperone	4	6.60E-04
ATP	5	0.1162
Annotation Cluster 3	Enrichment Score: 3.38	
Term	Count	PValue
glutathione transferase	6	1.07E-04
Annotation Cluster 4	Enrichment Score: 2.48	
Term	Count	PValue
cellular carbohydrate biosynthetic process	6	4.79E-04
Annotation Cluster 5	Enrichment Score: 2.07	
Term	Count	PValue
cofactor metabolic process	11	8.09E-04

Figure 4.2: Immunity pathway is highly upregulated in *Wolbachia*-infected cells compared to *Wolbachia*-free cells.



(A) Transcriptional profiling of AMPs of the Imd pathway shows that they are highly upregulated in *Wolbachia*-infected cells compared to the *Wolbachia*-free cells. Drosomycin is an AMP of the Toll pathway (B) mRNA levels of *Rel*, the transcription factor of the Imd pathway are upregulated in *Wolbachia*-infected cells compared to *Wolbachia*-free cells. Bar graphs represent the means of three replicates. All bars shown here are statistically significantly different at $p<0.05$, statistical tests performed using cufflinks (Trapnell et al., 2013, Trapnell et al., 2012)

Figure 4.3: Rel is predominantly in the active form in *Wolbachia*-infected JW18 cells.



Relish is synthesized as an inactive precursor that is activated by the cleavage of the inhibitory ankyrin domain when the Imd pathway is active. Full-length Rel is 110kDa, and the cleaved C-terminal inhibitory ANK domain is 49kDa. Show here is a western blot on the replicates of cell lysates of JW18 and JW18DOX cell lines using antibodies against the C-terminal portion of Rel. The ratio of the cleaved to full-length Rel (both the bands near 110KDa) is shown at the bottom.

4.2 *Wolbachia*'s presence in flies does not alter gut immune effectors

Since *Wolbachia* can alter the composition of the fly microbiome, but cannot directly interact with the gut commensal microbes, we hypothesized that *Wolbachia*-induced changes in host immunity might be responsible for this phenotype. Evidence from the cell line model shows that the Imd pathway is up-regulated in the presence of *Wolbachia*. Furthermore, Imd is the major pathway responsible for the production of AMPs in the fly gut (Buchon et al., 2013a, Tzou et al., 2000). Therefore, we first asked whether the presence of *Wolbachia* alters the Imd pathway in the L3 larval gut, where we observe the maximal effect of *Wolbachia* on the gut microbiome.

4.2.1 *Wolbachia* does not affect the expression of Imd pathway components in the larval gut

To examine the possibility of *Wolbachia* altering the gut immune responses in the L3 larval guts, we first generated triplicates of axenic organisms that are either *Wolbachia*-free or infected. We then isolated whole guts of ten axenic L3 larvae for each replicate, extracted whole RNA, and performed quantitative RT-PCRs on several immunity-related genes. We first tested for the expression of the Immune deficiency (*imd*) and *Rel* genes, which are the key components in actuating the signal in the major immunity pathway in the gut tissue, and found

that there were no significant transcriptional differences due to the presence of *Wolbachia* (Fig. 4.4 A). Since antimicrobial peptide (AMP) expression is a well-characterized immune response readout, we measured the expression levels of all Anti-Microbial Peptides (AMPs) that are downstream of the Imd pathway. Expression of *AttB*, *AttD*, *CecA2*, *CecC*, *DptB*, and *Dro* was not different between *Wolbachia*-free and infected L3 larval gut tissue (Fig. 4.4 B). Other AMPs downstream of Imd, such as *AttA*, *AttC*, *CecA1*, *CecB*, and *Dpt* were either not expressed or expressed at low levels. These results indicate that in the axenic conditions, *Wolbachia* alone do not activate Imd signaling.

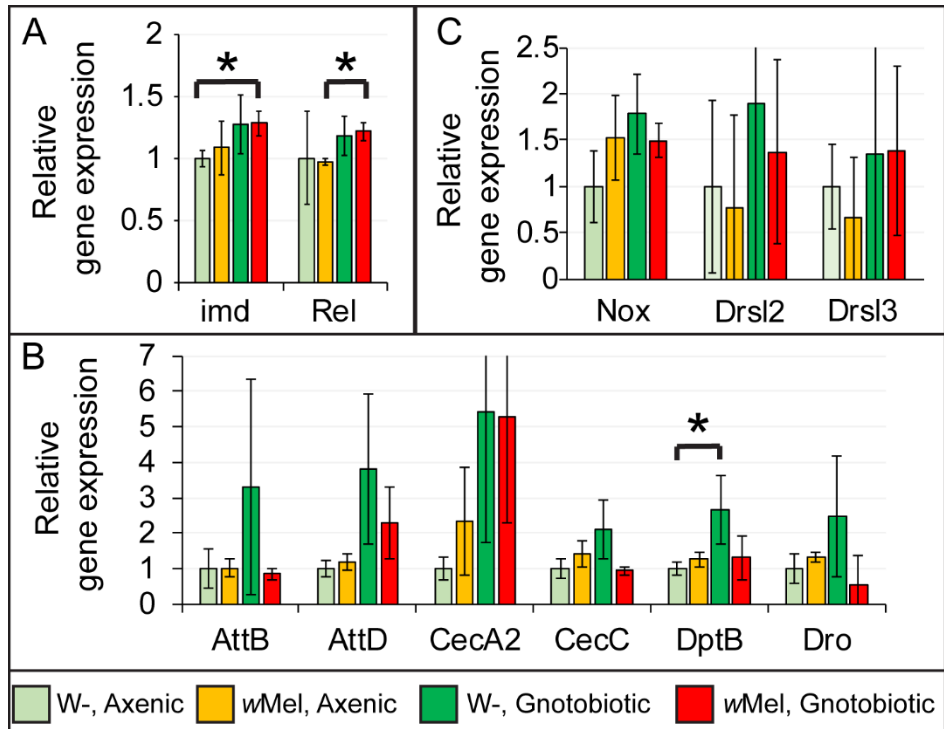
To quantify the expression of Imd pathway genes in the presence of gut bacteria, we generated gnotobiotic larvae by seeding germ-free larvae with *A. pasteurianus* and *L. plantarum* (see Materials and methods). Compared to the axenic larvae, the expression levels of *imd* and *Rel* were moderately upregulated in the presence of gut microbes (Fig. 4.4). Importantly, this upregulation was independent of the *Wolbachia* infection status. In the gnotobiotic flies, *imd* and *Relish* expression was similar in *Wolbachia*-free and infected larval guts (Fig. 4.4 B). Regarding AMP production, again, *AttA*, *AttC*, *CecA1*, *CecB*, and *Dpt* were either not expressed or expressed at low levels. For expressed AMPs, the introduction of commensal bacteria caused an overall increase in the levels of

AMPs expressed. Due to the high levels of variability in expression levels, only *Diptericin B* (*DptB*) was statistically significant. The presence of *Wolbachia* did not result in significant changes in AMP genes expression in the gnotobiotic flies.

In addition, the JAK/STAT pathway is also relevant for gut innate immunity, and can regulate the expression of some AMPs (Osman et al., 2012, Buchon et al., 2009b). We tested *Drsl 2* and *Drsl 3* (also known as *dro 2* and *dro 3*) transcriptional levels under different *Wolbachia* and microbiome status. Again, we found no significant differences due to the presence of *Wolbachia* in axenic or gnotobiotic L3 larvae (Fig. 4.4 C).

Together these results suggest that *Wolbachia* modulation of the microbiome is independent of AMP production in the gut.

Figure 4.4: *Wolbachia* infection does not affect expression of Imd pathway components and ROS producing oxidases



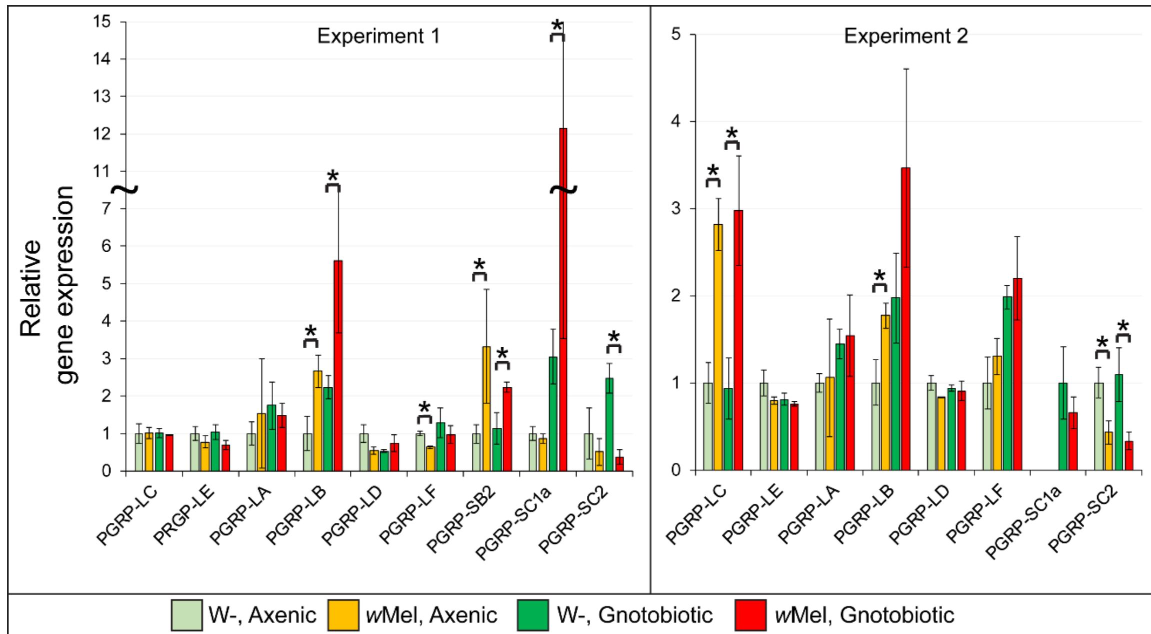
Relative expression of Imd pathway signal transducer *imd* (A), the transcription factor *Rel* (A), AMPs (B), *Nox* (C), and AMPs downstream of JAK/STAT signaling (C), both in axenic and gnotobiotic L3 larval guts in the presence or absence of *Wolbachia* infection, determined by quantitative RT-PCR. All conditions are normalized to *Wolbachia*-free axenic L3 larval guts. Bar graphs show means (N=3 biological replicates of 10 larvae each) and error bars are standard deviations. Asterisk shows statistical significance of P<0.05 (student t-test).

4.2.2 *Wolbachia* affect the expression of negative regulators of Imd pathway

Negative regulators of the Imd pathway are critical for the maintenance of the gut homeostasis in flies, as they prevent chronic and over-activation of the immune responses and inflammation (reviewed by (Buchon et al., 2014)). The Imd pathway is activated in response to microbial stimuli either systemically or in the gut. The negative regulators are necessary to limit the time and extent of the anti-microbial responses, as over activation of immune responses can affect host development and reduce lifespan (Zaidman-Remy et al., 2006, Bischoff et al., 2006, Paredes et al., 2011). The negative regulators work in two major ways: 1) They inhibit the signal transduction within the cell (such as PGRP-LF, which dimerizes with the receptor PGRP-LE and inactivates it (Persson et al., 2007)), or 2) they are secreted outside the cell to degrade the bacterial components that activate the Imd pathway (PGRP-SC (Bischoff et al., 2006) and PGRP-LB (Zaidman-Remy et al., 2006) degrade the intact peptidoglycan molecules) (Paredes et al., 2011, Costechareyre et al., 2016). To examine the possibility of *Wolbachia* altering the expression of these negative regulators in the L3 larval guts, we performed duplicate experiments using qRT-PCRs on all peptidoglycan recognition proteins in the RNA derived from the midguts of ten axenic and gnotobiotic, *Wolbachia*-free and infected L3 larvae. *PGRP-LB* and *PGRP-SC2* are

the most consistent and significantly affected genes in the presence of *Wolbachia* (Fig. 4.5). *PGRP-LB* expression is upregulated and *PGRP-SC2* expression is down-regulated in the presence of *Wolbachia*, in both axenic and gnotobiotic L3 larval guts. Both PGRP-LB and PGRP-SC2 are amidases that negatively regulate the Imd pathway and are important in the maintenance of gut homeostasis by suppressing constant inflammatory response to innocuous gut bacteria (Paredes et al., 2011). Even though, *PGRP-SC2* is expressed at much higher levels compared to *PGRP-LB* throughout the gut (Dutta et al., 2015) and current study), PGRP-LB is more functionally relevant in regulating the Imd pathway in the gut (Costechareyre et al., 2016). These results show that the presence of *Wolbachia* leads to the upregulation of a negative regulator of the Imd pathway, which may explain the slight (even though statistically not significant) reduction in the AMP levels in *Wolbachia*-infected gnotobiotic L3 larvae.

Figure 4.5: *Wolbachia* affects the expression of negative regulators in the L3 larval gut.



Relative expression of Imd pathway receptors and negative regulators, both in axenic and gnotobiotic L3 larval guts in the presence or absence of *Wolbachia* infection, determined by quantitative RT-PCR. Two separate experiments were performed and all conditions are normalized to *Wolbachia*-free axenic L3 larval guts. Bar graphs show means (N=3 biological replicates of 10 larvae each) and error bars are standard deviations. Asterisk shows statistical significance of P<0.05 (student t-test).

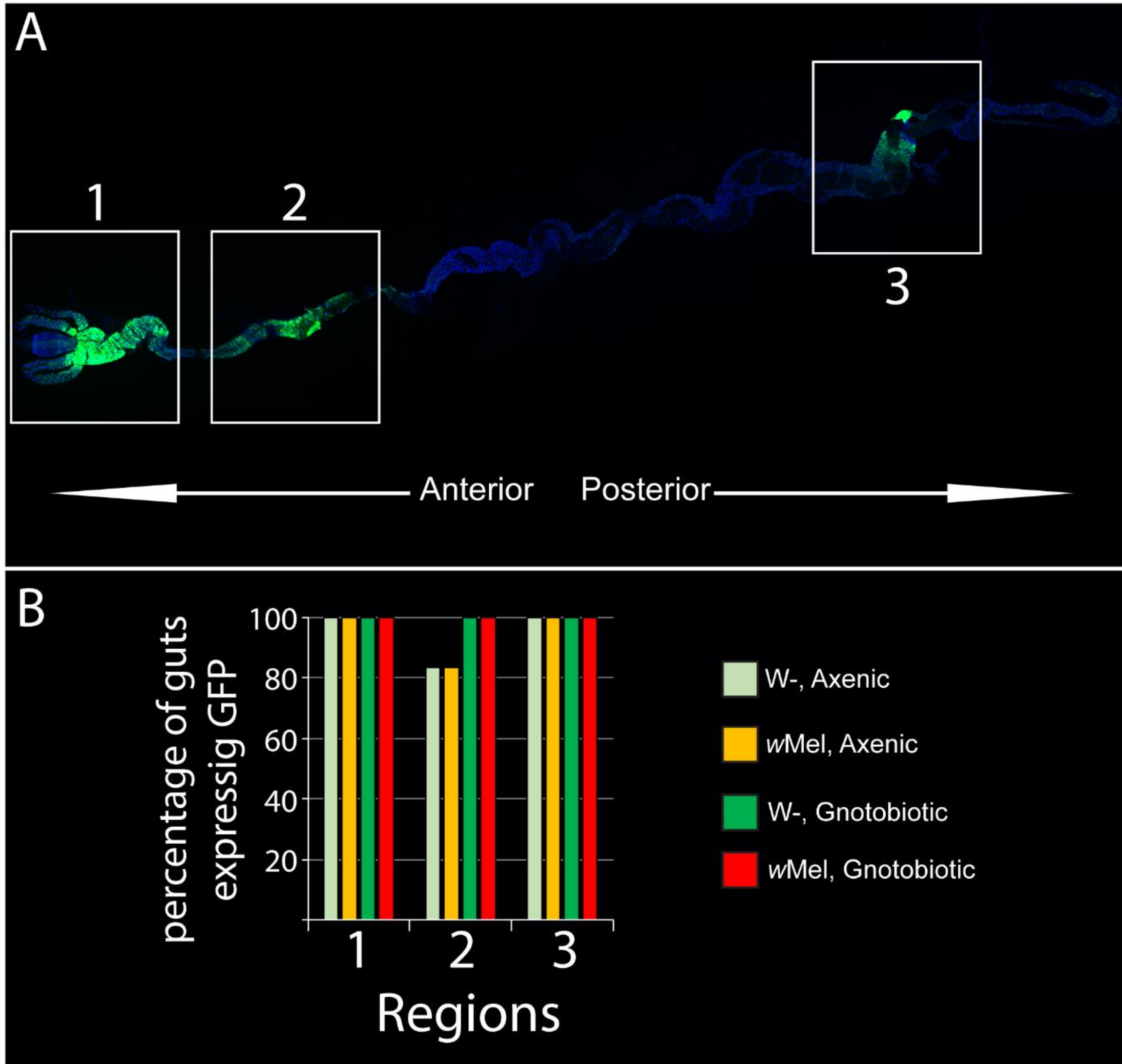
4.2.3 Neither *Wolbachia* nor the microbiota affect expression of ROS effectors or the levels of ROS in the larval gut

Another key defense mechanism in the gut is the production of reactive oxygen species (ROS). A readout for the immune activation of ROS is upregulation of *Nox* and *Duox* genes (Bae et al., 2010). *Duox* was not expressed in the guts and and levels of *Nox* expression were not different between *Wolbachia*-free and infected guts (Fig. 4.4 C).

However, ROS are also generated by cellular respiration in the mitochondria and the presence of *Wolbachia* could affect the cellular metabolism. To measure the levels of ROS-related stress in the gut, we utilized a transgenic construct with the promoter of *GstD1* fused to GFP coding sequence as a readout (Sykiotis and Bohmann, 2008). Since the *GstD1* promoter is active in the presence of oxidative stress, elevated levels of ROS due to *Wolbachia* infection can be observed by imaging the GFP expression in guts. We introduced the GFP reporter transgene into the *upd*>hPABP-Flag by mating with the GstD1-GFP flies and utilizing the first generation of a cross between the two genotypes for this analysis. We produced axenic and gnotobiotic, *Wolbachia*-free and infected L3 larvae, dissected six organisms from each condition, and imaged the guts to examine the expression of GFP (Fig. 4.6 A). We observed similar patterns of GFP

expression in the gut across all conditions irrespective of the presence of *Wolbachia* or gut commensal microbiota (Fig. 4.6 B) suggesting that *Wolbachia* do not significantly alter the redox status in the gut.

Figure 4.6: ***Wolbachia*** **do not alter ROS levels in the gut**



(A) Representative image of the GFP expression in L3 larval gut in response to oxidative stress. The regions in the boxes are the signatures of ROS levels across all conditions used in the experiment. (B) Quantification of the percentage of guts expressing GFP in the three regions specified in (A) (Bar graphs show mean of 6 samples, no statistically significant differences between any groups, Chi-square test with Yate's correction).

4.3 Microbiome composition in *Relish* mutants

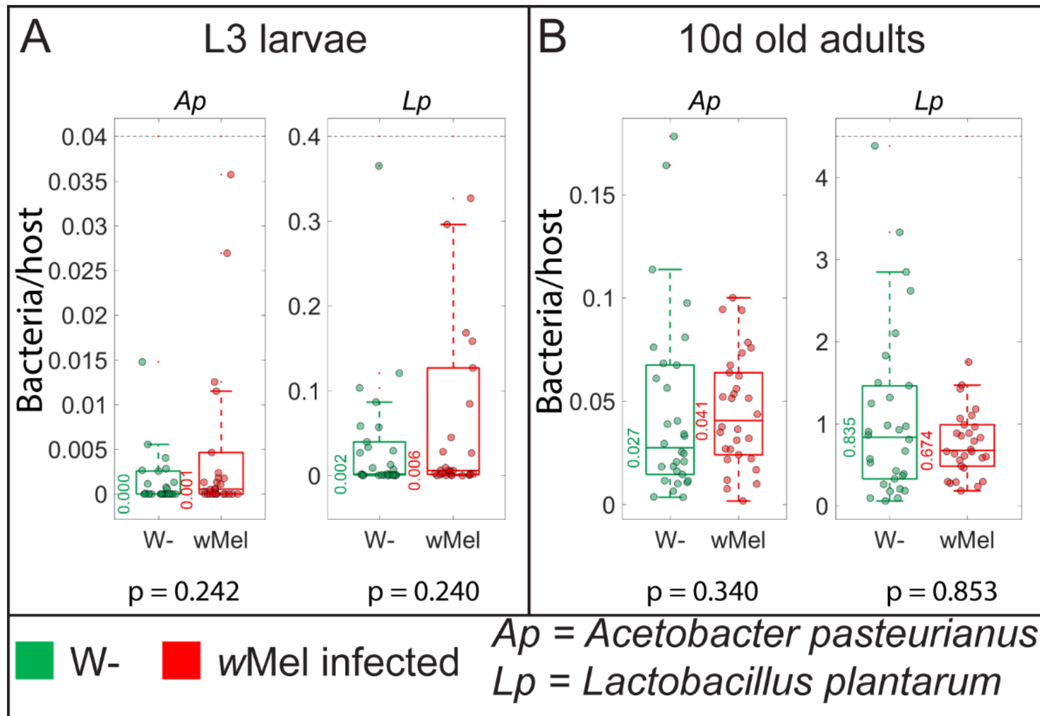
Previous experiments indicate that *Wolbachia* do not alter the expression of most AMPs downstream of the Imd pathway, but affects some of the negative regulators, such as *PGRP-LB* and *PGRP-SC2* (Fig. 4.5). However, this does not eliminate the possibility that the post transcriptional regulation of these components could be affecting the microbiome composition. To address the functional role of the Imd pathway in altering the microbiome composition, we introduced *Wolbachia* into a widely used mutant strain of *Rel*, rel^{E20}. To introduce *Wolbachia* into this stock, we mated the males of the rel^{E20} with the females of either *Wolbachia*-free or infected *w*; CyO/Sco; TM6b/MKRS and selected for *w*; CyO/+; MKRS/rel^{E20}. Then we mated these first generation offspring with the males of rel^{E20} again to obtain *w*; ;rel^{E20} flies that are either *Wolbachia*-infected or free. Using these flies, we generated axenic and gnotobiotic embryos. We then extracted whole DNA from 30 individual organisms from the L3 larval stage and from 10 day old adults. We then measured the amount of each of the species of the gut bacteria per host using qPCR as descried below.

4.3.1 *Wolbachia* induced microbiome changes are absent in Relish mutants

Gnotobiotic organisms were produced and individual organisms were sampled to assay the levels of each gut bacteria relative to the host. Three

separate experiments were performed, and 10 individuals (at L3 larval stage and 10 day old adults) were sampled from each experiment. On performing qPCR with species-specific primers and comparing to the host DNA, we found that the levels of both species of gut bacteria, *A. pasteurianus* and *L. plantarum*, are not statistically different between *Wolbachia*-free and infected L3 larvae or 10 day old adults (Fig. 4.7). However, the levels of both the bacteria in the L3 larvae of the *rel*^{E20} genotype were significantly lower than in the L3 larvae of the *upd*>hPABP-Flag genotype (about 100 fold). Hence, the reduction in the levels of *A. pasteurianus* due to the *Wolbachia* infection that was observed in the *upd*>hPABP might not be apparent in the *rel*^{E20} genotype, as the levels of *A. pasteurianus* in both the *Wolbachia*-free and infected larvae are very low.

Figure 4.7: *Wolbachia* have no effect on microbiome composition in *Rel* mutants



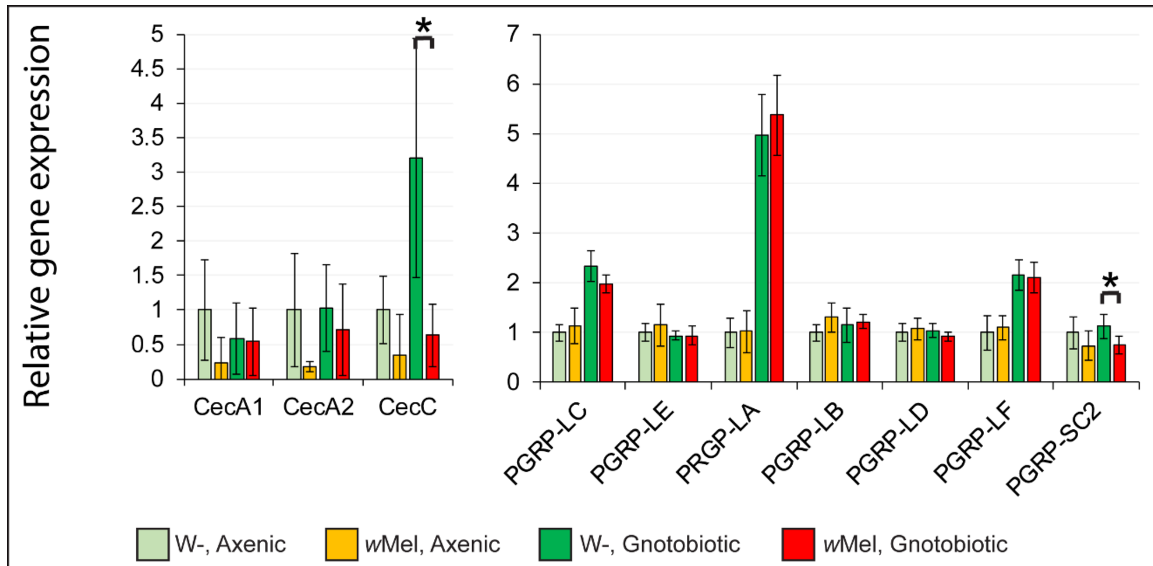
Measurement of levels of *A. pasteurianus* and *L. plantarum* in rel^{E20} L3 larvae. (A, B) Box-Whisker plot of levels of each bacterium in L3 larval (A) and 10d adult stages (B) (N = 30, median values are shown next to the boxes. p values of two-sided Wilcoxon rank sum test are reported).

4.3.2 Transcriptional changes induced by *Wolbachia* on the negative regulators of the Imd pathway are dampened in Relish mutants

We sampled the guts of the gnotobiotic organisms produced in 4.3.1 to measure the expression levels of various Imd pathway components by qRT-PCR. Since Rel is the major transcription factor that controls the expression of most AMPs in the IMD pathway, rel^{E20} mutants did not express most AMPs (Fig. 4.8). *CecA1*, *CecA2*, *CecC*, were expressed, but at extremely low levels.

On measuring the transcription profiles of the PGRP family of genes, we observed no statistically significant effects of *Wolbachia* on any gene except *PGRP-SC2*. However, interestingly, the gut microbiota induced an up-regulation of transcripts in *PGRP-LC*, *PGRP-LA*, and *PGRP-LF* ($p<0.05$, student t-test), independent of *Wolbachia* status (Fig. 4.8). Since the expression of PGRP-SC2 is also closely linked to other stress responses induced by the FOXO transcription factor (Guo et al., 2014), the effect of *Wolbachia* on this gene can be observed even in *Rel* mutants.

Figure 4.8: Effect of *Wolbachia* on the expression of Imd pathway effectors and negative regulators



Relative expression of Imd pathway AMPs and negative regulators, both in axenic and gnotobiotic L3 larval guts in the presence or absence of *Wolbachia* infection, determined by quantitative qRT-PCR. All conditions are normalized to *Wolbachia*-free axenic L3 larval guts. Bar graphs show means (N=3 biological replicates of 10 larvae each) and error bars are standard deviations. Asterisk shows statistical significance of P<0.05 (student t-test).

4.4 *Wolbachia* dependent changes in the microbiome are host genotype dependent

Since *Wolbachia*-dependent effects on the microbiome are absent in *Rel* mutants, we asked whether this is a non-specific effect due to other factors in the *Rel* mutant genotype or if it is specifically due to the mutation in *Rel*. The *upd*>hPABP-Flag genotype has two balancer chromosomes with severe gene rearrangements and inversion, and it expresses the human PABP in certain tissues where the *upd* gene is expressed, and these are absent in the *Rel* mutant flies. To address this question of the effect of host genotype on the *Wolbachia*-induced microbiome change, we began by testing other genotypes for this effect.

We first utilized an isogenized *white* homozygous fly genotype (w^{1118}) (see Materials and Methods). We performed the assays in section 3.5 with individual organisms. We generated axenic and gnotobiotic (with equal proportions of *A. pasteurianus* and *L. plantarum*, see Materials and Methods) embryos. Three separate experiments were performed, and 10 individuals (at L3 larval stage and 10 day old adults) were sampled from each experiment and their DNA was extracted. We performed qPCR with species-specific primers and compared to the host DNA to measure microbial levels and variation in L3 larvae and in adult flies (Fig. 4.9). The fraction of L3 larvae that harbored no *A. pasteurianus* were

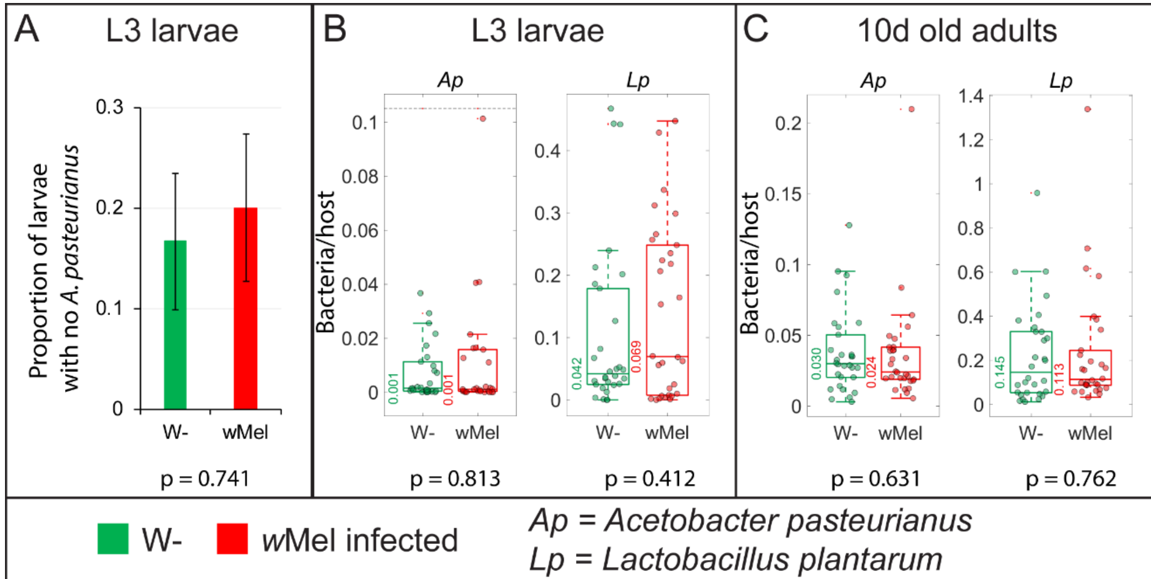
comparable between *Wolbachia*-free and infected flies (20% vs 17% respectively, p = 0.741) (Fig. 4.9 A). There was also no *Wolbachia*-dependent effect on the levels of either *A. pasteurianus* or *L. plantarum* relative to the host (Fig. 4.9 B, C). The bacterial levels on the food were similar between *Wolbachia*-free and infected bottles at both the L3 larval and 10day old adult stages (Fig. 4.10). However, the levels of bacteria in this genotype were at least 10 fold lower than in the *upd*>hPABP-Flag genotype, even though similar amounts of bacteria were seeded to produce the gnotobiotic organisms. These results show that the effect of *Wolbachia* on the gut microbiota can be sensitive to the host genotype.

We then sampled the guts of the gnotobiotic to measure the expression levels of various Imd pathway components by qRT-PCR. We observed no statistically significant effects of *Wolbachia* on any gene. However, the presence of gut microbiota affected the expression of the AMPs, and some negative regulators (PGRP-LB, and PGRP-SC1a) (Fig. 4.11). This shows that the presence of *Wolbachia* affects gene expression in different host genotypes dissimilarly, and thus could also affect the microbiome composition differently.

This is the first study to date to consider the effect of the host genotype on the effects of *Wolbachia* on the microbiome composition. Further, no other study has used gnotobiotic animals to carefully control for the starting conditions.

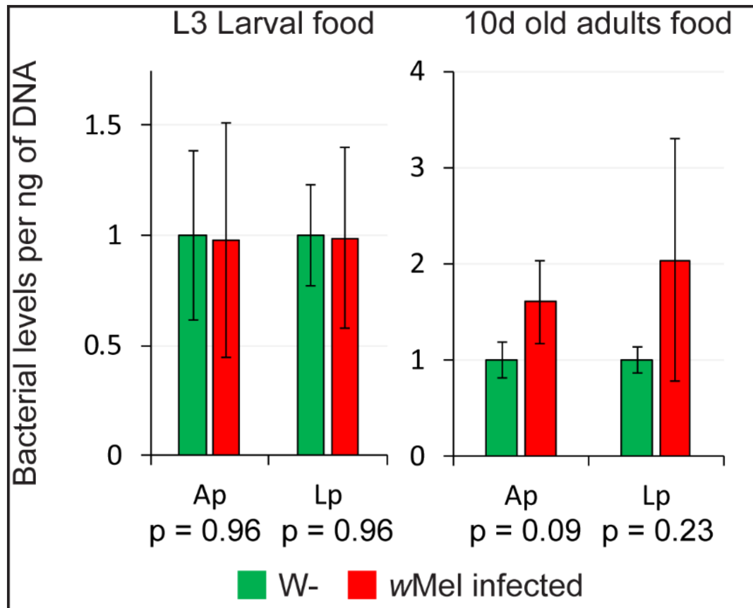
However, thorough future investigations are required to discover host and microbial genetic and epigenetic determinants that can alter the tolerance and carrying capacity of a wide range of host species.

Figure 4.9: *Wolbachia* effect on microbiome is genotype dependent.



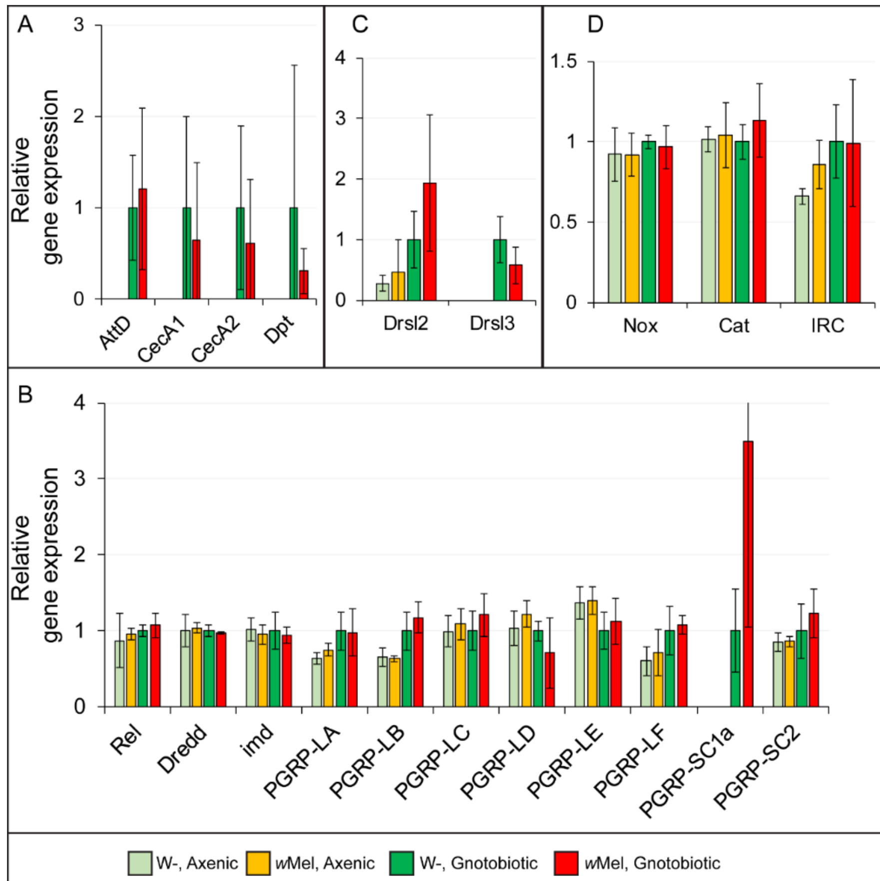
Measurement of *A. pasteurianus* levels in flies of a different genetic background (*white*). (A) Measurement of proportion of individual L3 larvae of different genetic background (*white*) that had no *A. pasteurianus* in the gut (N = 30, Chi-squared test, error bars are confidence intervals). (B, C) Box-Whisker plot of levels of each bacteria in L3 larval (B) and 10d adult stages (C) (N = 30, median values are shown next to the boxes. p values of two-sided Wilcoxon rank sum test are reported).

Figure 4.10: Bacterial levels in the food of w^{1118} (white eyed) flies.



Levels of each bacterial species per nanogram of DNA obtained from the food, normalized to the levels in food inhabited by *Wolbachia*-free flies. Bar graphs represent the means of 3 experiments, error bars are standard deviations. p values are from student t-tests.

Figure 4.11: Effect of *Wolbachia* on the expression of Immune effectors and negative regulators in w^{1118} genotype



Relative expression of Imd pathway AMPs (A); signal transducer *imd*, caspase *Dredd*, transcription factor *Rel*, and PGRP-family of genes (B); AMPs downstream of JAK/STAT signaling pathway (C); *Nox*, and ROS scavengers (D), both in axenic and gnotobiotic L3 larval guts in the presence or absence of *Wolbachia*-infection, determined by quantitative qRT-PCR. All conditions are normalized to *Wolbachia*-free gnotobiotic L3 larval guts. Bar

graphs show means (N=3 biological replicates of 10 larvae each) and error bars are standard deviations.

4.5 Impact of *Wolbachia* and microbiota on the gut pH environment.

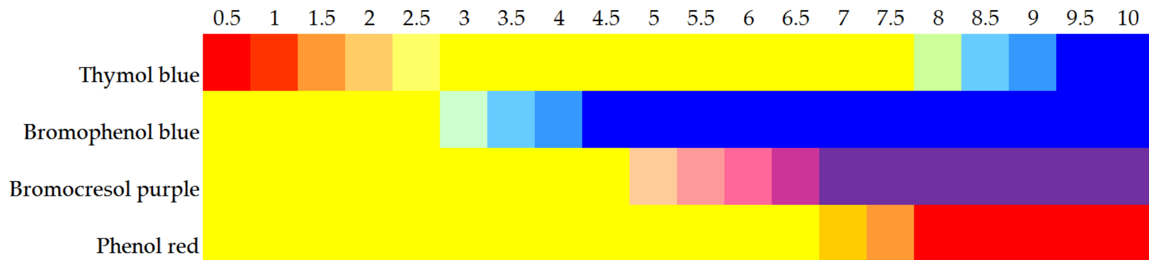
Low pH in the gut of many animals is required for protein digestion, absorption of calcium, iron and vitamin B12, and also important to eliminate ingested pathogens and parasites (Yao and Forte, 2003). A portion of the fly anterior midgut is acidic with pH at around 2, which is analogous to a vertebrate stomach. The generation of acids is mediated by H^+ V-ATPase class of proteins. The gut pH has been shown to influence the microbiome composition and conversely the presence of microbes in the gut can influence the gut pH (Overend et al., 2016). Here we asked if the presence of *Wolbachia* causes a significant change in the gut pH, which in turn can affect the composition of the microbiome.

To address this question, we developed a protocol based on the literature (Overend et al., 2016) to assess the pH of various regions of the gut. Axenic or gnotobiotic feeding L3 larvae were generated as previously described (see section 3.5, and materials and methods), and they were picked out of the food and exposed to apple juice agar plates saturated with a pH indicator for three hours. On dissecting their guts, various regions of the gut assumed different hues depending on the pH of that region. The color hues were utilized to infer

the pH of the region based on a calibration curve previously generated (Fig. 4.13).

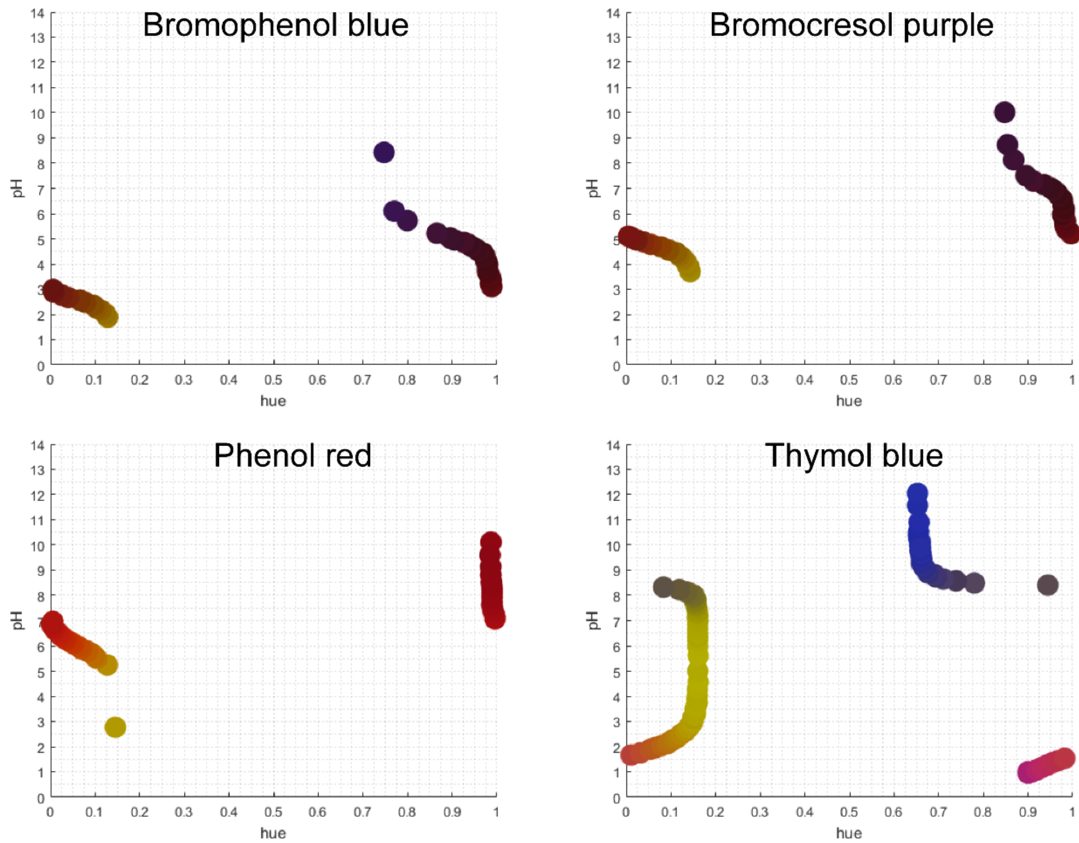
pH indicators have a stable color profile above a certain pH and a different color below a certain pH, and the color transition is not very sharp. In general, the color change occurs over 1-2 pH units, where the intermediates are not easy to interpret. So, we chose several pH indicators to eliminate the problem of having to interpret these colors in the transient pH ranges. The pH indicators were chosen such that the transition region of one pH indicator would be a part of the stable color region of at least one other pH indicator. So, we chose thymol blue, bromophenol blue, bromocresol purple, and phenol red (Fig. 4.12) for this analysis. We then generated calibration curves of the hue of the pH indicator versus the pH of the saturated dye solution as determined by a pH probe, for all four dyes (see materials and methods 2.10.2) (Fig 4.13).

Figure 4.12: pH indicators were chosen based on the orthogonality of their transition regions.



The top row of numbers are the pH values. Four different dyes were used for this analysis: Thymol blue, Bromophenol blue, Bromocresol purple, and Phenol red. pH indicators have a stable color hue above a certain pH and another color hue below a certain pH. The transition regions are harder to define and thus harder to infer the pH values. For this purpose, we utilized several pH dyes with non-overlapping transition regions to avoid having to interpret the transition regions of any pH dye. With the gut pH profile data from all the dyes, we can infer which pH ranges of the gut are affected due to *Wolbachia* or gut microbiota.

Figure 4.13: Calibration curves of pH versus hue of the pH indicators



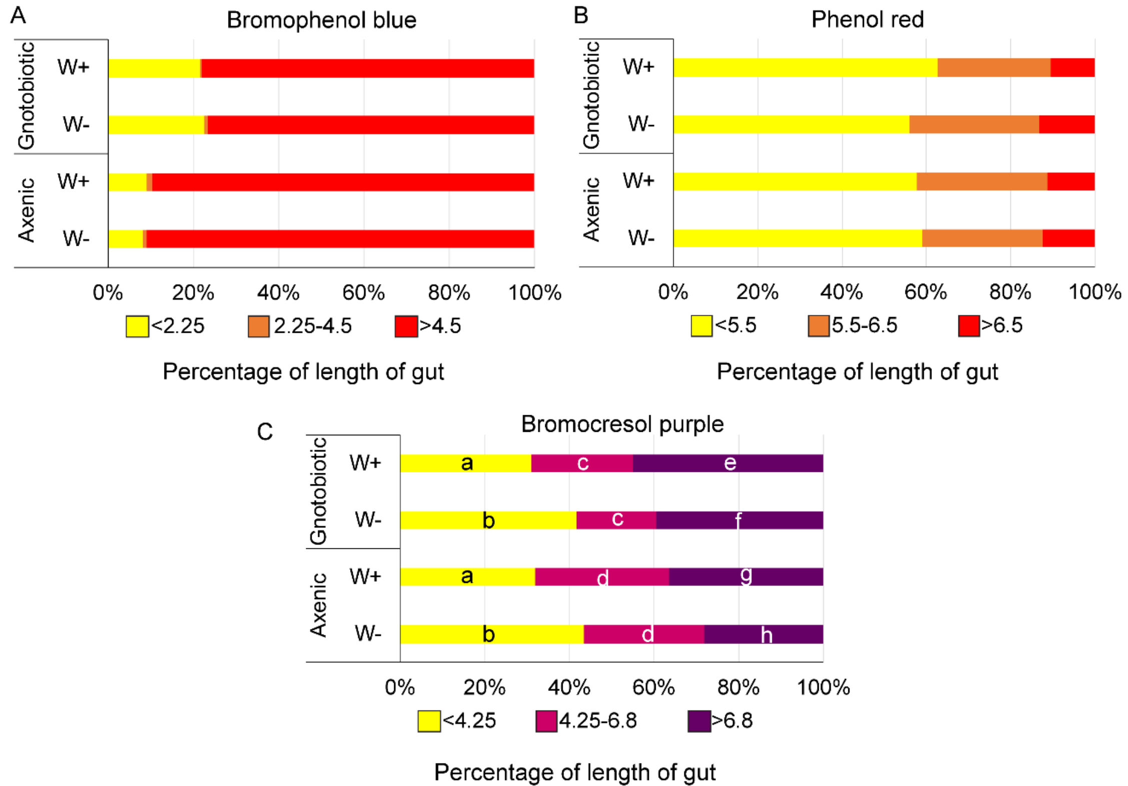
The hue of 200ul of a saturated solution of each of the pH indicator was measured at pHs ranging from 1-12. The pH dye solutions at various pHs were placed in a clear 96 well plate, and images of individual wells were taken using a stereomicroscope. The hues of the dyes were plotted against the pH of the solution at which the image was captured.

4.5.1 Presence of *Wolbachia* reduces the portion of acidic region in the gut

Using the pH indicators and their calibration curves, we then asked if the presence of *Wolbachia* and the microbiome can affect the gut pH. We generated axenic and gnotobiotic *Wolbachia*-free and infected L3 larvae and transferred them to apple juice plates saturated with each of the pH indicators separately. After three hours of feeding, we dissected 20 guts for each condition for each pH indicator plate in 0.9% saline and imaged them at the settings described in 2.10.1. Using a custom script written in MATLAB, we extracted the length of the mid-gut region, and the length of the portions of mid-gut at each hue. Using the calibration curves (Fig. 4.13), we then inferred the fraction of the length of the gut at various pH values (Fig. 4.14). We eliminated thymol blue from the analysis as the L3 larvae on thymol blue food did not feed enough to resolve the pH values. We observed no statistically significant changes in the pH profiles of the guts using bromophenol blue and phenol red (Fig. 4.14 A, B). However, we found statistically significantly changes in the pH profile of guts using bromocresol purple, where *Wolbachia*-infection causes a slight reduction in the fraction of the acidic region of the gut and a slight increase in the basic portion compared to *Wolbachia*-free larval guts (Fig. 4.14 C). We also found that the microbiome affects the pH profile by increasing the portion of the basic region of the gut and

reducing the transition region. The acidic region of the gut was not affected by the presence of gut bacteria (Fig. 4.14 C).

Figure 4.14: Effect of *Wolbachia* on the pH profiles of the gut



The pH profiles of the guts of L3 larvae fed with bromophenol blue (A), phenol red (B), and bromocresol purple (C). The bars of each color of every pH dye represent the proportion of the gut length at a certain pH that is indicated by the legend at the bottom of each panel. There are no statistically significant differences between the pH profiles of the guts fed with bromophenol blue and phenol red due to *Wolbachia*-infection. The gut with bromocresol purple has statistically significant differences in the pH profiles. The proportion of the gut with a pH below 4.25 is significantly affected by *Wolbachia*-infection (a vs b) irrespective of the gut microbial status. The proportion of gut length above pH 6.8 is affected by both *Wolbachia* and the gut microbial status (e vs f vs g vs h).

Finally, the proportions of the gut in the transition regions of pH between 4.25 and 6.8 are affected only the gut microbiome and not by *Wolbachia* (c vs d). Taken together, we can infer from bromocresol purple and bromophenol blue that the proportion of the length of the gut that is between 2.25 and 4.25 is statistically significantly altered due to *Wolbachia*-infection. The percentage of guts at each pH range are compared across all infection conditions, the letters on the bar graphs represent statistical significance at $p<0.05$, Chi-square test.

4.6 Discussion

To determine the mechanism of *Wolbachia*-induced changes in the microbiome composition, we investigated several possibilities. We first asked whether *Wolbachia*-infection alters the immune homeostasis of the host, which in turn could lead to altered microbiome composition. We addressed this question in both a cell line system and in intact guts of the host. *Wolbachia*-infected cell lines showed an elevated Imd pathway response compared to *Wolbachia*-free cells at both transcriptional and post-transcriptional levels. However, we did not observe any *Wolbachia*-induced elevated levels of Imd pathway effectors such as AMPs in the gut. On the contrary, expression of certain negative regulators was affected by the presence of *Wolbachia*. To test whether these negative regulators were the cause of the altered microbiome, we obtained mutants of the major transcription factor of the Imd pathway, Relish. The Relish mutants did not show any *Wolbachia*-dependent effects on the levels of *A. pasteurianus*. However, the levels of *A. pasteurianus* in both the *Wolbachia*-free and infected larvae were very low (about 100-fold lower than in the *upd*>hPABP-Flag larvae). The lack of any significant changes could be masked by the absence of any considerable levels of *A. pasteurianus*. This shows that the host genetic background is also a significant factor in determining the abundance and composition of the microbiome.

To further test the role of host genotype, we determined the effect of *Wolbachia* on the microbiome of w^{1118} mutant flies. Again, we observed that the levels of *A. pasteurianus* were significantly lower in the w^{1118} genotype compared to the *upd*>hPABP-Flag larvae, and the presence of *Wolbachia* does not affect the levels of *A. pasteurianus*.

We looked at the second major innate immune response in the gut, ROS. To observe the levels of ROS, we utilized a fly strain that harbors a GstD-GFP construct (GFP downstream of GstD promoter), which expresses GFP under oxidative stress. We crossed this fly strain with the *upd*>hPABP-Flag genotype to make sure that the resultant genotype resembles the *upd*>hPABP-Flag genotype as closely as possible. We did not observe any changes in the pattern and intensity of GFP expression in the gut due to *Wolbachia*-infection, ruling out the possibility that the changes in the microbiome are due to altered ROS levels in the gut.

Finally, we asked whether *Wolbachia*-infected larvae have altered gut physiology, such as gut pH, compared to *Wolbachia*-free larvae. L3 larvae were fed with food containing several pH dyes, their guts were dissected and imaged to determine the pH ranges. We observed no significant *Wolbachia*-induced changes in the pH profiles using Bromophenol blue and Phenol red. However,

using Bromocresol Purple revealed that the presence of *Wolbachia* slightly reduces the fraction of the length of acidic regions of the gut between pH 2.25 and 4.5, and increased the fraction of the length of the basic regions of the gut beyond pH 6.8. However, this change accounted for about 10%-20% of the gut length and cannot explain the drastic changes in the microbiome composition.

It is unclear whether any one pathway is responsible for *Wolbachia*-induced changes in the microbiome composition. Given that the negative regulators of the Imd pathway are differentially expressed and the gut pH profile is slightly altered due to *Wolbachia*-infection, it is hard to definitively point towards one isolated mechanism. Despite any clear mechanism, these results are important for the *Drosophila* and the *Wolbachia* fields. It is necessary to delineate the contributions of various microbes independent of each other on host phenotypes, especially when *Wolbachia*-infected flies can have a microbiome that is different from *Wolbachia*-free flies.

CHAPTER 5

Effects of *Wolbachia* and the microbiome on fly development rate

Wolbachia has a significant effect on the microbiome composition. Though this phenotype is sensitive to host genotype, these findings are relevant for studies investigating the phenotypic consequences of *Wolbachia*-infection on the host. Previously *Wolbachia* have been shown to alter many phenotypes in insects, such as fecundity (Fast et al., 2011, Ikeya et al., 2009), insulin signaling and metabolism (Ikeya et al., 2009), immunity and resistance to pathogens (Bian et al., 2010b, Hedges et al., 2008, Hughes et al., 2011a, Kambris et al., 2009, Kambris et al., 2010b, Moreira et al., 2009, Rances et al., 2012, Teixeira et al., 2008), stem cell activity (Fast et al., 2011), and lifespan (Min and Benzer, 1997). With growing evidence that resident gut microbes are also capable of altering many of these phenotypes (Brummel et al., 2004, Joseph et al., 2009, Sharon et al., 2010, Storelli et al., 2011, Shin et al., 2011, Ridley et al., 2012, Newell and Douglas, 2014), it is important to delineate the relative contributions of each of the different commensal bacterial species to the phenotypic changes.

Several studies have shown the essential role of microbiome on host development (Shin et al., 2011, Chaston et al., 2014, Storelli et al., 2011); however, the contributions of *Wolbachia* to this phenotype have not been studied. Further,

development rate is a good read out of overall host fitness as many of the processes mentioned above are required for proper development. Here we use a developmental rate assay to determine if, and to what extent, *Wolbachia* and the microbiome independently affect host development.

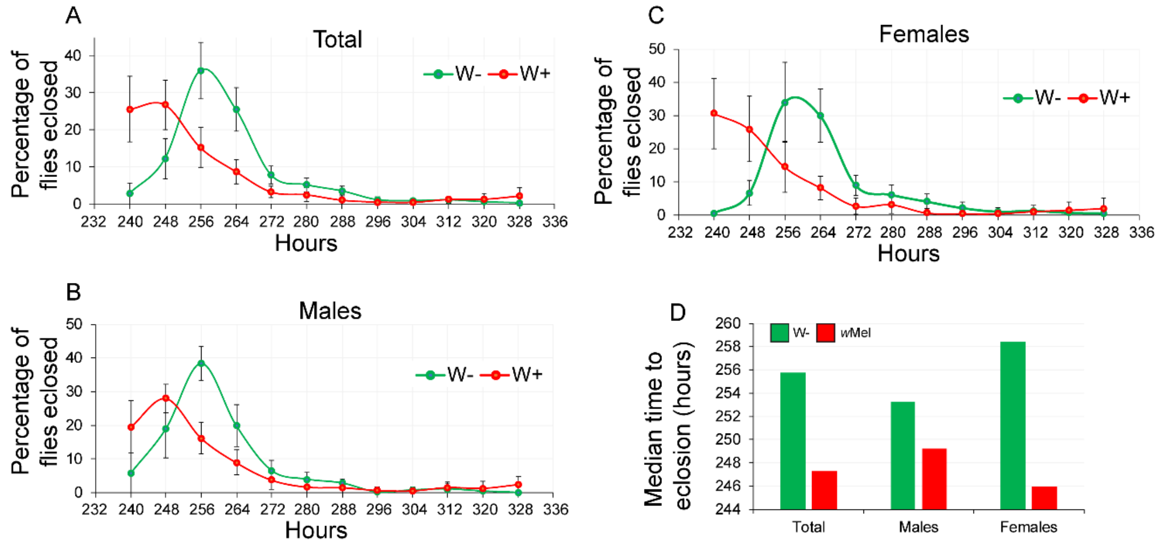
5. 1 *Wolbachia*-infected axenic flies develop faster than their *Wolbachia*-free counterparts

To determine if *Wolbachia* affects the development rate of the flies, we generated axenic *Wolbachia*-free and infected *upd*>hPABP-Flag embryos, maintained them in sterile conditions, and counted the eclosing adults every eight hours for four days from the time of eclosion of the first adult. We also sexed the eclosing flies to determine the role of sex on development rate. Two separate experiments were performed with four replicates for each condition and the data were pooled for analysis. We generated frequency distributions of the eclosing flies and obtained the median time for eclosion.

We found that *Wolbachia* speeds up development from embryos to adult eclosion by 8.4 hours (Fig. 5.1, $p = 1.4e-7$) when considering all flies irrespective of their sex. Further, when the sex of the flies was considered, *Wolbachia* speeds up the development of females (Fig. 5.1C, D, 12.4 hours, $p = 7.6e-12$) more than in males (Fig. 5.1 B, D, 4 hours, $p = 4.1e-4$). While *Wolbachia*-free females develop

slightly slower than their male counterparts, the females develop faster than males when they harbor *Wolbachia* (Fig. 5.1 D).

Figure 5.1: ***Wolbachia*** **speeds up development of axenic flies**



Frequency distribution of eclosing axenic flies (A-C). *Wolbachia* influences the development rate of (A) Unsexed (p = 1.4e-7), (B) Males (p = 4.1e-4), and (C) Females (p = 7.6e-12) flies. (D) Median times of eclosion for all the three categories. Line graphs represent means of eight replicates from two separate experiments, error bars are standard deviations. p Values are from Kolmogorov-Smirnov test. Bar graphs represent medians.

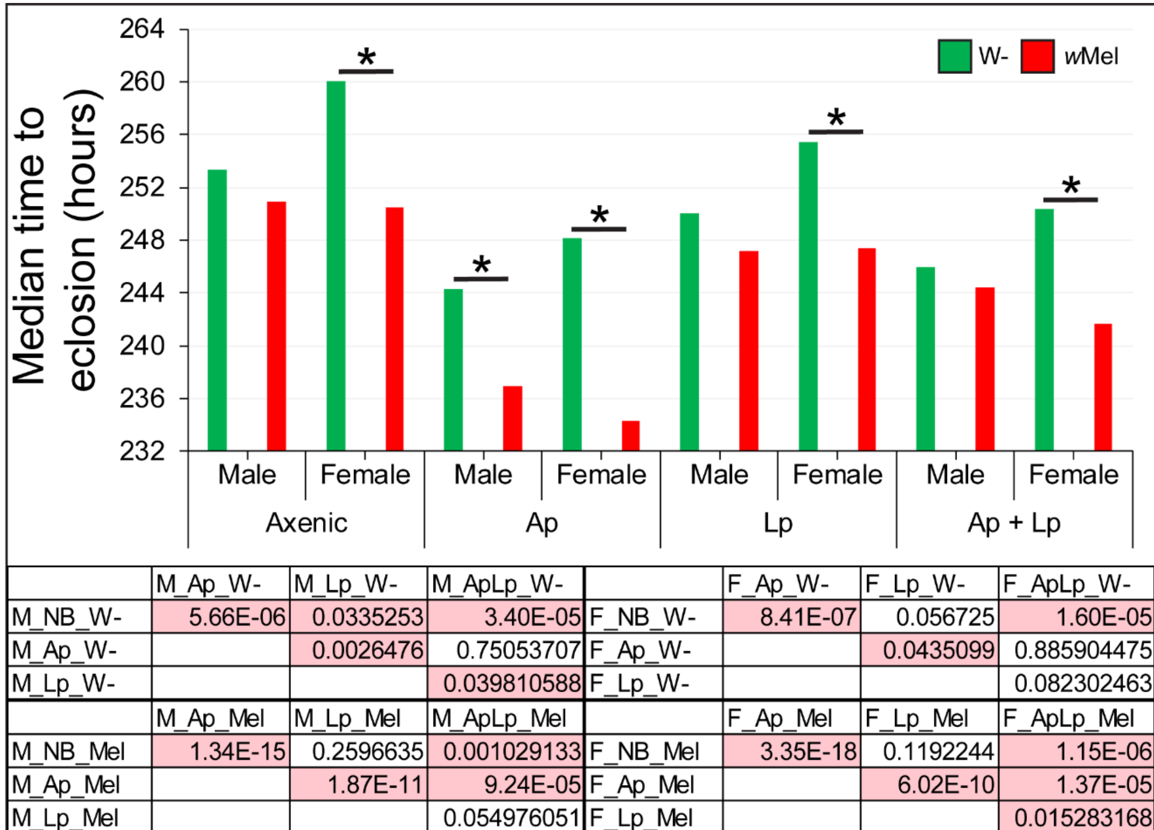
5. 2 Gut microbiota speed up development independent of *Wolbachia*, and their effects are additive

Various species of gut microbiota have been shown to affect the development rate of flies (Chaston et al., 2014, Storelli et al., 2011, Shin et al., 2011). However, since there are no reports on the combined effects of both *Wolbachia* and the gut microbiota, we studied this process using the two species of gut microbiota found in the *upd*>hPABP-Flag fly strain. We generated *Wolbachia*-free and infected axenic, mono-associated gnotobiotic or gnotobiotic embryos with both the gut bacteria. Since *Wolbachia*-infected larvae exclude *A. pasteurianus*, we added excess of the bacteria to ensure that the bacteria can reach the gut. 10^5 CFUs of each species (100 fold more than what was used for generating the gnotobiotic organisms in Chapters 3 and 4) were used for seeding the axenic embryos to generate the gnotobiotic organisms. The eclosing adults were counted every 8 hours for 4 days from the time of eclosion of the first adult. We generated frequency distributions of the eclosing flies and obtained the median time for eclosion (Fig. 5.2).

We first observed that in every condition, the presence of *Wolbachia* sped up development in both males and females. Like the axenic condition, the effect of *Wolbachia* was stronger in females compared to males. When considering the

presence of gut bacteria, *A. pasteurianus* was more effective in speeding up development than *L. plantarum*, in both *Wolbachia*-free and infected flies. These results suggest that the effect of *Wolbachia* on development is independent of the gut microbiota. Further, the advantage of harboring both *Wolbachia* and gut microbiota is additive.

Figure 5.2: *Wolbachia* and the microbiome independently increase development rate.



	M_Ap_W-	M_Lp_W-	M_ApLp_W-		F_Ap_W-	F_Lp_W-	F_ApLp_W-
M_NB_W-	5.66E-06	0.0335253	3.40E-05	F_NB_W-	8.41E-07	0.056725	1.60E-05
M_Ap_W-		0.0026476	0.75053707	F_Ap_W-		0.0435099	0.885904475
M_Lp_W-			0.039810588	F_Lp_W-			0.082302463
	M_Ap_Mel	M_Lp_Mel	M_ApLp_Mel		F_Ap_Mel	F_Lp_Mel	F_ApLp_Mel
M_NB_Mel	1.34E-15	0.2596635	0.001029133	F_NB_Mel	3.35E-18	0.1192244	1.15E-06
M_Ap_Mel		1.87E-11	9.24E-05	F_Ap_Mel		6.02E-10	1.37E-05
M_Lp_Mel			0.054976051	F_Lp_Mel			0.015283168

Median times of eclosion of *Wolbachia*-free and infected flies under axenic and gnotobiotic conditions. Bar graphs represent median time to eclosion. Asterisks represent p-value < 0.05, Kolmogorov-Smirnov test on the eclosion rate curves that were used to calculate the median time to eclosion. The table shows Kolmogorov-Smirnov test p-value of comparisons of various treatment groups.

5.3 Discussion

Wolbachia affect many phenotypes in the host, but the contributions of the microbiome in the context of *Wolbachia*-infection have not been addressed. Here we asked how *Wolbachia* and the microbiome affect the development rate of the flies independently and in the presence of each other. First, we observed that the presence of *Wolbachia* significantly increases the development rate of the flies. We then generated both *Wolbachia*-free and infected gnotobiotic organisms with just one or both the gut bacterial species. We observed that the gut microbiota also contributes toward reducing the developmental time. However, this effect is only statistically significant in female flies. Further, the contributions of *Wolbachia* and the microbiome are also additive.

When comparing the effects of *Wolbachia* and the microbes on the host phenotypes, it is important to delineate their individual effects. For instance, when comparing the developmental rates of *Wolbachia*-infected organisms to *Wolbachia*-free organisms, we need to compare the *Wolbachia*-infected flies harboring *L. plantarum* (since *A. pasteurianus* is depleted in *Wolbachia*-infected larvae) and the *Wolbachia*-free flies harboring both *A. pasteurianus* and *L. plantarum* (since *Wolbachia*-free organisms have both *A. pasteurianus* and *L. plantarum*). Statistically, there is no difference between these two groups in either

males (p-value = 0.313623686) or females (p-value = 0.435320149). That is the individual effects of *Wolbachia* and the microbiome can confound each other. We can observe the developmental advantage conferred by *Wolbachia* and *A. pasteurianus* individually, but when analyzed together, we can see that their effects are confounded. Hence, when measuring phenotypic contributions of *Wolbachia* on the host, it is important to assess the relative contribution of the *Wolbachia*-associated microbiome.

CHAPTER 6

Discussion and Future Directions

(Portions of this chapter previously published in Simhadri et al., 2017)

The interaction between resident microbes is a key determinant in the microbial species present in a host. In the insect gut, pathogenic organisms can be excluded by resident microbes (reviewed by Cirimotich et al., 2011). The insect microbiome can also influence symbiont vertical transmission. A recent study by Hughes and collaborators showed that in *Anopheles* mosquitoes, the intracellular bacteria *Wolbachia* are robustly excluded from vertical transmission by an *Asaia,* an acetic acid bacterium (Hughes et al., 2014). Here, we show for the first time a reciprocal aspect of commensal microbiome bacteria and symbionts: our data indicate that *Wolbachia* bacteria in *Drosophila melanogaster* can alter the composition of resident microbes compared to *Wolbachia*-free hosts.

6.1 *Wolbachia* modulate the *Drosophila* microbiome

We initially surveyed the composition of the resident bacterial species in a *Drosophila* stock reared in our lab – *upd*>hPABP-Flag, and we found *Acetobacter pasteurianus* and *Lactobacillus plantarum* are the only two species in this strain of flies. This is in general agreement with reports from other groups that list *Acetobacter* and *Lactobacillus* as genera commonly associated with *Drosophila*

reared in the lab and wild caught fly strains (Chandler et al., 2011, Ren et al., 2007a, Corby-Harris et al., 2007). We compared the microbial composition of another stock with the same genetic background that was not infected with *Wolbachia*. Surprisingly, the levels of *A. pasteurianus* are lower in the *Wolbachia*-infected flies compared to the *Wolbachia*-free flies in both males and females. By validating this result using PCR with species-specific primers, we also showed that the levels of *A. pasteurianus* are consistently reduced in adults and larval stages.

These differences in the microbiome could be due to the presence of *Wolbachia* or could reflect a *Wolbachia*-unrelated event that is maintained across generations. Since the microbiome is transmitted vertically via the feces of the fly to the next generation, the difference in the levels of bacteria that we observed could be just due to a lower seeding from previous generations. To eliminate the effect of parental transmission of microbes to the offspring, we studied the effects of *Wolbachia* on flies raised from gnotobiotic embryos. The results show that *Wolbachia*-infection is sufficient to reduce the levels in *A. pasteurianus* at certain developmental stages. Specifically, *A. pasteurianus* is considerably lower in the *Wolbachia*-infected larvae compared to the *Wolbachia*-free counterparts. We show that this reduction is due to the complete lack of *A. pasteurianus* in a significant

fraction of the *Wolbachia*-infected L3 larvae compared to the *Wolbachia*-free larvae. Further, by performing FISH to probe for *A. pasteurianus* in L3 larval guts, we observed a marked absence of *A. pasteurianus* in a majority of *Wolbachia*-infected guts, but not in *Wolbachia*-free guts. Recent work in *Anopheles stephensi* mosquito also shows variation of *Wolbachia*-induced differences in the mosquito microbiome. Directly after a blood meal, *Wolbachia*-infected mosquitoes had reduced proportion of gammaproteobacteria compared to *Wolbachia*-free mosquitoes (see Fig. 2 in (Chen et al., 2016)). A week post blood meal, *Wolbachia* infected mosquitoes have a significantly diverse microbiota compared to the *Wolbachia*-free insects. However, when fed with just sugar meal or analyzed immediately post blood meal, there are no differences in the microbiota between *Wolbachia*-free and infected mosquitoes (see Table 1 in (Chen et al., 2016)). Another study in *D. melanogaster* showed that the presence of *Wolbachia* reduced the diversity of the gut microbiome (See Fig. 2B in (Ye et al., 2017)), and also reduced the abundance of *Acetobacter* genus (see Table 2 in (Ye et al., 2017)).

In contrast to the results in the larvae, the relative levels of *A. pasteurianus* in the young gnotobiotic adult flies were not significantly different from the *Wolbachia*-free adult flies. This could be a result of the loss of the microbiome during histolysis of the larval gut during pupation. In concordance, several

studies showed that newly eclosed adults have extremely low densities of resident bacteria and are recolonized by feeding (Wong et al., 2011a, Blum et al., 2013). Therefore, any *Wolbachia* induced reduction of *A. pasteurianus* in the larval stages is lost and must be re-established over time. This explains the large variability in the relative quantities of the gut microbes in both young *Wolbachia*-free and infected adults. On the other hand, the conventionally reared *Wolbachia*-infected adults have greatly reduced levels of *A. pasteurianus* since the bacteria are not externally introduced into the food. In the case of conventionally reared flies, the only source of the *A. pasteurianus* to the offspring is from the adults, which, from our data, are not detected or greatly reduced.

6.2 Effects of *Wolbachia* and microbiota on gut immunity and physiology

There are several possible mechanisms for the modulation of the microbiome by *Wolbachia*. *A. pasteurianus* levels could be reduced due to direct competition for nutrients, however this is unlikely since ingested nutrients are immediately available to *A. pasteurianus,* which are present in the lumen, while *Wolbachia* are intracellular. Additionally, factors derived from either *Wolbachia* or the host could inhibit *A. pasteurianus*.

Regarding *Wolbachia*-derived factors, *Wolbachia* contain a Type 4 secretion system (Wu et al., 2004), which can be utilized to secret factors that subvert host

cell biology to favor bacterial survival and growth. However, there is no evidence of a *Wolbachia*-derived factor that could directly influence the presence of other bacteria. To be effective against *A. pasteurianus*, such a factor would have to be exported into the gut lumen. Further, since the same strain of *Wolbachia* was present in both the host genotypes used in this study, and we do not observe similar outcomes of microbial composition, it shows that any putative factors are unlikely to reach the lumen if they were secreted by *Wolbachia*. Therefore, we favor host-derived factors as the most likely mechanism.

A second possible mechanism for the modulation of the microbiome by *Wolbachia* is an indirect inhibition of *A. pasteurianus* by *Wolbachia* via the host. The host immune system, specifically Antimicrobial peptides (AMPs), could be playing a role in altering the microbiome in response to *Wolbachia*-infection. Previous studies on host immunity showing that *Wolbachia* upregulates the immune response were performed in non-native hosts that were trans-infected with *Wolbachia* from another host (Kambris et al., 2009, Moreira et al., 2009, Bian et al., 2010a, Rances et al., 2012). However, similar studies performed on a host such as *Drosophila melanogaster* natively infected with *w*Mel strain of *Wolbachia* did not show any systemic upregulation of immunity (Rances et al., 2012, Wong et al., 2011b). Though both the native and non-native *Wolbachia*-infected hosts

exhibit robust antiviral response to single-stranded RNA viruses (Teixeira et al., 2008, Hedges et al., 2008, Moreira et al., 2009, Walker et al., 2011, Glaser and Meola, 2010), the native host (*Drosophila*, in this case) did not show any anti-bacterial activity when infected with pathogenic strains of bacteria via injury (Wong et al., 2011b, Rottschaefer and Lazzaro, 2012). No previous study addresses *Wolbachia*-induced anti-microbial effects on commensal gut microbes or *Wolbachia* putative immune regulation specifically in the digestive tract. Regulation of immune response in the fly gut differs in several aspects from systemic immunity. It is possible that *Wolbachia*-infection generates an intestine-specific immune response acting to destabilize the microbiome. We tested this hypothesis in both axenic and gnotobiotic organisms that are either *Wolbachia*-free or infected. We first asked whether *Wolbachia*-infection alters the expression of the transcription factor of the Imd pathway *rel*, and the signal transducer *Dredd*. We found that the levels of both *rel* and *Dredd* transcripts are not affected by either *Wolbachia* or the gut microbiota. However, the activation of the Imd pathway also relies on post transcriptional modifications of the Rel protein, and the lack of changes in the transcript levels does not equate to an inactive Imd pathway. Since AMPs have been used as a readout for the activation of the Imd pathway, we then tested whether *Wolbachia*-infection alters the expression of

AMPs that are regulated by the Imd pathway. We observed no statistically significant alteration in the expression of AMPs controlled by the Imd or the JAK/STAT pathways in any of the conditions tested. However, we do see a slight reduction in the expression of some of the AMPs in the Imd pathway in the guts of gnotobiotic larvae in the presence of *Wolbachia* compared to the *Wolbachia*-free larvae.

Since the AMP expression levels are also controlled by the negative regulators of the Imd pathway, we assayed for the expression levels of the regulatory components of the Imd pathway. From two separate experiments, we consistently observed that two amidases that act as the negative regulators of the Imd pathway, PGRP-LB and PGRP-SC2 are differentially expressed due to *Wolbachia*-infection. While PGRP-LB is upregulated in *Wolbachia*-infected flies regardless of the gut bacterial status, PGRP-SC2 is downregulated. Given that PGRP-LB is more contextually important in the gut as a negative regulator (Costechareyre et al., 2016), and the slight reduction in the AMP expression in the presence of *Wolbachia*, the Imd pathway seems to be suppressed by *Wolbachia*-infection.

To test whether the differential expression in the negative regulators is a contributor to the *Wolbachia*-induced alteration of microbiome composition, we

utilized mutants of the Rel protein where *Wolbachia*-induced differences in the transcript levels of the negative regulators are absent. We introduced *Wolbachia* into the *rel*^{E20} mutant background and quantified the levels of the gut bacteria in the presence and absence of *Wolbachia*. We found that there are no significant differences between the microbiome composition of the *Wolbachia*-free and infected gnotobiotic flies at both L3 larval and 10d old adult stages. However, we noticed that the levels of *A. pasteurianus* in the larvae of this fly background are far lower (100 fold) than the levels we observed in the *upd*>hPABP-Flag larvae. This could potentially confound the effect of *Wolbachia* on the reduction of *A. pasteurianus* as the control larvae themselves have very low *A. pasteurianus* levels. Other confounding factors are the presence of balancer chromosomes, and transgenes like the UAS-hPABP-Flag and *upd*-Gal4, that are absent in the *rel*^{E20} mutant genotype.

To assess whether the fly genotype also plays role in the *Wolbachia*-mediated changes in the microbiome composition, we used another genotype, *w*^{1118}, which does not have any transgenes or balancers. We did not detect any significant effect of *Wolbachia* on either *A. pasteurianus* or *L. plantarum* levels at L3 larval stage or in 10day old adult flies. Since the levels of *A. pasteurianus* in the *w*^{1118} genotype are significantly lower than in the *upd*>hPABP-Flag genotype,

Wolbachia-induced effects on *A. pasteurianus* might be harder to detect, if any. Further, we also observed a significantly different pattern of gene expression of the Imd pathway components compared to the *upd*>hPABP-Flag larvae.

Thus, host genotype can play a role in determining the outcome of microbial populations in the gut. Findings presented here are in accordance with mounting evidence showing a complex interaction of *Wolbachia* and commensal microbial composition influence by several variables including host genotypes. (Ye et al., 2017, Dittmer et al., 2014, Rossi et al., 2015, Chen et al., 2016, Hughes et al., 2014). A thorough future investigation into host and microbial genetic and epigenetic determinants are needed that can alter the tolerance and carrying capacity of the host for each of the colonizing microbes across a wide range of insect species.

Since the host genotype is also a determinant in the *Wolbachia*-mediated phenotype, we continued to look for other mechanistic aspects in the *upd*>hPABP-Flag larvae that could explain the *Wolbachia*-induced microbiome composition changes. Reactive oxygen species (ROS) could be playing a role in modulating the microbiome in response to infection from intracellular bacteria like *Wolbachia*. There are several papers indicating that *Wolbachia* upregulate ROS in the insect host (Andrews et al., 2012, Pan et al., 2012, Brennan et al., 2012,

Brennan et al., 2008, Bian et al., 2013). In these earlier studies, most of the measurements were done utilizing the whole organism. The amplitude of the response varies according to the tissue: data from naïve hosts suggest that while changes in major ROS effector genes Nox and Duos can be significantly affected by *Wolbachia* at the systemic level, in the gut there are no significant differences (Pan et al., 2012). In agreement, we also did not observe *Wolbachia*-driven changes in the levels of *Nox* or *Duox* expression in the fly gut. These results suggest that the mechanism of *Wolbachia*-modulation of the microbiome does not operate through simple changes of gene expression of the two major classes of key effectors of gut immunity, AMPs and Nox.

Other physiological aspects such as gut pH have been shown to be affected by commensal microbiota (Overend et al., 2016). Differences in gut pH due to *Wolbachia*'s presence could selectively influence the presence of *A. pasteurianus* and *L. plantarum*. We utilized a pH indicator based assay to stain the guts of the *upd*>hPABP-Flag larvae to determine the pH profile of various gut regions. We fed the larvae with apple juice agar containing various pH dyes, imaged the guts after dissection, and analyzed the color hues across the entire midgut region. We found that when using bromocresol purple, the acidic (<4.25) portion of the gut is shorter and the basic (>6.8) portion is longer in *Wolbachia*-

infected guts compared to *Wolbachia*-free guts. Though this result is statistically significant, the difference in the length of the acidic region is about 10%, and this difference alone is not sufficient to explain the drastic difference in the microbiome composition due to *Wolbachia* infection. These findings highlight the complexity of *Wolbachia* interaction with their hosts.

Our data show that the presence of *Wolbachia* has a significant effect on the microbiome composition for the bacteria species, in certain host genotypes in lab conditions. Though this effect might not be generalizable to every fly genotype and host species, these finding are relevant for studies investigating the phenotypic consequences of *Wolbachia*-infection on the host. Previously *Wolbachia* have been shown to be capable of altering many phenotypes in insects, such as fecundity (Fast et al., 2011, Ikeya et al., 2009), insulin signaling and metabolism (Ikeya et al., 2009), immunity and resistance to pathogens (Bian et al., 2010b, Hedges et al., 2008, Hughes et al., 2011a, Kambris et al., 2009, Kambris et al., 2010b, Moreira et al., 2009, Rances et al., 2012, Teixeira et al., 2008), stem cell activity (Fast et al., 2011), and lifespan (Min and Benzer, 1997). With growing evidence that resident gut microbes are also capable of altering many of these phenotypes (Brummel et al., 2004, Joseph et al., 2009, Sharon et al., 2010, Storelli et al., 2011, Shin et al., 2011, Ridley et al., 2012, Newell and Douglas, 2014), it is

important to delineate the relative contributions of each of the bacteria to the phenotypic changes (under study).

6.3 Effects of *Wolbachia* and the microbiome on fly development rate

While the role of microbiome on the host phenotypes such as development has been well documented (Chaston et al., 2014, Ridley et al., 2012, Storelli et al., 2011, Shin et al., 2011), the role of *Wolbachia* on fly development has not been studied. Here we investigated the role of both the gut bacteria and *Wolbachia* independently, as well as in conjunction.

We generated axenic *upd*>hPABP-Flag embryos that were either *Wolbachia*-free or infected and measured the time to adult eclosion. We also measured the temporal distribution of eclosion by counting the number of flies eclosing every eight hours for four days. We found that in axenic flies, the presence of *Wolbachia* speeds up development by almost a half a day. Since the total time to develop from embryonic stage to adulthood is about 10 days, a reduction of half a day is a very strong phenotype. Further, when we also included the sex of the flies as a variable, we observed that *Wolbachia* conferred female flies a greater advantage in speeding up development than in males.

We then measured the effect of having various gut microbes in conjunction with *Wolbachia* on the development rate of the flies. We generated

gnotobiotic *Wolbachia*-free and infected embryos with either only *A. pasteurianus*, or only *L. plantarum*, or with both the bacteria. On measuring the temporal distribution of adult eclosion, we observed that *A. pasteurianus* speeds up development significantly more than *L. plantarum*, and the benefit conferred by either bacteria is additive to the fitness advantage provided by *Wolbachia*-infection.

Finally, this experiment provides a great example for the need to consider the phenotypic contributions of each of the microbial species independently. When we consider the effect of *Wolbachia* alone on the development rate without paying close attention to the microbiome composition, we can potentially have confounding effects of the microbiome. A stable stock of the *Wolbachia*-infected *upd*>hPABP-Flag flies harbor only *L. plantarum*, while the *Wolbachia*-free flies harbor both *A. pasteurianus* and *L. plantarum*. From Fig 5.2, we would be comparing the times to eclosion of the *L. plantarum* harboring *Wolbachia*-infected flies with the *Wolbachia*-free flies harboring both the microbes. There is no statistically significant difference between these two conditions in the median time to eclosion. While we can see a strong fitness advantage due to the presence of both *Wolbachia* and *A. pasteurianus* in these flies when their phenotypic

contributions are considered independently, we fail to see this effect when we ignore the contributions of the microbiome towards a *Wolbachia*-mediated effect.

The modulation of the microbiome by *Wolbachia* may have dramatic effects in other aspects of host fitness as well. For example, in terms of immunity, it is known that the certain composition of the microbiome confers protection against pathogens in several organisms, from plants to humans (Mazmanian et al., 2008, Emmert and Handelsman, 1999). In *Drosophila*, it has been described that higher relative rates of *L. plantarum* promote protection against *Serratia marcesens* and *Pseudomonas aeruginosa*, two known *Drosophila* pathogens that also cause opportunistic infections in humans (Blum et al., 2013). These findings raise the possibility that *Wolbachia* changes host defense indirectly by affecting microbiome composition. Altered immune competence can play a key role in survival of host populations in nature.

The results shown here are also relevant for the development of bacterial-based approaches in vector control. Several studies have shown that gut bacteria and *Wolbachia* inhibit the presence of human pathogens in insect vectors, including *Plasmodium falciparum*, Dengue, West Nile and Chikungunya virus (Teixeira et al., 2008, Hedges et al., 2008, Hughes et al., 2011b, Moreira et al., 2009, Kambris et al., 2009, Kambris et al., 2010a, Bian et al., 2010a, Rances et al., 2012,

reviewed by Bourtzis et al., 2014, and Cirimotich et al., 2011). Therefore, it is important to understand interactions of *Wolbachia* with other bacteria that inhibit disease transmission, in order to determine their synergistic or antagonistic interactions towards vector control.

Appendix 1

JW18 cell lines

The JW18 cell line is the only *Drosophila melanogaster* cell line to date that is stably infected with *Wolbachia* (Serbus et al., 2012). It is a non-homogenous population of embryonically derived cells from a *Wolbachia*-infected fly stock in William Sullivan's lab. A *Wolbachia*-free line called JW18DOX was then generated from the JW18 cells by treatment with doxycycline. Both cell lines are invaluable in high throughput studies to understand host-*Wolbachia* interactions (Serbus et al., 2012, White et al., 2017).

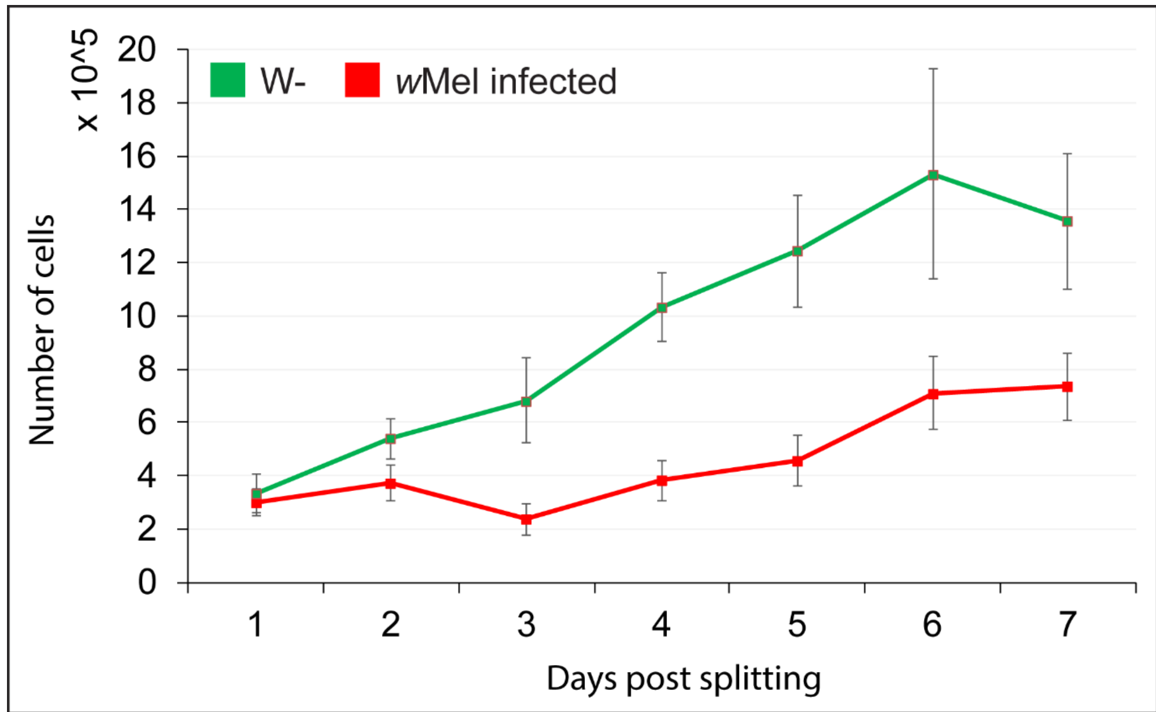
To elucidate the transcriptional changes in the host due to *Wolbachia*, we performed RNA-seq of the JW18 cells with and without *Wolbachia*. Further, to eliminate the effects of doxycycline treatment on the transcriptional differences between JW18 and JW18DOX cells, we extracted *Wolbachia* from the JW18 cells and re-infected the JW18DOX cells to generate another stable *Wolbachia*-infected line called JW18R. This new JW18R line is *Wolbachia*-infected and has the same genetic background as the JW18DOX line. On performing RNA-seq and pathway enrichment, we found that Imd, the innate immunity pathway is most significantly affected by *Wolbachia*-infection. Specifically, all the immune effectors of the pathway – AMPs are significantly up-regulated in *Wolbachia*-infected cells

(Fig. 4.3). The full list of genes that are expressed and are statistically significantly different between at least two of the three groups (JW18, JW18DOX, and JW18R) along with their gene ontology is provided in the (attach the dataset once uploaded)

We also characterized certain phenotypic characteristics such as growth rate and density profiles of *Wolbachia* across time in the cell lines. The *Wolbachia*-infected JW18 cells grow at approximately one half the rate of the *Wolbachia*-free JW18DOX cells, presumably due to the burden of hosting high densities of *Wolbachia* (Fig. A1.1). This is in agreement with the published data about the mitotic rate of the JW18 cells being two times higher than the JW18DOX cells (Serbus et al., 2012). We also measured the densities of *Wolbachia* across time to determine the temporal stability of the infection. We measured the number of copies of *Wolbachia* DNA per the number of JW18 DNA copies using qPCR. Simultaneously, we also measured the *Wolbachia*/host DNA copies after treating the JW18 cells with 50mg/ml of rifampicin as a control for reducing *Wolbachia* densities. We found that the *Wolbachia* levels stay temporally constant in the JW18 cells, while the rifampicin-treated cells have decreasing levels of the bacteria over time (Fig A1.2). In addition to qPCR, we also measured the number of *Wolbachia* per host cell using confocal imaging and custom scripts for image

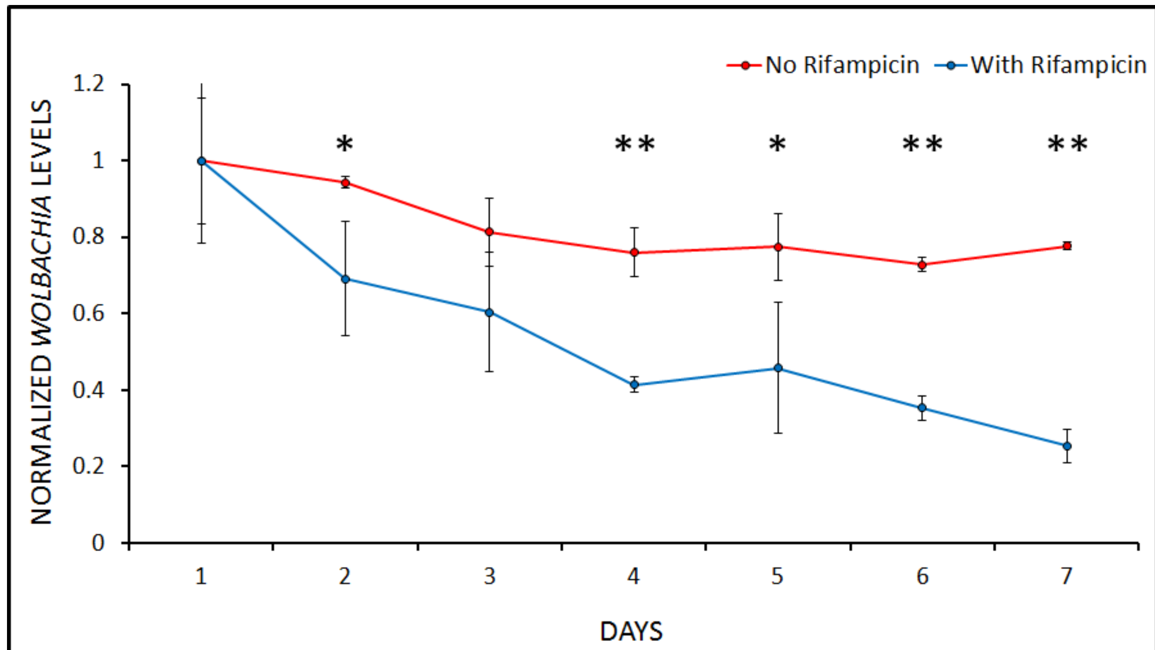
analysis in MATLAB (https://github.com/RamaSimhadri/Bacteria_counting). We observed that the number of *Wolbachia* per host cell remained constant over the course of a week after splitting the cells, consistent with the qPCR results.

Figure A1.1: Growth rates of JW18 and JW18DOX cell lines



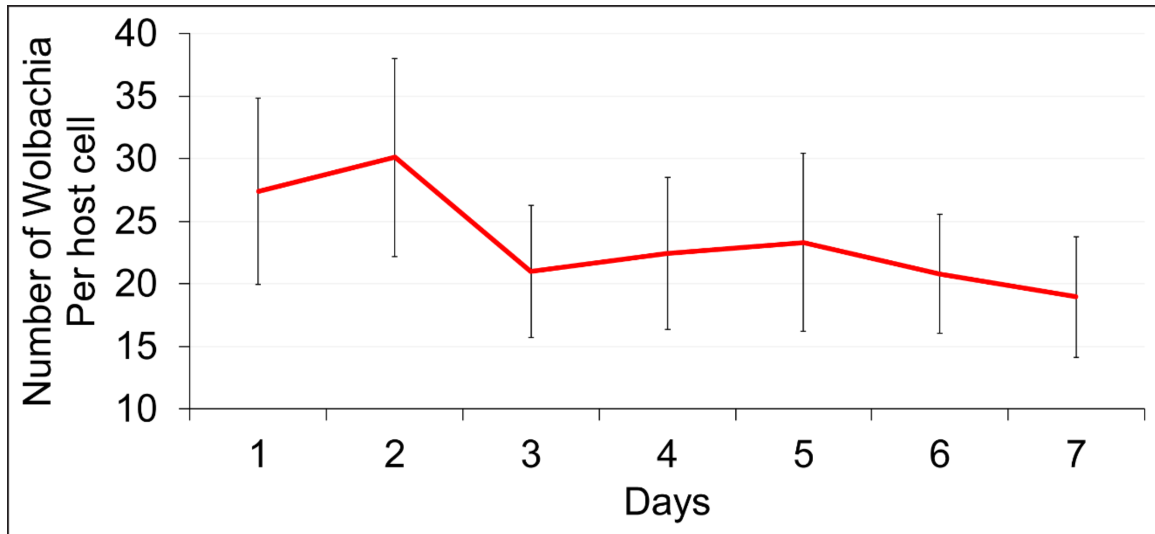
Growth rate of the JW18 and JW18DOX cell lines measured as number of viable cells under a hemocytometer per day after splitting each cell line to the same density. Both the JW18 and JW18DOX cell lines were split to a density of about 3x10^5 cells into multiple wells of a 24 well plate. Then samples were collected from one of the wells of each cell line every day to count the number of viable cells under a hemocytometer. By the end of 7 days, the *Wolbachia*-free JW18DOX cell line grew to nearly 14x10^5 cells and the *Wolbachia*-infected JW18 cell line grew to 7x10^5 cells. The *Wolbachia*-free JW18DOX cell line grows twice as fast the *Wolbachia*-infected JW18 cells.

Figure A1.2: Temporal dynamics of *Wolbachia* density in JW18 cells determined by qPCR



Wolbachia density measured as the number of copies of *Wolbachia* DNA to the number of host DNA copies by qPCR. JW18 cells were split to a density of around 3x10^5 cells into a 24 well plate. Cells from three wells were collected every day for seven days, and the total genomic DNA was extracted. qPCR of this DNA using primers against *Wolbachia* and the host can be used to calculate the density of *Wolbachia* per host cell. Densities of *Wolbachia* on cells treated with rifampicin are shown in the blue line, and the red line represents cells grown without rifampicin. Error bars represent standard deviation. * represent statistical significance at p-value < 0.05, and ** represent p-value <0.005, student t-test.

Figure A1.3: Temporal dynamics of *Wolbachia* density in JW18 cells determined by imaging



Number of *Wolbachia* cells were counted per host cell over a course of one week after splitting the cells. Confocal imaging of JW18 cells stained with a FISH probe against *Wolbachia*, followed by image analysis to count the number of *Wolbachia* per host was performed using custom image analysis scripts on MATLAB. Error bars represent standard deviation. None of the *Wolbachia* counts at each of the time points were statistically significantly different from each other.

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List of Abbreviated Journal Titles

Acta Trop – Acta Tropica

Adv Parasitol – Advanced Parasitology

Am J Physiol Gastrointest Liver Physiol – American Journal of Physiology-Gastrointestinal and Liver Physiology

Annu Rev Cell Dev Biol – Annual Review of Cell and Developmental Biology

Annu Rev Entomol – Annual Review of Entomology

Annu Rev Genet – Annual Review of Genetics

Annu Rev Immunol – Annual Review of Immunology

Annu Rev Microbiol – Annual Review of Microbiology

Annu Rev Physiol – Annual Review of Physiology

Appl Environ Microbiol – Applied and Environmental Microbiology

Biochem Biophys Res Commun – Biochemical Biophysical Research Communications

BMC Microbiol – BMC Microbiology

Ecol Evol – Ecology and Evolution

Cell Metab – Cell Metabolism

Cell Rep – Cell Reports

Chem Immunol Allergy – Chemical Immunology and Allergy

Dev Cell – Developmental Cell

Dis Model Mech – Disease Models & Mechanisms

Ecol Lett – Ecology Letters

EMBO Rep – EMBO Reports

Environ Microbiol – Environmental Microbiology

FASEB J – The FASEB Journal

FEMS – Federation of European Microbiological Societies

FEMS Microbiol Let – FEMS Microbiology Letters

FEMS Microbiol Rev – FEMS Microbiology Reviews

Genes Dev – Genes & Development

Infect Immun – Infection and Immunity

Insect Mol Biol – Insect Molecular Biology

J Biol Chem – The Journal of Biological Chemistry

J Cell Sci – Journal of Cell Science

J Exp Bot – Journal of Experimental Botany

J Immunol – The Journal of Immunology

J Innate Immun – Journal of Innate Immunity

J Insect Sci – Journal of Insect Science

J Invertebr Pathol – Journal of Invertebrate Pathology

Mech Dev – Mechanisms of Development

Microb Ecol – Microbial Ecology

Mol Ecol – Molecular Ecology

Nat Commun – Nature Communications

Nat Protoc – Nature Protocols

Nat Rev Immunol – Nature Reviews Immunology

Nat Rev Microbiol – Nature Reviews Microbiology

Nucleic Acids Res – Nucleic Acids Research

Parasit Vectors – Parasites & Vectors

PLoS Biol – PloS Biology

PLoS Genet – PloS Genetics

PLoS Pathog – PloS Pathogens

Proc Biol Sci – Proceedings of the Royal Society B: Biological Sciences

Proc Natl Acad Sci U S A – Proceedings of the National Academy of Sciences of the United States of America

Sci Rep – Scientific Reports

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