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Impacts of environment-derived microbiota on vector competence of

Aedes aegypti for Zika virus

By

William Louie

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

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in the

OFFICE OF GRADUATE STUDIES

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DAVIS

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2022

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ABSTRACT

Arthropod borne viral (Arboviral) disease accounts for 17% of the total infectious disease burden, afflicting over 100 million people annually. Global expansion of mosquito-borne arboviruses demands integrated approaches to vector control and public health surveillance. However, disparate outcomes in laboratory vector competence studies complicates risk assessment of mosquito species as vectors. While the contribution of mosquito and viral genetics has enjoyed much attention, the effects of mosquito microbiota on arboviral transmission potential are poorly understood. For *Aedes aegypti*, which is an effective vector for many arboviruses including Zika virus (ZIKV), the microbiota is primarily environmentally derived and dominantly resides in the gut. Chapter 1 reviews the current knowledge of *Ae. aegypti* vector competence for Zika virus as well as known effects that mosquito microbiota have on vector competence. Chapter 2 assesses the impact of microbes acquired from the larval habitat on *Ae. aegypti* development and ZIKV transmission. Adult female mosquitoes that emerged from microbially rich larval water derived from cemetery headstones were found to harbor more diverse microbiota and consistently lower ZIKV infection and transmission rates than their laboratory counterparts reared in laboratory tap water. However, microbial community compositions varied between experiments despite a consistent phenotype. Together, the results suggest that wild *Ae. aegypti* are likely less competent vectors than conventionally determined in the lab where larvae are typically reared in tap water, and that this effect is mediated by mosquito interactions with their microbiota. Chapter 3 investigates the reversibility of larval microbe-mediated refraction of ZIKV after developmental maturity. A higher dissemination rate was observed in *Ae. aegypti* depleted of gut microbes during pupation, and this was linked to reduced blood digestion efficiency. Results of this work suggest an immuno-metabolomic mechanism by which gut microbes confer resistance to ZIKV dissemination, by way of nonstructural midgut modifications. Overall, work presented in this dissertation emphasizes the

importance of environmental microbes as a source of variation in infection susceptibility that

demands consideration when conducting vector competence studies. It also highlights the

complex interactions between mosquito, virus, and all the symbionts in between that play shape

transmission out in nature.

CHAPTER 1

INTRODUCTION AND BACKGROUND

Aedes aegypti vector competence for Zika virus and determinants of vector competence

1   Zika virus biology and transmission

1.1   Arbovirus overview

The term arbovirus (arthropod-borne virus) is an ecological classification of viruses characterized by their requirement for cycling between an arthropod vector and a vertebrate host. Arboviruses span many phylogenetically distinct viral families (Examples: *Flaviviridae*, *Togaviridae*, *Bunyaviridae*) and can use numerous different vectors, such as mosquitoes and ticks, as well as vertebrate hosts such as primates, rodents, and birds (1). Almost all known arboviruses are RNA viruses (the only known DNA arbovirus being African swine fever virus (2)), which means they lack the ability to error-correct during genome replication. The absence of an error repair mechanism results in high mutation rates (~1 mutation per genomic replication cycle) and rapid evolution (3). Consequently, arboviruses can quickly gain new phenotypes that increase their transmission ability and facilitate outbreaks, thus warranting their study to curb the next global pandemic.

Rapid global expansion of mosquito-transmitted arboviruses poses a massive threat to human public health. The World Health Organization estimates 100 million arbovirus infections annually, most coming from mosquito-borne pathogens, accounting for roughly 17% of all infectious disease burden worldwide (4, 5). It is also estimated that 3.9 billion people living in tropical and subtropical areas worldwide are at risk, with that number expected to escalate with continued urbanization, global travel, climate change, deforestation, and a myriad of other sociopolitical environmental issues (6–9). These primarily anthropogenic problems have expanded the frequency, duration, and intimacy of direct interactions between humans and wildlife; this facilitates ideal conditions for zoonotic spillover, which can lead to the emergence and reemergence of arboviruses in urban populations (10). These issues also feed into an

expanding habitat of vector mosquitoes that are necessary to sustain the transmission cycles of many arboviruses (8). Arboviruses of concern include, but are not limited to, flaviviruses (*Flaviviridae*, *Flavivirus*) such as yellow fever virus (YFV), Japanese encephalitis virus (JEV), West Nile virus (WNV), dengue virus (DENV) and Zika virus (ZIKV), alphaviruses (*Togaviridae*, *Alphavirus*) like chikungunya virus (CHIKV) and Venezuelan equine encephalitis virus (VEEV), and bunyaviruses (*Bunyavirales*) like Rift Valley Fever Virus (RVFV) which have already caused outbreaks in the recent past (11). Only YFV has an approved vaccine, so vector control and surveillance remain the primary methods of disease mitigation.

1.2   Arbovirus transmission

Complex transmission dynamics are arguably the most fascinating part of arbovirus biology. They typically exist in three flavors: an enzootic cycle, a rural epizootic cycle, and an urban epidemic cycle (10). Arboviruses originated in sylvatic enzootic cycles, which were and still are transmitted between forest-dwelling vectors and nonhuman reservoir hosts (Figure 1). The full host range of any single arboviral species is unknown and impossible to determine in practice, complicating efforts to mitigate arboviral outbreaks as the pool of reservoir hosts and potential vectors is so immense and often inaccessible. Human activity that disrupts, displaces, or removes sylvatic hosts and their habitats or that increases interaction with sylvatic vectors, at an interface sometimes called the zone of emergence, pose serious risks of zoonotic spillover that can seed rural epizootic or urban epidemic cycles of transmission.

Enzootic cycles can spill into a rural setting where vectors feed on domesticated animals or migratory birds which then become the primary amplifying hosts. In these scenarios, humans can become infected when fed on by mosquitoes or ticks that acquired a virus from an infected viremic animal, though the transmission cycle usually ends at this step as humans often do not

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sustain high enough viremias to propagate back to the vector, making humans dead-end hosts (Figure 1). For example, though WNV typically cycles between *Culex spp.* mosquitoes and birds like crows and house sparrows, humans can also become infected if bitten by a transmitting *Culex spp.* mosquito that previously fed on a viremic bird. An infected human, however, will not develop an adequate viremia to infect another mosquito that feeds on them afterwards.

Urban epidemic cycles are events where a virus circulates between mosquito populations and humans living in urban centers (Figure 1). This results in an epidemic, which is defined as elevated prevalence of a pathogen in a region where the pathogen is not normally present. Epidemic cycles are accompanied by a spike in clinical cases of disease that places enormous strain on medical and overall societal infrastructure until the cycle ends or the virus establishes endemicity in the region. Epidemic cycles typically resolve after public health control measures successfully mitigate transmission from vector mosquitoes or sufficient herd immunity in humans is achieved through vaccination or enough people attaining infection-mediated immunity. Of particular interest is the flavivirus ZIKV which has garnered worldwide attention in 2015-2016 when it found itself in urban epidemic cycles across the globe.
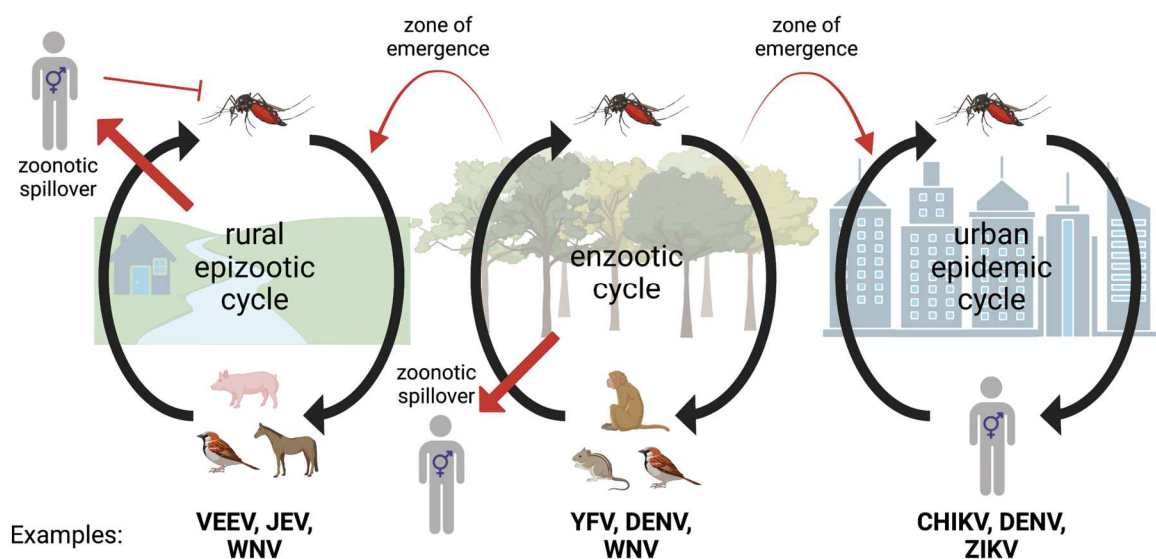
Figure 1. Arbovirus transmission cycles. Information adapted from Weaver et al. 2004, Weaver et al. 2016, and Anez et al. 2019 (1, 10, 12). Straight Red arrows indicate spillover into humans outside the normal transmission cycle. Created with BioRender.com

1.3 Flavivirus biology

Viruses belonging to the genus *Flavivirus* share genome structures and replication strategies. Flaviviruses are small (~50 nm), round, and enveloped positive-sense (+) single-stranded RNA viruses (13). Their genomes are ~10-11 kilobases (kb) and encode 10 total genes from a single open reading frame (ORF) flanked by 5' and 3' untranslated regions (UTRs). The 10 genes include 3 structural (capsid [C], precursor membrane [prM], and envelope [E]) and 7 nonstructural (NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5) proteins (14). Direct translation of the ORF yields a single polyprotein that is cleaved by viral and host proteases into the distinct proteins, though only the structural proteins are present in the final infectious virion product while the remaining nonstructural proteins are involved in replication and immune evasion (15).

Flaviviruses infiltrate target host cells by receptor-mediated endocytosis. The wide host range and numerous tissue types these viruses must traverse to complete their life cycle means there are many host receptors required for flaviviral entry, and the full range is not completely understood. While the exact receptors vary by viral species and between arthropod and mammalian cells, common ligands found to bind flaviviral E proteins include C-type lectins, $\alpha_v\beta_3$ integrins, and phosphatidylserine receptors such as TIM, TYRO3, AXL and MER (16). Upon internalization, endosomal acidification facilitates viral fusion with the host membrane that releases the nucleocapsid protein and RNA genome into the cytoplasm. This is followed by translation of viral RNA into the viral polyprotein, cleavage of the polyprotein into its constituent structural and non-structural proteins, and RNA replication in virus-induced replication

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organelles, also called vesicle packets, derived from the host rough endoplasmic reticulum (rER) (13, 15). Next, RNA and nucleocapsid components are assembled into immature virions that then enter the Golgi apparatus for packaging. Stepwise acidification of the Golgi stimulates both E protein reorganization and furin-induced cleavage of prM, forming mature infectious virions which are then exocytosed from the cell (15). Furin cleavage is inefficient for many flaviviruses, however, and this results in heterogeneous mixtures where only a small fraction of released viral particles are infectious (17).
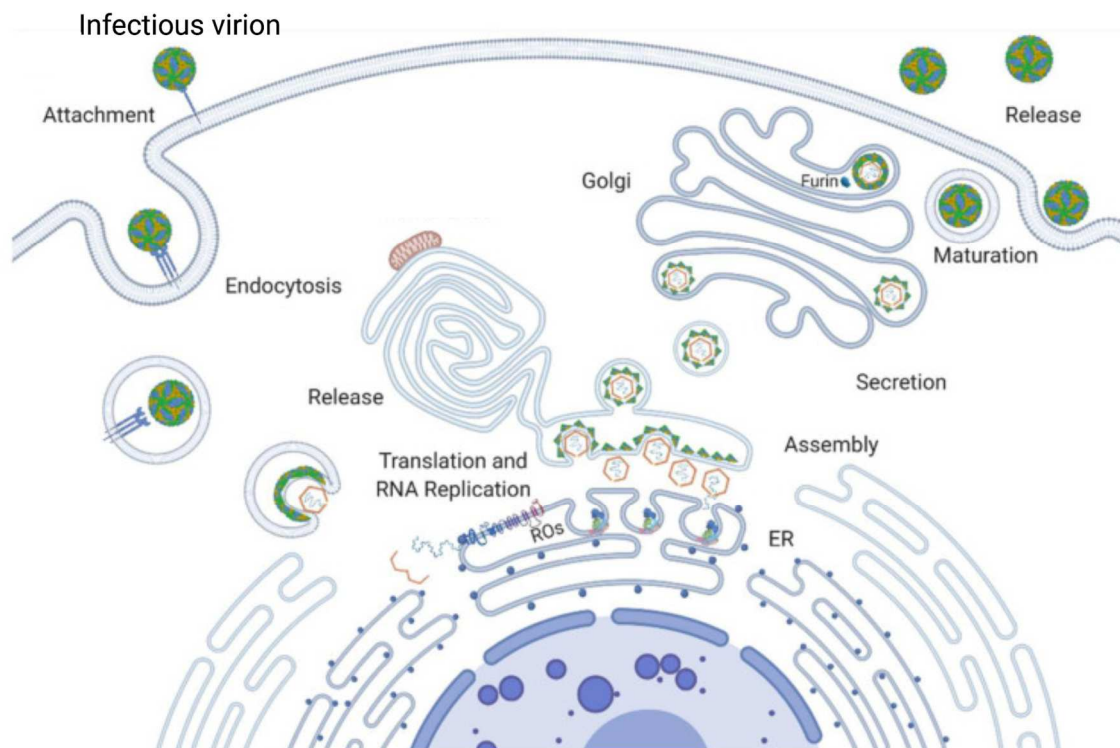
Figure 2. Flavivirus replication cycle. Attachment of an infectious virion to replication, packaging, and export of mature infectious progeny are shown. Adapted from van den Elsen *et al.* 2021 (15) via Biorender.com. ER = endoplasmic reticulum

1.4   Zika virus – A re-emerging threat

Zika virus (ZIKV) was hardly a pathogen of notoriety for over 60 years since its isolation from sentinel rhesus macaques in the Ziika Forest of Uganda (18). This was in 1947, and its discovery was a mere by-product of the yellow fever surveillance program led by the Rockefeller Foundation Program (19). The ancestral African strain was designated ZIKV MR-766 (20). Unsurprisingly, YFV dominated the spotlight for Western researchers, as this disease posed a tremendous burden for imperialist expansion and, more importantly, for the native and displaced peoples exploited by these enterprises (21). While there were fewer than 20 reported human cases of ZIKV for many decades, an explosive epidemic from 2015 to 2016 brought worldwide attention to this emergent virus (22). In the Americas, this epidemic ZIKV was primarily spread to humans by *Aedes aegypti* and *Aedes albopictus* mosquitoes (23). While cases of sexual transmission and blood transfusion-derived transmission were also reported, diagnostic tests that distinguish these cases from vector-borne infection are lacking (24). As of 2017, the cumulative confirmed cases of ZIKV passed 220,000 across 52 American countries and territories (PAHO Zika Cumulative Cases; 4 January 2018). Like other flaviviruses, most clinical cases of ZIKV are met with mild, nonspecific symptoms that include fever, headache, rash, and general lethargy that is usually self-limiting (18). Unlike other flaviviruses, however, ZIKV infection is also accompanied by unusual teratogenic and neurotrophic outcomes. Women infected with ZIKV during pregnancy exhibit prolonged viremias and a risk of transmitting ZIKV to their fetus, which sometimes causes spontaneous abortion and stillbirth (25). Vertical transmission from mother to child can also result in congenital birth defects, now termed Congenital Zika Syndrome (CZS) (26). Brazil was especially impacted in 2015 when the prevalence of microcephaly skyrocketed to 54.6 per 100,000 live births, almost ten times the normal occurrence in a given year (26). These cases have long-lasting effects, with hearing and vision loss, limb weakness, and delayed neurodevelopment observed in children born with CZS

(27, 28). With these clinical manifestations came worldwide news coverage and a terrified populace that bolstered government initiatives to support ZIKV research.

Although the ZIKV pandemic of 2015-2016 has ended, the World Health Organization now classifies 84 countries as ZIKV endemic (WHO Newsroom, 20 July 2018), with potential for renewed epidemic activity given the periodic nature of flavivirus emergence, where new outbreaks may be facilitated by increased numbers of immunologically naïve human populations and geographic expansion of invasive vector *Aedes spp.* mosquitoes (29, 30). It is likely that ZIKV was silently circulating across the globe for decades, avoiding detection in areas where nonspecific symptoms were misdiagnosed as cases of endemic arboviruses like DENV (18). Several outbreaks prior to 2015 were also reported in Yap, Federated States of Micronesia in 2007 and French Polynesia in 2014. The outbreak in Yap was traced to the most abundant *Aedes* mosquitoes on the island, *Aedes hensilli*, and the outbreak in French Polynesia was most likely facilitated by both the recently invaded *Aedes aegypti* and the endemic *Aedes polynesiensis* mosquitoes (31, 32). As for Brazil and other large countries, ZIKV outbreaks were preceded by the invasion of *Aedes aegypti* and *Aedes albopictus*, both of which are efficient ZIKV vectors (23). Thus, the pattern will probably hold that emergence and expansion of competent mosquito vectors will facilitate the next arboviral pandemic. Although local ZIKV transmission has not been reported in the continental United States (US) except for Miami, FL and environs in 2016 (33), rapid spread and proliferation of *Ae. aegypti and Ae. albopictus* mosquitoes poses a heightened risk of ZIKV pandemics in the future. Invasion of California by *Ae. albopictus* in 2011 and *Ae. aegypti* in 2013 (34, 35) as well as in other areas of the US, has led to increased state surveillance efforts for early detection and response to future ZIKV epidemics.

2   Arboviral vectors

At a general level, the most significant determinant of ZIKV infection risk in any given region is the abundance of *Ae. aegypti* (Figure 3). In most countries with a 2015-2016 ZIKV, *Ae. aegypti* was identified as the main vector culprit. But how was this determined? Assessing which vector population is likely behind an outbreak requires fulfillment of several import ecological and biological criteria that are described below.

Figure 3. U.S. map of estimated ZIKV cases with *Ae. aegypti* distribution in 2016. Entire figure is taken from Monaghan *et al.* 2016 (36). Since 2016, *Ae. aegypti* has expanded beyond the listed cities and at higher abundance.

2.1   Incriminating a vector

Elucidating the emergence or re-emergence of an arbovirus entails finding the vector culprit(s). For a mosquito species to become a successful vector, several criteria need to be met. First, the mosquito needs to exist at a high enough abundance to maintain transmission at a population level. No matter the transmissibility of a virus by a single mosquito, transmission will not be maintained if the vectors are too rare. Second, the mosquito must be able to become infected by and transmit virus, also referred to as vector competence. This includes not only infection susceptibility but also expectorating infectious virus in saliva during blood feeding, and this will be discussed in further detail. Third, the mosquito population must exhibit a high enough vectorial capacity for arboviral transmission. Statistical modelling of vectorial capacity utilizes laboratory vector competence and accounts for the extrinsic incubation period (EIP) of the virus within mosquito tissues, the biting rate, and the probability that an infected mosquito will survive long enough to transmit virus in a subsequent feed. Calculation of vectorial capacity can be conducted with the formula below (Equation 1) (37, 38). Lastly, the vector must spatially and temporally coexist with the vertebrate host in a permissive environment. Fulfillment of all four criteria is necessary for a vector species to facilitate a local transmission cycle.

$$VC = \frac{ma^2bp^n}{-log_e p}$$

Equation 1. Vectorial capacity formula. m = number of female mosquitoes per host, a = daily blood feeding rate, b = transmission rate among exposed mosquitoes, p = probability of daily survival, and n = extrinsic incubation period (EIP)

2.2   Assessing vector competence

Laboratory determination of vector competence for arboviruses is no simple endeavor. This first requires the capture and propagation of wild mosquitoes in a laboratory or insectary, and in many cases, experiments are deemed infeasible due to failure to establish healthy mosquito colonies. Additionally, there is always a worry that genetic inbreeding during the colonization process could reduce mosquito fitness and thus stray resulting studies from applicability. In the fields of genetic modification and paratransgenesis of mosquitoes for the purposes of population replacement or suppression, this caution is especially apparent (39). Given that acknowledgement, much of these cautions are speculative, and empirical evidence suggesting that genetic inbreeding alone will reduce fitness and impose detrimental phenotypes is dubious at best (40–43). Upon attaining sufficient adult female mosquitoes of choice, vector competence experiments can commence.

Experimental oral exposure of a mosquito can be done in two ways. The first, and more cumbersome, method is feeding mosquitoes on an anesthetized viremic animal. Aside from the obvious practical challenges with rearing and maintaining another animal, this requires timing the feed where the animal is at peak viremia to maximize infection success. This imposes another challenge of choosing an appropriate animal model for feeding. In the case of ZIKV, for example, Ifnar$^{-/-}$ knock-out mice are commonly used because immunocompetent mice do not generate viremias for wild-type ZIKV (44). The second feeding method is by presentation of virus-spiked blood via an artificial membrane feeder. While logistically much easier than the viremic animal approach, membrane feeding often has reduced feeding success and introduces another degree of separation to biological reality. Membrane feeding bypasses all tissues by which a mosquito's mouthparts interact, though the consequences of using artificial feeders as opposed to organisms on infection outcomes are not entirely clear. Regardless of approach, titration of the blood is essential to confirming the ingested viral dose by blood-engorged female mosquitoes.

Infection of mosquitoes is typically assessed 3-14 days post-blood feed. Detection of virus in the body or dissected midgut is confirmatory of an infected mosquito. However, an infected mosquito is not necessarily transmitting virus to its host. Viral presence in the hemolymph, the arthropod equivalent of blood, is indicative of a disseminated infection, and this can be determined by detecting virus in appendages like the legs and wings. To ascertain transmission, virus must be present in saliva, indicating infection of the salivary glands. In practice, separation of a single mosquito individual into its main body, pulled legs and wings, and expectorate for independent assays is standard (23, 45). To collect expectorate, or saliva, one simply places the proboscis of an immobilized mosquito into a capillary tube or pipette tip containing buffer or oil to force salivation. Detection of virus in saliva confirms that a mosquito is not only infected but also transmitting. The most common assays for assessing infection of tissues are quantitative revert transcription polymerase chain reaction (RT-qPCR), which detects viral genomic RNA, and plaque assay, which detects virus capable of infection. While positive detection of viral RNA by RT-qPCR is not always indicative of active infection in a mosquito body, concurrent detection of RNA in accessory tissues like the legs and wings or salivary glands of the same mosquito reliably suggests infection of the body by infectious virus.

2.3   Anatomical barriers

An arbovirus faces several anatomical hurdles within the mosquito before reaching its salivary destination. The virus must first infect a midgut cell from the gut lumen after blood meal ingestion and escape through the opposite (basal laminal) side of the midgut epithelium. This is often called the midgut infection barrier (cell entry) and the midgut escape barrier (cell exit) (Figure 4). It has been observed for many arboviruses that only a few midgut cells, about 1 to 15 per ~100 cells, are initially infected among those susceptible, and these patches of infected

cells seed the resulting infection (46, 47). Upon release to the hemocoel, which is the open hemolymph-filled body cavity, the virus must find its way to the salivary glands where infection and escape must happen yet again. This is called the salivary gland infection barrier (cell entry) and the salivary gland escape barrier (cell exit) (Figure 4).
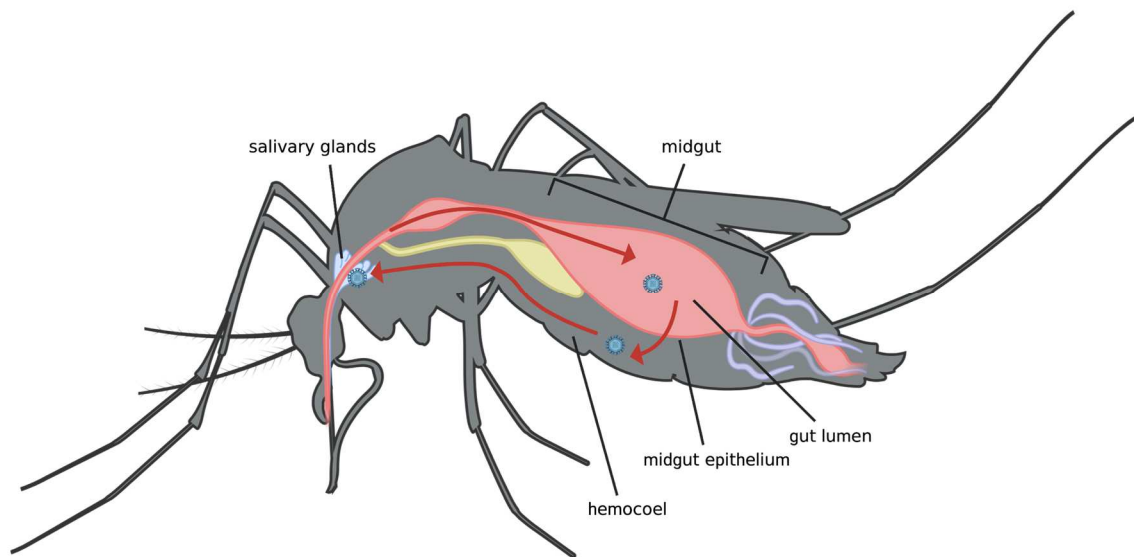
Figure 4. Arbovirus infection route in a mosquito. Blue spheres represent an arbovirus. Information is adapted from Franz *et al.* 2015 (46). Created with BioRender.com

The path a virus takes shapes not only its replication kinetics but also its evolution. In contrast to single-host viruses, arboviral evolutionary trajectories are constrained due to cycling between disparate hosts. Acquisition of beneficial mutations in a vertebrate is often nullified by a reduced fitness in the vector, dampening the evolutionary momentum of an otherwise highly mutable virus (48). In DENV, adaptive evolution in mosquitoes is even suggested to be slower than in vertebrate cells (49). Additionally, genetic bottlenecking resulting from consecutive tissue-specific purifying selection accelerates the accumulation of mutations within a single organism (50). An example of this phenomenon is a study which found that *Cx. quinquefasciatus* and *Cx. tarsalis* mosquitoes can expectorate unique WNV populations between feeding episodes,

though most genetic variants are quickly removed when transmitted to birds (51). This demonstrates an evolutionary trend where mutants quickly arise among the viral mutant swarm yet are equally quickly selected against, and this phenomenon molds the way researchers consider the arrival of new genetic variants in arbovirology.

2.4   Confounding factors

There are many variables that can affect vector competence of a mosquito relative to a specific arbovirus, and while genetic determinants have long been the subject of study (52–54), non-genetic factors are less understood and will be discussed here.

Vector competence levels can be easily biased by the laboratory approach conducted. While the saliva capture method is a standard process for determining transmission potential, it may not accurately reflect actual transmission levels. Forced salivation was recently shown to underestimate transmission titers in natural feeding patterns by *Aedes aegypti* infected with ZIKV, *Culex quinquefasciatus* infected with WNV, and *Aedes triseriatus* infected with La Crosse virus (*Peribunyaviridae*, *Orthobunyavirus*) (55). Detection of infectious virus or viral RNA in the legs and wings or detection in mosquito excreta, a novel proxy method for assessing dissemination, are more reliable predictors of transmission than previously thought (55, 56). Additionally, successive virus-spiked bloodmeals, which are more reflective of the repetitious feeding behavior of *Ae. aegypti*, unsurprisingly increase laboratory infection and transmission rates relative to a single blood meal (57). These subtle yet consequential experimental readouts complicate cross-study comparisons.

Environmental variables can have profound impacts on arboviral transmission in mosquitoes. It is established that incubation temperature drastically affects infection kinetics of the ectothermic

arthropods, with increasing temperatures generally reducing the EIP of a given arbovirus (58). This phenomenon further complicates translatability of older studies as climate change is driving higher average daily temperatures worldwide with more extreme fluctuations (58, 59). Immature mosquitoes that undergo nutritional stress or extreme larval competition become more competent vectors for some arboviruses (60–62) but not others (63). In *Ae. aegypti*, adults that overcame treatment by pyriproxyfen, a larval growth inhibitor, were less susceptible to ZIKV infection (64). Among studies with our arbovirus-mosquito pairing of interest, *Ae. aegypti* and ZIKV, there is high variation in the infection and transmission potential among geographically disparate mosquito populations. Studies conducted encompass *Ae. aegypti* from across the globe including, but not limited to, Singapore (65, 66), Australia (67, 68), Brazil (69, 70), and the United States (70, 71). While genetic differences among the globally distributed *Ae. aegypti* and inbreeding of colony mosquitoes likely plays a role in this variation, an alternate explanation is the variable effects of their microbiota on ZIKV vector competence. The interactions between mosquitoes and their microbial symbionts are elusive and complex, and this will be discussed in further detail in Sections 3 and 4.

3   *Aedes aegypti* microbiota

Ae. aegypti origins can be traced to sub-Saharan Africa. Ancestral forms were likely tree-dwelling mosquitoes that shifted their breeding strategies to human settlements (72). Transport of *Ae. aegypti* to the "New World" on ships carrying enslaved African peoples was one of the many grave legacies brought on by European colonization. From this arrival came centuries of adaptation, proliferation, and terrorization as *Ae. aegypti* catalyzed numerous YFV outbreaks in the Americas (73, 74). In the last century, *Ae. aegypti* larvae and eggs have also hitchhiked

domestically through trade vessels (boats, planes, buses, cars) (75, 76) and the transport of used tires (77).

Ae. aegypti and *Ae. albopictus* mosquitoes are the primary vector species for ZIKV transmission. The combination of aggressive anthropophilic feeding patterns (78), ability to reproduce in a wide range of human-associated water containers (30, 79, 80), and impressive desiccation resistance of their eggs has facilitated their invasion and expansion across the globe (81, 82). *Ae. aegypti* has established a greater presence in urban environments, likely due to its higher preference for abundant artificial containers among dense population centers (83). In contrast, *Ae. albopictus* is more environmentally plastic and tends to predominate in rural areas, though several studies suggest interspecies competition between *Ae. albopictus* and *Ae. aegypti* within the same habitat (84, 85). Nevertheless, *Ae. aegypti* and *Ae. albopictus* can coexist in peri-domestic environments and areas with heterogenous larval habitats (86, 87). The aggressive host seeking behavior of *Ae. aegypti* in urban and suburban neighborhoods, has prompted local government responses resulting in the establishment of numerous laboratory colonies. This has allowed for ease of specimen production, and made *Ae. aegypti* the focus of my work.

3.1   *Aedes aegypti* life cycle

The *Ae. aegypti* life cycle consists of an aquatic and a terrestrial phase. A gravid female will lay ~40-80 eggs at the water's edge in containers holding standing water (88). The eggs dehydrate upon water evaporation and can remain dormant for months thanks to a serosal cuticle layer that protects the embryo from desiccation (89). This feature is highly effective in protecting the eggs through the dry seasons and is believed to explain the successful global proliferation of *Ae. aegypti* even out of tropical environments where they originated (23, 90). Reconstitution in

water in concert with a reduced concentration of dissolved oxygen acts as a stimulus for egg hatching (91). The larvae feed on organic detritus and microalgae as they develop over a time range between four days and several weeks depending on available food, temperature, and water chemistry (92). As the larvae grow, they molt three times and get progressively larger with each molt, with the molting periods marking their transition to the next instar (L1-L4) (93). Once large enough, larvae metamorphose into pupae which during this two-day period remain motile, but do not feed (94). Adult emergence, or eclosion, from pupae marks the transition from the aquatic to terrestrial stage of the *Ae. aegypti* life cycle.

Adult female mosquitoes exhibit both sugar feeding and blood feeding behavior. Males, however, feed exclusively on sugar and lack the mouthpart adaptations required to blood feed (95). Laboratory studies and field surveys have implicated floral and extrafloral nectaries as sugar sources for *Anopheles spp*. and *Culex spp*. mosquitoes, but little is known about sugar feeding sources and behavior in *Ae. aegypti* (96–99). Older studies suggested *Ae. aegypti* rarely sugar feed and rely primarily on blood sources from which they take frequent bites (100, 101). Newer studies are more conflicting, however, with highly variable yet modest detection of sugar-ingested *Ae. aegypti* in the field (102, 103). Nevertheless, female *Ae. aegypti* are adept at blood-seeking and are highly anthropophilic (88, 94). While sugar sources provide the energy needed for flight and basic metabolism, blood provides the dietary protein needed for egg production and maturation (104, 105). A female *Ae. aegypti* that lands on a host will use its proboscis, to inject saliva and extract blood (94). Mosquito saliva contains a protein cocktail that is vital for anti-coagulation, anti-inflammation, and anesthetization of the host at the bite site (106). Ingested blood is stored in the posterior midgut where proteins are broken into their constitutive amino acids over the course of 2 to 3 days; these are used for yolk protein synthesis, secretion and uptake by developing oocytes in a process called vitellogenesis (107, 108). This process is usually completed by 3-4 days post-blood meal, after which, the female is

ready to oviposit in another water-collection receptable. This constitutes one gonotropic cycle, and a single female can have multiple cycles provided she survives long enough for multiple feeds.

There are many factors affecting the duration and efficiency of the *Ae. aegypti* life cycle. Abiotic factors like temperature and environmental chemistry have long been acknowledged as contributing factors, yet the microbiological side to this story had been vastly underappreciated until the last two decades. With new methodological tools and an explosion of interest in microbiome studies, *Ae. aegypti* biology can be more thoroughly explored in the context of its microscopic partners.

3.2   *Aedes aegypti* holobiont and its origins

Like every animal studied, mosquitoes are holobionts that live in close association with their microbial symbionts (109). The mosquito as well as its assemblage of bacteria, archaea, fungi, protozoa, and viruses exists as a super-organism that is more than the sum of its parts. However, within the field of mosquito microbiota, most studies have focused on the bacterial members of the community while archaea, fungi, and insect-specific viruses are severely under-characterized. The microbiota of mosquitoes can influence life history traits including nutrition (110), development (111–114), fecundity (115), and even vector competence (109, 115, 116). Growing interest in the mosquito microbiome has been coupled with the increasing efficiency and shrinking costs of high-throughput Next Generation Sequencing (NGS) technology, allowing for more detailed probing of microbial community structure and function (109, 110, 117).

Whether *Ae. aegypti* harbor a core microbiome is subject to debate. Much variation exists among geographically distant populations, and ecological surveys suggest their microbiota are

primarily environment-derived (109, 112). Yet, bacteria belonging to phyla *Proteobacteria*, *Actinobacteria*, *Bacteroides*, and *Firmicutes* comprise >99% of the bacterial microbiota across a wide range of studies (109, 117, 118). For non-bacterial microbes, this question is even murkier.

3.2.1 Vertically transmitted microbes

Ae. aegypti acquire their microbes at several points in their life cycle. Vertical transmission of endosymbiotic bacteria and viruses from mother to progeny has been demonstrated under experimental conditions (119–121). Successful vertical transmission of *Wolbachia* bacteria among *Wolbachia*-colonized mosquitoes is instrumental to mosquito population replacement strategies (121). Alternately, coating of egg surfaces with bacteria from the ovaries, (i.e. transovarial transmission) is also a conceivable route of acquisition; this has not yet been experimentally proven, however. Bacterial genera that have been found on *Ae. aegypti* eggs include *Chryseobacterium*, *Delftia*, *Acinetobacter*, and *Stenotrophomonas* (111), though because many of these reported bacterial sequences are common reagent contaminants, we cannot yet conclude these are real members of the mosquito microbiota (122). Whether any microbes other than *Wolbachia* exist within the eggs is still unclear.

3.2.2 Microbial acquisition as larvae

The first major route of microbial colonization is through the aquatic environment as larvae (Figure 5). *Ae. aegypti* larvae are generalists that will feed on anything from plant debris to algae, protozoa, and organic detritus (123, 124). A portion of ingested microbes in the aquatic habitat survive digestion and colonize the larval gut. NGS studies of the larval gut often find a high abundance of cyanobacterial and microalgal DNA, though it is unclear what proportion represents live, symbiotic microbes and versus those that are mere artifacts from food

consumption (109, 125, 126). Most larval gut microbes are excluded from the mosquito body during the transformative process of pupation, and newly emerged adults are re-colonized by environmental microbes. Interestingly, while the microbial members of adults are mostly different from that of larvae, there are some shared bacterial species between these two life stages. Two hypotheses for larval microbe persistence in adults have been proposed. The first posits that pupation incompletely clears larval gut bacteria due to sequestration of bacteria along the meconial peritrophic membrane followed by newly emerged adults undergoing secondary succession by the surviving bacteria (Figure 5) (127). The second hypothesis posits that immediately upon eclosion, adults imbibe the larval water to re-seed their gut (Figure 5) (128, 129). Neither hypothesis precludes the other and both may be true to an extent.
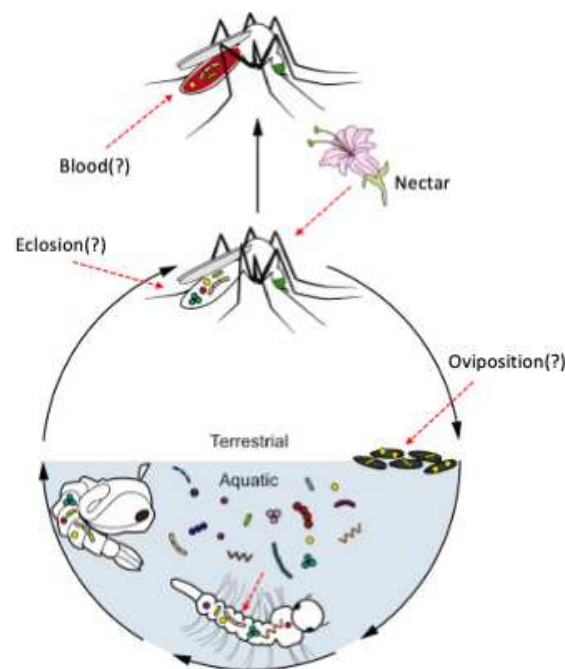
Figure 5. Origin of microbes in the mosquito life cycle. Diagram is adapted from Strand 2017 (130) and edited in Microsoft PowerPoint.

3.2.3   Microbial acquisition as adults

The second major route of microbial acquisition is via sugar feeding. As previously mentioned, *Ae. aegypti* sugar feeding behavior is poorly understood, complicating efforts to understand adult microbe acquisition. Nectar-derived microbes are likely to contribute to the adult mosquito microbiome, though confirmatory experiments are nonexistent (109, 131). Despite this, NGS studies and field surveys comparing microbial communities across life stages and across environments definitively show that adult mosquitoes harbor unique microbiota, and that specific composition is environmentally variable (109, 110, 117). Here it should be noted that the journey microbes undergo to enter an adult midgut is different than the journey to a larval midgut. Microbes ingested by larvae directly enter the midgut whereas microbes ingested by sugar-feeding adults are first routed to the sugar-storage organ, called the ventral diverticulum or simply the crop, after which they can be selectively directed to the midgut in a controlled manner (88, 94). This additional tissue barrier places another round of selection on adult-acquired microbes compared to microbes passed transstadially from the larval gut.

The last, and likely minor, potential route of microbial acquisition is through transfer of host skin microbiota during the blood uptake. Data currently supporting this route of exposure are lacking. This is likely due to the fact that the proboscis is not a single mouthpart but a sheath for six distinct needle-like structures: a gripping mandible pair, two piercing maxillae, a saliva-spitting hypopharynx, and a blood-sucking labrum (132). Ingestion of microbes via the labrum is not expected since the body parts that directly contact the host epidermis are the mandibles and maxillae.

3.3   Tissue specificity

Microbial symbionts dominantly reside in the mosquito gut. Some microbes can escape the gut and infect secondary tissues such as the salivary glands, testes, and ovaries, although these mechanisms are not well understood. While the exact microbial composition can vary, it has been proposed that some level of core microbiota exist within a single organism due to the presence of some shared bacterial genera across different tissues (109, 110, 117).

Ae. aegypti midguts typically harbor low bacterial diversity at the phylum level. Many surveys found primarily *Proteobacteria*, followed occasionally by *Bacteroidetes*, *Firmicutes* and *Actinobacteria* (116, 117). Common proteobacterial genera include *Asaia*, *Sphingomonas*, *Escherichia-Shigella*, *Pseudomonas* and *Serratia* (117, 133, 134). Gut bacterial diversity is dynamic and changes dramatically depending on mosquito feeding status. Adult female midguts show dramatic decreased bacterial diversity and increased bacterial abundance upon blood ingestion (Figure 6) (135). This is likely due to blood-induced changes in chemistry and pH which select against many bacterial species while also introducing dietary protein which allows others to proliferate (135, 136). The host blood source also seems to affect the gut microbial composition (137). Despite the apparent domination of a few bacterial taxa following a blood meal, diversity and abundance recover and stabilize at ~30 hours after blood feeding (117, 129, 135).
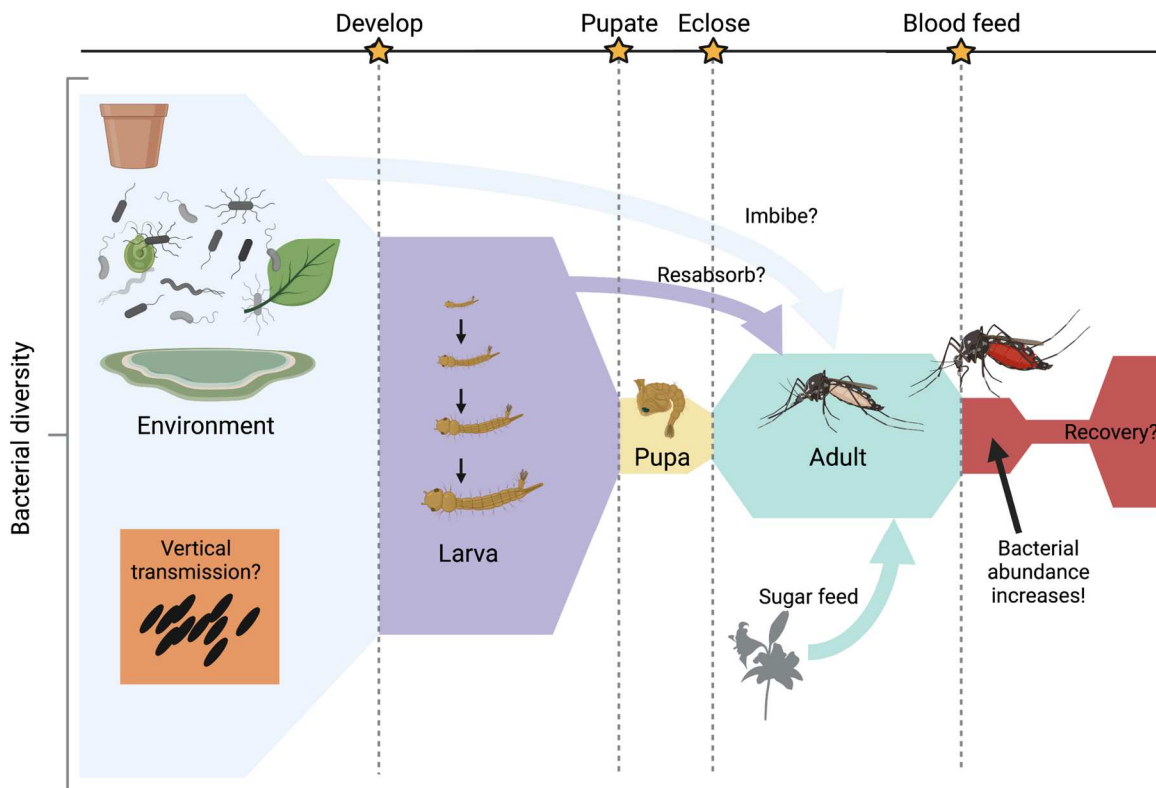
Figure 6. Bacterial diversity over the course of *Ae. aegypti* life history. Figure is presented as a funnel system where bacterial diversity gradually decreases over time. Heights indicate the level of bacterial diversity for each life stage. Information is adapted from Guégan *et al*. 2018 and Scolari *et al.* 2019 (109, 117). Created with Biorender.com.

The salivary glands also harbor bacteria, and this organ too is dominated by *Proteobacteria* in *Aedes app.* mosquitoes (118). Within this phylum, *Acetobacter*, *Burkholderia*, *Cupriavidus*, *Escherichia–Shigella*, *Pantoea*, *Serratia*, and *Sphingomonas* genera are often found (117). *Elizabethkingia spp.* bacteria, which have been found in midguts, salivary glands, and ovaries are also symbionts of interest. In *Ae. albopictus* (and potentially other species), *Elizabethkingia anophelis* is transmissible in saliva, and elevated levels of this bacterium were associated with reduced ZIKV vector competence (138, 139).

Invasion of mosquito reproductive organs opens the possibility of vertical transmission to offspring. Among them, *Serratia*, *Elizabethkingia*, and *Wolbachia* are of physiological interest (139–141). Extracellular bacteria can theoretically pass to progeny through surface coating of eggs, but this has not been confirmed (Figure 6). Intracellular bacteria can be inherited through infection of the germline cells, as shown in the case of *Wolbachia* (142). However, *Wolbachia* represents a unique case; this endosymbiont exhibits cytoplasmic incompatibility wherein only infected eggs can successfully be fertilized, thus ensuring transmission (143). Other forms of vertical transfer have not yet been widely described.

4 Mosquito-microbe interactions

4.1 Effects on development

Early investigation of larval microbes suggested that live bacteria are needed for development and pupation, as sterilized diets resulted in larval mortality (111). However, recent attempts to generate microbe-free mosquitoes (i.e. axenic) were met with some success in larval development into adults (144). A huge caveat is that these axenic mosquitoes pupated inefficiently and were smaller than gnotobiotic mosquitoes, those colonized with a single known bacterial isolate, and mosquitoes with an intact microbiome (144). These data suggest that, while not required, larval gut microbes nutritionally benefit their host and support growth. The initial explanation that live microbes stimulate pupation, through induction of hypoxia in the gut, is currently challenged by the axenic model of mosquito development (113). Much more work is needed to reconcile these findings.

4.2   Effects on blood feeding

Just as mosquito blood feeding affects gut microbial composition, the gut microbiota in turn influences host seeking and feeding behavior. In *Anopheles gambiae*, infection with the entomopathogenic fungus *Metarhizium anisopliae* led to a reduced appetite for blood proportional to the fungal load (145). In *Ae. albopictus*, artificial induction of fat body-specific vitellogenin was shown to stimulate host-seeking behavior; this coupled with a demonstrated gut bacterial stimulation of vitellogenin genes in insects provides a potential mechanism for microbial modulation of blood feeding behavior (146, 147).

Gut microbiota also contribute to blood digestion. Quickly after blood has been ingested, a semipermeable chitinous mesh called the peritrophic matrix (PM) is secreted by the gut epithelium and encases the blood bolus. This acts as a protective barrier from pathogens and blood-derived toxicity as well as a locale for digestive enzyme activity (148, 149). Antibiotic treatment of *Anopheles coluzzii* resulted in a malformed and perforated PM, showing that PM synthesis is dependent on gut microbiota (150). Antibiotic treatment of *Ae. aegypti* retarded blood protein digestion and reduced egg production, suggesting a role of gut microbes in nutrient metabolism as well (151). The full extent to which blood meal breakdown and nutrient uptake is facilitated by symbionts is unclear.

4.3   Immune interactions

Resident gut microbes exist in dynamic equilibrium with their mosquito hosts. It is proposed that status of this immune-microbe relationship shapes the success of arboviral infection. Mosquito innate immune responses typically fall into four major pathways: Toll, immunodeficiency (IMD), Janus kinase signal transducer and activator of transcription proteins (JAK/STAT), and RNA

interference (RNAi). Each pathway senses specific microbe-associated molecular patterns (MAMPs), triggering signal cascades that lead to generalized and specialized antimicrobial responses (Figure 7). Despite the specificity of microbial triggers for each pathway, there is growing evidence of cross-talk among these different responses (152). The four immune pathways are briefly described and contextualized below.

4.3.1   Toll pathway

Of the four pathways, Toll is arguably the best understood due to the plethora of comparative studies in other invertebrates like *Drosophila*. Put simply, recognition of Gram positive bacteria or fungi by membrane-bound Toll receptors activates the NF-kB transcription factor Rel1 (Figure 7). Toll recognition of MAMPs is facilitated by an intermediary protein, Spätzle, which is also suggested to sense viruses (153). Translocation of Rel1 into the nucleus is then proceeded by upregulation of antimicrobial genes, including antimicrobial peptides (AMPs) against bacteria and fungi (154). One such AMP, defensin, was also shown to suppress DENV infection in *Ae. aegypti* (155). A follow-up study found that biased upregulation of both Toll and JAK/STAT, which can occur during co-infection with the fungus *Beauveria bassiana*, also contributes to resistance to ZIKV (156, 157).

4.3.2   IMD pathway

The IMD pathway is a well-conserved pathway among mosquitoes responding to both Gram positive and Gram negative bacteria (153, 158). This pathway can be activated in response to midgut microbiota proliferation resulting from a blood meal (136, 158). Shown in anopheline mosquitoes, the MAMPs recognized are diaminopimelic acid (DAP)-type peptidoglycan (PG) in Gram negative bacterial cell walls and Lys-type PG in Gram positive bacterial cell walls (153,

159). Other cell wall components in various bacteria are also recognized, though in a more tissue dependent manner. In the example of Gram negative bacteria, binding of DAP-type PG to the host receptor PGRP-LC causes activation of transcription factor Rel2 that induces expression of AMPs (153, 159).

Bacteria, however, have adapted ways to survive the harsh immune effectors secreted by mosquitoes. Some gut bacteria coat themselves in C-type lectins acquired from the mosquito host to counteract AMP activity, thus maintaining gut homeostasis (160). Manipulation of AMPs is also a likely method of indirect control over pathogens. In *Ae. aegypti*, gut microbiota-induced AMP production via IMD and Toll resulted in lowered vector competence for Sindbis virus (SINV; *Togaviridae*, *Alphavirus*) (161). Similar relationships were also shown with specific bacteria like *Proteus spp*. which reduced DENV infection in *Ae. aegypti* (116).

4.3.3   JAK/STAT pathway

The JAK/STAT pathway is another mechanism for anti-pathogen defense. This multicomponent system is also evolutionarily conserved, with similar features to known processes in other animals. The transmembrane receptor Dome is a homolog to vertebrate type-1 cytokine receptor family interleukin-6 (162). Detection of MAMPs leads to Upd ligand binding with Dome, which then catalyzes autophosphorylation of signal transducer Hop, a Janus kinase (163). Recruitment and phosphorylation of transcription factor STAT by Hop then allows for expression of AMPs, nitric oxide, and opsonization factors (135, 153). Precise mechanisms, MAMPs, and accessory proteins involved with the JAK/STAT pathway are still unclear, but the effects on DENV susceptibility have been demonstrated (164). Upregulation of JAK/STAT by silencing of its inhibitor PIAS dramatically reduced DENV infection rates in *Ae. aegypti*, whereas knockdown

of either Dome or Hop saw increased DENV susceptibility (164). Infection with ZIKV has also

been shown to activate JAK/STAT to the same effect (156).

The relationship between JAK/STAT and other immune pathways is a topic of interest.

JAK/STAT is apparently inducible by Vago, a protein upregulated by Rel2 from the IMD pathway

(165), and Vago production inhibited WNV replication in *Culex* mosquitoes *in vitro* (166). In *An.*

gambiae, activation of STAT was observed during infection with *Escherichia coli* and

Micrococcus luteus bacteria, conferring moderate resistance to *Plasmodium* infection (167).
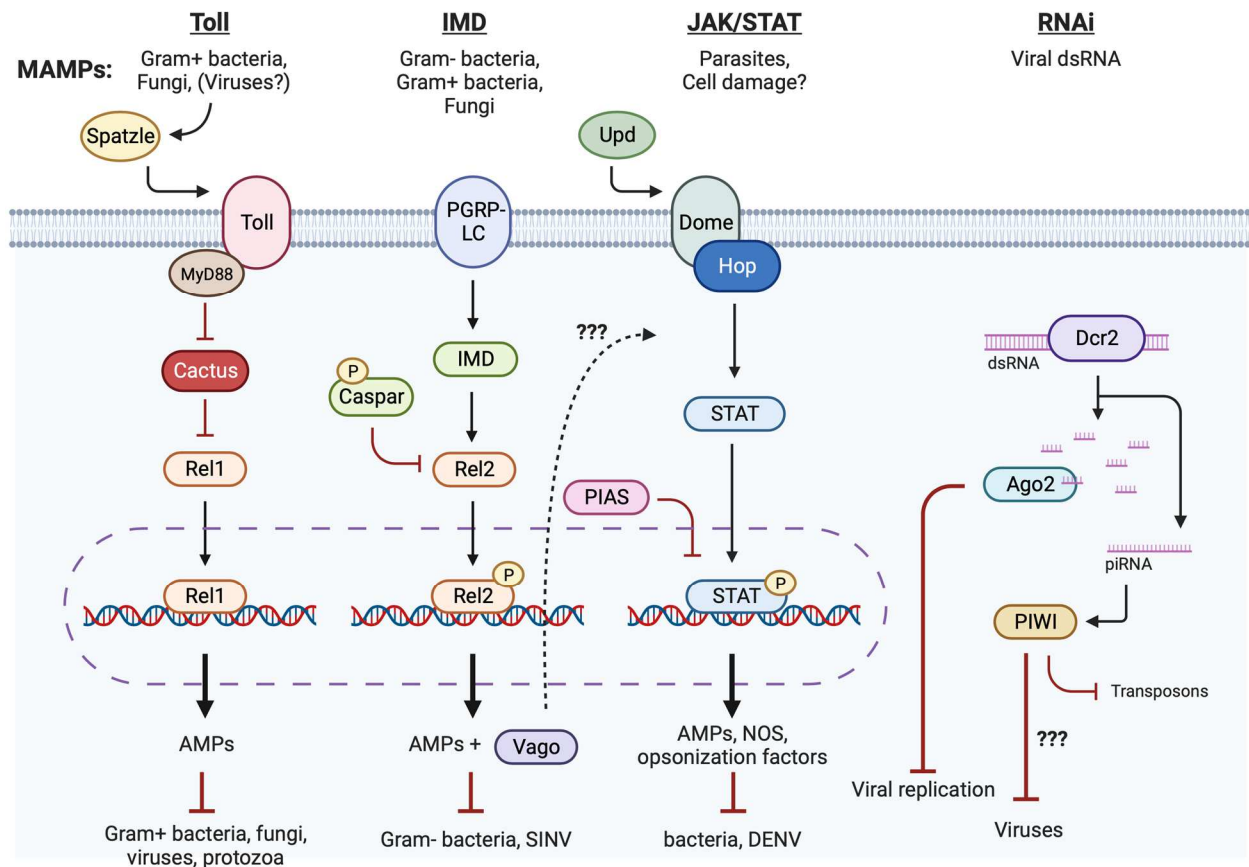
Figure 7. Four primary innate immune pathways in mosquitoes. Toll, IMD, JAK/STAT, and RNAi

pathways with their known and suspected MAMPs are sensed by pathogen recognition

receptors (PRRs). Dotted purple line represents the nucleus, and components bound to DNA

are transcription factors that regulate gene expression. Structures with a circular yellow P indicate phosphorylated forms of the protein. NOS = nitric oxide synthase. Information is adapted from Kumar *et al.* 2018, Caragata *et al.* 2019, and El-Sherbini *et al.* 2021 (152, 168, 169). Created with Biorender.com.

 4.3.4   RNAi pathway

While Toll, IMD, and JAK/STAT can be considered humoral pathways, RNAi is a primarily cellular immune pathway against viral infection. Viral RNA replication undergoes a double stranded RNA (dsRNA) intermediate step to generate the reverse complementary template for genomic synthesis (170). Host ribonuclease Dcr2 recognizes this dsRNA and cleaves it into 21 nucleotide (nt) fragments called small interfering RNAs (siRNAs) (171). These exogenous siRNAs are then loaded onto an effector protein Ago2 that seeks, binds, and degrades viral RNA in the cell (171). This mechanism has been shown to work against many RNA viruses including Semliki Forest virus (*Togaviridae*, *Alphavirus*), Flock House virus (*Nodaviridae*), bluetongue virus (*Reoviridae*), ZIKV, DENV, SINV, and WNV (171–176).

In addition to siRNAs, recent work has found that RNA fragments larger than 21 nt are also generated from some viral RNAs. Spanning 24-30 nt, these small RNAs are called PIWI-interacting RNAs, or piRNAs. Originally thought to be exclusively used against detrimental transposon activity in germline cells, piRNAs have now been found to be ubiquitous among somatic mosquito cells as well (177, 178). Characterized by their hallmark adenine bias at the 10th nt position and uracil bias on the 1st nt position, piRNAs exhibit template-dependent ping-pong amplification (178, 179). Additionally, production of piRNAs is Dcr2-independent, suggesting a separate function to the siRNA pathway (180). Exact roles of these piRNAs in the context of immunity are unclear, but production of piRNAs has an inhibitory effect against some

viruses and not others. In *Ae. aegypti*, infection by DENV-2 (181), ZIKV (182), CHIKV (178), and

Rift Valley fever virus (*Phenuiviridae*, *Phlebovirus*) (183) resulted in piRNA biogenesis.

4.4   Effects on vector competence

Several bacterial strains have been demonstrated to influence *Ae. aegypti* vector competence

for arboviruses. Most notably, *Ae. aegypti* infected with the bacterial endosymbiont *Wolbachia*

were shown to less efficiently transmit DENV and ZIKV (141, 184–186). While mechanisms

behind the blocking of arboviral transmission by *Wolbachia*-infected mosquitoes are still not

clearly understood, its effectiveness and vertical passage to subsequent generations has

prompted field trials and experimental release of *Wolbachia*-infected male mosquitoes as a

means of population replacement (187–189). Similarly, the bacterium *Chromobacterium Csp_P*

reduced transmission of DENV in *Ae. aegypti* and *Plasmodium falciparum* in *Anopheles*

gambiae respectively (190). Members of the bacterial genus *Asaia*, Gram negative symbionts

naturally found in *Aedes spp.* mosquitoes, are also being investigated for their high prevalence

in mosquitoes refractory to arboviruses and *Plasmodium* parasites (191–193). However,

bacteria appear also to increase mosquito transmission potential for arboviruses as well. These

divergent effects necessitate a thorough investigation into the mechanisms by which bacteria

modulate vector competence. *Ae. aegypti* colonized with *Serratia odorifera* are more susceptible

to infection by DENV-2 and CHIKV (140, 194), and follow-up studies with *Serratia marcescens*

suggest that bacterial infection undermines the gut epithelium integrity of *Ae. aegypti* via mucin-

degrading activity resulting in an increased rate of viral dissemination (195).

Microbes other than bacteria have also been found to modulate vector competence. *Ae. aegypti*

infected with the fungus *Beauveria bassiana* exhibit reduced life spans and DENV replication

efficiencies. In addition, infection with a fungal isolate of *Talaromyces* also increased

susceptibility to DENV (157, 196). *Culex quinquefasciatus* mosquitoes infected with the insect-specific flavivirus (ISV) Nhumirim virus demonstrated reduced competence for WNV, with this interaction also holding true in *Ae. aegypti* for ZIKV (197, 198). Another ISV, Yichang virus, inhibits infection of DENV-2 and ZIKV in *Ae. albopictus in vitro* and *in vivo* (199). Finally, an ISV alphavirus Eilat virus was shown to delay CHIKV midgut infection in *Ae. aegypti* (200). The coevolution of ISVs with their insect hosts has become a topic of interested in recent years for their potential as biocontrol agents akin to *Wolbachia* (201).

5 Discussion

5.1 Lingering questions

Investigation of mosquito microbiota in the context of arboviral transmission is still in its infancy. Most studies to on mosquito microbiota to date can be considered "observational", albeit with immense value. The range of microbial communities among both field-collected and lab-raised *Ae. aegypti* and *Ae. albopictus* has been surveyed in numerous countries including India (202, 203), Vietnam (204), Madagascar (205), France (204, 206), Australia (207), Gabon, Cambodia, Uganda (208), Brazil (134, 209), and the USA (133, 210, 211). From surveys like these, few bacterial candidates (*Wolbachia* (184, 212) and *Chromobacterium Csp_P* (190)) are currently being tested for their potential as arboviral vector competence modulators. However, the generalizability of their regulatory effects to the full range of arboviruses as well as their stability as long-term symbionts are virtually unknown.

Bioagents like these have become promising alternatives to traditional methods of vector control such as insecticides, which can exhibit off-target toxicity and environmental damage. Moreover,

insecticide resistance is a continuing problem in major targeted vectors (213, 214). In *Ae. aegypti*, resistance to all four major classes of insecticides (carbamates, organochlorines, organophosphates and pyrethroids) has been observed (215). Microbial symbionts are often less susceptible to resistance development when the bioactive agents are evolutionarily well-conserved and if adaptive evolution is allowed. For example, *Bacillus thuringiensis* svar. *israelensis* (Bti) has remained a potent larvicide for decades with little observed resistance (in *Ae. aegypti* at least) due to their diverse arsenal of functionally redundant Cry toxins (216–218). Thus, further exploration of microbial members as vector control agents and vector competence modulators is greatly warranted.

Tripartite interactions between arboviruses, mosquitoes, and their microbiota reflect many possible avenues for modulating vector competence as well as the complexities in selecting microbial candidates for application in the field. However, studies on specific microbial strains primarily involve inoculating mosquitoes with a pure culture isolate, usually after administration of antibiotics. How these candidates and any future candidates behave outside laboratory constraints where microbial resource competition, symbiosis, and coevolution run rampant remain open questions. Microbes rarely exhibit the same functional characteristics in isolation as they do in their natural environment, where they interact with diverse and dynamic communities with overlapping niches. For example, screening of *Asaia* and *Wolbachia* bacteria in laboratory-colonized and field-caught anophelines, *Ae. albopictus*, and *Cx. quinquefasciatus* found differential colonization successes between the two taxa as well as exclusion of *Wolbachia* from reproductive tissues where *Asaia* reside, hindering vertical transmission of the symbiont (219). An understanding of how the microbiome of mosquitoes influences arboviral vector competence in a microbial community context is lacking and must be reconciled prior to field applications.

5.2   Obligatory segue

In California (CA), USA, there is a real risk of ZIKV establishing a local transmission cycle. With the invasion of CA by *Ae. albopictus* in 2011 and *Ae. aegypti* in 2013, and the fact that CA is a central hub for domestic and foreign travel, surveillance efforts have expressed interest in epidemic forecasting and preemptive mitigation measures. From 2015 to 2021, CA has reported a total of 756 imported cases of ZIKV in travelers returning to the state (California Department of Public Health Monthly Update, as of Nov 1, 2022). CA *Ae. aegypti* have been determined to be competent ZIKV vectors (71), but whether these mosquito populations are uniformly competent is unknown. Lab colony mosquitoes are often more competent vectors than wild mosquitoes. The prevailing explanation for this phenomenon thus far is genetic bottlenecking of lab colony mosquitoes due to inbreeding, and there is some support for genetic variation impacting ZIKV infection susceptibility (68, 220–222). However, the potential role of homogenous insectary-derived microbiota in contributing to this observed difference in vector competence has not been investigated. Characterization of microbiota associated with ZIKV infection dynamics will elucidate biotic factors that determine ZIKV transmission success. In Chapter 2, I hypothesize that increased microbial exposure in laboratory mosquitoes reared in environmental water reduces their ZIKV vector competence.

The mechanisms by which microbiota modulate *Ae. aegypti* vector competence are incompletely understood. Activation of parallel immune pathways (such as Toll and JAK/STAT) by certain bacterial species can have downstream antiviral effects, but this has only been shown in pure culture inoculations or in gnotobiotic mosquitoes. Additionally, the route of microbial exposure and life stage in which microbes are introduced are variables that are not uniformly addressed across studies. Larval exposure to microbes in their aquatic habitat can have carryover effects on adult life history traits, which can theoretically regulate vector competence.

Whether these influences by transstadial microbes are immune-priming or merely developmental are unclear. Potential mechanisms for altered infection and transmission potential adjacent to immunity include nutritional supplementation (60), modification of midgut cell proliferation (223), triggering PM synthesis (150), triggering immune cell differentiation (224), and hormonally regulating midgut physiology (225). In Chapter 3, I hypothesize that larval-acquired microbes shape *Ae. aegypti* ZIKV vector competence by reinforcing midgut integrity.

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CHAPTER 2

Microbial Composition in Larval Water Enhances *Aedes aegypti* Development but

Reduces Transmissibility of Zika Virus

Microbial Composition in Larval Water Enhances *Aedes aegypti* Development but Reduces Transmissibility of Zika Virus

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ABSTRACT Arthropod-borne viruses comprise a significant global disease burden. Surveillance and mitigation of arboviruses like Zika virus (ZIKV) require accurate estimates of transmissibility by vector mosquitoes. Although *Aedes* species mosquitoes are established as competent ZIKV vectors, differences in experimental protocols across studies prevent direct comparisons of relative transmissibility. An understudied factor complicating these comparisons is differential environmental microbiota exposures, where most vector competence studies use mosquitoes reared in laboratory tap water, which does not represent the microbial complexity of environmental water where wild larvae develop. We simulated natural larval development by rearing Californian *Aedes aegypti* larvae with microbes obtained from cemetery headstone water compared to conventional tap water. *A. aegypti* larvae reared in environmental cemetery water pupated 3 days faster and at higher rates. Mosquitoes reared in environmental water were less competent vectors of ZIKV than laboratory water-reared *A. aegypti*, as evidenced by significantly reduced infection and transmission rates. Microbiome comparisons of laboratory water- and environment water-reared mosquitoes and their rearing water showed significantly higher bacterial diversity in environment water. Despite this pattern, corresponding differences in bacterial diversity were not consistently observed between the respective adult mosquitoes. We also observed that the microbial compositions of adult mosquitoes differed more by whether they ingested a bloodmeal than by larval water type. Together, these results highlight the role of transient microbes in the larval environment in modulating *A. aegypti* vector competence for ZIKV. Laboratory vector competence likely overestimates the true transmissibility of arboviruses like ZIKV when conventional laboratory water is used for rearing.

IMPORTANCE We observed that *A. aegypti* mosquitoes reared in water from cemetery headstones instead of the laboratory tap exhibited a reduced capacity to become infected with and transmit Zika virus. Water from the environment contained more bacterial species than tap water, but these bacteria were not consistently detected in adult mosquitoes. Our results suggest that rearing mosquito larvae in water collected from local environments as opposed to laboratory tap water, as is conventional, could provide a more realistic assessment of ZIKV vector competence since it better recapitulates the natural environment in which larvae develop. Given that laboratory vector competence is used to define the species to target for control, the use of environmental water to rear larvae could better approximate the microbial exposures of wild mosquitoes, lessening the potential for overestimating ZIKV transmission risk. These studies raise the question of whether rearing larvae in natural water sources also reduces vector competence for other mosquito-borne viruses.

KEYWORDS *Aedes aegypti*, Zika virus, arbovirus, microbiome, mosquito, susceptibility, transmission, vector competence

The global expansion of arthropod-borne viruses (arboviruses) poses a significant public health threat. Climate change and rapid urbanization may accelerate the zoonotic spillover or reemergence of arboviruses, increasing outbreaks in humans (1–3). Zika virus (ZIKV)

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(*Flaviviridae*, *Flavivirus*), which was understudied since its discovery in 1947 in Uganda (4), garnered worldwide attention following outbreaks in 2015 to 2016 (3, 5, 6). The wave of ZIKV epidemics, accompanied by newly recognized teratogenic phenotypes wherein ZIKV causes adverse outcomes in fetuses from infected pregnant mothers, now referred to as congenital Zika syndrome (7, 8), fueled efforts to better understand and mitigate transmission to curtail disease. Although the ZIKV pandemic of 2015 to 2016 has ended, ZIKV may reemerge via increased numbers of immunologically naive people and the geographic expansion of *Aedes* species vectors (9, 10).

Determining the ability of a mosquito to become infected by and transmit a virus (vector competence) is crucial for guiding surveillance and control, including identifying mosquito species to monitor and eliminate and for modeling outbreak risk. Evaluating vector competence in the laboratory entails exposing mosquitoes to an infectious bloodmeal, followed by the detection of viral RNA or infectious virus in mosquito tissues and saliva after an incubation period usually 3 to 14 days. Mosquito-borne arboviruses must escape the mosquito midgut, infect the salivary glands, and be secreted into saliva for transmission. Although the approach for assessing laboratory vector competence is standard, outcomes across studies vary greatly (6) and may be influenced by virus strain and passage history (11), virus dose (12), mosquito species (13), intraspecies mosquito genetics (14, 15), larval nutrition and competition (16, 17), and incubation temperature (18–20). Since 2017, many vector competence studies have been performed using *Aedes aegypti* and *Aedes albopictus* from various geographic origins and post-2015 strains of ZIKV (21–28). The absence of uniformity in the variables involved in laboratory vector competence makes direct comparisons across studies difficult. However, such comparisons are needed to assess reproducibility and identify differences in vector competence across geographies.

The mosquito microbiome is an important variable that influences arbovirus vector competence, wherein specific taxa can modify it. *A. aegypti* mosquitoes infected with the bacterial endosymbiont *Wolbachia* have a reduced ability to transmit ZIKV, dengue virus (DENV), and chikungunya virus (CHIKV), prompting field trials and experimental releases of *Wolbachia*-infected mosquitoes as a means of population replacement (29). Similarly, the bacterium *Chromobacterium* Csp_P reduces transmission of DENV by *A. aegypti* and *Plasmodium falciparum* by *Anopheles gambiae* (30). Members of the bacterial genus *Asaia* may also confer resistance of mosquitoes to arboviruses and *Plasmodium* (31–33). *A. aegypti* colonized with *Serratia* bacteria are more susceptible to infection by DENV and CHIKV *in vivo* but less susceptible to ZIKV *in vitro* (34–37). However, the functional roles of specific microbial strains in modulating the vector competence of mosquitoes in nature, where gut microbes exist as a community rather than as a monoculture, remain unclear. Examination of microbial strains in gnotobiotic mosquitoes requires repeatability in a microbial community context, including in the aqueous larval form. To address this gap, we analyzed the microbial structures of larval *A. aegypti* to elucidate the community dynamics of microbes that colonize larvae and adults, and we then assessed how differences in larval rearing environments and microbial composition affect ZIKV vector competence.

We modified the *A. aegypti* larval rearing environment by introducing microbes at different diversities and abundances. Since microbes in mosquitoes are primarily acquired through the environment (38, 39), rearing *A. aegypti* in different water sources provides control of microbial input to *A. aegypti* colonies in the laboratory (40, 41). Previous work showed that the bacterial microbiota of field-caught *Aedes* mosquitoes varies geographically (42) and that rearing field mosquitoes in a laboratory setting results in a convergence of the gut microbiota in just one generation (43). Moreover, larva-acquired microbes play a significant role in larval development, where axenically (raised as a single organism, free of any microbes) reared mosquitoes exhibit inconsistent pupation success and reduced adult size, likely due to a lack of nutritional supplementation by larval gut microbes (38, 44). Additionally, some larval gut microbes are passed transstadially to adults, suggesting symbiosis through multiple mosquito life

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stages (45, 46). Consequently, microbes acquired by larvae are expected to influence *Aedes* mosquito physiology and immune status (47–49), which, along with direct physical interactions by microbes, is expected to impact ZIKV vector competence (50, 51). We used larval rearing water that we determined contained a relatively low microbial content compared to microbe-rich water collected from outdoor environments in which *A. aegypti* larvae are naturally found to determine whether differences in water sources influence ZIKV vector competence in a controlled mosquito genetic background. Our data show that reduced microbial exposure in colonized mosquitoes reared in laboratory water (LW) versus environmental water (EW) modulates vector competence and could explain the variability in vector competence between laboratory and field mosquitoes.

RESULTS

Bacterial abundance and diversity decline during *A. aegypti* larval development. We began by assessing bacteria that persist through *A. aegypti* life stages. Persistence was defined as a bacterial taxon detected in more than one life stage, starting at the larval stage. A total of 31 mosquitoes reared in environmental cemetery water representing 4th-instar larvae (L4) ($n = 8$), pupae ($n = 8$), and adults (1 to 3 days posteclosion [dpe], $n = 7$; >7 dpe, $n = 8$) or pools of 100 to 200 eggs were sampled, and the numbers of bacterial amplicon sequence variants (ASVs) were compared among individuals and to the rearing water (Fig. 1A). Adult mosquitoes were divided into two age classes,1 to 3 dpe and >7 dpe, to compare young and old adults. Bacteria were scarce in washed eggs but significantly increased in L4 larvae ($P = 0.0008$ by a Kruskal-Wallis test). Although the bacterial abundance decreased across the totality of mosquito development ($P = 0.0002$ by a Kruskal-Wallis test), no difference in bacterial abundance between pupae and newly emerged adult females at 1 to 3 dpe ($P > 0.999$ by a Kruskal-Wallis test) was detected, nor was there a difference between young and old adult females at >7 dpe ($P = 0.7802$ by a Kruskal-Wallis test). The bacterial abundance in L4 larvae was significantly lower than that in adult females at 7 dpe, where a decrease in the mean 16S/RPS17 ratio from 129 (geometric mean = 50; geometric standard deviation [SD] = 6) to 1.4 (geometric mean = 0.4; geometric SD = 7) ($P = 0.0008$ by a Kruskal-Wallis test) was detected. A total of 200 ASVs were identified across all life stages (Table 1), with 102 observed in water, 124 in larvae, 125 in pupae, and 99 in adults (see Fig. S1A in the supplemental material). Thirty-one ASVs representing 19 bacterial genera were shared among the rearing water, larvae, pupae, and adults, and most belonged to the phylum *Bacteroidetes* (Fig. S1A and B). The microbial community compositions across life stages were also unique, shown by the distinct clustering of samples by life stage (Fig. 1B). The microbial compositions of larvae clustered close to water, while pupal compositions were more similar to those in adult mosquitoes. Concordant with the decline in microbial abundance and compositional shifts with life stage, a decline in alpha diversity (total observed species and Shannon diversity indices) was also detected, with the greatest difference in alpha diversity between L4 larvae and adults at >7 dpe ($P = 0.0009$ [observed species] and $P = 0.0036$ [Shannon] by a Kruskal-Wallis test) (Fig. 1C). The 10 most abundant ASVs accounted for nearly 80% of the L4 larval bacteria, with the proportion increasing to 90% as adults at >7 dpe (Fig. 1D). While *Flavobacterium* constituted the most common bacterial ASV in the rearing water (36%), *Elizabethkingia* was most common in larvae (two distinct ASVs, totaling 46%), while *Methylobacterium* expanded from 41% during pupation to 77% as adults at >7 dpe. At the phylum level, *Proteobacteria* were progressively significantly enriched with each developmental stage (larva, $16\% \pm 7\%$; pupa, $47\% \pm 9\%$; adult at 1 to 3 dpe, $73\% \pm 9\%$; adult at >7 dpe, $87\% \pm 13\%$ [$P < 0.0001$ by a Kruskal-Wallis test]), such that they comprised the majority of bacteria in adult mosquitoes despite comprising a smaller relative fraction in the rearing water ($10\% \pm 2\%$) (Fig. 1E). Taken together, these data show that the microbes in rearing water that colonize *A. aegypti* are at the highest abundance and diversity at the larval stage and then decrease in relative abundance during

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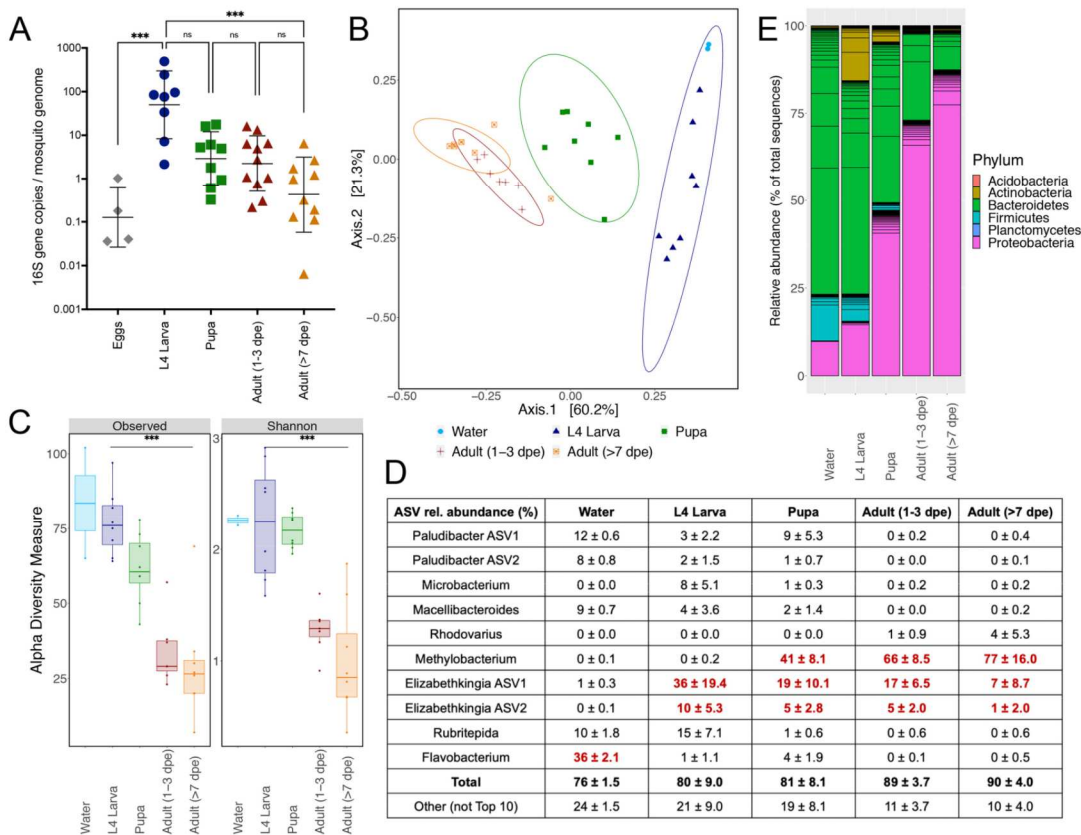
FIG 1 Microbial abundance decreases over the life of a mosquito, although some bacterial taxa persist. (A) Quantification of bacteria at each life stage of *A. aegypti* from Los Angeles, CA, normalized by the *A. aegypti* gene RPS17. Each dot represents a single mosquito or a pool of 100 to 200 eggs. dpe, days posteclosion. (B) PCoA at the amplicon sequence variant (ASV) level, by UniFrac distances of microbial composition within each life stage. (C) Alpha diversity of each life stage, using two metrics, observed ASVs and Shannon diversity. (D) Top 10 most abundant ASVs across all samples. Total indicates the sum of the top 10 ASVs, named by genus, while Other indicates the total remaining (non-top 10) ASVs. Red text highlights the ASVs that comprise the most common sequences or ASV types in the sample. (E) Relative abundances of all sequences, by ASV, colored by phylum. Samples for microbiome analysis in panels A to E were as follows: water ($n = 2$, sampled at the larval rearing midpoint [1 week]) and mosquitoes ($n = 8$ per life stage, except 1-3 dpe where $n = 7$; $n = 4$ pools of 100 to 200 eggs). For panels A and C, *** denotes significance at a P value of <0.001 using a Kruskal-Wallis test with multiple comparisons. ns, not significant ($P > 0.05$).

development. A fraction of the microbes, primarily in the phyla *Proteobacteria* and *Bacteroidetes*, detected in rearing water persist and are enriched in adult *A. aegypti*.

A. aegypti larvae reared in laboratory water exhibit delayed pupation relative to larvae reared in environmental water. We next asked whether the source and nature of larval water affected the kinetics and success of larval development. Eggs from colonized *A. aegypti* were surface sterilized, hatched, and reared to adulthood in standard laboratory water (LW) from the tap or environment water (EW) collected outdoors from cemetery headstones (Fig. 2A). Mosquitoes in both water types were reared at the same density and were supplemented with the same larval food quantity, which was standardized to eliminate differences in food availability and that was also sterilized to avoid introducing additional microbes. Larvae reared in LW exhibited significantly delayed pupation and first pupated on day 8, compared to EW-reared mosquitoes that pupated starting on day 5 ($P = 0.0005$ by a paired t test) (Fig. 2B). Furthermore, the percentage (50 to 81%) of larvae that pupated by day 14 in LW was significantly lower than in EW, where 100% of larvae pupated ($P = 0.0004$ by mixed-effect analysis of

TABLE 1 16S data sets used for microbiome analysis[a]

Dataset name	ZIKV Bloodmeal	Groups compared	16S region	Total sample size	Total # raw reads	Total ASVs (after filtering)	Mean # reads per sample (after filtering)
AM1019LS	none	Life stages (L4, pupa, adult 1-3 dpe / 7 dpe)	V3-V4	34	19.8 M	200	12,0872
AM1019ZE	PR15	LW vs. EW1*	V3-V4	60	15.6 M	1077	68,030
AM820ZE	BR15	LW vs. EW4	V4	77	17 M	221	5,228

[a]All data sets included Los Angeles *A. aegypti* mosquitoes with bacterial DNA from their respective rearing water samples. *, the data set contains samples for LW, EW1, and EW2. Group EW2 was excluded as after filtering; it failed to meet the threshold coverage level of 1,000 reads.

variance [ANOVA] with multiple comparisons). LW mosquitoes pupated slower than EW mosquitoes, even when the water was supplemented with *Saccharomyces cerevisiae* (baker's yeast) with or without antibiotics (adjusted $P = 0.0023$ by mixed-effect ANOVA with multiple comparisons), which is conventionally used to induce hatching via hypoxia (52), and also when vacuum hatching was added, also with the goal of increasing hatch rates (adjusted $P = 0.0022$ by mixed-effect ANOVA with multiple comparisons). This suggests that microorganisms in the environmental water promote pupation success and augment the larval growth kinetics of *A. aegypti*. We also assessed whether enhanced pupation was associated with a higher bacterial density in EW by comparing the bacterial levels in LW to those in four EW samples (EW1 to -4) collected from the rearing pans at 7 days posthatching. Surprisingly, bacterial DNA quantities in larval pans 7 days after hatching were not significantly different ($P = 0.078$ by a Kruskal-Wallis test) across LW samples or any EW sample (Fig. 2C), suggesting that the total microbial abundance did not influence the differences in the rates of larval development to pupation. Recognizing that gene sequencing does not represent living bacteria, we also cultured bacteria and compared the bacterial densities in LW and EW samples as well as in larvae, pupae, and adults (4 to 5 dpe) reared in both water types. The numbers of bacterial colonies culturable on LB agar were not significantly different between LW and EW ($P = 0.3143$) or between LW- and EW-reared larvae ($P = 0.1$), pupae ($P > 0.99$), or early adults ($P = 0.4286$ [all by a Mann-Whitney test]) (Fig. 2D), further suggesting that the abundance of culturable bacteria does not significantly impact larval development kinetics.

Given that the abundance of bacteria in the larval rearing water did not explain the differences in larval growth and pupation success, we next addressed whether other differences in EW versus LW were influencing mosquito growth. To control for exogenous micronutrient content and water chemistry that could confound the observed differences in larval development, we reared larvae in diluted EW to ablate the potential progrowth effect from EW due to these other factors. EW microbes were pelleted, washed five times in phosphate-buffered saline (PBS), and spiked into LW at different dilutions. Although the colony-forming bacterial quantities of EW dilutions ranged from 10^2 to 10^5 CFU/ml at day 0 (comparable at the lowest density to $10^{1.5}$ CFU/ml in LW), by day 7, the bacterial numbers in all EW dilutions and LW were not significantly different ($P = 0.1$ by a Kruskal-Wallis test) and reached $\sim 10^7$ CFU/ml (Fig. 3A). The pupation rates were not different ($F = 5.33$ and $P = 0.07$ by mixed-effect ANOVA) regardless of the EW dilution, and all EW groups exhibited 100% pupation by 10 dpe, which was in contrast to pupation from LW, where the mean was 62% (peak of 87%), which was significantly lower than those of all EW dilutions ($F = 17.19$ and $P = 0.0008$ by mixed-effect ANOVA) (Fig. 3B). Despite these differences in pupation rates, the quantities of colony-forming bacteria in L4 larvae were not significantly different with 1:500 or $1:10^4$ EW dilutions or with LW at 7 or 10 dpe (Fig. 3C), suggesting that larvae
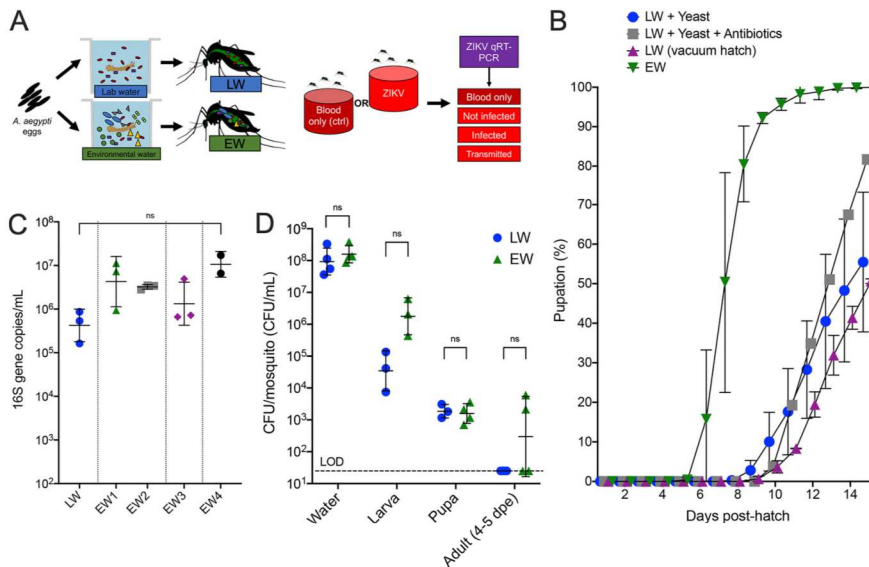
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FIG 2 Mosquitoes reared in environmental water develop faster than those reared in laboratory water. (A) Experimental design showing treatment of *A. aegypti* eggs with either laboratory water (LW) or environmental water (EW) from cemetery headstones. (B) Pupation kinetics and rates relative to the number of larvae that hatched in cohorts of 500 to 600 larvae per liter. LW was spiked with either live baker's yeast or yeast and antibiotics (penicillin-streptomycin-kanamycin at 50 μg/ml). Each symbol shows the mean cumulative percentage of pupated larvae on that day, with error bars denoting the range. Time course differences in pupation were determined by mixed-effects analysis (one-way ANOVA) with repeated measures and multiple comparisons. Each symbol represents the mean from replicate rearing experiments ($n = 3$). Individual pupation rates for replicate experiments are shown in Fig. S2A in the supplemental material. (C) 16S rRNA qPCR of rearing water at 7 days posthatching. EW was collected on 4 separate occasions (EW1 to -4). Each symbol shows the geometric mean of PCR results from DNA extracted from 200 μl water. Values were compared by a Kruskal-Wallis test with multiple comparisons. (D) Colony counts of bacteria represented as CFU cultured on LB agar at 37°C. Each symbol shows the average from five homogenized mosquitoes or 40 μl of water at the midpoint (7 days posthatch) of a rearing experiment. The absence of colonies detected is reported at the limit of detection (LOD) of 40 CFU/ml. Pairwise comparisons between LW and EW were performed by Mann-Whitney tests.

develop similar bacterial loads despite different initial exposure doses. The lack of a difference in larval development rates at various dilutions of EW microbes, together with the lack of a difference in microbial levels across EW and LW despite augmented pupation in EW, supports specific microbes, rather than absolute microbial levels, water chemistry, or nutrient content, as a driver of the faster and more efficient development of mosquitoes reared in water from the environment than in water from the laboratory.

Mosquitoes reared in environment water are less competent ZIKV vectors than mosquitoes reared in water from the laboratory. We next assessed the influence of the source of rearing water on the vector competence of *A. aegypti* for ZIKV. LW- and EW-reared female adult mosquitoes were presented with matched ZIKV titers or blood only in artificial bloodmeals and then assayed 14 days after bloodfeeding using quantitative reverse transcription-PCR (qRT-PCR) to detect ZIKV RNA in bodies as a marker of infection, legs and wings to indicate dissemination, and saliva to assess transmission (Fig. 4A). No ZIKV RNA was detected in any mosquito that ingested blood only (data not shown). LW-reared mosquitoes were significantly more susceptible to infection and transmitted ZIKV at significantly higher rates than EW-reared mosquitoes (Fig. 4B). This pattern was observed with 2015 ZIKV strains from Puerto Rico and Brazil and two Californian *A. aegypti* lineages. Although infection, dissemination, and transmission rates were higher in LW-reared mosquitoes, the mean ZIKV genome copies in bodies, legs/wings, and saliva did not significantly differ between the LW- and EW-reared groups (Fig. 4C and Fig. S3A and B [showing additional experimental replicates that
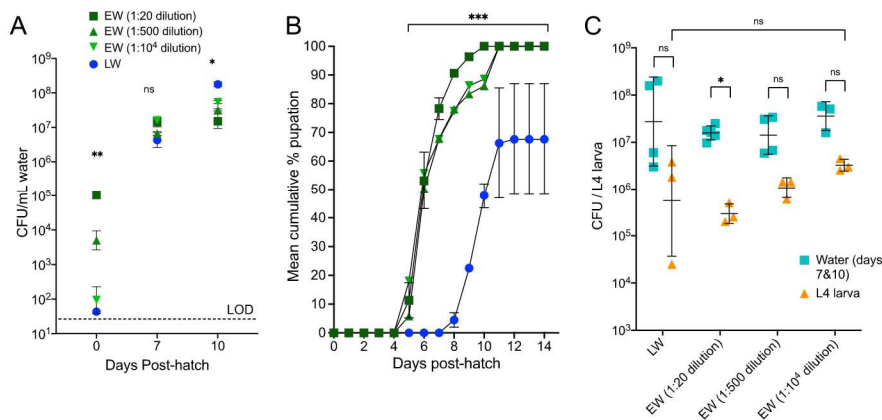
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FIG 3 Dilution of microbes pelleted from environmental water does not delay *A. aegypti* larval development, which is still faster than for larvae reared in laboratory water. Microbes pelleted and washed from 3 liters of EW were stored in glycerol stocks; diluted 1:20, 1:500, and 1:10^4; and then spiked into LW. (A and B) Bacterial growth for each water treatment (A) and pupation rates (B) were determined. Each symbol in panel B shows the mean cumulative percentage of pupation over time (individual rates for replicate experiments are shown in Fig. S2B in the supplemental material), with error bars denoting the range. Each symbol in panel A shows the geometric mean from triplicate measurements, and error bars denote the geometric standard deviations. Statistical tests were performed using a mixed-effects analysis (one-way ANOVA) with repeated measures and multiple comparisons. (C) Bacterial counts from water at days 7 and 10 were aggregated and are plotted with their respective 4th-instar larvae (L4) that were also sampled at the same time. Pairwise comparisons were performed using the Mann-Whitney test.

also revealed the same patterns]). Mosquitoes that contained >10^7 ZIKV RNA copies in their body were more likely to contain detectable ZIKV RNA in saliva (likelihood ratios [LRs] of 3.48 in LW and 3.50 in EW) (Fig. 4D).

To understand the dose response to ZIKV infection, *A. aegypti* mosquitoes reared in both water types were exposed to a range of bloodmeal titers below and above 10^5 PFU/ml (Fig. 5A). LW-reared mosquitoes became infected at a significantly lower bloodmeal titer than EW-reared mosquitoes ($F = 878$ and $P < 0.0001$ for comparison of fits [slope and *y* intercept] by nonlinear regression) (Fig. 5B). The infectious bloodmeal titer that produced ZIKV infections in 50% of the cohort (50% infectious dose [ID$_{50}$]) for LW-reared mosquitoes was 10$^{3.0}$ PFU/ml, compared to 10$^{5.6}$ PFU/ml for EW-reared mosquitoes, which represents a 400-fold difference. Mosquitoes reared in both water types followed a strong dose response to ZIKV infection ($R^2 = 0.33$ for LW and 0.85 for EW by nonlinear regression). Together, these data demonstrate that laboratory water-reared mosquito colonies are more susceptible to ZIKV infection and transmission than mosquitoes reared in water from the environment. The higher ID$_{50}$ of EW mosquitoes also suggests that these mosquitoes are less susceptible to infection by and transmission of ZIKV when ingesting a bloodmeal titer reflective of typical human viremia (53).

Larval water source does not differentiate bacterial compositions between adult mosquitoes as much as bloodmeal status. Although LW and EW *A. aegypti* mosquitoes that were not ZIKV exposed showed similar bacterial levels, we next questioned whether the same pattern would be observed in the context of ZIKV infection. Adult female mosquitoes reared in LW or EW that ingested ZIKV in bloodmeals were grouped into the following classes based on their infection outcomes: (i) not infected, where no ZIKV RNA was detected above the limit of detection of 65 ZIKV genomes/body; (ii) infected (low), defined as body titers of <10^6 ZIKV genomes/body; or (iii) infected (high), defined as body titers of >10^6 ZIKV genomes/body. The "high" and "low" infection states were defined based on the bimodal distribution of RNA levels observed in bodies (Fig. 4C). The reasoning for this grouping is that individuals with high ZIKV RNA levels in their bodies were more likely to have disseminated infections that lead to ZIKV RNA detection in saliva (Fig. 4D), a pattern also observed in previous studies with

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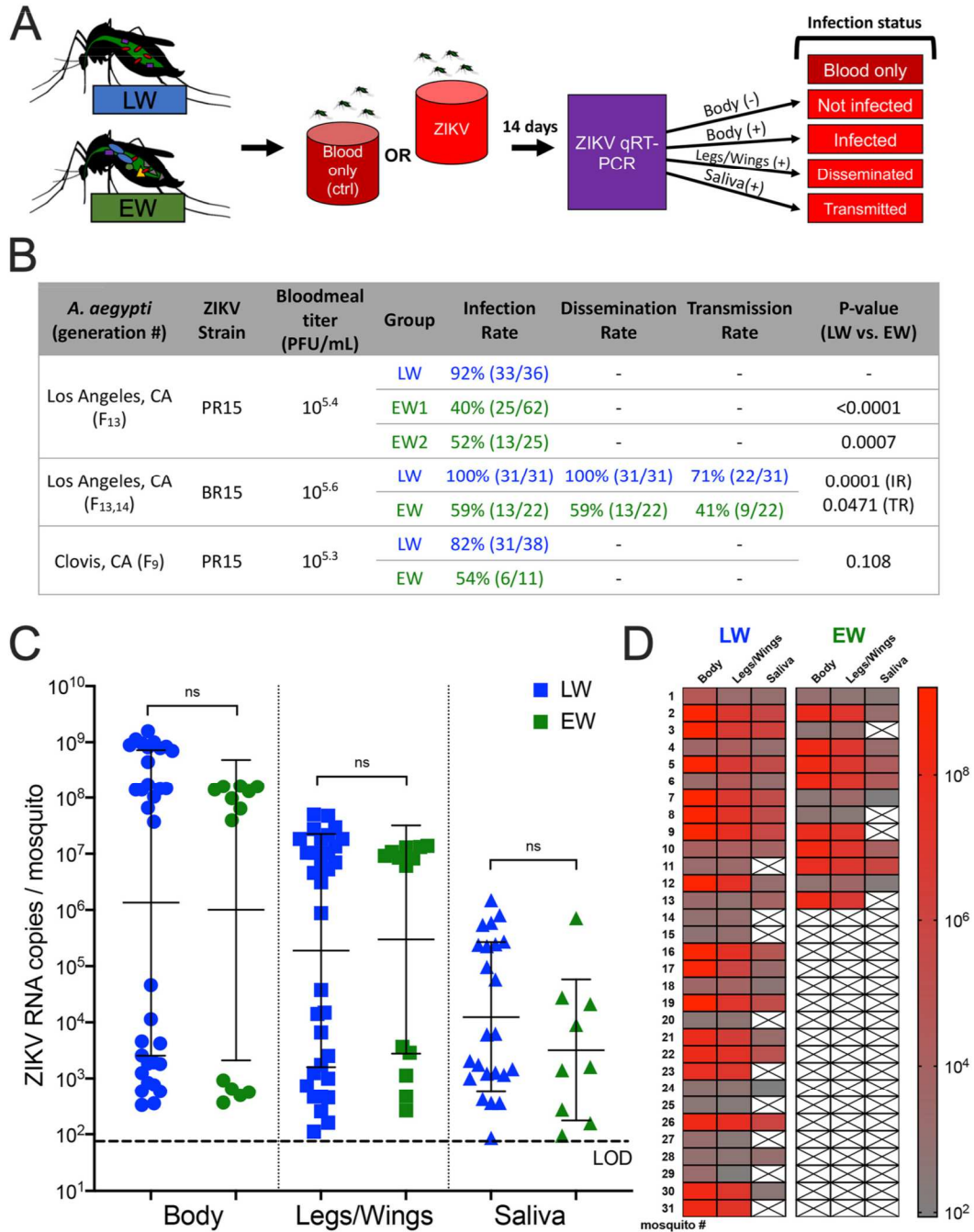
FIG 4 *A. aegypti* mosquitoes reared in environmental water are less competent ZIKV vectors than those reared in laboratory water. (A) Experimental overview of vector competence experiments showing control (no ZIKV) and ZIKV-exposed mosquitoes reared in either laboratory water (LW) or environmental water (EW) that
(Continued on next page)

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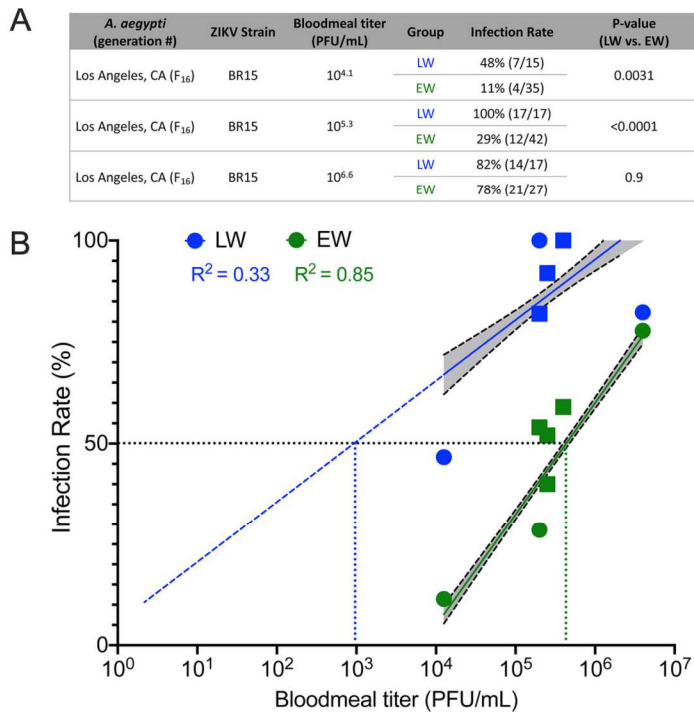
FIG 5 *A. aegypti* mosquitoes reared in environmental water require higher doses of ZIKV to become infected than those reared in laboratory water. (A) Summary of additional vector competence experiments using Los Angeles *A. aegypti* and ZIKV BR15 at three different bloodmeal titers. (B) Plot of infection rate versus bloodmeal titer. Squares represent the same data in Fig. 4B, and circles indicate the additional experiments in Fig. 5A. A best-fit nonlinear regression line with 95% confidence intervals (CIs) is shaded in gray. For LW, the slope is 14.9 (95% CI, 11.5 to 18.3), and the *y* intercept is 5.949 (95% CI, -12.57 to 24.44), and for EW, the slope is 27.6 (95% CI, 26.01 to 29.01), and the *y* intercept is -105.3 (95% CI, $= -113.8$ to -96.83).

A. aegypti from the same source colonies and that used the same ZIKV strains as the ones in this study (23). Mosquitoes that fed on blood without ZIKV or that had been presented with only sugar water were included as controls. Prior to a bloodmeal, where mosquitoes had been exposed to only sugar at 3 dpe, both LW and EW females had bacterial quantities in their bodies that were not significantly different ($P = 0.5476$ by a Mann-Whitney test), and the bacterial load was low (Fig. 6A). Ingestion of blood resulted in a 50- to 100-fold increase in bacterial levels in both groups compared to unfed mosquitoes of the same age ($P = 0.0005$ by a Kruskal-Wallis test [adjusted $P = 0.012$ for LW and adjusted $P = 0.0212$ for EW by multiple comparisons]). Bloodfed LW mosquitoes contained significantly higher bacterial levels than EW mosquitoes ($P = 0.0079$ by a Mann-Whitney test). Regardless of the infection outcome, both LW

FIG 4 Legend (Continued)
were incubated for 14 days after bloodfeeding and then harvested to assess infection (bodies), dissemination (legs/wings), and transmission (saliva). (B) Summary table of infection, dissemination, and transmission rates. Transmission rate (TR) refers to the number of individuals transmitting from the total number of individuals that ingested a bloodmeal with ZIKV. Infection experiments were repeated once for replication. Transmission was assayed for the Los Angeles *A. aegypti*-ZIKV BR15 combination. *P* values were calculated with Fisher's exact tests. IR, infection rate. (C) ZIKV RNA levels in Los Angeles *A. aegypti* mosquitoes infected with ZIKV BR15. Each symbol is for a single mosquito, and only mosquitoes that were ZIKV positive by qRT-PCR ($C_T < 40$) are shown. Error bars denote the geometric means and standard deviations among positive individuals. The Mann-Whitney test was used. The dotted line denotes the average limit of detection, 65 ZIKV RNA copies/mosquito or saliva sample, across all qRT-PCR plates. (D) Heat map matching individual mosquitoes with their respective tissues, colored by ZIKV RNA levels. X in LW and EW saliva column to mosquito 13 indicates sample was not tested; only 13 mosquitoes were in EW, so X for mosquitoes 14 to 31 indicates samples do not exist.

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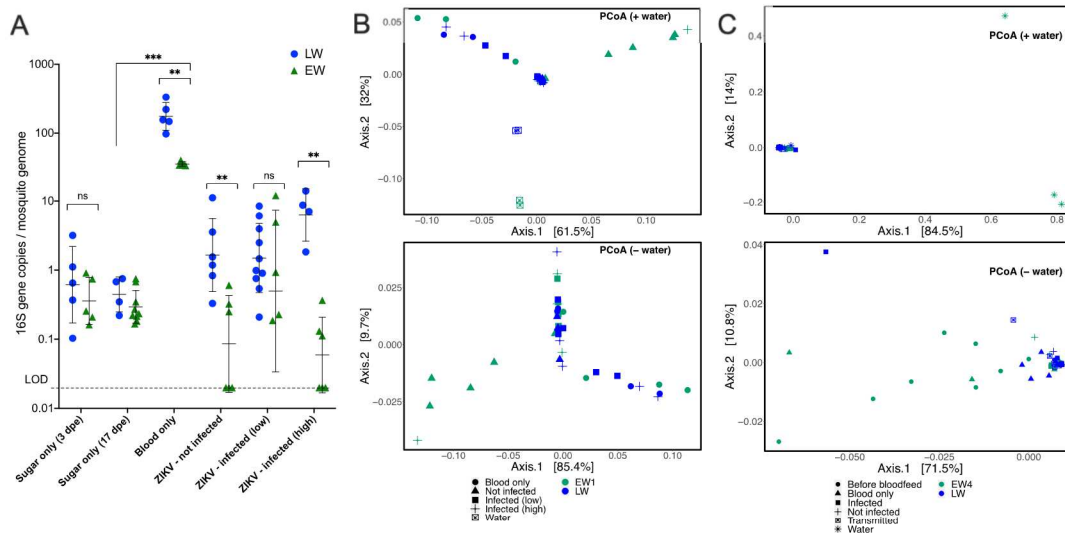
FIG 6 Microbial communities differ slightly by water type. (A) Bacteria in LW- or EW-reared *A. aegypti* mosquitoes exposed to sugar, blood only, or blood containing ZIKV quantified by 16S qPCR and normalized to the *A. aegypti* reference gene RPS17. Symbols refer to a single mosquito, and error bars denote the geometric means and geometric standard deviations. Statistically significant differences between LW and EW were determined by a Mann-Whitney test, while differences across treatments were determined by a Kruskal-Wallis test. Interaction effects between water type (LW and EW) and ZIKV infection states were investigated by two-way ANOVA with multiple comparisons, on $\log_{10}$-transformed values (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ns, not significantly different at the level of a P value of 0.05). (B and C) Principal coordinates of UniFrac distances, colored by water type. (B) A cohort of Los Angeles *A. aegypti* mosquitoes presented with ZIKV PR15 (data set AM1019ZE) (Table 1); (C) another cohort of Los Angeles *A. aegypti* mosquitoes presented with ZIKV BR15 (data set AM820ZE) (Table 1). Top plots include mosquito and rearing water samples, while bottom plots have water samples omitted for higher resolution.

and EW mosquitoes that ingested ZIKV showed lower bacterial levels than the blood-only groups ($F = 33.41$ and $P < 0.0001$ for infection state and $F = 40.30$ and $P < 0.0001$ for water type, by two-way ANOVA). Moreover, two-way ANOVA on bloodfed mosquitoes detected a significant interaction between water type and ZIKV infection state (interaction $F = 4.6$ and $P = 0.0032$). LW mosquitoes that were not ZIKV infected or that were infected at high levels contained higher bacterial levels than EW mosquitoes ($P = 0.0043$ and $P = 0.0095$, respectively, by a Mann-Whitney test).

Next, we examined the bacterial compositions of LW and EW mosquitoes, reasoning that the type of bacteria may influence vector competence more than the total bacterial load. We compared the relative bacterial abundances and taxonomic diversities of LW and EW adult mosquitoes as well as across mosquitoes that exhibited differential ZIKV infection states from two replicate vector competence experiments. Totals of 1,077 and 221 ASVs were identified in the first and second vector competence experiments, respectively (AM1019ZE and AM820ZE) (Table 1), most of which were detected in the rearing water (Fig. S4). EW1 and EW4 denote environmental water samples collected from the same cemetery in different months that were used for separate rearing experiments to account for the temporal variation in microbes within the same environment. Comparing EW1 and LW, we detected no significant differences in the clustering of samples by water type; however, when we compared EW4 and LW, we detected a difference in clustering ($F = 2.52$ and $P = 0.043$ [Adonis] and $F = 6.91$ and $P = 0.018$ [Betadisper]) by permutational multivariate analysis of variance [PERMANOVA]). The disparity in clustering patterns across different EW sample collections may be due to differences in community distributions in EW4 versus LW arising from the low number of ASVs, where most EW4 and LW mosquitoes in the AM820ZE data set were dominated by *Pseudomonas* (Fig. 6C, Fig. S4C, and Fig. S6C). Despite the identification of different ASVs between experimental iterations, EW had higher bacterial diversity and evenness than LW ($F = 17.25$ and $P < 0.0001$ [richness]

TABLE 2 Summary of contributions to microbial compositional differences by mosquito variable[a]

Group	Variable compared 1	Variable compared 2	Variable contribution	p-value
-	EW1	LW	22%	0.008**
EW1	ZIKV-exposed	Blood only	27%	0.019*
	Infected	Not infected	34%	0.011*
	Infected (low)	Infected (high)	10%	0.676
LW	ZIKV-exposed	Blood only	13%	0.619
	Infected	Not infected	8%	0.438
	Infected (low)	Infected (high)	10%	0.676
-	EW4	LW	5%	0.012*
EW4	Before bloodfeed	After bloodfeed	11%	0.074
	ZIKV-exposed	Blood only	12%	0.01**
	Infected	Not infected	8%	0.463
	Infected	Transmitted	9%	0.963
LW	Before bloodfeed	After bloodfeed	6%	0.692
	ZIKV-exposed	Blood only	9%	0.037*
	Infected	Not infected	7%	0.319
	Infected	Transmitted	5%	0.693

[a]Variable contribution, the percentage of the variance between samples associated with the metadata, was calculated using constrained analysis of principal coordinates. Statistical tests were performed by permutational ANOVA (*, $P < 0.05$; **, $P < 0.01$).

and $F = 12.8$ and $P < 0.0001$ [Shannon] by one-way ANOVA) (Fig. S4). When we compared mosquitoes only (and not their rearing water), the bacterial compositions between LW and EW mosquitoes were slightly different, as shown by partial sample overlaps, although clustering was not significantly different ($F = 1.87$ and $P = 0.111$ [Adonis] and $F = 0.15$ and $P = 0.707$ [Betadisper] by PERMANOVA) (Fig. 6B, bottom). Several EW mosquitoes that were refractory to ZIKV infection clustered together; these individuals had increased proportions of *Asaia* and *Flavobacterium* and significantly reduced proportions of *Rhodovarius*, *Micrococcus*, and *Neochlamydia* bacteria compared to infection-competent individuals, as determined by DESeq2 analysis and random forest modeling (Fig. S5A and Fig. S6A and B). The overall contribution of water type to differences in bacterial compositions across individual mosquitoes of either bloodfed status was 22%. Whether mosquitoes ingested ZIKV and whether mosquitoes that ingested ZIKV became infected were also important variables that explained 27% and 34%, respectively, of the differences in bacterial compositions across groups (Table 2). EW mosquitoes that ingested blood with ZIKV had a reduced abundance of *Serratia* compared to EW mosquitoes that ingested blood only (Fig. S5B and Fig. S6B). For both data sets, there were no significant differences in bacterial compositions in LW mosquitoes that ingested blood only or ZIKV; this may be an artifact of LW mosquitoes possessing few bacterial taxa such that differential abundances could not be detected (Table 2 and Fig. S6A and B). Additionally, no differences in bacterial composition were detected between mosquitoes with high and low levels of ZIKV RNA or between mosquitoes that were infected and those that transmitted (Fig. 6C and Table 2). Taken together, EW-reared adult females harbor different microbiota when reared in the same water source collected at different times. This suggests that despite differences in microbiota, adult female *A. aegypti* mosquitoes exhibit consistently reduced vector competence for ZIKV when reared in environmental water compared to laboratory water.

DISCUSSION

Here, we show that microbial diversity stemming from different water sources used to rear larvae in a laboratory environment modifies the vector competence of *A. aegypti* for ZIKV. Reduced vector competence in environmental water-reared *A. aegypti* was consistently

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observed using two lineages of Californian *A. aegypti* and two epidemiologically relevant ZIKV strains. These results suggest that modification of *A. aegypti* developmental conditions to reflect environmental water compared to laboratory tap water, which is conventional, decreases laboratory infection and, potentially, transmission rates for ZIKV. The use of laboratory water to rear larvae likely leads to overestimates of the transmission potential of ZIKV vectors in the environment. This pattern may apply to other vector-virus pairings as well, and future research should address this question. Due to the wide range of urban environments in which *A. aegypti* larvae develop, watering cans, bromeliads, potted plants, and abandoned tires, etc., and because each environment contains its own microclimate with unique microbial composition and nutrient content, studying whether larval development in different water containers also reduces vector competence would be of great interest.

Differences in pupation kinetics between EW- and LW-reared mosquitoes indicate that the type of bacteria, but not bacterial abundance, impacts the success of mosquito larval development; this mostly agrees with previous studies on gnotobiotically reared larvae with bacteria and yeast of similar densities (54). Since pupation in insectary environments typically occurs before 8 days, the earliest time that we observed pupation for LW larvae, we cannot exclude the presence of growth-inhibiting microbes in LW that were absent from EW larvae that pupated at higher rates and with faster kinetics. Alternately, by sterilizing the fish food to ensure that the microbes were derived from the water only, we may have hindered pupation rates and kinetics, where nonsterilized food, as is conventionally used, may be a requisite for rapid larval development. Our observation of wide variability in microbial contents in experiments using different collections of environmental water, but which all yielded 100% pupation success, suggests that there is likely functional redundancy in microbes needed to nutritionally support larval growth and stimulate pupation. While the bacterial diversities of laboratory and environmental water from natural mosquito larval habitats were different, bacterial taxonomic differences within mosquitoes reared in water from these respective sources were more subtle. This suggests that mosquitoes may harbor a relatively low number of species in a "core" microbiome (55), possibly explaining the low number of bacterial species detected and the lack of shared species across experimental replicates. In concordance with previous *A. aegypti* microbiome studies, we observed high relative abundances of *Proteobacteria* and *Bacteroidetes* in adult mosquitoes (38, 42). At the genus level, most adult mosquitoes were dominated by *Asaia*, *Flavobacterium*, *Elizabethkingia*, and *Pseudomonas* bacteria. These bacteria were also found in small quantities in their rearing water, suggesting that they are likely environmental in origin, except for *Elizabethkingia*, which was also detected in surface-sterilized eggs. Because the same ASVs matching *Elizabethkingia* were also identified in surface-sterilized eggs, the origin of *Elizabethkingia* in mosquitoes in this study cannot be determined. Bacteria from this genus are present in the environment, larvae, newly emerged adults, and also reproductive tissues of *Aedes* species mosquitoes (56) but have not yet been reported in eggs.

By varying the source of larval rearing water, we aimed to modify the microbiota of *A. aegypti* with the premise that mosquito microbes are acquired through the environment and especially larval water. We therefore expected that a sterile sugar diet and a single artificial bloodmeal provided to adults would narrow the microbial input of the mosquitoes to reflect larva-acquired microbes from the rearing water. While we detected differences in the microbiota in LW- versus EW-reared mosquitoes, the microbiota was more different between control bloodfed and ZIKV-bloodfed groups. Other studies have also measured strong relationships between bloodmeal status and microbiome composition (57, 58), with some showing greater differences in the expression of *A. aegypti* genes in mosquitoes that bloodfed than between axenic and conventionally reared mosquitoes (59). A functional limitation of this and previous work is the inability to account for all microbial sources in adult mosquitoes stemming from their natural field environment, including microbes acquired during sugar feeding of adults on flora.

Despite the lack of reproducible changes in the species composition of bacteria in adult *A. aegypti* mosquitoes reared in different aquatic environments, we observed a

substantial effect on vector competence, where EW-reared mosquitoes exhibited lower infection and transmission rates than LW-reared mosquitoes. As this is the first study examining the microbiota of Californian *A. aegypti* and one of the few mosquito studies using ASVs instead of operational taxonomic units (OTUs), where ASVs are gaining favor over OTUs due to their increased taxonomic resolution as well as their consistent labeling (60), direct comparisons to other *A. aegypti* microbiome studies should be made with caution. In addition to a "core microbiome" effect on mosquito vector competence, there could also be functional redundancy in the effects of the microbiota on mosquito physiology. Despite microbial variability in rearing water and mosquitoes observed in our experimental replicates, the increased infection and transmission of ZIKV by LW- compared to EW-reared mosquitoes was reproducible. Although we studied only fully bloodfed mosquitoes for ZIKV vector competence assays, we cannot exclude the possibility that EW mosquitoes ingested lower bloodmeal volumes than LW mosquitoes, which may have resulted in lower infection rates. However, even a 2-fold difference in the ingested viral dose is not expected to substantially impact infection rates since mosquito dose-response studies typically follow a log-linear dose-response relationship, which surpasses the likely magnitude of the variance in the bloodmeal volume. Finally, while the transmission rates by EW mosquitoes were demonstrated to be lower than those of LW mosquitoes in Los Angeles *A. aegypti* mosquitoes with ZIKV strain BR15 (Brazil 2015), it is not certain whether the reduced transmission potential in EW mosquitoes is true for ZIKV in *A. aegypti* in general. Since transmission was assayed in only one *A. aegypti*-ZIKV pairing, replication of this result in other *A. aegypti* colony-ZIKV strain combinations would be needed to definitively confirm the reduced transmission of multiple ZIKV strains by *A. aegypti*.

The overall reduction of bacterial levels in ZIKV-exposed mosquitoes relative to nonexposed mosquitoes suggests that ZIKV infection negatively impacts the mosquito microbiota. This could be due to interactions between the mosquito antiviral immune response and a generalized antimicrobial effect that indirectly kills bacteria within the mosquito gut. Another study with Brazilian *A. aegypti* found enrichment of *Rhodobacteraceae* and *Desulfuromonadaceae* in response to ZIKV infection (61), the former of which were not differentially abundant in our data set, while the latter were absent from both our mosquitoes and rearing water. These discrepancies imply that bacterium-mosquito interactions during ZIKV infection are region specific. Previous work on *A. aegypti* innate immunity implicated a link between antiviral and antibacterial immune responses to infection (48, 62, 63). For example, the Toll pathway, which recognizes bacterial cell walls in insects, also modulates responses to DENV infection (47). This implicates a nonspecific pan-arboviruses effect where elevated immune responses to the resident microbiota confer resistance to infection. Furthermore, additional life-history traits like adult body size are influenced by larval water conditions (16, 50), implicating a physiological modification that may indirectly result from microbial exposures of larvae. Moreover, gut microbes play a nutritional role in mosquito symbiosis (44, 59), and larval nutrition impacts mosquito size and development (64), although the role of size in the vector competence of *A. aegypti* and DENV and *Culex* species mosquitoes and West Nile virus is controversial (65–67). Carryover effects (49) of larval exposure to isolates of *Flavobacterium*, *Lysobacter*, *Paenibacillus*, and *Enterobacteriaceae* on adult lipid metabolism and DENV infection in *A. aegypti* corroborate our observations that bacterial exposure during the larval stage can influence adult mosquito traits. Interestingly, oral treatment of adults with antibiotics did not change their vector competence for ZIKV, suggesting that these carryover effects from larvae could become fixed after maturity (68). The influence of bacteria known to impact vector competence in a monoculture in the context of the complex microbial community should be a target of future research.

MATERIALS AND METHODS

Biosafety. All ZIKV experiments were conducted in a biosafety level 3 laboratory and were approved by the University of California, Davis, under biological use authorization number R1863.

Mosquitoes. Two sources of *A. aegypti* mosquitoes were used in this study. *A. aegypti* mosquitoes were field collected as larvae in Los Angeles, CA, or as eggs in Clovis, CA, in 2016 and reared under standard insectary conditions for several generations until F_{13-16} and F_9 eggs, respectively, were collected for use. Adults were morphologically identified by personnel trained in recognizing *A. aegypti*. Insectary conditions during the laboratory colonization process were 26°C, 80% humidity, and a 12-h/12-h light/dark cycle, with larvae maintained in 1 liter of deionized water (diH$_2$O) at 200 to 400 larvae per pan and provided 1 pinch of fish food (Tetra, Melle, Germany) every other day until pupation. Adults were maintained in 30- by 30- by 30-cm mesh cages (BugDorm; Megaview Science, Taiwan) with constant access to 10% sucrose, all under septic conditions.

Mosquito rearing. Urban-adapted *A. aegypti* larvae are known to develop within open containers, including cemetery headstones, plant pots, rain barrels, abandoned tires, and bromeliads, which tend to accumulate nutrients and organic matter (17, 69–71). Outdoor and laboratory water sources were used in this study (see Table S1 in the supplemental material). For the laboratory water, ethanol-cleaned plastic trays were filled with 1 liter of laboratory tap diH$_2$O in an insectary. Environmental water consisted of 2 to 3 liters per collection of stagnant water from headstone receptacles in Davis Cemetery (Davis, CA) after rainfall. Separate water collections were conducted prior to each experiment to encompass variation in outdoor environmental conditions over time. Collected water was used for two purposes, (i) as rearing water and (ii) pelleted to isolate microbes prior to inoculation in laboratory tap water, in separate experiments. The cemetery water was filtered through 1-mm mesh to remove insects, larvae, and large particulates and then centrifuged at 3,000 × *g* for 30 min to pellet microbes. The supernatant was discarded, and pellets were washed with sterile 1× phosphate-buffered saline (PBS; Thermo Fisher Scientific, Emeryville, CA) three times prior to creating glycerol stocks of pelleted microbes that were frozen for later use. Pellet aliquots were also plated onto LB agar plates in parallel (Sigma-Aldrich, St. Louis, MO) to estimate live bacterial quantities prior to freezing at −80°C.

Mosquito eggs were surface sterilized by submerging in 5% bleach (Clorox, Oakland, CA) for 10 min, washed twice in 70% ethanol (Thermo Fisher Scientific, Emeryville, CA), and dried for 10 min before hatching in diH$_2$O. A PBS wash on a subset of eggs after surface sterilization was cultured on LB medium to confirm the removal of live bacteria from egg surfaces. Hatching was stimulated either by a pinch of active dry yeast (Red Star Yeast, Milwaukee, WI) in larval water or by inducing negative pressure (Rocker 400 vacuum pump; Sterlitech Corp., Kent, WA) to reduce the dissolved oxygen content for 30 min. A total of ~2,500 larvae were transferred to six 1-liter pans to achieve a density of 400 to 500 larvae/pan. Food was prepared in agarose plugs that were made by mixing 1% agarose (Sigma-Aldrich, St. Louis, MO) with pulverized fish food (final concentration of 100 g/liter, or 10% [Tetra, Melle, Germany]) and rodent chow (final concentration of 80 g/liter, or 8% [Teklad Global 18% protein rodent diet; Envigo, Indianapolis, IN]), which was then autoclave sterilized before casting into 12-well plates, a modification of a previously described approach (44) for standardizing the larval diet. One plug was fed to larvae in each pan every other day. Pupae were counted once daily and transferred into plastic dishes containing sterile diH$_2$O within 30-cm^2 cloth cages. Once cages reached a mosquito density of about 500, adult females were transferred in batches of 100 to 32-oz plastic containers (Amazon, Seattle, WA) for vector competence experiments. Larval development experiments were repeated twice. Larval trays and adult mosquitoes were maintained at 26°C with 80% humidity and a 12-h/12-h light/dark cycle for the duration of the experiment. All trays and adult mosquitoes were housed in the same incubator. Adult mosquitoes were provided constant access to filter-sterilized 10% sucrose (Thermo Fisher Scientific, Emeryville, CA).

Virus sources and titrations. Two Asian-lineage ZIKV strains were used: PR15 (Puerto Rico 2015) (PRVABC59 [GenBank accession number KX601168]) and BR15 (Brazil 2015) (SPH2015 [GenBank accession number KU321639]), both of which were isolated from human serum and passaged 3 times in Vero cells (ATCC CCL-81; ATCC, Manassas, VA) before freezing in stocks. Stocks were titrated on Vero cells prior to bloodmeal presentation to confirm titers. The remaining bloodmeals were recovered after presentation to mosquitoes, frozen at −80°C, and back-titrated by a plaque assay on Vero cells to confirm the administered dose. For titrations, bloodmeals were serially diluted 10-fold in Dulbecco's modified Eagle's medium (DMEM), inoculated into one well, and incubated for 1 h at 37°C in 5% CO$_2$ with rocking every 15 min to prevent cell death due to desiccation. After 1 h, 3 ml of 0.5% agarose (Thermo Fisher Scientific, Emeryville, CA) mixed with DMEM supplemented with 2% fetal bovine serum (FBS) and penicillin-streptomycin (Thermo Fisher Scientific, Emeryville, CA) was added to each well to generate a solid agar plug. The cells were incubated for 7 days at 37°C in 5% CO$_2$, after which they were fixed with 4% formalin (Thermo Fisher Scientific, Emeryville, CA) for 30 min, plugs were removed, and wells were stained with 0.025% crystal violet (Thermo Fisher Scientific, Emeryville, CA) in 20% ethanol to visualize and quantify plaques. ZIKV bloodmeal titers were recorded as the reciprocal of the highest dilution where plaques were noted and are represented as PFU per milliliter of blood.

Zika virus vector competence experiments. Stock ZIKV inocula in DMEM, or DMEM with no virus as a control, were mixed at a 1:10 or 1:20 ratio with fresh heparinized sheep blood (HemoStat Laboratories, Dixon, CA) to achieve ZIKV titers of 10^4 to 10^6 PFU/ml for each experiment. Bloodmeals were presented to 200 to 300 female *A. aegypti* mosquitoes at 3 to 5 days posteclosion in cohorts of 100 per container with 2 to 3 containers per group, 24 h after sugar withdrawal. Bloodmeals were presented for 60 min through a collagen membrane that was rubbed with an artificial human scent (BG-Sweetscent mosquito attractant; Biogents USA) and heated to 37°C in a membrane feeder (Hemotek Ltd., Blackburn, United Kingdom). Fully engorged females (40 to 70 per group for each experiment) with blood in their abdomens visible at a ×10 magnification were cold anesthetized by holding for 4 min at −20°C, sorted into clean plastic containers at a density of 20 to 30 mosquitoes per container, and held at 28°C with 80% humidity and a 12-h/12-h light/dark cycle for 14 days, with constant access to filter-sterilized 10% sucrose. Fourteen

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days after bloodfeeding, mosquitoes were cold anesthetized and held immobile on ice. Legs and wings were removed before collection of the expectorate for 20 min into capillary tubes containing PBS (23). Each capillary tube was placed into a 1.5-ml tube containing 250 μl PBS and centrifuged at 8,000 × *g* for 1 min to recover saliva. Legs/wings and bodies were placed into 2-ml tubes (Thermo Fisher Scientific, Emeryville, CA) containing 500 μl PBS and a 5-mm glass bead (Thermo Fisher Scientific, Emeryville, CA). Surgical tools were washed once in Cavicide and twice in 70% ethanol between each dissection to minimize cross-contamination. For samples where microbial DNA from mosquito bodies was also analyzed in addition to viral RNA, the bodies were also washed twice in 70% ethanol and once in PBS prior to dissection to remove microbes on the surface of mosquitoes. Tissues were homogenized at 30 Hz for 10 min in a TissueLyzer (Retsch, Haan, Germany) before extracting viral RNA using a MagMax viral RNA extraction kit (Thermo Fisher Scientific, Emeryville, CA), into 60 μl elution buffer according to the manufacturer's recommendations. Detection and quantification of viral RNA in mosquito tissues and saliva were performed by quantitative reverse transcription-PCR (qRT-PCR) using TaqMan Fast virus 1-step master mix and a ZIKV-specific primer set (ZIKV 1086F/1162c) (probe, ZIKV 1107-FAM [6-carboxyfluorescein]) using established methodologies (23, 72). Cycle threshold (C_T) values from qRT-PCR were converted to RNA genome copies using standard curves established with known ZIKV RNA concentrations. Samples were assayed in technical duplicates and averaged together after conversion to RNA copies per milliliter. The limit of detection (LOD) was calculated from the standard curve linear regression line where the C_T value was 40; samples that did not yield a detectable C_T of <40 were reported at the LOD. Infection experiments were each repeated once.

16S amplicon sequencing and bioinformatics. DNA from individual mosquitoes (5 to 8 biological replicates per treatment) was extracted with a Quick-DNA Tissue/Insect Microprep kit (Zymo Research, Irvine, CA, USA), according to the manufacturer's instructions, and eluted in 30 μl elution buffer. DNA from larval water and bloodmeals was extracted with a DNeasy blood and tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. DNA extracted from individual mosquitoes was PCR amplified in either the V3-V4 (73) or solely the V4 (74) hypervariable region of the 16S rRNA gene. The presence and size of amplicons were confirmed by gel electrophoresis using a DNA ladder to identify the amplicon size (GeneRuler 1-kb Plus; Thermo Fisher Scientific, Emeryville, CA). Negative controls, including DNA extraction controls (extraction protocol with sterile PBS) and PCR controls (PCR with molecular-grade H_2O), were included in each library preparation. 16S amplicon libraries were prepared by the addition of Nextera XT index kit v2 set A adapter sequences (Illumina, San Diego, CA), which were cleaned using Kapa Pure beads (Roche, Basel, Switzerland), quantified by a Qubit double-stranded DNA (dsDNA) high-sensitivity (HS) assay (Thermo Fisher Scientific, Emeryville, CA), pooled to equimolar concentrations of 5 nM per sample, and sequenced at the University of California, Davis, DNA Core Laboratory using the Illumina MiSeq PE250 platform.

The bacterial composition of individual mosquitoes from different water types and that exhibited different ZIKV infection statuses was assessed by bioinformatic analysis of the 16S rRNA amplicon. Paired-end reads were filtered, trimmed, and processed using the DADA2 pipeline (package version 1.16.0) according to the recommended workflow (75, 76), which was handed to phyloseq (version 1.32.0) (77). Sequences were grouped into amplicon sequence variants (ASVs), a proxy for species (60), and assigned taxonomy using the Silva v132 reference database (78). Assigned taxa were filtered to remove environmental contaminants and sequencing artifacts. Contaminant and artifact ASVs were identified and removed if sequences were also present in the negative controls (DNA-extracted nuclease-free H_2O) or if reads aligned with "arthropod," mitochondrial, or chloroplast sequences.

Microbial ecology analyses were conducted using the R packages phyloseq (version 1.32.0) and vegan (version 2.5.7) (77, 79). To determine whether ASVs showed differential abundances across samples, differential expression analysis was conducted using DESeq2 (80). Random forest modeling was used to predict ASVs that distinguish mosquito cohorts, using the randomForest package (81). Sample reads were scaled to an even depth (mean number of reads per sample) prior to all analyses.

Microbial quantification. Both culture-dependent and culture-independent assays were conducted in parallel to quantify live and total bacterial loads in mosquitoes and their rearing water. Culture-dependent quantification of microbes was performed by culturing 40 μl of rearing water or 40 μl of 10-fold serial dilutions from individual mosquitoes (3 to 5 per treatment) homogenized in 500 μl PBS on LB plates at 37°C for 5 days. Plated dilutions that yielded distinct, countable colonies were enumerated for each mosquito sample. Each sample was plated in technical triplicates, and the mean colony count is reported. Culture-independent quantification of bacteria was performed by SYBR green real-time PCR (Thermo Fisher Scientific, Emeryville, CA) to amplify the 16S rRNA gene in samples from mosquitoes and water (5 to 10 per treatment). Bacterial culturing and quantitative PCR (qPCR) of mosquitoes were repeated twice for each rearing experiment. The mosquito data were normalized to an *A. aegypti* reference ribosomal protein S17 (RPS17) gene (82).

Statistical analyses. Differences in pupation kinetics were determined by mixed-effect ANOVAs with repeated measures. Bacterial abundance differences between groups were determined by either Mann-Whitney or Kruskal-Wallis tests. For 16S amplicon sequencing, differences in microbial communities were assessed using principal-coordinate analysis (PCoA) of weighted UniFrac distances and tested for significance by permutational multivariate analysis of variance (PERMANOVA). Quantification of the contribution of each variable to differences in microbial communities was conducted using constrained analyses of principal coordinates with the same UniFrac distances as those in the PCoA analyses.

Vector competence was assessed by quantifying infection, dissemination, and transmission rates, calculated as the number of individual bodies, legs/wings, or expectorates, respectively, that yielded detectable ZIKV RNA divided by the total number of individuals that ingested blood. The magnitude of ZIKV RNA in individual mosquito tissues is also reported. Differences in infection, dissemination, and

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transmission rates between mosquito groups were determined using Fisher's exact tests, and differences in RNA levels were assessed by Mann-Whitney tests. Calculation of the 50% infectious dose (ID_{50}) was performed using the nonlinear regression dose curve for LW and EW groups. All statistical analyses were performed using GraphPad Prism 9.0.2 (GraphPad Software, San Diego, CA).

Accession number(s). Raw sequencing data are available from the NCBI Sequence Read Archive under BioProject accession number PRJNA750810.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

FIG S1, EPS file, 2.9 MB.
FIG S2, EPS file, 0.2 MB.
FIG S3, EPS file, 0.2 MB.
FIG S4, EPS file, 0.7 MB.
FIG S5, EPS file, 0.2 MB.
FIG S6, EPS file, 0.5 MB.
TABLE S1, EPS file, 0.6 MB.

ACKNOWLEDGMENTS

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Water type	Collection source	Collection date	Figures (16S libraries)
EW1	Davis, CA, USA cemetery headstone	Aug 2018	Fig 1, 6B, Supplemental 3A, Supplemental 4A-B (AM1019LS, AM1019ZE)
EW2		Jun 2019	Fig 4B, Supplemental 3A
EW3		Nov 2019	Supplemental Fig 3B
EW4		Feb 2020	Fig 6C, Supplemental 4C-D (AM820ZE)
LW	Insectary tap (deionized)	At experiment start date	Matched with equivalent EW

Table S1. Water sources, collection dates, and data references for *Ae. aegypti* used in rearing experiments. LW is laboratory water, and EW is environmental water.
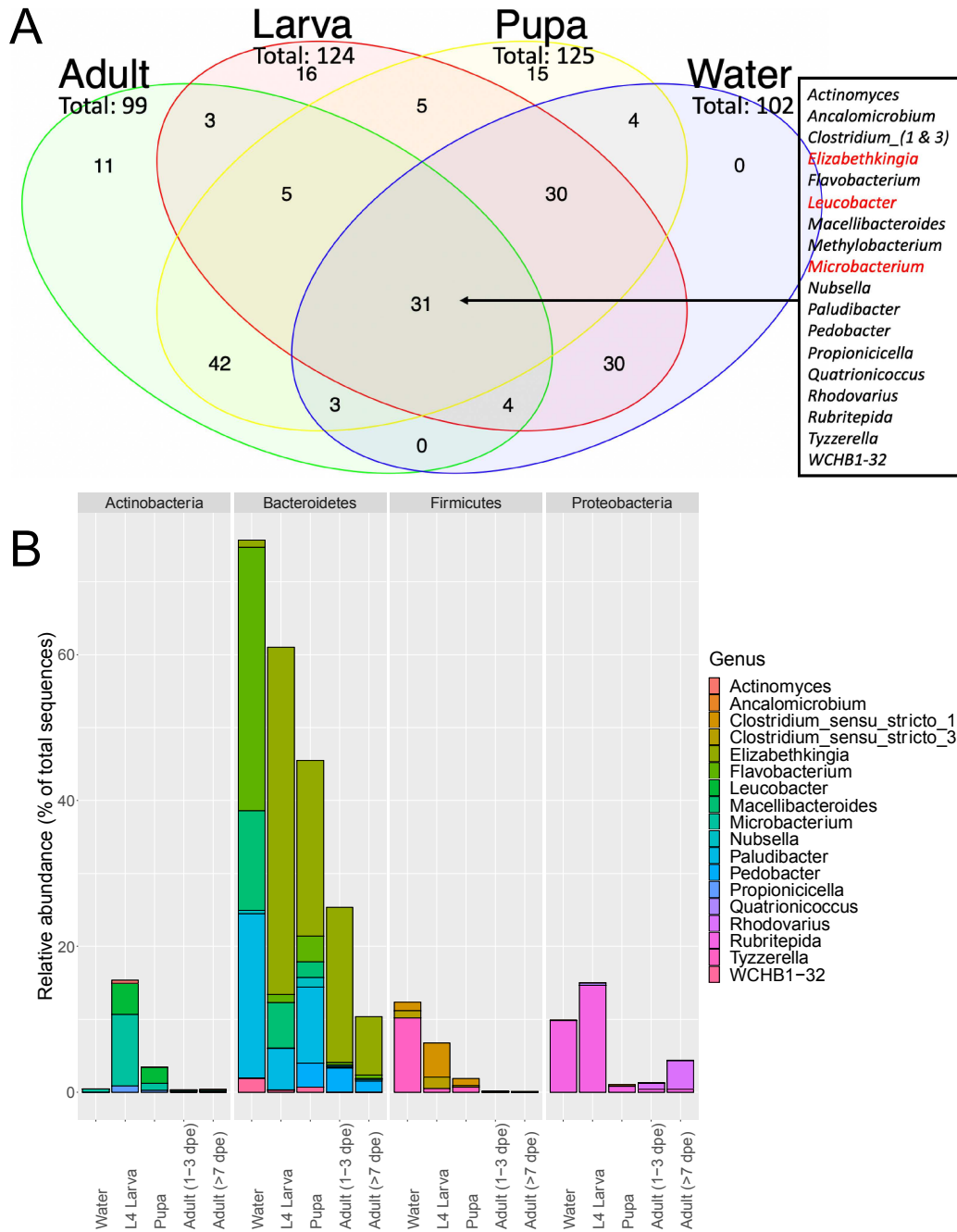
Figure S1. Bacterial ASVs shared in various life stages. A) Shared and distinct ASVs detected in rearing water, larvae, pupae, and adult mosquitoes. Text box lists shared bacteria by genus. Red text indicates genera that were also detected in eggs after surface sterilization. B) Relative abundance of the 19 genera shared among water, larvae, pupae, and adult mosquitoes grouped by bacteria taxa.
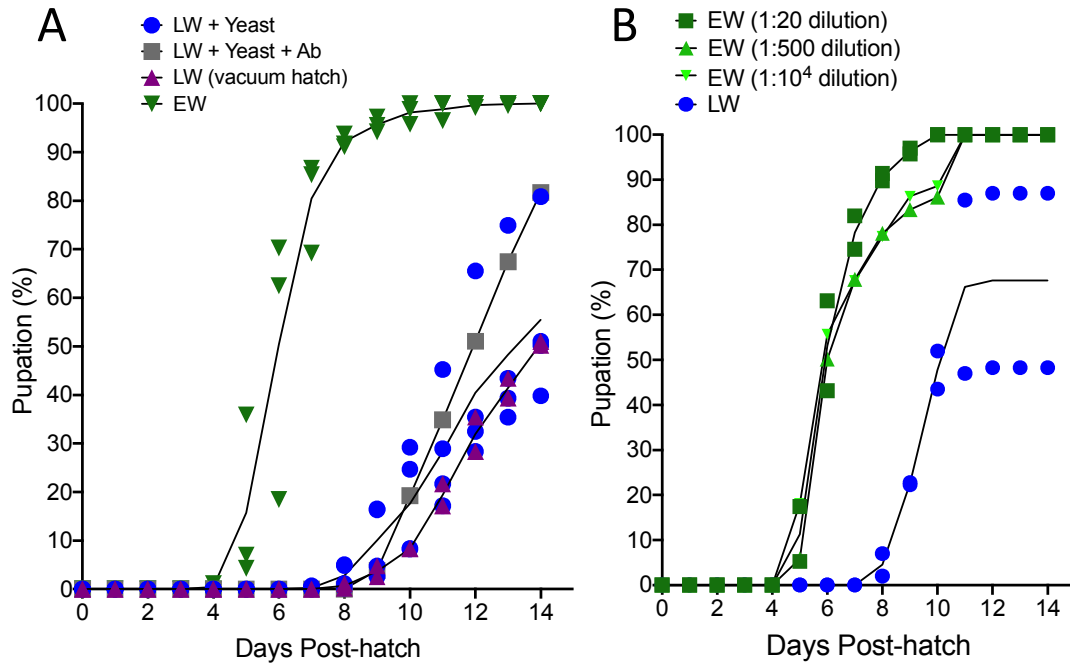
Figure S2. Development times of Los Angeles *Ae. aegypti*, plotted by individual experimental replicates. Pupation differences between A) EW vs. LW variations in Fig 2B, and B) LW vs. EW dilutions in Fig 3B. Black lines denote the average % pupation of replicate experiments.
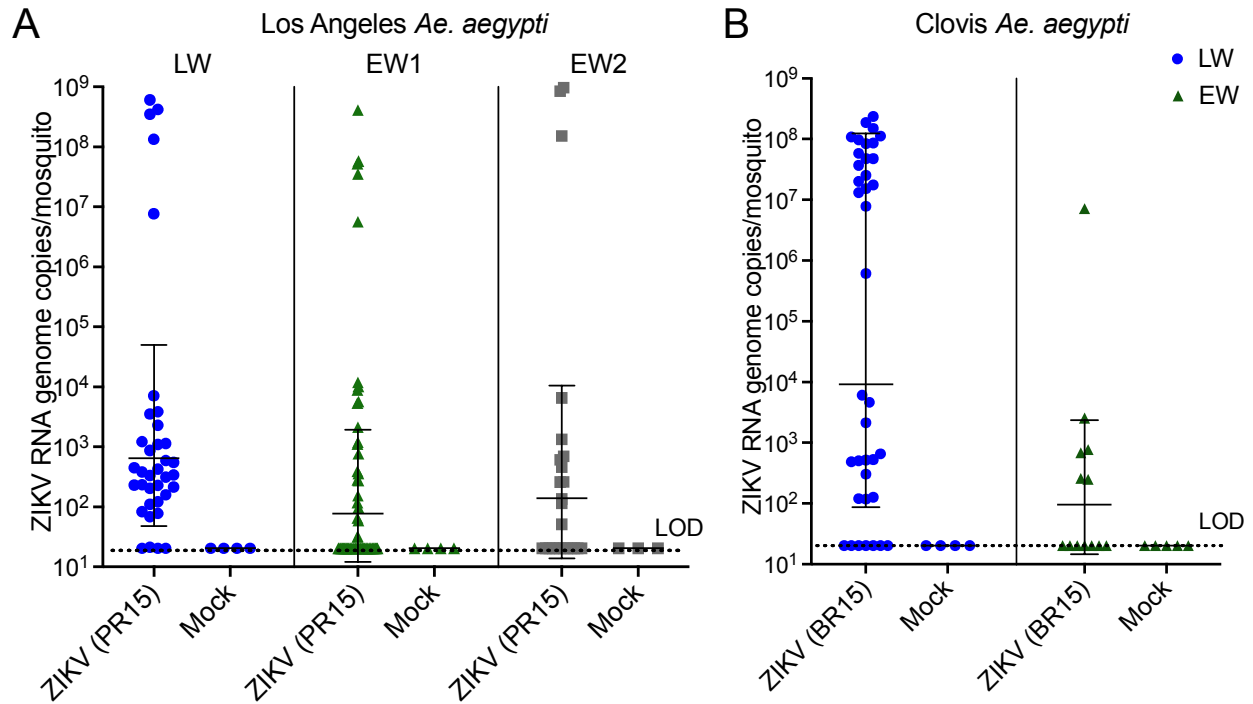
Supplemental Figure 3. ZIKV RNA levels in individual mosquitoes that ingested bloodmeals containing $10^{5.4}$ (A) or $10^{5.3}$ (B) PFU/ml ZIKV or blood with no virus (mock), summarized in Fig 4B. A) *Ae. aegypti* colonized from Los Angeles, CA were presented with PR15 ZIKV. B) *Ae. aegypti* colonized from Clovis, CA were presented with ZIKV BR15. Mosquitoes with no detectable ZIKV RNA are reported at the limit of detection (LOD) of the assay, which averaged $10^{1.3}$ ZIKV genome copies/mosquito in both A and B.
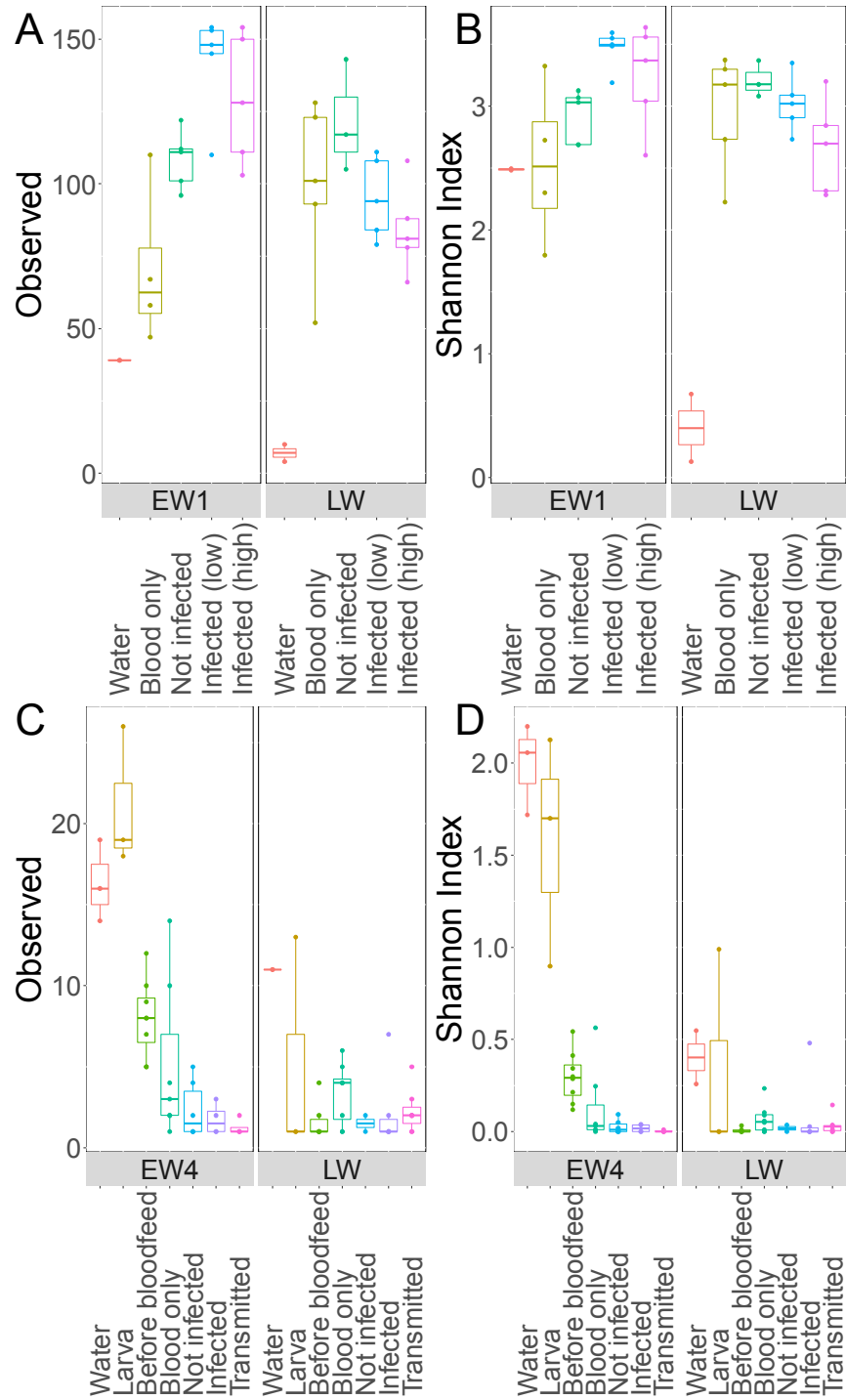
Figure S4. Alpha diversity of EW1, EW4, and LW mosquitoes.
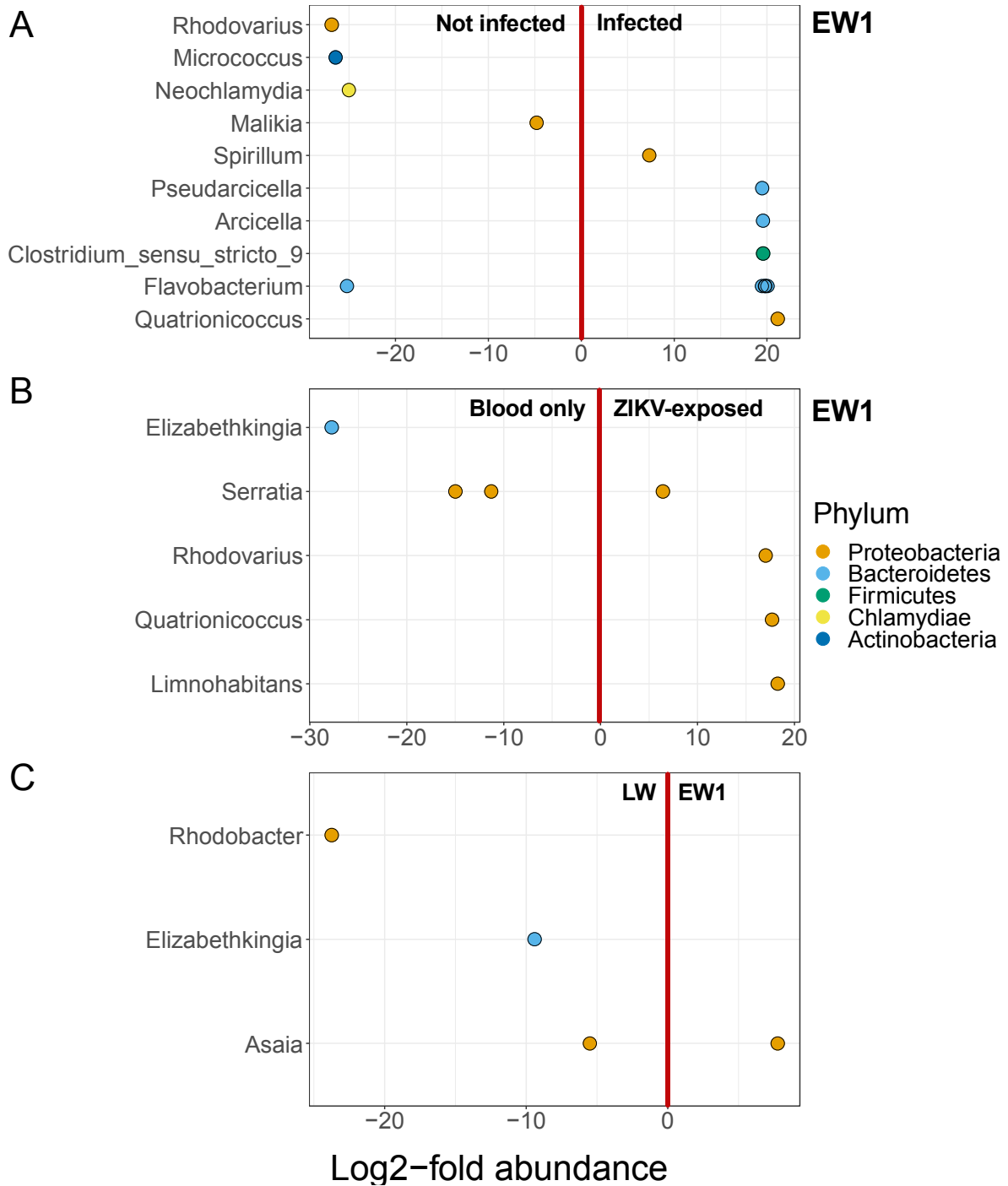
Figure S5. Differentially abundant taxa determined by DESeq2. Each dot represents an ASV

from the respective genus (y-axis), that was differentially abundant among mosquitoes

according to infection status or water type which are separated by the vertical red line. ASVs to

the left of the dividing line were more abundant in the left group while ASVs to the right of the

dividing line were more abundant in the right group. Bacterial taxa from *DESeq2* were significantly different if the adjusted p-value cut-off (alpha) was below 0.05. No differentially abundant ASVs were found between Not infected/ Infected and Blood-only/ZIKV-exposed mosquitoes in LW mosquitoes.
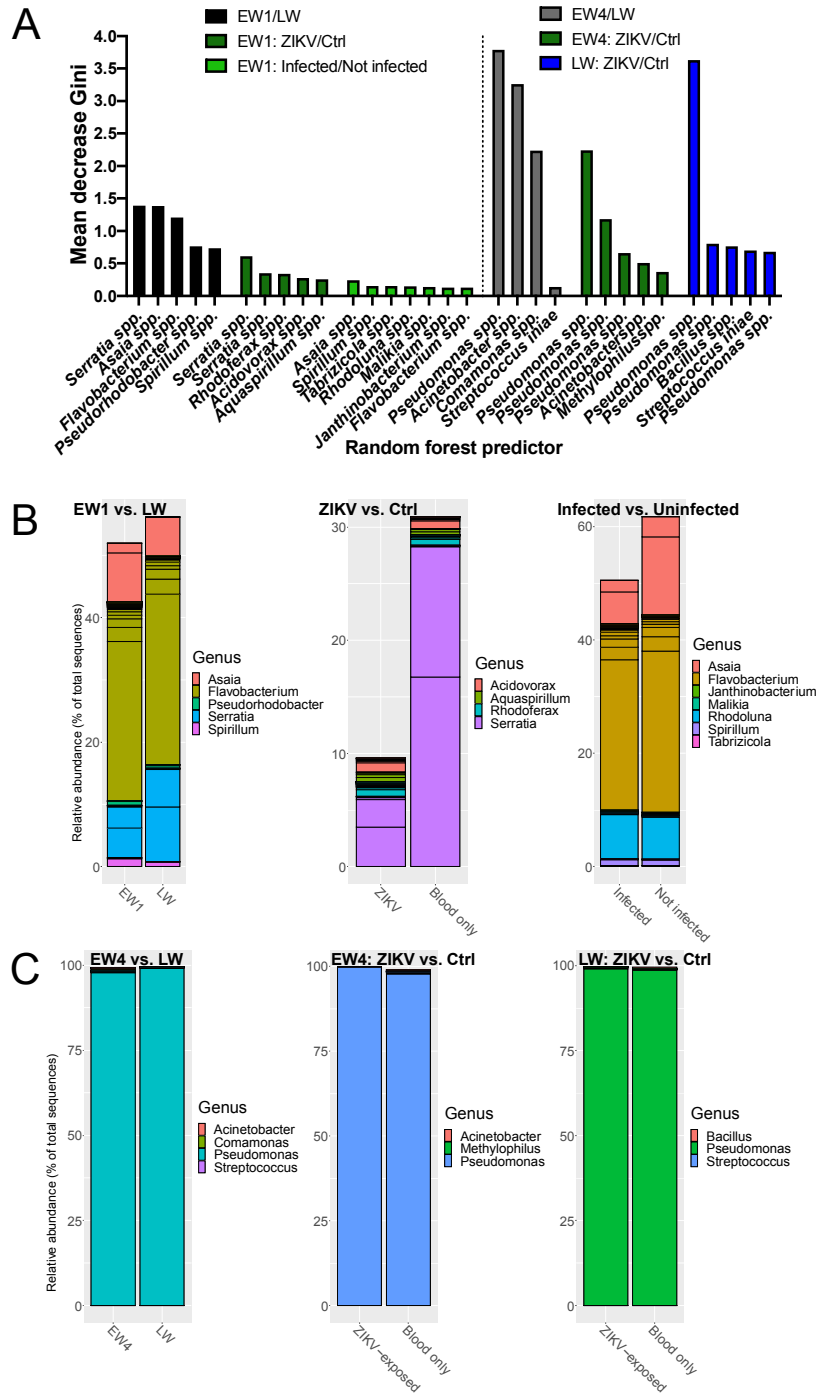
Figure S6. Random forest modelling of significant bacterial taxa. A) Top 5-7 predictors of mosquito groups were plotted, and B) relative abundance of the top 5-7 selected predictors were plotted using ggplot2. 'Ctrl' indicates mosquitoes that ingested blood only but no ZIKV.

CHAPTER 3

Microbes reduce susceptibility of *Aedes aegypti* to Zika virus by enhancing blood digestion and limiting midgut cell infection

1 **Microbes reduce susceptibility of *Aedes aegypti* to Zika virus by enhancing blood**

2 **digestion and limiting midgut cell infection**

3

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14 **Short Title:** Microbes mediate Zika virus susceptibility in mosquitoes

15

16 **Abstract**

17

18 The worldwide expansion of mosquito-borne pathogens necessitates improved control

19 measures, including approaches to restrict infection and transmission by mosquito vectors.

20 Reducing transmission is challenging because determinants of vector competence for viruses

21 like Zika (ZIKV) are poorly understood. Our previous work established that *Aedes (Ae.) aegypti*

22 larvae reared in microbe-rich environmental water are less susceptible to ZIKV as adults

23 compared to cohorts reared in microbe-deficient laboratory tap water. Here, we explain the

24 association by identifying a mechanism by which environment-derived microbes reduce

25 susceptibility of *Ae. aegypti* for ZIKV. Provided that the midgut represents the first barrier to

26 mosquito infection, we hypothesized that microbial exposure modulates midgut infection by

77

27   ZIKV. Since mosquitoes live in water as larvae and pupae and then transition to air as adults,

28   we also define the stage in the life of a mosquito when microbial exposure reduces ZIKV

29   susceptibility. *Ae. aegypti* larvae were reared in microbe-rich water and then treated with

30   antibiotics during the pupal and adult stages, adult stage only, or provided no antibiotics at any

31   stage. Vector competence was next evaluated in mosquitoes that ingested ZIKV-spiked

32   bloodmeals. Antibiotic treatment enhanced ZIKV infection and dissemination rates, especially in

33   *Ae. aegypti* treated as both pupae and adults. Antibiotic treated adult mosquitoes also had

34   increased midgut epithelium permeability, higher numbers of ZIKV-infected midgut cells, and

35   impaired bloodmeal digestion. Consistent with these changes, *Ae. aegypti* treated with

36   antibiotics as pupae and adults that ingested ZIKV in bloodmeals showed reduced expression of

37   genes associated with bloodmeal digestion and metabolism relative to mosquitoes that were not

38   antibiotic treated. Together, these data show that exposure to microbes throughout the life of

39   *Ae. aegypti* restricts ZIKV dissemination by facilitating blood digestion and limiting midgut cell

40   infection. Understanding the roles mosquito microbiota play in determining midgut physiology

41   and arbovirus susceptibility can lead to novel approaches to decrease mosquito transmission

42   and will improve understanding of vector competence in microbe-rich environmental habitats.

43

44   **Author Summary**

45

46   Mosquito-transmitted viruses like Zika continue to threaten human health. Absent vaccines or

47   treatments, controlling mosquitoes or limiting their ability to transmit viruses represents a

48   primary way to prevent mosquito-borne viral diseases. The role mosquito microbiota play in

49   shaping transmission of Zika virus has been limited to association-based studies. Our prior work

50   showed that *Aedes aegypti* mosquito larvae that develop in bacteria-rich water are less

51   susceptible to Zika virus compared to larvae reared in microbe-poor laboratory tap water. Here

52   we identify a mechanism that explains this association. Since mosquitoes are aquatic as larvae

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53   and pupae and terrestrial as adults, we also define the life stage when microbes need be

54   present to reduce Zika virus susceptibility. We used antibiotics to reduce environmental water-

55   derived microbes at pupal and adult or only adult stages and observed that, compared to

56   mosquitoes with microbes, antibiotic treatment increases Zika virus dissemination, increases

57   permeability and infection of the midgut, and impairs bloodmeal digestion. These data show that

58   microbial exposure throughout the life of a mosquito restricts Zika virus dissemination by

59   facilitating blood digestion and limiting midgut cell infection. These findings advance

60   understanding of microbiota-mosquito-virus interactions by defining how microbes reduce

61   susceptibility of *Aedes aegypti* to Zika virus.

62

63   **Introduction**

64

65   *Aedes (Ae.) aegypti* and *Ae. albopictus* mosquito-transmitted viruses, including dengue (DENV),

66   chikungunya (CHIKV), and Zika (ZIKV), pose a massive burden on human health, especially in

67   tropical regions. Absent licensed vaccines or specific treatments for these pathogens, vector

68   control represents the primary means to reduce the diseases they cause. Vector competence

69   studies, which assess the capacity for mosquitoes to become infected with, develop

70   disseminated infections, and transmit viruses in laboratory environments, are foundational to

71   defining which species maintain arbovirus transmission cycles. For *Ae. aegypti,* prior studies

72   show that temperature (1,2) and geographic origin (3,4) are major determinants of ZIKV vector

73   competence. Other factors, including the microbial environment of mosquito vectors, have

74   historically been overlooked. The microbiota of *Aedes spp.* mosquitoes primarily derive from the

75   environment (5–7), reside dominantly in the midgut (7,8), and can influence physiology and life

76   history traits through nutritional supplementation (5,6,9,10) and immune stimulation (11–13).

77   Prior studies from our group and others' show that microbiota influence ZIKV competence for

78   *Ae. aegypti* (14,15), and *Ae. albopictus* (16). We discovered that when *Ae. aegypti* larvae reared

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79   in microbe-rich water sourced from cemeteries were exposed orally to ZIKV as adults, they

80   became infected, developed disseminated infections, and transmitted virus less efficiently than

81   cohorts where larvae were reared in microbe-deficient laboratory tap water (14). We also

82   determined that the presence of microbes accelerates mosquito development. Another study

83   demonstrated that ablation of microbiota via oral treatment of adult *Ae. aegypti* with antibiotics

84   prior to exposure to ZIKV in an artificial bloodmeal did not affect vector competence (17).

85   Together, these findings suggest that the influence of microbiota on ZIKV vector competence

86   may be specific to the mosquito developmental stage (larval, pupal, adult) during which the

87   microbes are present. Beyond ZIKV, microbial effects on mosquito life-stage development and

88   metabolism that condition pathogen susceptibility may be generalizable to an array of mosquito-

89   borne pathogens. Depending on the bacterial species, larval gut microbiota can modify lipid

90   metabolism in *Ae. aegypti*, including to decrease DENV susceptibility, possibly via metabolism-

91   driven fitness changes (18). Bacterial infection in *Anopheles (An.) gambiae* larvae increases

92   hemocyte activity in adults, which correlates with elevated *Plasmodium falciparum* susceptibility

93   in vectors (19). To understand whether reduced ZIKV vector competence after larval

94   development in microbe-rich water is conferred transstadially from larvae to pupae to adults,

95   one goal of this study was to determine whether exposure to microbes throughout life is

96   required to reduce ZIKV susceptibility in *Ae. aegypti*.

97

98   Microbes are likely also involved in maintaining physical barriers that restrict access of an

99   ingested virus or parasite to the mosquito midgut. Treatment of the malaria vector *An. coluzzi*

100   with antibiotics hinders the synthesis of the peritrophic matrix (PM), a chitinous mesh that

101   sequesters the bloodmeal and furnishes digestive enzymes used in bloodmeal digestion (20).

102   Given that the mosquito midgut represents the first infection barrier for most vector-borne

103   pathogens, we hypothesized that microbial exposure affects midgut integrity, which in turn

104   conditions susceptibility to ZIKV. Physiological interactions between microbes and the mosquito

105 midgut that influence vector competence are supported by prior studies. The commensal

106 bacterium *Serratia marcescens* increases susceptibility of *Ae. aegypti* for CHIKV by secreting a

107 protein that digests membrane-bound mucins coating the gut epithelium, rendering the

108 epithelium more accessible to the virus (21). The bacterium *Bacillus thuringiensis* svar.

109 *israelensis* (Bti) expresses δ-endotoxins that create pores in midgut cells of larval mosquitoes,

110 which is lethal and has been a mainstay of larvicidal vector control for decades (22,23). We

111 predicted that elimination of microbes disrupts midgut integrity, increasing ZIKV access to or

112 traversion of the midgut epithelium, resulting in increased midgut cell infection and higher

113 dissemination rates *Ae. aegypti*. We reared *Ae. aegypti* larvae in microbe-rich environmental

114 water. Half of the experimental cohorts were administered antibiotics in water during the pupal

115 stage to ablate microbes. Some cohorts of adults were also treated with antibiotics administered

116 in sugar water after eclosion and prior to exposure to ZIKV-spiked bloodmeals. We measured

117 physiological differences in the midgut in blood-engorged mosquitoes across cohorts treated

118 with or without antibiotics and treated at different life stages. Microscopy was used to evaluate

119 midgut anatomy and integrity and to quantify ZIKV-infected midgut cells. The magnitude and

120 rate of bloodmeal digestion was measured by quantifying hemoglobin levels. RNA sequencing

121 (RNA seq) was used to identify differential gene expression in cohorts of *Ae. aegypti* treated

122 with or without antibiotics at different life stages and in response to blood with or without ZIKV.

123 We observed that *Ae. aegypti* treated with antibiotics as pupae and adults and as adults only

124 are more susceptible to ZIKV infection and dissemination compared to mosquitoes that were not

125 antibiotic-treated, and that susceptibility is highest after antibiotic exposure at both pupal and

126 adult stages. Antibiotic treatment of pupae and adults also increased midgut permeability, the

127 number of ZIKV-infected midgut cells, and impaired bloodmeal digestion. Gene ontology

128 analyses of ZIKV-exposed mosquitoes revealed enrichment of PM-associated genes, with more

129 genes related to blood protein metabolism upregulated in mosquitoes harboring microbiota.

130 Together our results show that exposure to a microbe-rich community throughout the life of *Ae.*

131   *aegypti* facilitates blood digestion which reduces midgut cell infection and restricts ZIKV

132   dissemination.

133

134   **Materials and Methods**

135

136   **Biosafety**

137   All ZIKV infection experiments were conducted in a biosafety level 3 (BSL3) laboratory and were

138   approved by the University of California, Davis Institutional Biosafety Committee under

139   Biological Use Authorization #R1863.

140

141   **Mosquito sources**

142   *Ae. aegypti* mosquitoes colonized from field collections from 2 locations in California were used

143   in this study. Mosquitoes were field-collected in 2016 as larvae (Los Angeles) and eggs (Clovis).

144   Mosquitoes from both locations were maintained for several generations at 26°C, 80% humidity,

145   and a 12 h:12 h light:dark (L:D) cycle with 10% sucrose provided to adults and periodic

146   presentation of sheep blood to stimulate egg laying, as described in our previous study (14).

147   Generation F_{20} or F_{22} mosquitoes were used for these studies.

148

149   **Mosquito rearing and antibiotic treatments**

150   *Microbe source and preparation.* Microbes from water collected outdoors in cemeteries were

151   added to water in which *Ae. aegypti* larvae were reared, using an approach detailed in our

152   previous study (14). Briefly, 1 liter (L) of collected water was filtered through a 1 mm mesh to

153   remove large debris, centrifuged at 3000 g to pellet microbes, washed 3 times with sterile 1X

154   phosphate buffered saline (PBS, Thermo Fisher Scientific, Emeryville, CA), and frozen in

155   glycerol at -80°C. Microbe aliquots were thawed at room temperature and placed in laboratory

156   tap water prior to addition of mosquito eggs.

157   *Larval rearing.* Mosquito eggs were hatched in 1 L deionized (diH$_2$O) water spiked with 1 mL of

158   microbes from a glycerol stock aliquot. Approximately 1500 larvae were reared in three pans at

159   a density of ~500 larvae/pan. Each pan contained an agarose plug that was prepared by mixing

160   1% agarose (Sigma-Aldrich, St. Louis, MO) with pulverized fish food (final concentration of 100

161   g/L, or 10% [Tetra, Melle, Germany]) and rodent chow (final concentration of 80 g/L, or 8%

162   [Teklad Global 18% Protein Rodent Diet, Envigo, Indianapolis, IN]), which was autoclave-

163   sterilized before casting into 12-well plates. One to 2 plugs were added to larval pans every

164   other day until day 15 post-hatch. Larval trays were kept in environmental growth chambers

165   (Binder, Bohemia, NY) at 26°C, 80% humidity, and a 12 h:12 h L:D cycle.

166   *Antibiotic treatments to pupae and adults.* Pupae or adults were divided into three treatments: 1)

167   mosquitoes exposed to antibiotics (Abx) both as **p**upae and **a**dults (AbxPA), 2) mosquitoes

168   exposed to antibiotics as **a**dults (AbxA) only, and 3) mosquitoes not exposed to antibiotics (No

169   Abx), where the latter represents the control group. First, pupae were picked and randomly

170   divided into 2 batches each day. The AbxPA batch of pupae was washed in diH$_2$O containing an

171   antibiotic cocktail consisting of 50 μg/mL each of penicillin, streptomycin, and kanamycin

172   (Sigma-Aldrich, St. Louis, MO) for 10 minutes before transfer to another water vessel containing

173   fresh antibiotic cocktail. The other batch of pupae, from which the AbxA and No Abx groups

174   were derived, was transferred to a separate cage without washing or antibiotics. Adults in the

175   AbxPA group were maintained on the antibiotic cocktail dissolved in 10% sucrose (Thermo

176   Fisher Scientific, Emeryville, CA) *ad libitum*. Adults in the other batch were initially maintained

177   on filter-sterilized 10% sucrose without antibiotics *ad libitum* until eclosion was complete. Once

178   adults reached a density of ~500, females were aspirated out and transferred in cohorts of 80-

179   120 mosquitoes to 32-ounce plastic containers (Amazon, Seattle, WA) with mesh lids. At this

180   point, the second non-antibiotic treated batch was further divided into two treatments: AbxA was

181   provided 10% sucrose *ad libitum* with the antibiotic cocktail, and No Abx was fed 10% sucrose

83

182 *ad libitum* without the antibiotic cocktail. Adult mosquitoes were maintained at 26°C, 80%

183 humidity, and 12 hour:12 hour L:D cycle throughout the experiment. All mosquitoes were

184 housed in the same incubator. Antibiotic treatment experiments were repeated twice and

185 represented as the "high" and "low" ZIKV dose experiments.

186

187 **Bacterial quantification by 16S qPCR**

188 We measured the efficacy of antibiotic treatment for removing bacteria from AbxA and AbxAP

189 groups by performing 16S qPCR on larvae, pupae, and adults 3-5 days post-eclosion (dpe) from

190 all groups, as well as in L4 larvae and pupae before antibiotic treatment. DNA from 5 L4 larvae,

191 5 pre-treatment pupae, 5 No Abx, 10 AbxA, and 10 AbxAP individuals was extracted with the

192 Quick-DNA Tissue/Insect Microprep Kit (Zymo Research, Irvine, CA, USA), according to the

193 manufacturer instructions and eluted in 30 μL elution buffer. Extracted DNA was PCR-amplified

194 in the V3-V4 (24) hypervariable region of the 16S rRNA gene, as previously described (14,24).

195 Quantification of bacteria was performed by SYBR Green Real-Time PCR (Thermo Fisher

196 Scientific, Emeryville, CA) to amplify the 16S rRNA gene in whole mosquitoes. Each sample

197 was tested in technical duplicates. 16S quantities were normalized to an *Ae. aegypti* reference

198 ribosomal protein S17 gene (RPS17) and reported as a 16S:RPS17 ratio (25).

199

200 **Hemolytic activity**

201 Hematophagous bacteria can directly lyse blood cells and confound hemoglobin digestion

202 measurements. Bacterial isolates from adult female *Ae. aegypti* were screened for hemolytic

203 activity via culturing on agar plates. Individual mosquitoes were homogenized (TissueLyser,

204 Retsch, Haan, Germany) in 500 μL PBS and 40 μL of supernatant was plated on either R2A

205 agar (Sigma-Aldrich, St. Louis, MO) or sheep blood agar plates (Biological Media Services,

206 University of California, Davis School of Veterinary Medicine) for 3 days at 37°C. Up to 10

207 colonies from each mosquito were streaked on media plates 2-3 additional times to generate

84

208   axenic cultures. Resulting colonies were streaked on sheep blood agar at both 30°C and 37°C

209   and screened for clearing zones around colonies over the course of 3 days. The viability of this

210   assay was tested in-house with hemolytic *Streptococcus spp.* isolates from the UC Davis

211   School of Veterinary Medicine.

212

213   **Virus source and titrations**

214   We used ZIKV strain BR15 (Brazil 2015, SPH2015, GenBank Accession # KU321639), the

215   same strain we used previously for mosquito infection experiments (14). This virus strain was

216   originally isolated from human serum from a patient in Brazil in 2015 and passaged 3 times in

217   Vero cells (CCL-81, ATCC, Manassas, VA) before freezing in stocks. Stocks were titrated on

218   Vero cells prior to bloodmeal presentation to approximate stock titers, and ZIKV-spiked sheep

219   blood was also titrated after bloodfeeding to confirm the dose presented in each bloodmeal. For

220   titrations, serial 10-fold dilutions of cell culture supernatant containing virus stock or ZIKV-spiked

221   blood were inoculated into multi-well plates containing confluent Vero cells at one dilution per

222   well and incubated for 1 hour at 37°C in 5% CO_2 with rocking every 10 minutes. After

223   incubation, each well was filled with an agar overlay (0.5% agarose [Thermo Fisher Scientific,

224   Emeryville, CA] in Dulbecco's modified Eagle's medium [DMEM, Thermo Fisher Scientific,

225   Emeryville, CA], + 2% fetal bovine serum [FBS, Genesee Scientific, San Diego, CA] +

226   penicillin/streptomycin [Thermo Fisher Scientific, Emeryville, CA]). The cells were incubated for

227   1 week at 37°C in 5% CO_2, then fixed with 4% formalin (Thermo Fisher Scientific, Emeryville,

228   CA). Fixed cells were stained with 0.025% crystal violet (Thermo Fisher Scientific, Emeryville,

229   CA) in 20% ethanol and visualized on a light table to count plaques. ZIKV bloodmeal titers are

230   represented as plaque-forming units (PFU) per mL of blood.

231

232   **ZIKV vector competence experiments**

233   Stock ZIKV in DMEM, or DMEM with no virus as a control, were diluted in 10-fold increments

234   with fresh heparinized sheep blood (HemoStat Laboratories, Dixon, CA) to achieve ZIKV titers

235   of 10^4 and 10^6 PFU/mL. Bloodmeals were presented to female *Ae. aegypti* at 3-5 days post-

236   eclosion in cohorts of 80-120 per container, at 2-3 containers per treatment. Cohort sizes were

237   very large to maximize the numbers of bloodfed mosquitoes in the case of low feeding rates.

238   Mosquitoes were sucrose-starved 18-24 hours before the presentation of bloodmeals.

239   Mosquitoes were offered bloodmeals for 60 minutes through a collagen membrane rubbed with

240   an artificial human scent (BG-Sweetscent mosquito attractant, Biogents USA) and heated to

241   37°C in a membrane feeder (Hemotek Limited, Blackburn, United Kingdom). Bloodmeals were

242   presented to mosquitoes inside a plexiglass glove box containing an open bottle of boiling water

243   to maximize humidity. After feeding, mosquitoes were immobilized with CO_2 for 5 seconds and

244   then immobilized on a chill table (Bioquip (R.I.P.), Compton, CA, USA) to assess their

245   bloodfeeding status using a microscope at 10X magnification. Fully engorged females (18-45

246   per treatment for each experiment) were sorted into fresh plastic containers at a density of 10-

247   20 mosquitoes per container and held at 28°C with 80% humidity and 12 h:12 h L:D cycle for 14

248   days. No Abx cohorts were provided 10% sucrose *ad libitum,* while AbxPA and AbxA cohorts

249   were maintained on 10% sucrose with the antibiotic cocktail *ad libitum*.

250

251   At 14 days post-bloodfeed (dpf), surviving mosquitoes were CO_2-anesthetized and immobilized

252   on a chill table. Bodies, legs/wings, and saliva were collected to assess infection, dissemination,

253   and transmission rates, respectively. Legs/wings were removed before collection of expectorate

254   into capillary tubes containing PBS by forced salivation, using an established approach (26). To

255   minimize cross-contamination, surgical tools were washed once in CaviCide® (Metrex, Orange,

256   CA) and twice in 70% ethanol between dissections. Each capillary tube was placed in a 1.5-mL

257   tube containing 250 μL PBS and pulse-centrifuged at 8,000 g to recover saliva. Bodies and

258   legs/wings were placed into 2-mL tubes containing 500 μL PBS and a 5-mm glass bead

86

259   (Thermo Fisher Scientific, Emeryville, CA), then homogenized at 30 hertz for 5 minutes in a

260   TissueLyser (Retsch, Haan, Germany). Viral RNA was extracted using a MagMax Viral RNA

261   Extraction Kit (Thermo Fisher Scientific, Emeryville, CA), into 60 μL elution buffer following the

262   manufacturer's recommendations.

263

264   Detection and quantification of viral RNA in mosquito tissues and saliva were performed by

265   quantitative reverse transcription polymerase chain reaction (RT-qPCR) using a TaqMan Fast

266   Virus 1-Step Master Mix and a ZIKV-specific primer set (ZIKV 1086F/1162c, probe: ZIKV 1107-

267   FAM) using established methodologies (26,27). Cycle threshold (Ct) values from the RT-qPCR

268   were converted to RNA genome copies using standard curves established with ZIKV RNA of

269   known concentration. Each sample was assayed in technical duplicates and converted to copies

270   per mosquito before calculating the average value. The limit of detection (LOD) was calculated

271   from the standard curve linear regression line where the Ct value = 40. Samples that did not

272   yield a detectable Ct of less than 40 were reported at the LOD. Infection, dissemination, and

273   transmission rates are all reported as the number of ZIKV-positive individuals in their respective

274   tissues divided by the number that ingested ZIKV-spiked blood.

275

276   **Midgut permeability assessments**

277   A low molecular weight dextran-FITC (MW 4000, Sigma-Aldrich, St. Louis, MO) was co-spiked

278   into ZIKV-bloodmeals and used to measure midgut permeability in No Abx, AbxA, and AbxPA,

279   groups. Permeability was assessed by measuring the fluorescent intensity of dextran-FITC

280   inside versus outside at three locations in the midgut for each mosquito for a total of 5

281   mosquitoes per treatment. A stock solution of 20 μg/mL dextran-FITC dissolved in PBS was

282   spiked into sheep blood at a 1:20 dilution to achieve 1 μg/mL, the concentration used in a

283   previous study (28). Dextran-spiked blood mixed with 10^5 PFU/ml ZIKV and presented to

284   mosquitoes as described above for vector competence assessments. Blood-engorged

285   mosquitoes were visualized by fluorescence microscopy to detect FITC in tissues. Samples

286   were covered in foil for all post-dissection steps to minimize photobleaching. Quantification of

287   FITC fluorescence was then performed on Fiji (ImageJ) (29) by measuring intensity relative to

288   background by subtracting fluorescence of an equal-sized region outside the sample area.

289   Fluorescence intensity ratios between the gut lumen and hemocoel were calculated at three

290   randomly drawn areas of equal size around the gut perimeter, then averaged together. Each

291   average was also conducted across three slices per sample to minimize focal plane variation in

292   fluorescence.

293

294   **Fluorescence imaging of mosquito midguts**

295   Mosquitoes in No Abx, AbxA, and AbxPA groups that ingested ZIKV-spiked bloodmeals were

296   imaged using immunofluorescence microscopy to examine midgut structure and to quantify the

297   number of ZIKV-infected midgut epithelial cells. Legless and wingless mosquito bodies or

298   dissected midguts were placed in 10% formalin in PBS for 24 hours, then prepared for various

299   image-based assessments as described below. Mosquitoes were imaged at the University of

300   California, Davis Advanced Imaging Facility using a Leica TCS SP8 STED 3X inverted

301   microscope.

302   *Midgut structure*: Mosquito bodies were washed once in PBS, submerged in PBS with 30%

303   sucrose overnight, and then molded and frozen at -80°C in Tissue Plus OCT medium (Thermo

304   Fisher Scientific, Emeryville, CA). Tissue molds were cut into 10 μm slices at the median sagittal

305   plane (at a depth where the blood bolus reaches maximum diameter, determined with test

306   samples) using a Leica CM1860 Cryostat Microtome (Leica Biosystems, Deer Park, IL, USA)

307   and embedded onto charged microscope slides. A single drop of ProLong Gold Antifade

308   Mountant with DAPI (Thermo Fisher Scientific, Emeryville, CA) was used to mount each slide.

309   *ZIKV-infection of midguts:* Dissected midguts in 10% formalin were washed twice in PBS.

310   Tissues were submerged in a blocking solution consisting of 1X PBS, 0.2% Triton X-100

311   (PBS+T) (Bio-Rad Laboratories, Hercules, CA), 1% bovine serum albumin (BSA, Sigma-Aldrich,

312   St. Louis, MO), and 10% normal goat serum (Lampire Biological Laboratories, Pipersville, PA)

313   for 10 minutes before staining with a primary monoclonal antibody that reacts with multiple

314   flaviviruses (Flavivirus treatment antigen antibody D1-4G2-4-15, Novus Biologicals, Littleton,

315   CO) at a 1:1000 dilution in blocking solution at 4°C overnight. Midguts were washed five times

316   via direct tube transfer in PBS+T before staining with the secondary antibody (Goat anti-mouse

317   Alexa Fluor 594, Abcam, Cambridge, United Kingdom) at a 1:1000 dilution in blocking solution

318   for 1 hour at room temperature. Midguts were washed 5X in PBS+T before mounting onto

319   microscope slides with ProLong Gold Antifade Mountant with DAPI. ZIKV detection and

320   quantification were done by manually counting distinct red patches, using blue DAPI as a

321   reference for individual cells across three slices of a single midgut, and averaging the values.

322   FITC, DAPI, and Alexa Fluor 594 were each visualized (and FITC was quantified) in their

323   respective channels before merging.

324

325   **Histologic analyses**

326   Histological images at 20X and 40X magnification were used to visualize mosquito anatomy and

327   to validate that sample preparation for immunofluorescence microscopy did not mechanically

328   disrupt the midgut, which could affect interpretations of midgut integrity. Blood-engorged

329   mosquitoes with their legs and wings removed were placed in 10% formalin in PBS for 24 h,

330   then encased within tissue cassettes (Thermo Fisher Scientific, Emeryville, CA) submerged in

331   70% ethanol. Samples were submitted to the University of California, Davis School of Veterinary

332   Medicine Anatomic Pathology Service, where whole mosquitoes were paraffin-embedded, cut

333   into 5 μm slices, and stained with hematoxylin and eosin (H&E). Slides were visualized at 20X

334   and 40X magnification under a Zeiss Axio Vert A1 inverted light microscope connected to an

335   Axiocam 208 color camera (Carl Zeiss, Jena, Germany). Although we evaluated sections from 5

336   individual mosquitoes each for No Abx, AbxA, and AbxPA, groups, we used histology readouts

337   as a qualitative approach to evaluate gross midgut anatomy. Anatomical structures were

338   identified by an expert in mosquito anatomy. Representative images for each of the three

339   groups are shown.

340

341   **Blood digestion analyses**

342   Quantification of hemoglobin in bloodfed mosquitoes using a hemoglobin colorimetric detection

343   kit (Thermo Fisher Scientific, Emeryville, CA) was used to measure blood digestion. At 0, 1, 2,

344   or 3 dpf, 10-12 individual mosquitoes each in No Abx, AbxA, and AbxPA, groups exposed to

345   blood only or ZIKV-spiked blood with their legs/wings removed were homogenized in 300 μL

346   hemoglobin diluent, 100 μL of which was used for the assay, per manufacturer's instructions.

347   Absorbance at 570 nm was measured from each sample once in 96-well plates using a

348   SpectraMax iD5 Multimode Microplate Reader (Molecular Devices, San Jose, CA). Absorbance

349   values were converted to hemoglobin concentrations via standard curves generated from

350   hemoglobin standards and serial dilutions of fresh sheep blood used for feeding. Output

351   absorbance values were normalized to homogenized, non-bloodfed mosquito negative controls,

352   and units were converted to μg per mosquito body. Before conducting the assay, we validated

353   and optimized this kit for bloodfed mosquitoes, including ensuring that heat incubation of

354   mosquitoes at 60°C for 10 minutes to destroy ZIKV infectivity did not affect hemoglobin

355   quantification (**Figure S1**).

356

357   **RNA sequencing and bioinformatics**

358   Mosquito messenger RNA was sequenced to determine differences in gene expression

359   associated with antibiotic treatments after ZIKV exposure. Total RNA was extracted from adult

360   female mosquitoes in triplicate pools of 5 individuals each for No Abx, AbxA, and AbxPA groups

361   using the Quick-RNA Tissue/Insect Microprep kit (Zymo Research, Irvine, CA, USA). RNA

362   extracts were eluted into 20 μL elution buffer and quantified using a Qubit fluorometer RNA

90

363   high-sensitivity (HS) assay (Thermo Fisher Scientific, Emeryville, CA). Approximately 1 μg of

364   total RNA per sample was submitted to Genewiz Inc. from Azenta Life Sciences (Chelmsford,

365   MA, USA), where poly-A selection, library preparation, and paired-end Illumina HiSeq 2x150 bp

366   were performed. Raw FASTQ data files were received and treated by filtering and trimming

367   reads with *cutadapt v3.2* (30) using default parameters (Quality cutoff >= 30, minimum length of

368   100 bp). Next, *FastQC* (31) was used to visualize quality scores. Processed reads were then

369   aligned to version AaegL5.2 of an *Ae. aegypti* transcriptome obtained from VectorBase (32)

370   using *bowtie2 v2.4.3* (33). Aligned reads were imported into R and analyzed with the package

371   Differential Expression Analysis using *DESeq2 v1.28.1* (34). Default parameters were used to

372   determine the differential abundance of each gene using an input experimental design of

373   [antibiotic treatment + bloodmeal status + interaction]. Genes with a significance (adjusted P

374   value) below 0.05 are reported. Significant genes were mapped to biological processes using

375   the Gene Ontology program *topGO* (35).

376

377   **Statistical analyses**

378   The 16S:RPS17 ratios to verify antibiotic efficacy from qPCR were compared using a Kruskal-

379   Wallis test with multiple comparisons for adult mosquito treatments and across life stages.

380   Differences in ZIKV infection, dissemination, and transmission rates between antibiotic-treated

381   groups were determined using Fisher's exact tests. Differences in ZIKV RNA levels in bodies,

382   legs/wings, and saliva across antibiotic-treated groups were assessed by Kolmogorov-Smirnov

383   tests. Differences in hemoglobin levels across antibiotic-treated groups were evaluated using

384   Kruskal-Wallis tests with repeated measures. Relative fluorescence values and numbers of

385   nuclei were averaged in triplicate and tested for significance by one-way ANOVA tests.

386   Differences in numbers of ZIKV-infected midgut epithelial cells were assessed using Fisher's

387   exact tests. For RNA-Seq, statistical tests built into the *DESeq2* package were used. In short,

388   each sample was normalized by library size, and the geometric mean for each gene was

91

389   calculated. Negative binomial generalized linear models (glm) were used for each gene to

390   determine differential expression across antibiotic-treated groups. The Wald test was used for

391   statistical significance, with correction for the false discovery rate (FDR) set to 0.05. For

392   principal components analyses (PCA), variance stabilized transformation was implemented

393   before plotting. All statistical analyses, except RNA-Seq, were performed using GraphPad

394   PRISM 9.0.2 (GraphPad Software, San Diego, CA). RNA-Seq analyses were conducted in R

395   *v3.6* (36). P-values of less than 0.05 were considered statistically significant. For gene ontology

396   (GO), Fisher's exact tests were used on GO terms and corrected for the false discovery rate.

397

398   **Accession number(s).** Raw sequencing data are available from the NCBI Sequence Read

399   Archive under BioProject entry PRJNA818687. Scripts for the RNA-Seq analysis are available

400   on Github (DOI: 10.5281/zenodo.7259822).

401

402   **Results**

403

404   **Reduced *Ae. aegypti* susceptibility to ZIKV conferred by microbial exposure is life stage-**

405   **dependent**

406   To understand whether reduced ZIKV vector competence after larval development in microbe-

407   rich water that we observed previously (14) is conferred transstadially from larvae to pupae to

408   adults, one goal of this study was to determine whether exposure to microbes throughout life

409   (i.e. at larval, pupal, and adult stages) is required to reduce susceptibility of *Ae. aegypti* to ZIKV.

410   *Ae. aegypti* larvae were reared in laboratory water spiked with microbe-rich water sampled from

411   cemeteries that we characterized in a previous study (14) and was dominated by over 1000

412   bacterial species from the phyla *Bacteroides*, *Firmicutes*, and *Proteobacteria*. Control

413   mosquitoes were not provided antibiotics (No Abx) at any life stage. To assess the effects of

414   microbe depletion at pupal and adult life stages, cohorts of pupae and the adults they

415   developed into were administered an antibiotic (Abx) cocktail (AbxPA) that was provided to

416   pupae in water and orally in sucrose to adults from eclosion to 3 days of age (**Figure 1A**). To

417   study the effects of antibiotic treatment on adult mosquitoes, we also included cohorts where

418   adults were provided antibiotics (AbxA) orally for 3-5 days post-eclosion. AbxA mosquitoes were

419   not antibiotic-treated before 3 days of age. After each treatment, mosquitoes were orally

420   presented 3-5 days post-eclosion with low or high-dose ZIKV-spiked bloodmeals for vector

421   competence assessments. To maximize bloodfeeding success, sugar with and without

422   antibiotics was withdrawn from mosquitoes 24 hours prior to bloodmeal presentation. We first

423   assessed the efficacy of antibiotic treatment via qPCR quantification of 16S DNA. Antibiotics

424   significantly reduced the number of prokaryotes in all treated groups. (Kruskal-Wallis [K-W] with

425   multiple comparisons, $P = 0.002$ between No Abx and AbxA [adj. $P = 0.002$] and No Abx and

426   AbxPA [adj. $P = 0.006$]) (**Figure 1B**). Significantly higher 16S:RPS17 ratios, our proxy for

427   prokaryote levels, were observed in No Abx L4 larvae and pupae relative to adult mosquitoes

428   (K-W multiple comparisons, $P = <0.0001$ between No Abx and larva [adj. $P = 0.0012$] and No

429   Abx and pupa [adj. $P = 0.0079$]). This is consistent with ours (14) and others' (6,12) previous

430   observations that microbial abundance decreases during mosquito development. Antibiotic

431   treatment of pupae and adults significantly increased ZIKV infection and dissemination rates at

432   both low (3.2×10^4) and high (4.8×10^6) PFU/ml ZIKV bloodmeal doses compared to no

433   antibiotic treatment (Fisher's exact test, infection: $P < 0.0001$, dissemination: $P < 0.0001$).

434   Although it increased infection rates, antibiotic treatment of adults did not significantly increase

435   dissemination rates compared to no antibiotic treatment (Fisher's exact test, infection: $P =$

436   0.067, dissemination: $P = 0.71$) (**Figure 1C**). The AbxPA mosquitoes exhibited a more robust

437   dose response to infection, achieving larger increases in rates of infection and dissemination

438   compared to No Abx and AbxA across the two ZIKV doses (**Figure 1D**). Despite increased

439   infection and dissemination rates, transmission rates across antibiotic treatments and at the two

440   ZIKV doses did not differ significantly. Even though transmission rates were not different, levels

93

441 of ZIKV RNA levels in saliva were higher in AbxPA compared to AbxA (Kolmogorov-Smirnov

442 test, P = 0.009) at the higher dose, but not higher than No Abx (Kolmogorov-Smirnov test, P =

443 0.71) (**Figure 1E-F**). These results show that antibiotic treatment during both pupal and adult life

444 stages increases rates of ZIKV infection and dissemination, transmitted titers, and susceptibility

445 to higher ingested doses. These data also reveal that microbes acquired by larvae must be

446 maintained as pupae to reduce ZIKV susceptibility. The observation that antibiotic treatment of

447 adult mosquitoes is not sufficient to enhance ZIKV dissemination suggests that microbe-

448 mosquito interactions that influence ZIKV susceptibility must occur in the aqueous phase of a

449 mosquito's life.

450

451 **Antibiotic treatment increases midgut permeability**

452 To understand how microbial exposure of larvae and pupae reduce susceptibility of *Ae. aegypti*

453 to ZIKV, we next evaluated midgut integrity. Our rationale for focusing on the midgut is that it

454 represents the first barrier to infection of *Ae. aegypti* by ZIKV after oral exposure. We asked

455 whether higher infection and dissemination rates in AbxPA and AbxA groups compared to the

456 No Abx group are explained by modified epithelial structure or reduced midgut integrity resulting

457 from microbe depletion. We first assessed permeability by directly visualizing and enumerating

458 midgut epithelial cell density in DAPI-stained sections. As a second step, we added FITC-

459 dextran (which is small enough that it can traverse cell membranes) into ZIKV-spiked

460 bloodmeals to visualize and quantify the relative fluorescent intensity of the marker inside

461 versus outside the midgut, where higher fluorescence inside versus outside is indicative of

462 higher midgut integrity. We compared fluorescent intensity across antibiotic-treated groups

463 immediately after ingestion of a bloodmeal with ZIKV (**Figure 2A**). The midgut epithelium was

464 less organized, as evidenced by reduced contiguity in DAPI-stained midgut epithelial cell nuclei

465 in both AbxA and AbxPA compared to No Abx groups (**Figure 2B**). These qualitative

466 observations of structural disorganization of the midgut were also supported quantitatively,

94

467   where fewer cells in a 100 μm length of midgut epithelium were detected in AbxPA compared to

468   No Abx groups (**Figure 2C**, P = 0.078). For this assessment, five mosquitoes were examined

469   per group, and three separate sections for each mosquito were randomly selected, quantified,

470   and averaged (**Figure 2D**). The relative dextran-FITC fluorescence inside versus outside of the

471   midgut lumen for the AbxPA group was significantly lower than the No Abx group (P = 0.009),

472   and the AbxA group trended lower (although not significantly) than the No Abx group, indicating

473   that antibiotic treatment increases midgut permeability (**Figure 2E**). To rule out physical rupture

474   or other disruptions to the midgut during sample preparation that may have reduced structural

475   integrity, we treated and stained midgut sections from a separate cohort of similarly treated

476   mosquitoes with DAPI or H&E. At 10X, 20X, and 40X magnification, midgut epithelia across

477   treatments did not appear structurally different (**Figures 2G**, **S1**). Together these data support

478   antibiotic treatment, especially of both pupal and adult *Ae. aegypti* life stages, increasing midgut

479   permeability in adult mosquitoes after ingestion of ZIKV-spiked bloodmeals.

480

481   **Antibiotic treatment increases ZIKV infection of the midgut epithelium**

482   Next, we asked whether disruptions to midgut integrity with antibiotic treatment translate to

483   greater infection success, measured as higher numbers of ZIKV-infected midgut epithelial cells.

484   We detected ZIKV in midguts by staining sections with a flavivirus binding antibody 2 or 3 dpf

485   and counting the total number of cells in each midgut that were stained with fluorescent anti-

486   ZIKV antibody (**Figure 2A**). The number of ZIKV-positive cells in midguts was significantly

487   higher in the AbxPA group compared to the No Abx group (Brown-Forsythe ANOVA test with

488   Dunnett's multiple comparisons test, P = 0.048, AbxPA versus No Abx P = 0.086) (**Figure 2F**).

489   At 3 dpf, more infected cells were visible in AbxPA compared to AbxA and No Abx (**Figure 2G**).

490   These data support the role of microbes in larvae and pupae in maintaining the integrity of the

491   mosquito midgut, which in turn reduces the susceptibility of the midgut epithelium to infection by

492   ZIKV.

493

Microbial disruption via antibiotic treatment impairs blood digestion

Since midgut integrity can affect physiology of blood digestion in addition to ZIKV susceptibility,

we next assessed whether elevated midgut permeability after antibiotic treatment affects blood

digestion. We employed a commercially available hemoglobin colorimetric assay as a proxy for

blood protein digestion by quantifying the reduction of hemoglobin levels in blood-engorged

mosquitoes on 1 and 2 dpf compared to immediately after feeding on 0 dpf. Before performing

the assay, which is typically used for human blood, we first validated and optimized the

approach for use with homogenized mosquitoes that ingested sheep bloodmeals. We observed

that sheep blood absorbance varies linearly as a function of hemoglobin concentration, like the

kit standard (**Figure S2A**) and that absorbance decreases reproducibly in serially diluted

mosquitoes at both 560 and 580 nm wavelengths. The assay was unaffected by the 10 minute

60°C heat treatment of homogenized fed mosquitoes used to inactivate ZIKV infectivity in the

samples (**Figure S2B**). Blood-engorged females were homogenized and assayed for

hemoglobin immediately after bloodfeeding at 0 dpf to assess whether different groups ingested

the same bloodmeal volume, as well to measure the kinetics of bloodmeal digestion 1, 2, and 3

dpf (**Figure 3A**). Although hemoglobin levels that approximate bloodmeal size varied in

individual mosquitoes, there was no significant difference in mean ingested hemoglobin levels

on 0 dpf across antibiotic-treated groups regardless of whether the bloodmeal contained ZIKV,

which shows that antibiotic treatment did not influence bloodmeal size (K-W test with multiple

comparisons, P = 0.522) (**Figure 3B, C**). After ingestion of blood without ZIKV, mean

hemoglobin levels in the AbxPA group were higher than in the No Abx group 2 dpf (K-W test, P

= 0.0063). By 3 dpf, mean hemoglobin levels in the AbxPA group were higher than both AbxA

and No Abx groups (K-W test with multiple comparisons, adjusted $P_{AbxPA\text{-}No\ Abx}$ = 0.0347,

adjusted $P_{AbxPA\text{-}AbxA}$ = 0.051) (**Figure 3B**). The same pattern was observed after ingestion of

blood with ZIKV: AbxPA showed a reduced rate of blood digestion compared to No Abx 1 dpf

96

519   (K-W test with multiple comparisons, adjusted $P_{AbxPA\text{-}No\ Abx}$ = 0.0368, adjusted $P_{AbxPA\text{-}AbxA}$ =

520   0.0035) and both No Abx and AbxA 2 dpf (K-W test with multiple comparisons, adjusted $P_{AbxPA\text{-}}$

521    $_{No\ Abx}$ = 0.0037, adjusted $P_{AbxPA\text{-}AbxA}$ = 0.0027) (**Figure 3C**). The 3 dpf time point for individuals

522   that ingested blood with ZIKV was not assayed for hemoglobin since uneven bloodfeeding rates

523   across cohorts and the desire to maintain statistically robust group sizes led us to prioritize use

524   of 3 dpf mosquitoes for infection and dissemination assessments. We also compared mean

525   hemoglobin levels across treatments and days post-feed for mosquitoes that ingested blood

526   with or without ZIKV. Although levels did not differ immediately after bloodmeal ingestion on 0

527   dpf, mean hemoglobin digestion was significantly faster in No Abx and AbxA on 1 dpf (K-W test,

528    $P_{No\ Abx}$ = 0.0163, P_{AbxA} = 0.0009) but not AbxPA (K-W test, P = 0.25) in ZIKV-exposed

529   mosquitoes compared to mosquitoes that ingested blood without ZIKV (**Figure 3D**). These data

530   show that ablation of larval and pupal microbes reduce bloodmeal digestion, and that blood with

531   ZIKV is digested more quickly than blood lacking virus.

532

533   **Reduced bloodmeal digestion promotes susceptibility of *Ae. aegypti* ZIKV dissemination**

534   **out of the midgut**

535   We hypothesize that, in addition to disrupting midgut cell integrity, antibiotic mediated reduction

536   of microbes in mosquitoes via reduced bloodmeal digestion provides the virus more time to bind

537   and enter midgut epithelial cells, manifest as increased ZIKV infection and dissemination.

538   Increased magnitude of ZIKV dissemination in antibiotic-treated groups was confirmed by

539   measuring ZIKV RNA levels in legs/wings from mosquitoes from each treatment that ingested

540   ZIKV-spiked blood 3 days earlier. The rates of ZIKV dissemination were marginally higher in the

541   AbxPA group (33%) compared to the AbxA (16%) and No Abx (11%) groups (Fisher's exact

542   test, P = 0.06) (**Figure 3E**). In contrast to No Abx and AbxA, all ZIKV-infected individuals in

543   AbxPA also had disseminated infections. To determine whether bacteria with hemolytic activity

544   contribute to bloodmeal digestion in the No Abx groups, bacteria from 5 No Abx treated adult

97

545   mosquitoes were cultured on two types of agar and then Sanger sequenced. Although different

546   species of bacteria were detected, none showed hemolytic activity (**Table S1**), suggesting that

547   bloodmeal digestion is not directly mediated by bacteria in the mosquito midgut. Together, these

548   data show that microbes enhance the ability of *Ae. aegypti* to digest blood, likely via an indirect

549   mechanism since the culturable bacteria recovered from mosquitoes that were not antibiotic

550   treated were not hemolytic. The increased rate of blood digestion in mosquitoes with an intact

551   microbiome reduces ZIKV susceptibility.

552

553   **Microbial promotion of bloodmeal digestion and reduction in ZIKV susceptibility is**

554   **manifest across genetically distinct lines of *Ae. aegypti***

555

556   To evaluate whether our observations of impaired blood digestion and increased ZIKV

557   dissemination with antibiotic treatment are generalizable to *Ae. aegypti* from a different genetic

558   background than Los Angeles, CA, we performed the similar analyses with *Ae. aegypti* obtained

559   from Clovis, CA, where prior genome sequencing shows that the *Ae. aegypti* from Los Angeles

560   and Clovis are genetically distinct (37), and were probably derived from separate independent

561   introductions into California since 2013 when the species became established in the state. *Ae.*

562   *aegypti* from Clovis were reared under the same antibiotic treatments as for Los Angeles

563   mosquitoes. Blood digestion kinetics and ZIKV vector competence were then quantified in

564   Clovis mosquitoes (**Figure 4A**). As observed for mosquitoes from Los Angeles, AbxA and

565   AbxPA groups from Clovis demonstrated lower mean blood digestion relative to the No Abx

566   group 2 dpf (K-W test with multiple comparisons, adjusted $P_{AbxPA-No\ Abx}$ = 0.0013, adjusted P_{AbxPA-}

567    $_{AbxA}$ = 0.0271) (**Figure 4B**). Also consistent with vector competence data from Los Angeles

568   mosquitoes, the Clovis AbxPA group exhibited a marginally but not statistically significantly

569   higher infection rate (34%) than both the Clovis AbxA (26%) and No Abx (15%) groups (Chi-

570   square [χ^2] test, P = 0.106). The dissemination rate in the Clovis AbxPA group (26%) was

98

571   significantly higher than the No Abx (11%) and AbxA (10%) groups (χ^2, P = 0.04), although

572   mean RNA levels in ZIKV-positive individuals were not different for any treatment (**Figure 4C**).

573   These results from genetically distinct *Ae. aegypti* support the generalizability of our

574   observations, where microbes augment blood digestion and reduce ZIKV susceptibility in *Ae.*

575   *aegypti* from more than one genetic lineage.

576

577   **Antibiotic treatment, blood ingestion, and ZIKV exposure influence the *Ae. aegypti***

578   **transcriptome**

579

580   To identify gene expression differences underlying increased ZIKV susceptibility after antibiotic

581   treatment of pupae and adults, we employed RNA-Seq analyses on pooled adult female *Ae.*

582   *aegypti* from Los Angeles before bloodfeeding and 2 dpf. We also analyzed females that

583   ingested blood only or ZIKV-spiked blood (**Figure 5A**). On average, 94.5% (min: 92.7, max:

584   95.7%) of reads from all samples aligned at least once to the Liverpool *Ae. aegypti* (LVP_WG)

585   transcriptome. PCA showed tight clustering of mosquitoes prior to bloodfeeding distinct from

586   mosquitoes that ingested blood with or without ZIKV (**Figure 5B**, **Figure S3A**). While most of

587   the differences in gene expression related to bloodfeeding status, the presence of ZIKV in the

588   bloodmeal also yielded differentially expressed genes (DEGs) for each antibiotic treatment. At

589   baseline (without introduction of blood or ZIKV), 20 significant DEGs were found between

590   AbxPA and No Abx (**Table S2**). The most downregulated gene in AbxPA relative to No Abx was

591   uncharacterized (AAEL021374, adjusted P = 0.019), but other downregulated genes in AbxPA

592   included membrane components (AAEL020586, adjusted P = 0; AAEL003626, adjusted P =

593   0.047) and ATP binding proteins (AAEL019793, adjusted P = 0.026; AAEL010711, adjusted P =

594   0.005). After bloodfeeding, over 1/3 of total genes in the *Ae. aegypti* genome were differentially

595   expressed, consistent with other RNA-Seq studies (38). At 2 dpf, 7846 genes in No Abx, 9356

596   genes in AbxA, and 7846 genes in AbxPA were differentially expressed compared to sugar-fed

99

597 only. Among them, 6676 genes were shared among No Abx, AbxA, and AbxPA, representing an

598 overwhelming majority of the total DEGs (**Figure S3B**). Between ZIKV-bloodfed and bloodfed

599 only comparisons, differences were more visible. The AbxA and No Abx groups each had more

600 DEGs between ZIKV-bloodfed and blood only individuals (No Abx: 1258 DEGs, AbxA: 1974)

601 than AbxPA (62 DEGs) (**Figure 5C**, only DEGs that differed significantly across antibiotic

602 treatments are shown [adjusted P < 0.05 by Wald test]). Most shared DEGs were between the

603 AbxA and No Abx groups; AbxPA expressed a unique set of DEGs. Assignment of Kyoto

604 Encyclopedia of Genes and Genomes (KEGG) pathway identity (IDs) to DEGs identified many

605 unknown genes. Hypothetical protein products with no homology to genes with functional

606 annotations were common (No Abx: 526, AbxA: 792, AbxPA: 29), as were genes involved in

607 metabolic processes (No Abx: 371, AbxA: 599, AbxPA: 25) and thermogenesis (No Abx: 277,

608 AbxA: 452, AbxPA: 9) (**Figure 5D**). Taken together, these data suggest that bloodfeeding has a

609 larger effect than microbial exposure on *Ae. aegypti* gene expression. In ZIKV bloodfed

610 mosquitoes, antibiotic treatment of both pupal and adult stages reduces differential gene

611 expression more than treatment of adults only or no antibiotic treatment, indicating that pupal-

612 derived microbes have a greater effect on the gene expression profile than adult-derived

613 microbes.

614

615 Provided that microbes mediate immunomodulatory effects in mosquitoes (12,39,40), we

616 theorized their presence could influence ZIKV susceptibility by modifying the expression of

617 immune response pathways where some respond to both bacteria and viruses. We identified

618 immunity-related DEGs across antibiotic-treated mosquitoes 2 dpf in the RNA-Seq data.

619 Transcripts representing components of various immune pathways were accessioned from

620 existing literature and used to filter the dataset before analyses of DEG profiles. Representative

621 genes examined include regulators from Toll (41–43), RNAi (44–47), JAK-STAT (42,48,49), and

622 Imd pathways (50,51), as well as general immune and immune-adjacent genes such as

100

623   hormones (52–55), antimicrobial peptides (AMPs) (49,56), reactive oxygen species (ROS)

624   (57,58), and known DENV host factors (49,56). There were few DEGs demonstrating significant

625   changes in transcript abundance in immune pathways between AbxA and AbxPA, No Abx and

626   AbxA, or No Abx and AbxPA groups (**Figures 5E, S4**). Exceptions are gene CTP4H32

627   (AAEL007812, cytochrome p450 (38)) which was elevated in AbxA, and Unkn566

628   (AAEL000566, transmembrane protein in the JAK-STAT pathway (49)) which was elevated in

629   AbxPA (**Figure 5E**). Comparison of immune genes between No Abx and AbxA and between No

630   Abx and AbxPA, revealed a handful of genes that passed the statistical threshold; these are in

631   the JAK-STAT pathway (Ukn566 between No Abx and AbxA; Ukn566 and Ukn7703 between No

632   Abx and AbxPA) (**Figure S4**). These data suggest that gene expression changes after antibiotic

633   treatment in ZIKV-exposed *Ae. aegypti* that show different susceptibility to disseminated

634   infection are not related to known immune response pathways.

635

636   **Gene ontology assignments reveal the role of microbiota in stimulating expression of**

637   **protein digestion-associated genes in *Ae. aegypti* orally exposed to ZIKV**

638

639   Given that RNA-Seq revealed no major differences in expression of immune response genes

640   with antibiotic treatment, we next performed Gene Ontology (GO) analyses to predict enriched

641   functions in non-immune associated DEGs. We identified DEGs displaying significant changes

642   in abundance between the antibiotic treatments in mosquitoes that ingested ZIKV in blood or

643   that ingested blood only. The AbxA and No Abx groups that ingested ZIKV in blood showed

644   increased expression of genes enriched in general translation processes (AbxA: 125, adjusted

645    $P < 10^{-10}$; No Abx: 127, adjusted $P < 10^{-20}$) and proteolysis (AbxA: 84, adjusted P = 0.0006; No

646   Abx: 56, adjusted P = 0.015). In contrast, the AbxPA group showed few upregulated genes

647   resulting in a lack of enriched functions (**Figure 6A**). Conversely, enrichments in genes

648   downregulated in mosquitoes that ingested ZIKV in blood versus mosquitoes that ingested

101

649   blood only revealed that the ZIKV the AbxPA treatment group showed reduced transcript

650   abundance for genes associated with DNA metabolism (8) and redox processes (4). AbxA and

651   No Abx were depleted in genes for DNA replication (N=48 and 34, respectively) and DNA repair

652   (N=18 and 2, respectively). All treatment groups that ingested blood with ZIKV compared to

653   blood only exhibited downregulation of carbohydrate metabolism genes (AbxPA: N=8, adjusted

654   P = 0.006; AbxA: N=6, adjusted P = 0.0002; No Abx: N=16, adjusted P = 0.017) (**Figure 6B**).

655   These data suggest that microbiota during early ZIKV infection promote expression of genes

656   associated with proteolysis and translation while downregulating genes relating to DNA

657   synthesis.

658

659   The magnitude of change in DEGs in mosquitoes that ingested ZIKV in blood or blood only for

660   each antibiotic treatment were also compared. Gene AAEL008117, the most depleted transcript

661   in AbxA and No Abx groups (~20 $\log_2$-fold reduced), corresponds to nompC, a

662   mechanosensitive ion channel that senses balance and touch in *Drosophila melanogaster* (59).

663   The most common DEGs that changed in response to ZIKV exposure matched to proteolytic

664   and PM-associated enzymes, including endopeptidases, collagenases, and dehydrogenases

665   (60). Multiple serine-type endopeptidases were differentially expressed depending on antibiotic

666   treatment (No Abx: AAEL001693, all three: AAEL008769, AbxA, and AbxPA: AAEL013284,

667   AAEL001690) (**Figure 6C**). In addition, some vitellogenesis associated proteins were more

668   enriched in antibiotic treated mosquitoes compared to the No Abx group (AbxA and AbxPA:

669   AAEL014561 [vitelline membrane protein 15a-3], AbxPA: AAEL013027 [vitelline membrane

670   protein 15a-1]). In AbxPA, the gene demonstrating the biggest reduction in transcript abundance

671   corresponds to a m1 zinc metalloprotease (AAEL003012), showed increased expression in the

672   No Abx group. Overall, GO analyses revealed *Ae. aegypti* with disrupted microbiota are

673   deficient in genes that influence blood protein metabolism.

674

675 **Discussion**

676

677 In this study, we found that microbial exposure in two genetically distinct populations of *Ae.*

678 *aegypti* facilitates midgut integrity and enhanced bloodmeal digestion. Together these effects

679 reduce ZIKV infection of the midgut epithelium and result in less efficient dissemination out of

680 the midgut (**Figure 7**). Reduced microbial colonization during both pupal and adult *Ae. aegypti*

681 life stages, together with higher bloodmeal titers, produced the largest increases in

682 susceptibility. These findings indicate that microbial exposure during the aqueous pupal stage of

683 mosquito development is most important for conditioning susceptibility to ZIKV. These data

684 show that larval microbial exposure confers developmental resistance to ZIKV dissemination out

685 of the midgut by reinforcing midgut integrity after bloodfeeding.

686

687 Augmented rates of ZIKV infection and dissemination but not transmission in *Ae. aegypti* after

688 antibiotic treatment suggest that microbial effects act at the midgut infection and escape barriers

689 but not at salivary gland infection or escape barriers. Hematophagous feeding introduces a

690 unique problem in that engorgement immensely distends the midgut. Midgut distention can

691 result in breaches in the integrity of the epithelial barrier, presenting an opportunity for a virus

692 ingested with blood to bypass infection of the epithelium. Prior studies detected bloodmeal-

693 induced perforations in the midgut basal lamina, which do not fully recover after digestion,

694 supporting a process where successive bloodmeals increase rates of ZIKV dissemination (61).

695 Similar studies also found that ZIKV disseminates from the midgut after digestion is complete,

696 which contrasts with the alphavirus CHIKV, which disseminates during digestion (62).

697 Considering that we observed increased magnitude of and rate of bloodmeal digestion in ZIKV-

698 exposed mosquitoes without antibiotics compared to antibiotic-treated pupae and adults, we

699 envision a process where microbes accelerate digestion, decreasing the window of time the

700 virus has to escape the bloodmeal, traverse the PM, and access the midgut epithelium, which

103

701   results in reduced ZIKV dissemination out of the midgut. We did not observe gross structural

702   differences in the midgut epithelium with 10-40X DAPI and H&E staining, so we theorize that

703   microbes do not impact midgut permeability by physically modifying the midgut epithelium. We

704   acknowledge that increased midgut permeability may also allow for extracellular ZIKV traversion

705   of the midgut epithelium, a process we did not measure in these studies.

706

707   Given that there are conserved elements of mosquito antiviral defenses and gut microbial

708   homeostasis, including immune signaling and off-target suppression by ROS and AMPs (11,39),

709   we were initially surprised that gene expression analyses did not detect differences in antiviral

710   immune responses in response to antibiotic treatment. Rather, our RNA-Seq analyses confirm

711   prior observations that show protein digestion and vitellogenesis dominate mosquito

712   physiological processes after bloodfeeding (38). Our results are also similar to another

713   transcriptomic study which found immense upregulation of proteases and trypsins 2 days post-

714   ZIKV exposure (63). It stands to reason that blood digestion and vitellogenesis are hampered

715   without the presence of microbial symbionts. Midgut symbionts may also indirectly reduce

716   mosquito vector competence in multiple ways. These include supplementation of larval nutrition

717   and development to increase mosquito fitness (9,64–67), stimulation of bloodfeeding behavior

718   resulting in larger bloodmeals (although bloodmeal size differences were not observed in our

719   studies) and increased nutrition (68), and regulation of midgut epithelial cell proliferation, where

720   the physical microbe-midgut interface can block virus access to midgut infection (69) [although

721   this may be pathogen specific as increased midgut infection has also been observed (21,70)].

722

723   Both microbes and ZIKV in bloodmeals resulted in increased blood digestion relative to

724   antibiotic treated mosquitoes that ingested blood without virus. It is unclear why ZIKV enhances

725   digestion; this may result from the virus modulating cell metabolism to benefit viral replication, a

726   pattern shown for many viruses (71). In human cells, for example, infection with DENV elevates

727   glycolysis to meet cellular energy needs (72). Conceivably, a similar trend is true for ZIKV-

728   infected mosquito midgut cells, where increased energy demand produced by viral infection is

729   met by increasing blood metabolism. Absent ZIKV in the bloodmeal, antibiotic-treated

730   mosquitoes exhibited digestive deficiencies during late digestion, defined here as beyond 2 dpf.

731   Since blood ingestion is followed by rapid proliferation of midgut microbes that results from a

732   heme-mediated reduction in ROS (73), the delayed digestion kinetics in antibiotic treated

733   mosquitoes could be influenced by the near absence of microbes to promote digestion. Since

734   blood digestion occurs in 2 phases where disparate enzymes dominate each phase (74), it is

735   also possible that the digestive enzymes that have different efficiencies were differentially

736   expressed across antibiotic treatments. We observed late trypsins and serine-type

737   endopeptidases (enzyme types commonly found in the latter phase of digestion) (74–77) in all

738   bloodfed antibiotic groups at 2 dpf; greater resolution of how digestion enzymes change over

739   time is warranted to test this hypothesis. While over 300 serine protease-like proteins have been

740   described in *Aedes spp.* (78,79), very few have been studied experimentally. One study

741   implicated matrix metalloproteases and collagenase activity in degrading the basal lamina,

742   which increased dissemination of CHIKV from the midgut (80). Our finding of a downregulated

743   metalloprotease (AAEL003012) in mosquitoes with disrupted microbiota as pupae and adults,

744   as well as an elevated serine collagenase precursor (AAEL007432) in mosquitoes that ingested

745   ZIKV in blood supports a role of digestive enzymes in promoting arboviral dissemination. The

746   potential interaction between blood protein metabolism and arboviral infection of mosquitoes

747   warrants further study.

748

749   Whether blood digestion genes, including those that are differentially upregulated in mosquitoes

750   exposed to antibiotics, can also modulate mosquito immune responses is currently unclear. A

751   link between metabolism and immunity is possible (81). Some proteases have immune

752   functions (82,83), including cleavage of proenzymes that activate the prophenoloxidase

105

753   pathway required for melanization (84). Metabolites influence and regulate immune pathways

754   like Toll and Imd, and metabolic regulators balance microbial resistance and tolerance to gut

755   symbionts in arthropods, including mosquitoes (63,81). In *Ae. aegypti*, blood-derived heme

756   induces iron- and ROS-dependent signaling that also regulates immune genes, affecting DENV

757   susceptibility (85). Metabolism of *Bacteroides* associates with elevated ZIKV susceptibility and

758   conditions insecticide resistance (86), implicating a complex metabolic network that can also

759   influence arbovirus susceptibility. Further investigation of the relationship between blood

760   digestion and immunity could uncover novel determinants of mosquito vector competence.

761

762   Determining how microbes modulate the susceptibility of mosquitoes to arboviruses can help

763   identify approaches to reduce transmission and decrease human disease. Microbial effects may

764   also explain variability in vector competence across laboratories and geographic origins where

765   each has different microbial communities (87), together with conventionally acknowledged

766   factors like inbreeding during colonization (88,89). Our previous work, together with a growing

767   body of mosquito microbiome studies, suggests that mosquito-microbe-virus interactions are

768   environmentally variable and warrant investigation in more field-relevant contexts (14,18,67,90).

769   A limitation of our studies is the use of a single microbial community and ZIKV strain. We

770   acknowledge that disparate microbial species and community structures and other ZIKV strains

771   could impact susceptibility differently. We also acknowledge that unculturable bacterial species

772   could potentially play a role in mediating hemolytic activity that could have impacted blood

773   digestion assays. Our use of antibiotics, which incompletely eliminates microbiota, most closely

774   represents a dysbiotic phenotype. Newer tools including auxotrophic symbionts (91) and axenic

775   mosquitoes (9) can be used to further investigate the metabolic roles of microbes in influencing

776   arboviral infection of mosquitoes. Future studies of infection dynamics at the microbe-mosquito

777   midgut interface could prove useful in reconciling disparate vector competence outcomes and

778   discovering novel vector control approaches.

106

779

789

790

791   **Author Contributions**

792   Conceptualization: WL, LLC

793   Data Curation: WL, LKM, ETK

794   Formal Analysis: WL, LKM, ETK

795   Funding Acquisition: WL, LLC

796   Investigation: WL,

797   Methodology: WL, ALR, LLC, GMA

798   Project Administration: LLC

799   Resources: LLC

800   Supervision: LLC, GMA

801   Validation: WL

802   Visualization: WL, ALR, LLC

803   Writing-Original Draft: WL

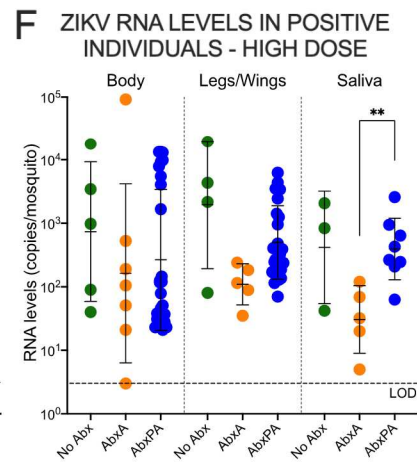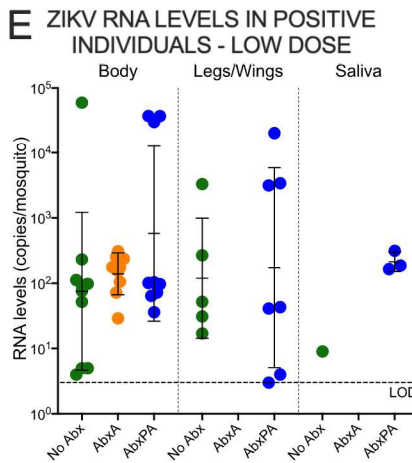804   Writing-Reviewing and Editing: WL, ALR, LLC, LKM, ETK, GMA

A

L4 larvae — N = 5 — Pupae (before Abx) — N = 5 — Adults (3-5 dpe) — N = 5, 10, 10 — DNA extraction — 16S qPCR — Body

Los Angeles, CA *Ae. aegypti* — Environmental microbes — pick pupae — diH₂O / Antibiotics (Abx) diH₂O — eclose — sucrose — No Abx (no antibiotics) / AbxA (antibiotics to adults) / AbxPA (antibiotics to pupae) — LOW 3.2 ×10⁴ PFU/mL / HIGH 4.8 × 10⁶ PFU/mL — ZIKV RT-qPCR — Body — Legs/Wings — Saliva

Rear larvae | Pupate in diH₂O +/- Abx | Incubate + / +/- | Blood feed + incubate | Vector competence
Larvae | Pupae | Adults 3-5 dpe | Adults 14 dpf

B VERIFICATION OF ANTIBIOTIC EFFICIENCY

16S:RPS17 ratio

**** ** ** **

No Abx (adult), L4 larva, Pupa, AbxA, AbxPA (adult) — LOD

C VECTOR COMPETENCE RATES

BODIES — Infection Rate (%): No Abx 13% / 28%, AbxA 26% / 39%, AbxPA 39% / 87% — * ***

LEGS / WINGS — Dissemination Rate (%): No Abx 11% / 22%, AbxA 0% / 33%, AbxPA 25% / 87% — ** ***

SALIVA — Transmission Rate (%): No Abx 4% / 17%, AbxA 0% / 28%, AbxPA 11% / 27% — ns ns

Generation	Group	ZIKV dose (PFU/mL)	Infection Rate	Dissemination Rate	Transmission Rate	Infection Sig. (P)	Dissemination Sig. (P)	Transmission Sig. (P)
F₂₀	No Abx	3.2 x 10⁴	13% (6/45)	11% (5/45)	4% (2/45)	0.0398	0.0081	0.1376
	AbxA		26% (9/34)	0% (0/34)	0% (0/34)			
	AbxPA		39% (11/28)	25% (7/28)	11% (3/28)			
F₂₂	No Abx	4.8 x 10⁶	28% (5/18)	22% (4/18)	17% (3/18)	< 0.0001	< 0.0001	0.6767
	AbxA		39% (7/18)	33% (6/18)	28% (5/18)			
	AbxPA		87% (26/30)	87% (26/30)	27% (8/30)			

D CHANGE IN INF/DIS/TRAN RATE BY DOSE

Change in rate — No Abx: Body 15, L/W 11, Saliva 13; AbxA: Body 13, L/W 33, Saliva 28; AbxPA: Body 48, L/W 62, Saliva 16

E ZIKV RNA LEVELS IN POSITIVE INDIVIDUALS - LOW DOSE

RNA levels (copies/mosquito) — Body, Legs/Wings, Saliva — No Abx, AbxA, AbxPA — LOD

F ZIKV RNA LEVELS IN POSITIVE INDIVIDUALS - HIGH DOSE

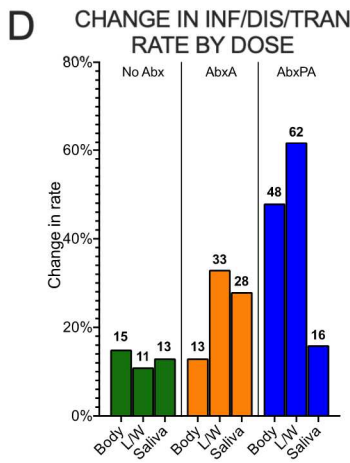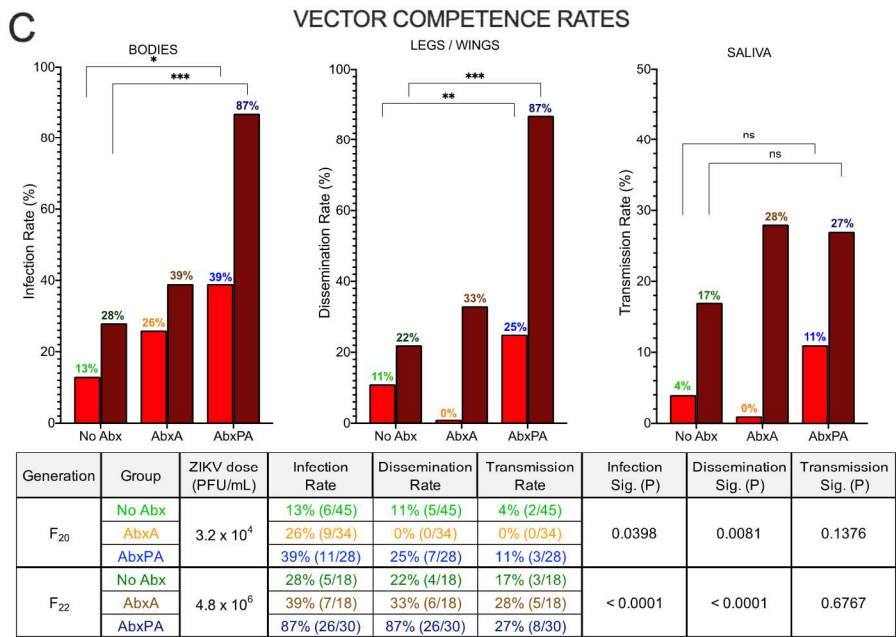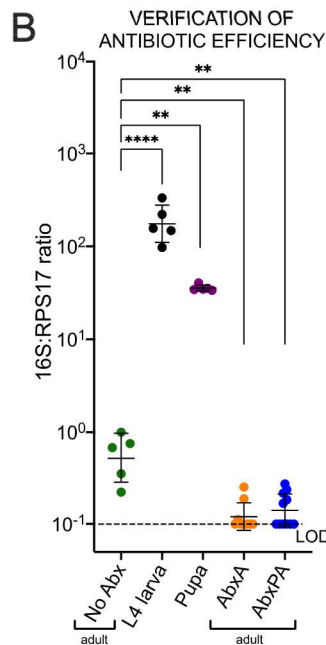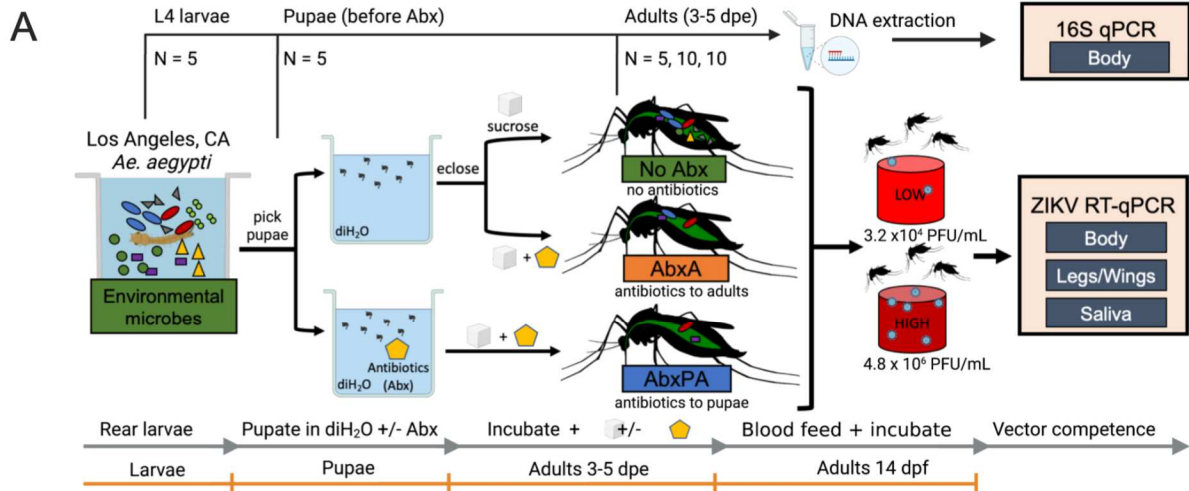RNA levels (copies/mosquito) — Body, Legs/Wings, Saliva — No Abx, AbxA, AbxPA — ** — LOD

Figure 1. Antibiotic treatment of *Ae. aegypti* pupae and adults from Los Angeles, California, increases ZIKV susceptibility. A) Experimental design. Microbes from cemetery headstones were added to tap water during larvae rearing. Pupae in the antibiotic (Abx) groups were treated by adding an antibiotic cocktail to pupal water. No antibiotics (No Abx) groups received no antibiotics. Antibiotics were delivered orally in sugar to adult Abx (AbxA) groups; the No Abx group received sugar only. Mosquitoes from each group and life stage were sequenced to verify the efficacy of antibiotic treatment. Adult mosquitoes were presented with either of 2 doses of ZIKV spiked into bloodmeals, and ZIKV vector competence was assessed by measuring ZIKV RNA levels in bodies to determine infection, legs/wings to assess dissemination, and in saliva to measure transmission, which were measured as rates in cohorts and mean levels from individuals. **B) Antibiotic treatment reduces bacterial DNA in mosquitoes.** Bacteria was quantified in mosquitoes by 16S qPCR. Each point represents a single mosquito (N larvae, pupae, No Abx = 5; N AbxA, AbxPA = 10). 16S copies were normalized to the *Ae. aegypti* RPS17 gene. The limit of detection (LOD) was a 0.1 16S:RPS17 ratio; below this value, 16S was not detected. Statistical significance across all groups was determined by a Kruskal-Wallis test with multiple comparisons. **C) Vector competence of antibiotic treated mosquitoes for ZIKV.** Bar graphs show ZIKV infection, dissemination, and transmission rates in individual mosquitoes as determined by ZIKV RNA RT-qPCR. The summary table shows the same data as in the bar graphs. Rates were calculated as the number of bodies, legs/wings, or expectorates, respectively, that yielded at detectable ZIKV RNA, divided by the total number of individuals that ingested blood within a cohort. Statistical significance across all treatments was determined by Fisher's exact test. Vector competence experiments were conducted once. **D) Dose response to infection, dissemination, and transmission. Differences in** infection, dissemination, and transmission rates after ingestion of high versus low dose ZIKV bloodmeals (data from panel C). **ZIKV RNA levels in mosquito tissues after ingestion low (E)** and **high (F) dose ZIKV-spiked bloodmeals**. Each dot shows

the mean value for an individual mosquito based on triplicate measurements. The average LOD across all experiments was 3 copies per mosquito. For B, D, and E, geometric means are shown, and error bars show standard deviations among positive individuals. *denotes $P < 0.05$ **denotes $P < 0.01$ Kolmogorov-Smirnov tests were used to compare RNA levels across antibiotic groups.
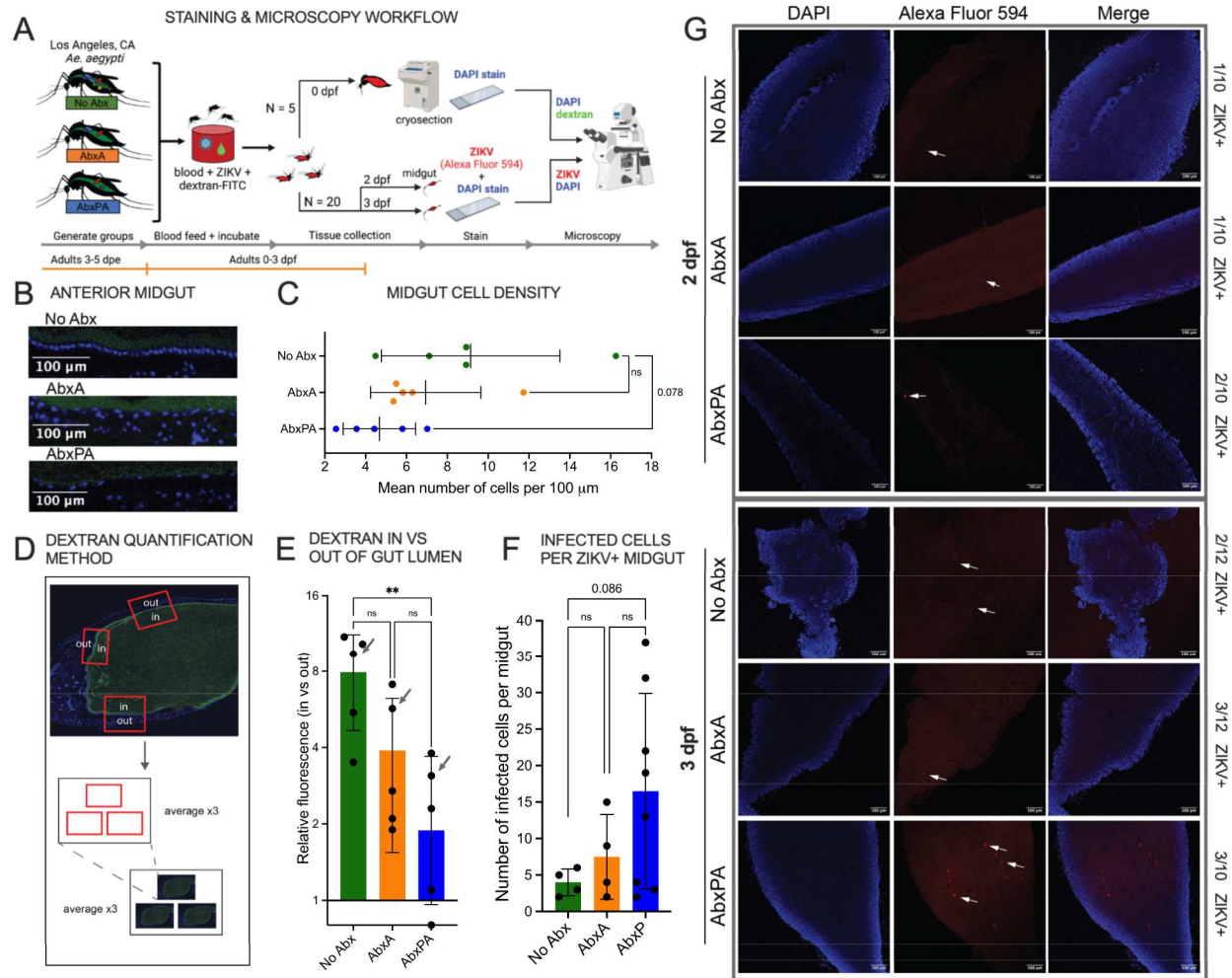
Figure 2. Antibiotic treatment modifies the structure and increases permeability and ZIKV infection of the *Ae. aegypti* midgut. A) Schematic of bloodfeeding experiment and sample preparation for immunofluorescence microscopy. Bloodmeal contained 1 µg dextran-FITC (green) and 3.3 x 10^6 PFU/mL ZIKV. Whole mosquitoes were harvested 0 days post-feed (dpf), cryosectioned, DAPI stained, and examined using fluorescence microscopy. Midguts from the same cohort were harvested at 2, and 3 dpf, and ZIKV antigen and DAPI-stained before fluorescence microscopy. **B) Antibiotic treatment disrupts regularity of DAPI staining in midgut epithelium.** Anterior-zoomed cross sections of whole, *Ae. aegypti* at 0 dpf about 30 to 60 minutes post-engorgement visualized at 10X magnification. **C) Antibiotic treatment of pupae and adults reduces midgut cell density.** Cell density was calculated as the number of

cell nuclei (blue) per 100 µm length of gut. Each symbol represents one mosquito. **D) Dextran fluorescence quantification method** that was used to generate data in panel E. Fluorescence ratios were calculated in triplicate randomly selected areas in a single midgut slice, and each point represents the average of three slices in a single mosquito. **E) Antibiotic treatment of pupae and adults increases midgut permeability, evidenced by reduced relative dextran inside versus outside the gut lumen**. Dextran permeation was calculated as the ratio of fluorescence intensity inside the gut lumen to fluorescence intensity outside the gut lumen. Each symbol represents 9 measurements from one mosquito. Grey arrows indicate the individual represented in panel B. **F) Antibiotic treated pupae and adults have more ZIKV-infected midgut epithelial cells**. The number of ZIKV-infected cells, as determined by ZIKV antigen staining, were counted for each ZIKV-positive midgut on 2 and 3 dpf. Data from both days is shown. Each symbol represents one mosquito. **G) Antibiotic treated ZIKV-positive midguts at 2 and 3 dpf.** Midguts examined under immunofluorescence at 10X magnification, at 2 and 3 dpf. Cell nuclei (blue) and ZIKV antigen (red) are shown with the combined (merged) channels. Images reflect representative observations for each antibiotic treatment. Lines show means and error bars show standard deviations. * denotes $P < 0.05$, ns is not significantly different at $p < 0.05$.
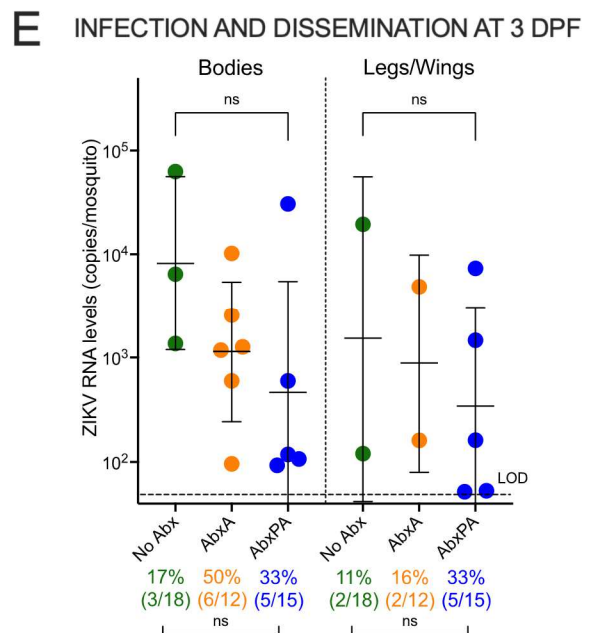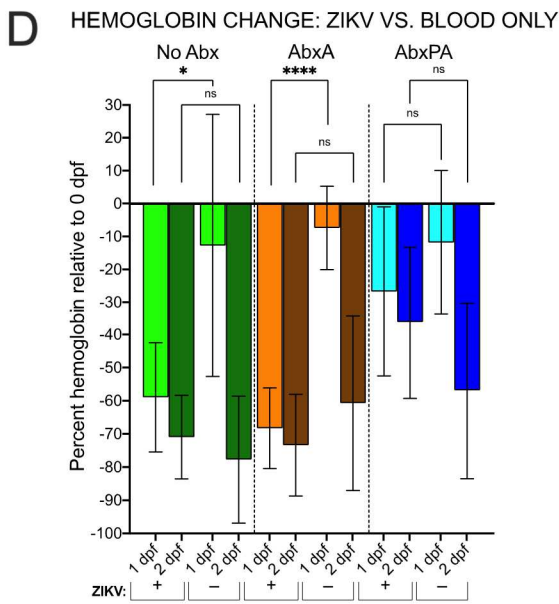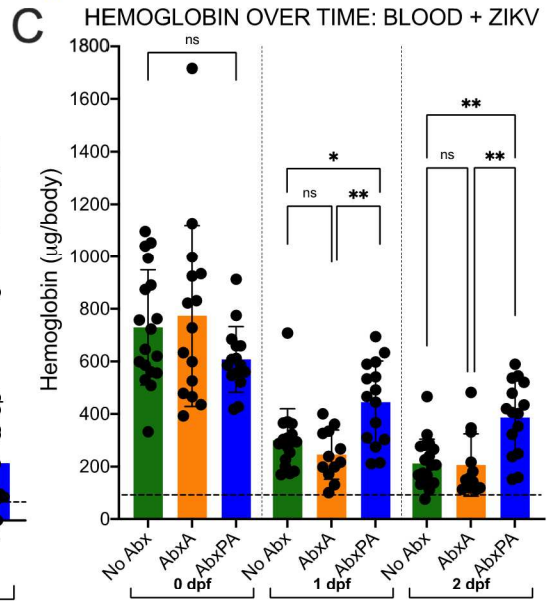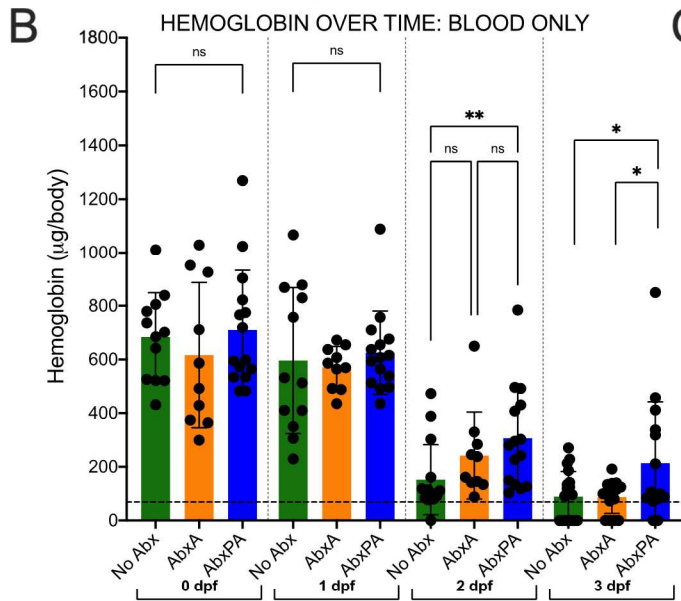
A HEMOGLOBIN ASSAY WORKFLOW

Los Angeles, CA
Ae. aegypti

No Abx
AbxA
AbxPA

sheep blood
0 dpf   N = 10-12
1 dpf   N = 10-12
2 dpf   N = 10-12
3 dpf   N = 10-12

sheep blood + ZIKV
0 dpf   N = 12-15
1 dpf   N = 12-15
2 dpf   N = 12-15

homogenize + heat inactivate

hemoglobin assay

Generate groups | Blood feed + incubate | Whole body collection | Virus inactivation | Digestion assay
Adults 3-5 dpe | Adults 0-3 dpf

B HEMOGLOBIN OVER TIME: BLOOD ONLY

C HEMOGLOBIN OVER TIME: BLOOD + ZIKV

D HEMOGLOBIN CHANGE: ZIKV VS. BLOOD ONLY

E INFECTION AND DISSEMINATION AT 3 DPF

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Figure 3. Bloodmeal digestion kinetics in antibiotic treated *Ae. aegypti*. A) Experimental design for detection of hemoglobin in homogenized antibiotic treated mosquitoes that fed on blood with or without ZIKV. B) Despite ingesting equivalent bloodmeal sizes, antibiotic treatment reduces blood digestion in the absence of ZIKV at 2 and 3 dpf. *Ae. aegypti* that fed on blood (N: No Abx = 12, AbxA = 10, AbxPA = 15 per day) were assayed 0, 1, 2 and 3 dpf. **C) Despite ingesting equivalent bloodmeal sizes, antibiotic treatment reduces blood digestion with ZIKV at 1 and 2 dpf.** *Ae. aegypti* that fed on blood spiked with 2.1×10^6 PFU/mL ZIKV (N: No Abx = 18, AbxA = 12, AbxPA = 15 per day) were assayed 0, 1 and 2 dpf. **D) Mosquitoes not treated with antibiotics or treated only as adults that ingested ZIKV show increased blood digestion compared to mosquitoes treated with antibiotics as pupae and adults.** The bar graphs show the percent reduction in hemoglobin at 1 and 2 dpf compared to the 0 dpf, calculated for all Abx groups and between ZIKV challenged and unchallenged groups. Bars show mean and error bars indicate the standard deviation (raw values are shown in panels B and C). **E) Antibiotic treatment of pupae and adults results in higher ZIKV dissemination rates 3 dpf.** Legs and wings of individual bloodfed females from the same cohort were assayed by ZIKV RT-qPCR at three dpf. * denotes $P < 0.05$; ** denotes $P < 0.01$; ns is not significantly different. Each symbol in B, C and E shows the mean value for 1 mosquito.
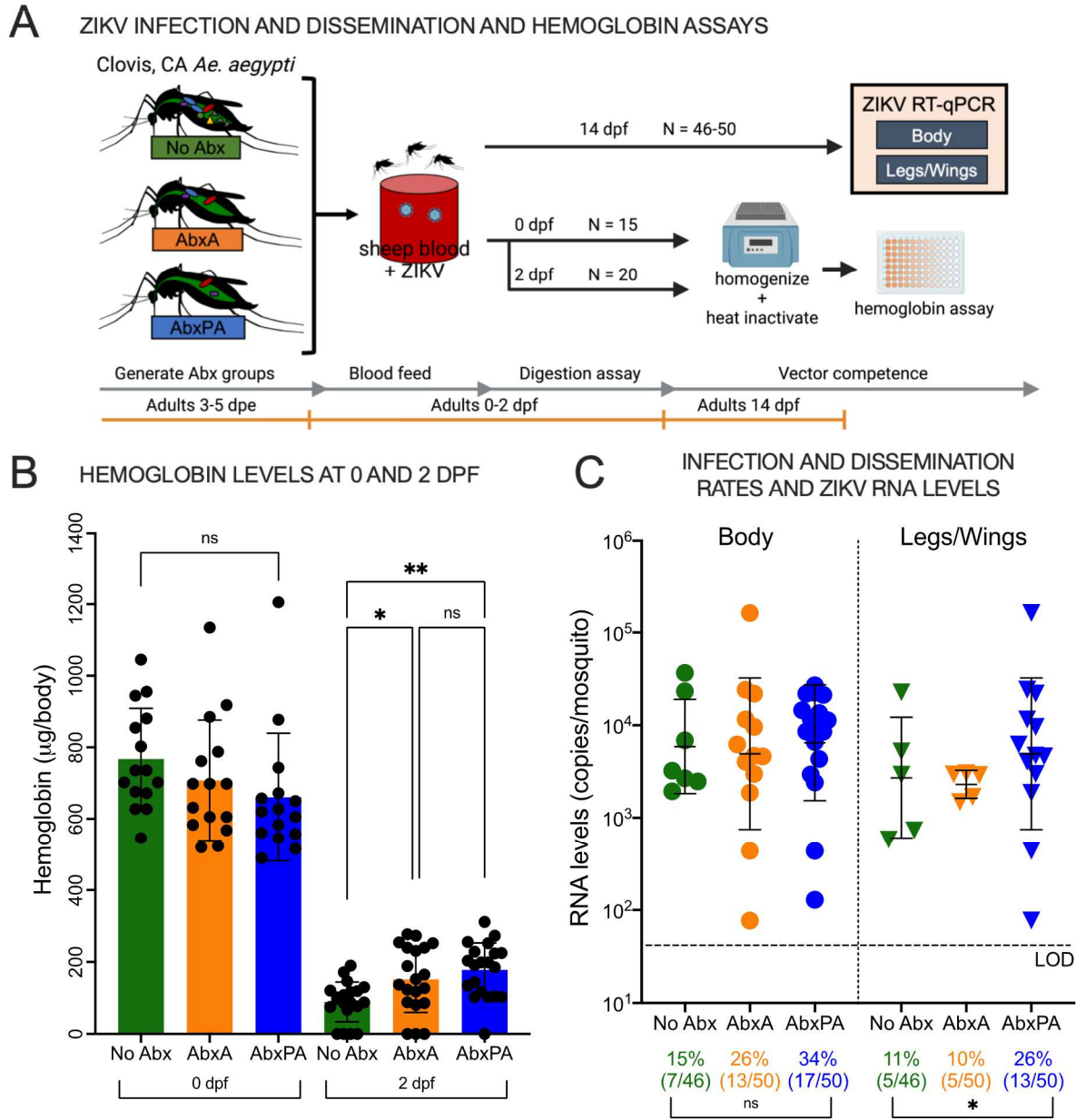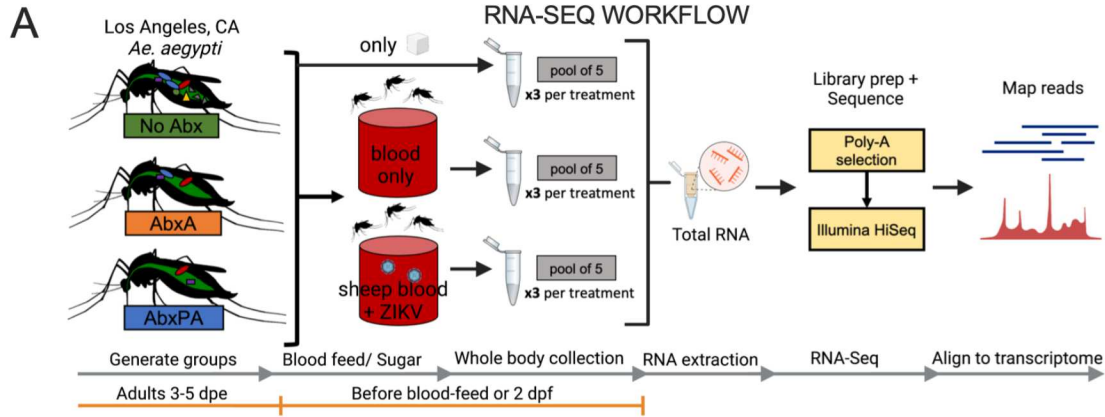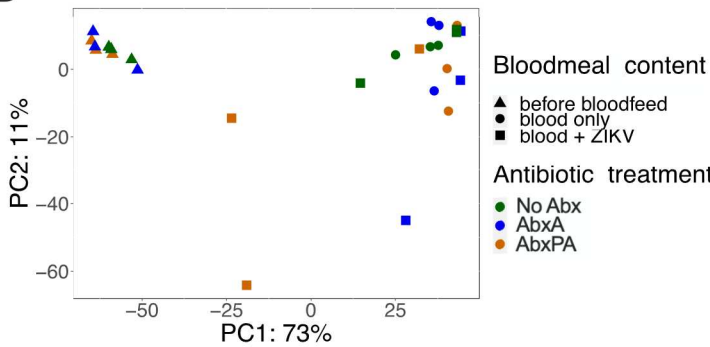
Figure 4. Antibiotic treatment of *Ae. aegypti* pupae and adults from Clovis, California, decreases bloodmeal digestion and increases ZIKV susceptibility. A) Experimental design using the same assays as in Figures 1 and 3 applied to an *Ae. aegypti* colony from Clovis, CA. **B) Despite ingesting equivalent bloodmeal sizes, antibiotic treatment reduces blood digestion with ZIKV at 2 dpf in Clovis mosquitoes.** Error bars show the means and standard deviations (N = 15 for each treatment per day). **C) Antibiotic treatment of pupae and adults results in higher ZIKV dissemination rates at 14 dpf in Clovis**

mosquitoes. ZIKV RNA levels from bloodfed Clovis *Ae. aegypti* at 14 dpf. Error bars denote the

geometric means and standard deviations. The dotted line denotes the average LOD at 62 RNA

copies/mosquito. The number of positives per number exposed is listed under the x-axis.

Samples under the LOD included in rate measurements are not shown in panel C. (* is $P < 0.05$;

** is $P < 0.01$; ns is not significantly different). Each symbol in B and C shows the mean value for
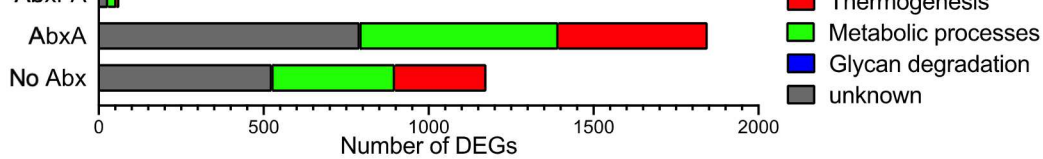
1 mosquito.

A — RNA-SEQ WORKFLOW

B — PCA: BLOOD MEAL STATUS & ABX TREATMENT

C — DEGs: ZIKV vs. BLOOD ONLY
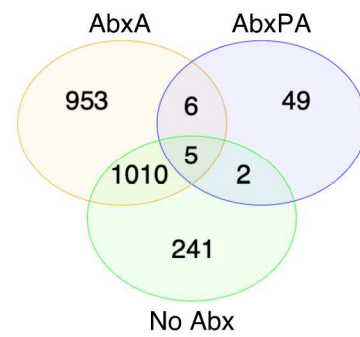
D — KEGG ASSIGNMENT OF DEGs

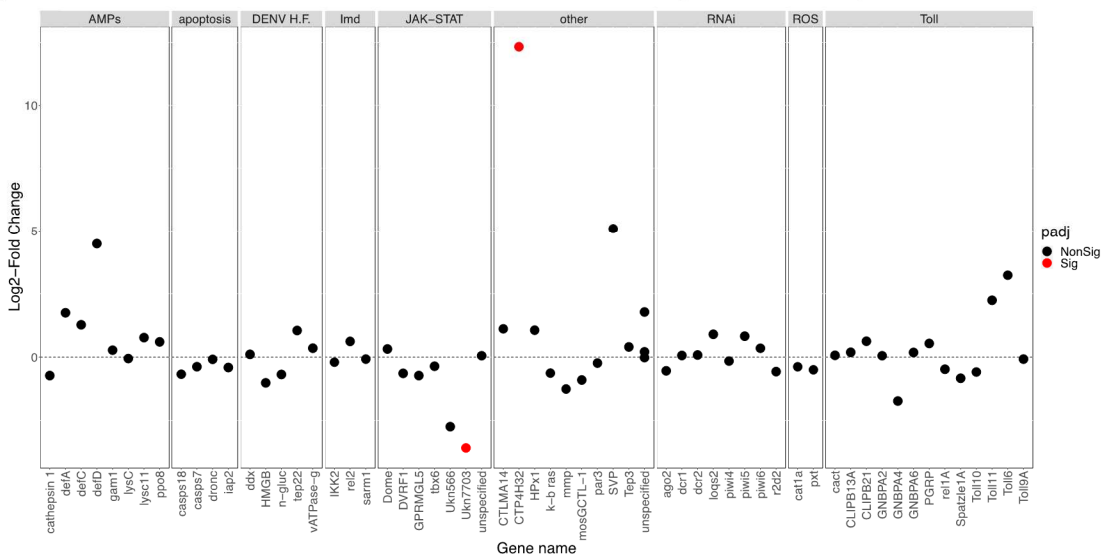**E** — LACK OF IMMUNE GENES AMONG DEGs (AbxA vs. AbxPA)

Figure 5. Differentially expressed genes between antibiotic-treated adult *Ae. aegypti* females before and after a bloodmeal. A) Experimental design showing *Ae. aegypti* from Los Angeles, CA, pooling and RNA sequencing approach. For each antibiotic treatment and each bloodfeeding state, triplicate pools of 5 whole, legless mosquito bodies were RNA-extracted, pre-processed, and sequenced. **B) Transcriptome profile differs more by bloodmeal status than by antibiotic treatment.** PCA plot after variance stabilized transformation. Shapes indicate the bloodmeal status while colors represent the antibiotic treatments. Separate PCAs for individual variables, for ease of visibility, are shown in Figure S3. **C) ZIKV-responsive DEGs are shared among between *Ae. aegypti* not antibiotic-treated and antibiotic-treated as adults.** Venn diagram of DEGs before and after bloodfeeding by *Ae. aegypti* for each antibiotic treatment. **D) DEGs correspond to metabolic processes, thermogenesis, and unknown genes for all antibiotic treatments.** KEGG Pathway ID assignment of DEGs in panel C. **E) DEGs between mosquitoes treated with antibiotics as pupae and adults compared to as adults only and ZIKV-bloodmeals or blood only do not correspond to known immune response genes.** Comparison of immune response gene expression between AbxA and AbxPA groups. Relative expression of select known and suspected immune-related genes from relevant literature (41–50,53–58). Expression is higher in AbxA if the $\log_2$-fold change is positive, while expression is higher in AbxPA if $\log_2$-fold change is negative. Points are considered significant (red) if the adjusted P value is < 0.01. Comparisons of the same genes between AbxA or AbxPA and the control treatment, No Abx, are shown in Figure S4.
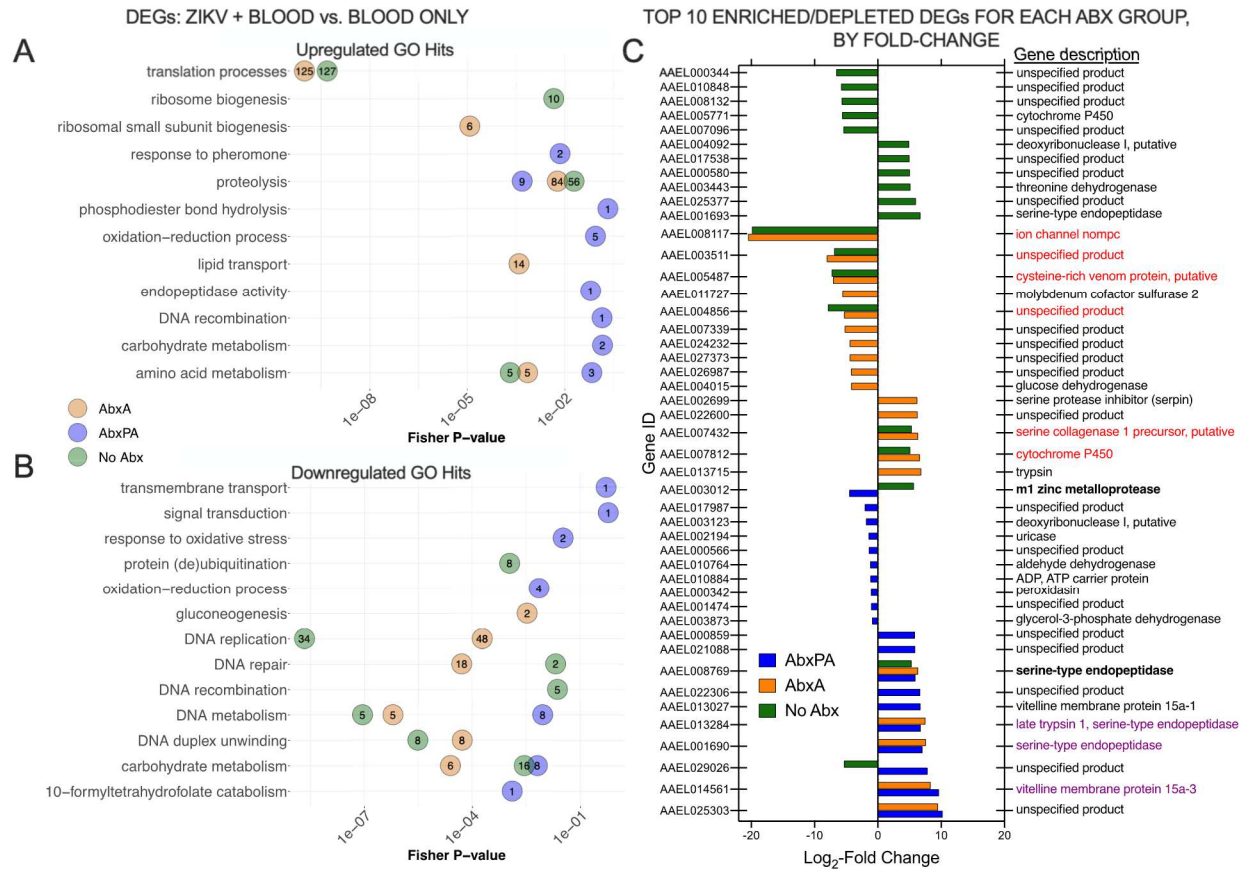
Figure 6. Gene Ontology assignment of DEGs and identity of most enriched/depleted genes. Gene Ontology (GO) analysis of significant DEGs comparing antibiotic treatment mosquitoes that ingested ZIKV in bloodmeals versus blood only. **A) Upregulated GO hits for mosquitoes treated with antibiotics as adults or untreated primarily map to general translational processes and proteolysis.** Mosquitoes treated with antibiotics as pupae and adults demonstrate a reduced complement of proteolysis associated genes, and **B) GO enrichments in genes showing reduced transcript abundance for all antibiotic treatments primarily map to DNA synthesis and repair mechanisms.** GO terms for the DEGs (determined from Figures 5C-D) and their descriptions on the y-axis, and statistical significance (P-values, Fisher's exact test) of the GO term on the x-axis, with the exact number of DEGs within the colored circles. DEGs farther to the left (lower P-values) are more significant. **C) ZIKV-responsive DEGs demonstrating the highest magnitude fold change are distinct**

between AbxPA and No Abx treatment groups. The top 10 upregulated and top 10 downregulated DEGs, by magnitude $\log_2$-fold change for each antibiotic treatment. A positive $\log_2$-fold change refers to more transcripts after a ZIKV-spiked bloodmeal, while a negative value refers to fewer transcripts. The right y-axis is the gene description from annotations retrieved from transcriptome metadata as well as VectorBase (32). Red text indicates the top genes shared between No Abx and AbxA. Purple text indicates the top genes shared between AbxPA and AbxA.
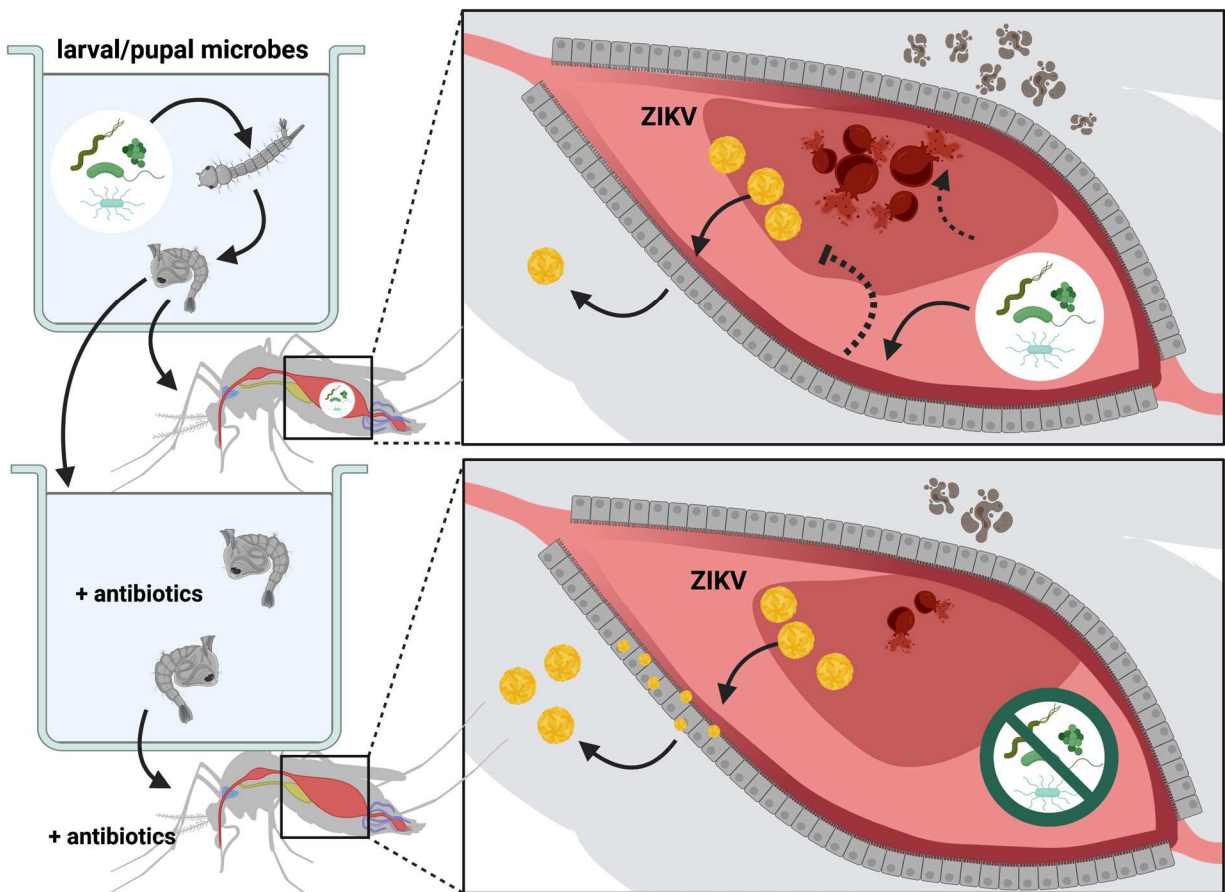

Figure 7. Working model of *Ae. aegypti* microbiota effects on ZIKV dissemination.
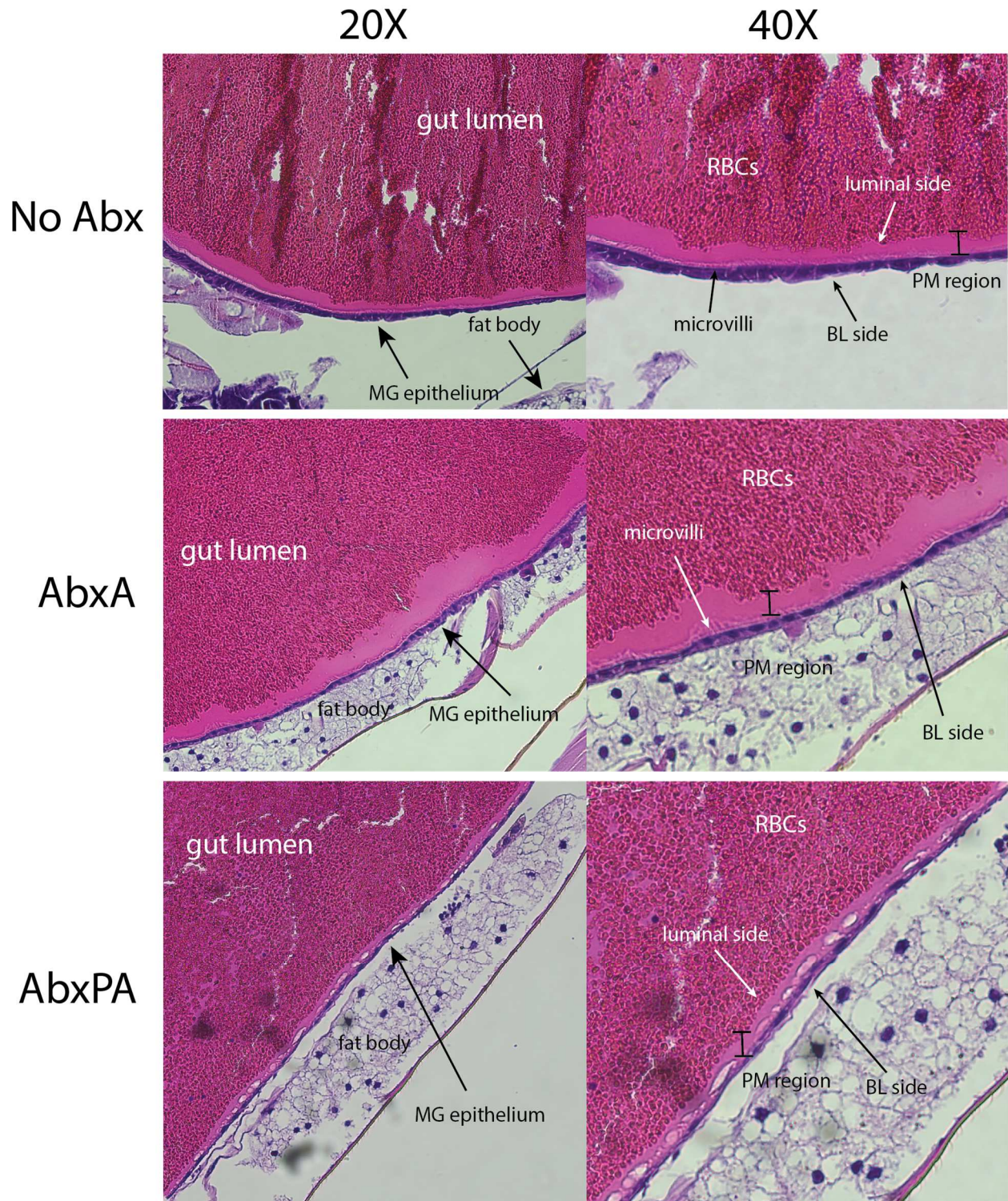
Figure S1. Histology of bloodfed female midguts. Cross sections of whole, bloodfed female

Ae. aegypti along the median sagittal plane. H&E staining and bright field microscopy with a

focus on the blood bolus perimeter was used to assess and identify potential structural artifacts

during sample preparation that could confound immunofluorescence observations. Both 20X and 40X magnification of the same section are shown side-by-side. MG epithelium = midgut epithelium. PM region = peritrophic matrix region (where the fully formed PM would be seen). RBCs = red blood cells, from bloodmeal.
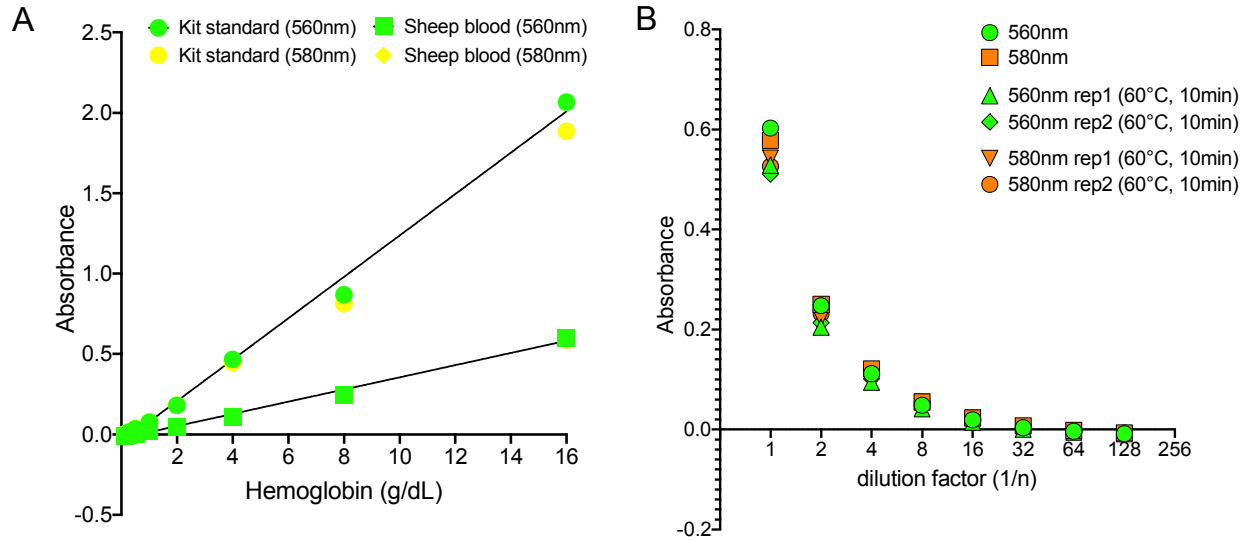

Figure S2. Validation of hemoglobin colorimetric assay kit for bloodfed mosquitoes. A) Serial 1:2-fold dilutions of heparinized sheep blood were tested in parallel with the kit hemoglobin standard at the absorbance extremes of 560nm and 580nm. Lines indicate standard curves generated from linear regression that were used in the hemoglobin assays. **B)** Absorbance values of sheep blood hemoglobin after heat-treating at 60°C for 10 minutes in duplicates (rep = replicate).

A
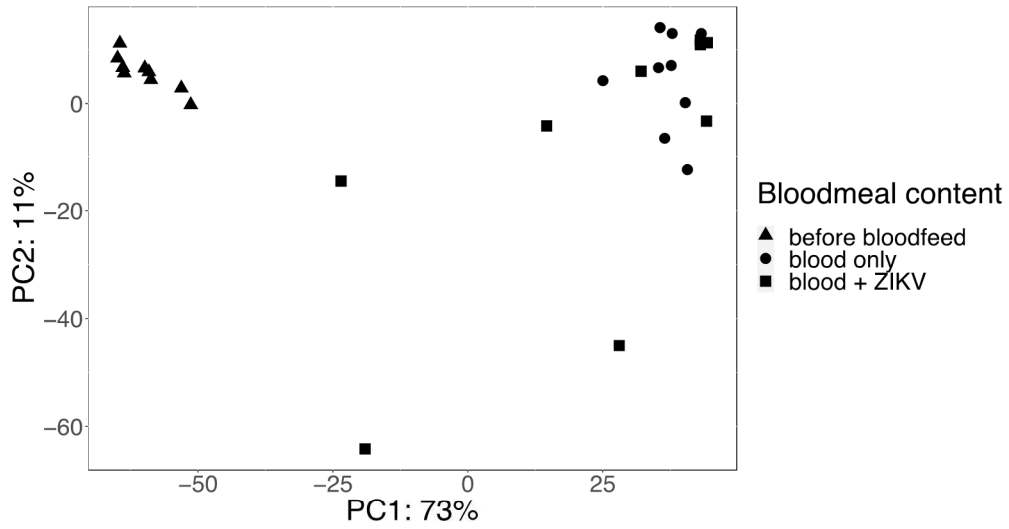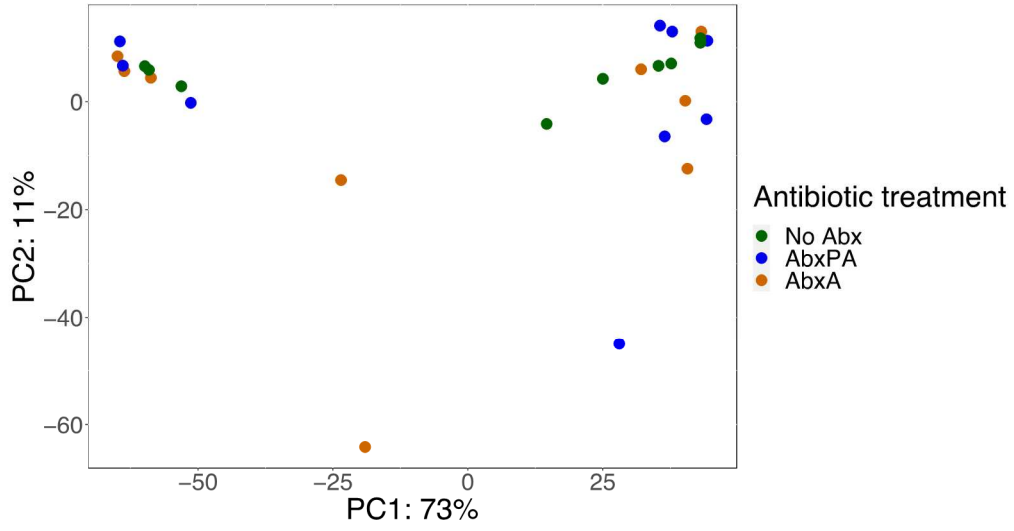
B DEGs BEFORE vs AFTER BLOODFEED

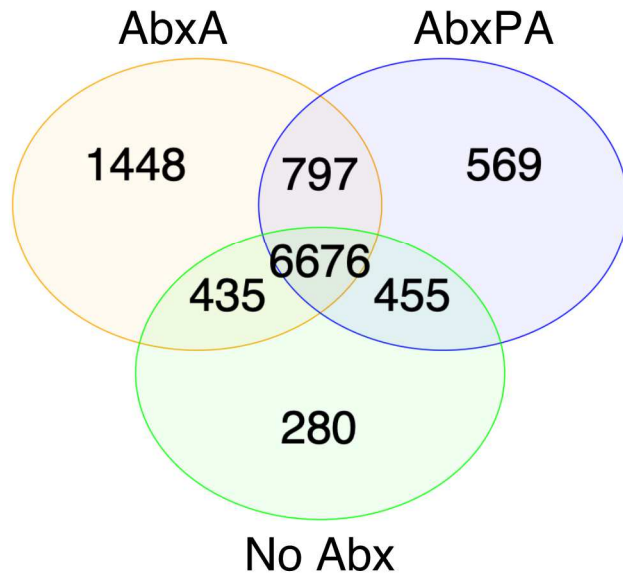**Figure S3. DEGs from bloodfed antibiotic-treated mosquitoes, without ZIKV. A)** Individual

PCA plots, by a single variable, representing the same data in Figure 5B. Shapes indicate the

bloodmeal status while colors represent the antibiotic treatments. **B)** DEGs between bloodfed
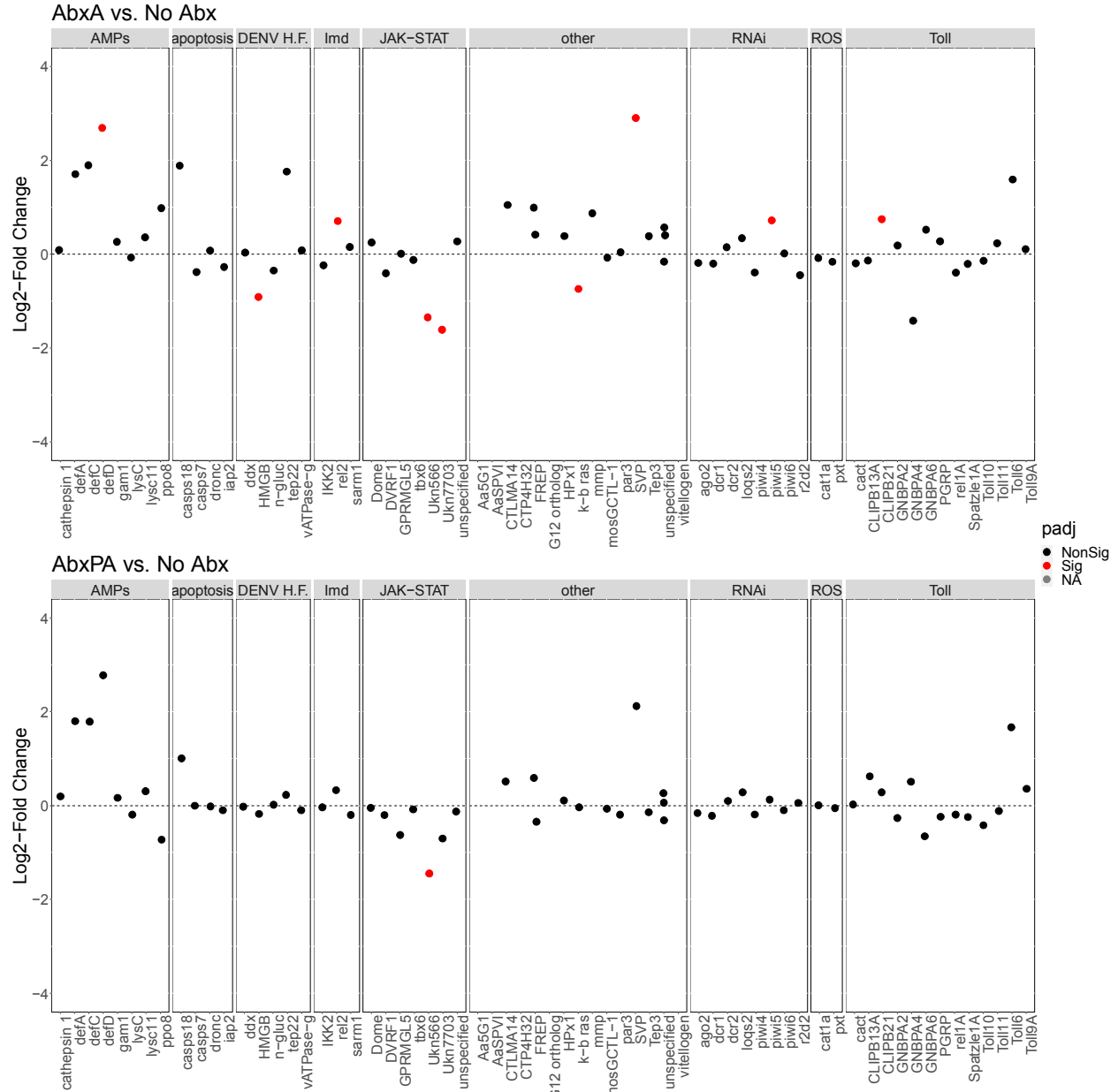
and sugarfed *Ae. aegypti*.

Figure S4. Change in immune response gene expression across antibiotic-treated groups of *Ae. aegypti*. A positive log$_2$-fold change indicates enrichment in AbxA or AbxPA, while a negative log$_2$-fold change indicates enrichment in No Abx.

Closest bacterial species to sequences from *Ae. aegypti* not treated with antibiotics	E-value	Percent identity	Accession Number	Culture medium	Hemolytic activity?
Elizabethkingia ursingii strain EM514-03	3.00E-124	99%	ON714894.1	R2A agar, sheep blood agar	no
Elizabethkingia bruuniana strain KMUH55	3.00E-124	99%	ON714884.1	R2A agar, sheep blood agar	no
Elizabethkingia miricola strain BM10	7.00E-56	85%	CP011059.1	R2A agar, sheep blood agar	no
Pseudomonas sp. MF0	3.00E-125	99%	AY331340.1	R2A agar	NA
Pseudacidovorax intermedius strain HMF4787	1.00E-124	99%	MN595029.1	R2A agar	NA
Salmonella clone (uncultured)	8.00E-126	99%	EF605247.1	R2A agar, sheep blood agar	no

Table S1. Bacterial isolates from adult *Ae.* aegypti that were not treated with antibiotics (No Abx). Cultured and isolated bacterial colonies from adult female *Ae. aegypti*, 3-5 dpe were Sanger sequenced at the V4 region of the 16S gene. Sequences were input into nucleotide BLAST, and the closest hits are reported. Hemolytic activity was tested by presence of ring of clearance around colonies (from lysed red blood cells) when cultured on sheep blood agar at 37°C. NA indicates hemolytic activity was not evaluated.

Gene	Log2Fold Change	Adjusted P	GO accession	GO term	Gene description
AAEL021374	-4.99	0.019	NA	NA	NA
AAEL020586	-2.88	0.000	GO:0016020	membrane	NA
AAEL003626	-1.27	0.047	GO:0016021	integral component of membrane	sodium/chloride dependent amino acid transporter
AAEL019793	-1.00	0.026	GO:0005524	ATP binding	NA
AAEL010711	-1.00	0.005	GO:0005524	ATP binding	eph receptor tyrosine kinase
AAEL020123	-0.95	0.040	NA	NA	NA
AAEL019444	-0.87	0.022	GO:0003723	RNA binding	NA
AAEL004715	-0.86	0.041	GO:0008285	negative regulation of cell population proliferation	b-cell translocation protein
AAEL017280	-0.85	0.040	GO:0003677	DNA binding	NA
AAEL007041	-0.83	0.033	GO:0005509	calcium ion binding	low-density lipoprotein receptor (ldl)
AAEL027453	1.49	0.019	GO:0004550	nucleoside diphosphate kinase activity	NA
AAEL012832	1.88	0.026	GO:0031902	late endosome membrane	cytochrome B561
AAEL000384	3.58	0.000	GO:0055085	transmembrane transport	vesicular acetylcholine transporter
AAEL012340	4.56	0.026	GO:0016788	hydrolase activity, acting on ester bonds	lipase 1 precursor
AAEL029026	8.16	0.026	NA	NA	NA
AAEL014561	8.47	0.040	GO:0005576	extracellular region	NA

Table S2. DEGs between sugarfed *Ae. aegypti* without antibiotics and antibiotic-treated as pupae and adults. A negative Log$_2$fold-change refers to downregulation in AbxPA vs No Abx, while a positive Log$_2$fold-change refers to upregulation in AbxPA vs No Abx.

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CONCLUDING REMARKS

The culmination of this work demonstrates *Ae. aegypti* vector competence for ZIKV is microbially dependent. Moreover, *Ae. aegypti* microbiota exhibit taxonomic variability even across experimental replicates, though their effects on an infection phenotype are consistent. This suggests functional redundancy wherein disparate microbial community compositions can influence mosquito physiology in similar ways. The effects that microbiota have on ZIKV dissemination susceptibility are life-stage dependent and likely through nonstructural modifications of the mosquito midgut. Many interesting questions arise about the mechanisms and generalizability of these interactions. How much microbial variation exists across larval microhabitats? Do differences in these microhabitats affect vector competence in different ways? What nonstructural modifications do microbiota bestow to mosquito gut physiology, and how important is immunometabolism in this regard? And finally, how does this translate to heterogeneity in intrapopulation transmission potential among *Ae. aegypti*? Probing the differential effects of larval microhabitats, each with their own unique microbial communities, on ZIKV transmission potential is an interesting future endeavor.

The upcoming decades are expected to experience the most tenacious arbovirus activity that we have ever seen in human history. Even as researchers continue to study genetic determinants of arbovirus emergence, development of pan-arbovirus vaccines, novel vector control techniques, and advanced sequencing and surveillance tools, of which all have merit, however, these endeavors alone will not suffice. Global climate change is increasing the range of permissive environments for major vector mosquitoes, including *Ae. aegypti*, and societal resource stratification is shifting environmental and societal dynamics in ways that are likely to alter vector borne disease transmission. Thus, integrated public health endeavors that combine the described efforts with environmental considerations (temperatures, microhabitat diversity, and microbiome interactions) are required.

Unfortunately, complete elimination of mosquito-borne diseases is unlikely, at least in the foreseeable future. To do so requires marriage of thorough biological knowledge (transmission dynamics, vector species range, environmental drivers, and arbovirus evolution) with accessible medical technology (i.e. vaccines, diagnostic systems), all within an agreeable sociopolitical landscape. However, multi-tiered, multi-faceted public health systems have the potential to aggressively mitigate the disease burden of major arboviruses at the local level. This work represents only a minute fraction of the potential for microbe-mediated suppression of arboviruses.

The works described in this dissertation represent an underappreciated approach to studying viral transmission by mosquitoes. We found that *Ae. aegypti*, when reared as larvae in microbe-rich water more reflective of their natural habitat, become less competent vectors for ZIKV than previously thought. This is mediated by microbial exposure during the early developmental stages, though the resulting acquired microbiota is highly variable. Maximizing microbial exposure through developmental maturity is required for reducing ZIKV dissemination susceptibility. The mechanism by which this occurs is linked to gut functionality and blood digestion, though specific molecular interactions remain nebulous. Further investigation of mosquito-microbe-arbovirus interactions can drive more holistic approaches to vector control that are desperately needed for sustainable and equitable public health.