



Original Research Paper

THE MOSQUITO HOLOBIONT: MICROBIOME–VIRUS INTERACTIONS AS DETERMINANTS OF VECTOR COMPETENCE AND ARBOVIRUS EVOLUTION

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Abstract

Mosquitoes are not autonomous vectors, but rather a collection of organisms and their surrounding environments (ecological units). The phenotype of mosquitoes is a result of the interactions of the insect host, surrounding microorganisms, invasive viruses, and the surrounding environment. This paper aims to describe the effects on mosquitoes from their bacterial microbiota, fungal associates, insect viruses, and other microbes on mosquito physiology, antiviral immunity, vector competence, and the evolution of arboviruses. From studies done on *Aedes* mosquitoes, it is shown that certain gut microbes can modify nutrient availability, epithelial barrier integrity, signaling pathways that deal with reactive oxygen, antimicrobial responses, and the crossing of an arbovirus from the gut to the salivary glands. The best example is *Wolbachia*. Decreases in arbovirus replication have been demonstrated in several laboratory strains. These laboratory strains have also been able to diminish dengue cases in field deployments, though this effect is conditional on strain density, temperature, tissue distribution, and the host genetics. Some insect-specific viruses can inhibit or even reverse replication of arboviruses through superinfection exclusion, but more studies are required for the *Arbomycota*. The Holobiont approach defines the limits of vector competence. This approach defines vector competence as a system of interacting factors rather than as the traditional fixed trait of a mosquito and a virus. This approach also poses evolutionary questions for the factors that influence arbovirus selection and diversity during transmission. More consistent field studies, longitudinal studies, integrated omics, and greater emphasis on causation over correlation are required.

Keywords:

Mosquito holobiont; microbiome; arbovirus; vector competence; *Wolbachia*; virome; innate immunity; arbovirus evolution

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1. INTRODUCTION

Controlling mosquito-borne diseases is challenging because one or many of a number of biological processes, which can vary by context and be highly specific between different mosquito populations, may be involved in vector transmission [1-3]. Vector competence is generally defined as a mosquito's ability to become infected; infection maintenance as the sheath of the proboscis is maintained; dissemination; and the infection of the salivary glands. Although this definition is useful, it is too simplistic when considering the vector as a sterile container [4-5]. Both adult and immature mosquitoes host vertically transmitted, transstadially transmitted and larvally acquired bacterial, fungal, viral and other microbial pathogens. Mosquitoes can alter the development, feeding, reproduction, survival, digestion, and the immune response and host susceptibility to pathogenesis of the organisms it carries. Integrating vectors with their associated microorganisms supports the holobiont concept. The holobiont is not static. The structure of the microbiota of the mosquito is dependent on the species and genotype of the mosquito, its age and sex, whether it has taken a blood meal, its location, the laboratory colony, the temperature, and the quality of the habitat [6-7]. For these reasons, the microbiota observed in a particular experimental context cannot be generalized to other contexts, and therefore the holobiont perspective should be adopted to understand vector competence [8–10].

2. Composition, Acquisition, and Spatial Organization of the Holobiont

The mosquito microbiome is compartmentalized. Different communities of bacteria have been described to colonize the midgut, salivary glands, reproductive tissues, crop, Malpighian tubules, and the cuticle.

Wolbachia, *Asaia*, *Serratia*, *Enterobacter*, *Pseudomonas*, *Elizabethkingia*, and *Chromobacterium* are some of the mostly described bacteria in mosquitoes. Diversity of other organisms including fungi and viruses specific to insects is greater. These organisms are established in the breeding water and detritus, consumed by mosquito larvae. During metamorphosis, many organisms are lost due to an ecological filter. Some are able to cross the filter, but most are lost and must be recolonized after emergence. The adult mosquito's blood, sugar, and even oviposition site can have a large effect on the bacterial community. Blood feeding is the most important biological factor [8,10]. The introduction of blood into the digestive system begins to change the digestive system and immune system within hours. This rapid response can change the pH, redox state, and availability of nutrients. Blood feeding also has an effect on the microbial community. The microbial community that is present during feeding is often different from that present at the establishment of a virus. It has been noted that insectary rearing of mosquitoes alters the microbiome of the organism. The strongest effect on the microbiome is from the geographic location of the organism. To determine the role of specific microbes in vector competence, longitudinal and tissue-resolved sampling is essential.

Table 1. Major components of the mosquito holobiont

Component	Examples	Principal functions	Relevance to transmission
Bacteria	<i>Wolbachia</i> , <i>Asaia</i> , <i>Serratia</i> , <i>Enterobacter</i>	Nutrition, immune tone, metabolite production	Can inhibit or enhance arbovirus establishment
Insect-specific viruses	<i>Flavivirus</i> -, <i>alphavirus</i> -, <i>bunyavirus</i> - like ISVs	Persistent infection, viral interference	May alter replication through superinfection exclusion
Fungi	Yeasts and filamentous fungi	Metabolism, competition, immune stimulation	Functional effects remain understudied
Host tissues	Midgut, salivary glands, reproductive tract	Barrier function and tissue- specific immunity	Define infection, dissemination, and transmission bottlenecks
Environment	Larval water, diet, temperature, chemicals	Shapes microbial acquisition and stability	Modifies both microbiome and vector competence

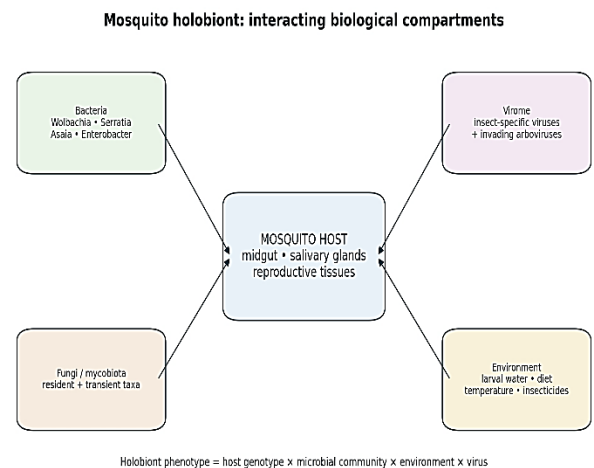


Figure 1. Major interacting biological components of the mosquito holobiont and their tissue and environmental context.

3. Immune Homeostasis and Mechanisms of Microbiome–Virus Interaction

Microbes that colonize mosquitoes elicit the same pathways as arboviruses when they engage the mosquito's innate immune system. Signal transduction pathways Toll, IMD, JAK-STAT, RNAi, and the complement cascade will all be activated, and microbial reactive oxygen species and

Table 2. Selected microbial taxa and plausible effects on arbovirus competence

Taxon	Reported interaction	Mechanistic theme	Interpretive caution
<i>Wolbachia</i>	Often suppresses dengue/Zika replication	Lipid competition, membrane remodeling, immune/redox effects	Strain-, density-, tissue-, and temperature-dependent
<i>Serratia</i>	Can alter colonization, gut barrier, and infection	Community interaction and epithelial effects	Strong strain dependence
<i>Asaia</i>	Stable colonizer; candidate paratransgenic chassis	Tissue colonization and engineered delivery	Field persistence and biosafety require evaluation
<i>Chromobacterium</i>	Some strains produce anti-pathogen metabolites	Secreted metabolites	Dose and isolate specificity
<i>Enterobacter/Pseudomonas</i>	Variable antiviral or physiological effects	Immune stimulation and metabolites	Genus-level labels are insufficient

4. Bacterial Determinants Beyond Wolbachia

Beyond Wolbachia, certain bacterial species promote, while others impede, infection by arboviruses. *Serratia* behaves contextually. Some strains of this species can efficiently colonize the gut, either through direct interactions with other members of the gut bacterial community, or indirectly through interactions with other members of gut bacterial community, or by interacting directly with epithelial integrity. Other strains of *Asaia* are examples of stable colonization strains and can be used in paratransgenesis [24]. Also, certain strains of *Enterobacter*, *Chromobacterium*, and *Pseudomonas* are capable of producing antimicrobial or antiviral metabolites [25-28]. Labeling a genus can lead one to believe products that are secreted in a similar manner, which can be misleading, since closely related isolates can have differences in secretions, colonization, and even immunostimulation and persistence. The phenotype that a high dose of a bacterial species is able to produce may not occur at lower, natural, levels of colonization [29]. A strong causal statement would require the isolation of candidate strains, natural recolonization at numbers that are close to natural levels, and testing of several replicated strains in more than one mosquito genetic background. Recent studies that connect microbial sphingolipid metabolism to *Aedes* vector competence are applicable and important in this context, since they connect correlation to a possible functioning mechanism [30-31]. These studies would also help justify the use of metabolites as biomarkers instead of broad taxonomic surveys. However, these survey types would need to be conducted at additional fermentable substrate levels and across multiple multispecies communities in order to consider microbial species appropriate candidate control agents. [32-35]

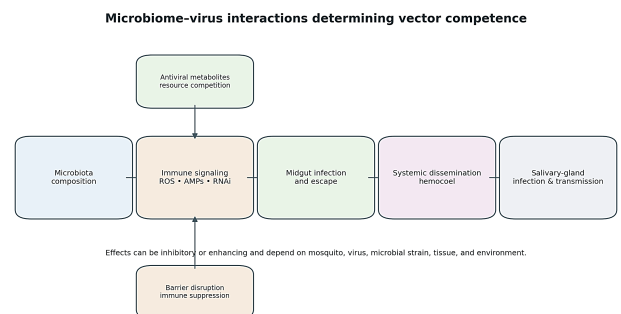


Figure 2. Mechanistic routes by which microbiome composition can inhibit or enhance arbovirus acquisition, dissemination, and transmission.

5. Wolbachia as a Model of Microbiome-Mediated Pathogen Blocking

Presently, the most advanced microbial example for the control of arboviruses (dengue and Zika viruses) is Wolbachia. Some Wolbachia strains induce cytoplasmic incompatibility, enabling population replacement and elimination of dengue and Zika infections in *Aedes aegypti*. There is not a singular, defined pathway for blocking, and the available data suggest a variety of mechanisms, including competition for lipids and cholesterol, alteration of the endoplasmic-reticulum membrane, change in redox biology, immune priming, resource competition in the cells, and density dependent exclusion. The best evidence for Wolbachia blocking viruses is when it is present at viral density in tissues involved in reproduction and in tissues involved in virus replication and transmission. There are combinations of Wolbachia strain and mosquito

Table 3. Wolbachia evidence relevant to dengue control

Evidence level	Representative finding	Strength	Remaining issue
Laboratory	Reduced dengue/Zika infection in transinfected <i>Aedes</i>	Mechanistically reproducible	Host background and thermal sensitivity
Field entomology	Stable introgression and high prevalence in target populations	Demonstrates ecological feasibility	Long-term density and spatial heterogeneity
Randomized community trial	Lower virologically confirmed dengue after wMel deployment	Strong causal epidemiological evidence	Transferability to other settings
Operational programs	Incidence reductions reported with replacement or suppression strategies	Real-world scalability	Cost, surveillance, and implementation variation

6. Virome and Insect-Specific Viruses

Some viromes of mosquitoes encompassing insect-specific flaviviruses, alphavirus-like and bunyavirus-like viruses, densoviruses and others, have been analyzed and described using metagenomics. The detection of these viruses can not be considered insignificant merely for cataloguing. One of the medically important viruses can be maintained as a persistent infection in a mosquito. Persistent infection of a mosquito alters cellular pathways and the environment of the insect's immune system along with the milieu in which the mosquito-transmitted virus replicates. When a cell is superinfected with a second virus, it is able to block the replication of the first virus. This has been shown in the laboratory where an insect-specific virus has been found to block the replication of either dengue or any other arbovirus, however the effect was weak or even absent in some cases. Vertical transmission of an insect-specific virus leads to the formation of different mosquito populations and structured viromes within different regions and geographic areas, where differences in vector competence may be observed. Infecting an arbovirus may lead to changes to the resident insect viruses and vice versa. This reciprocal effect will complicate the causality assessment. More studies are needed to determine the quantity of viral loads rather than their presence. To determine the virome state of insects, defined viral isolates along with the whole laboratory population should be studied. Contamination, index hopping and unassigned low abundance sequences should be validated.

Comparative viromics can help describe population structures of mosquitoes and will improve population ecology of mosquitoes [11–23, 68–76].

The main goal of this type of research is to develop descriptions and tools that help with the diagnosis and control of vector-borne pathogens. Such tools and descriptions will help researchers understand the prevalence of vector-borne diseases [11–23, 68–76].

Wolbachia-mediated arbovirus blocking: convergent mechanisms

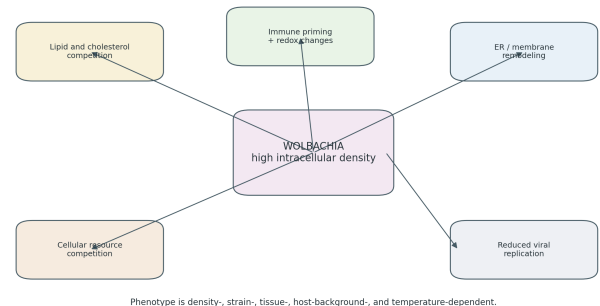


Figure 3. Convergent mechanisms proposed for Wolbachia-mediated arbovirus blocking in *Aedes* mosquitoes.

7. Mycobiome and Other Understudied Partners

Research on the fungi seen in mosquito-related microbiomes lags behind that of bacteria. Environmental fungi like yeasts and molds can grow on breeding sites and feed on nectar, and entomopathogenic fungi can negatively affect survival and alter the immune response. Fungal metabolites can potentially interact with bacteria and/or viruses. Many studies lack sufficient details and are vulnerable to low biomass contamination. Vector-competence models integrate protozoan associates and other eukaryotic microbes even less. A holobiont perspective should not treat the microbiome as the bacteriome, and should study cross-kingdom interactions, and if fungi or protists can reshape microbiota, modify nutritional or immune responses [33–35].

8. Environmental Modulation of Microbiome Effects

Temperature, larval nutrition, chemistry of water, the presence of pesticides and antibiotics, crowding, humidity,

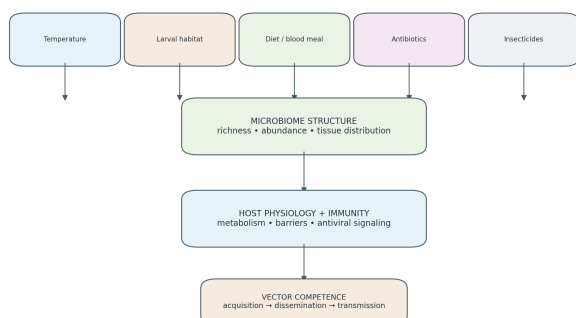
Figure 4. Environmental determinants that restructure mosquito microbial communities and thereby modify host physiology and vector competence.

and the source of blood meals all can change both microbial communities and the traits of mosquitoes involved in pathogen transmission. Temperature has particularly profound effects on most of the processes involved in microbial and viral replication and the intrinsic or extrinsic incubation periods of some pathogens. Thus, bench top studies may observe microbial associations that are unstable under field conditions. A microbial association that is observed in the laboratory and maintained at a constant temperature may, in the field, change, reverse, or intensify. Antibiotic exposure may deplete some pathogenic species, while selection of resistant taxa and a change in host metabolism may occur. Insecticides may also lead to changes in community structure of microbial species associated with insects. Larval habitat may imprint an adult microbial state; however, this signal may be greatly diminished by metamorphosis. Blood-meal host species may change the nutrient supply and may select different microbial communities in the digestive system. The combined factors of microbial state, genotype of the mosquito, and genotype of the virus along with environmental factors limit the extent of one-factor laboratory studies. Longitudinal field studies are most informative when used in conjunction with the previously mentioned factors [25–35, 64–67, 85–91].

Table 4. Experimental approaches for mosquito holobiont research

Method	Primary output	Strength	Limitation
16S/ITS amplicon sequencing	Bacterial/fungal community profiles	Scalable and relatively inexpensive	Composition al and limited functional resolution
Shotgun metagenomics	Genes, pathways, strain information	Higher functional resolution	Cost and host-DNA burden
Metatranscriptomics/viromics	Active transcripts and viral community	Captures functional activity	Low abundance and contamination challenges
Metabolomics	Small-molecule mediators	Connects microbes to mechanism	Annotation and causal attribution
Gnotobiotic reconstruction	Controlled causal perturbation	Direct mechanistic testing	Artificial ecology and rearing effects

Environmental modulation of the mosquito microbiome-virus axis



9. Vector Competence as a Barrier-by-Barrier Systems Phenotype

An arbovirus must traverse many infectious barriers after a blood meal. These barriers include digestion, infection of the midgut epithelium, invasion of the hemocoel, distribution to tissues, invasion of the salivary glands, and attainment of the saliva at an infectious concentration. All stages are susceptible to influencing factors, such as midgut bacteria. They impact the epithelial barrier, the peritrophic matrix, availability of metabolites and signaling components of the immune system. Additionally, systemic microbes or products may have a role in the dissemination while tissue resident symbionts may influence the salivary gland infection. Therefore, the infection rate, dissemination rate, and transmission rate must be analyzed individually. Protection against infection of the midgut may be perceived as strong protection, while protection against invasion of the salivary gland may leave the body infection unaltered while decreasing the transmission. Standardized methods will allow comparisons of studies. Measurements of salivary output rather than the mosquito bodies will give more epidemiologically relevant results. Host genetics will also define the barriers, explaining the differences in effects of microbial interventions in different populations of mosquitoes [1-10, 77-87].

10. Arbovirus Evolution Within the Holobiont

Mosquitoes act as evolutionary filters on viruses. Viral populations are restricted at each of the following steps: crossing the midgut, escaping the midgut, dissemination to other tissues, migration to the salivary glands, and even during saliva transmission. Changes in the microbiome can influence the strength and even the direction of bottlenecks via alterations in host-immunity, host-metabolism, and host-tissue. Repeated persistent exposure to Wolbachia or other microbes that would activate the host's immune response would likely favor more fit viral variants. Conversely, severe bottlenecks would reduce viral fitness and adaptability. Of the two, documented fitness effects of the microbiome on viruses far outnumber documented evolutionary effects. Thus, separating evolutionary theory from observed genotype-frequency distributions should be facilitated. Using experimental evolution in combination with deep-sequencing of multiple generations of mosquitoes and viral populations will provide insight into the evolutionary trajectories of a microbiome intervention. Long-term control strategies rely on the ability to predict the evolution of the viral genome. This necessitates a rapid response to ensure control strategies minimize the selection pressure exerted on the target genome. Control strategies should focus on the viral genome and ensure subsection both the density of infected mosquitoes and the genetic diversity of the virus is minimized [55, 71–76, 86–91].

Table 5. Microbiome-based vector-control strategies

Strategy	Biological principle	Potential advantage	Key risk/limitation
<i>Wolbachia</i> replacement	Cytoplasmic incompatibility plus pathogen blocking	Demonstrated dengue reduction	Thermal, spatial, and evolutionary stability
<i>Wolbachia</i> suppression	Incompatible mating reduces mosquito abundance	No permanent introgression required	Requires sustained release logistics
Paratransgenesis	Engineered symbiont delivers anti-pathogen effector	Potential tissue-specific action	Containment, stability, horizontal transfer
Natural inhibitory strains	Select or augment protective bacteria	May use native ecology	Colonization can be inconsistent
Microbial metabolite targeting	Manipulate functional molecules rather than taxa	Potentially more standardized	Delivery and off-target effects

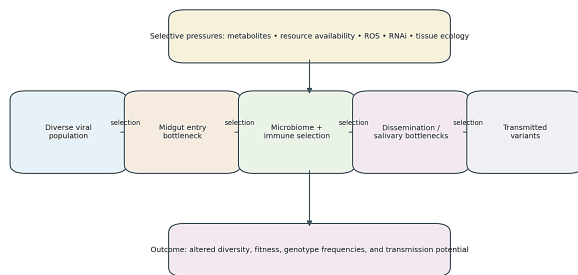
The mosquito holobiont as an evolutionary filter for arboviruses

Figure 5. Conceptual model of the mosquito holobiont as an evolutionary filter imposing sequential selection and transmission bottlenecks on arbovirus populations.

11. Experimental and Multi-Omic Approaches

There isn't a single method to study the community of the mosquito holobiont as a whole. The analysis of the communities of bacteria and fungi by amplicon sequencing is relatively cheap, but it suffers from a lack of resolution and composition bias. DNA-based shotgun metagenomics and RNA-based shotgun metatranscriptomics offer more resolution of pathways and strains, respectively. To characterize community virome, viromics is employed. The structural and functional connections of the community are characterized by functional proteomics and functional metabolomics, respectively [29-35]. To define community structure and interactions of mosquitoes with other members of different compartments, the whole mosquito homogenates are used. These whole mosquito homogenates

provide a composite picture of community structure from all the compartments and thus, limit the functional interactions. The main causality question can be addressed by gnotobiotics, as these methods define microbiota of choice for a given host [59-61]. Full axenic rearing can disturb the development and the physiology of the host. Gnotobiotics for mosquitoes address many of the drawbacks associated with other methods used to eliminate microbes [62-64]. Different techniques need to be used to achieve the goal of proposing a hypothesis based on the mechanisms that are likely to be operative and testing it, rather than taking the presence of associational networks to be an adequate representation of mechanisms [65-67].

12. Microbiome Engineering, Paratransgenesis, and Translational Control

The advantages of microbiome engineering include all techniques that reduce vector abundance or pathogen transmission by controlling the abundance and composition of microbes found on or in an organism. Techniques include introducing the microbiome to manipulate or replace a population with *Wolbachia*, designing symbionts that produce antivirals, selecting naturally occurring microbial inhibitors, modifying the microbiome, integrating microbial, sterile-insect, genetic, and other control methods. Although paratransgenesis is an attractive approach, a culturable symbiont is much easier to manipulate than the mosquito genome. Additionally, the symbiont must colonize target tissues, persist through the life stages, and retain genetic stability and predictable spread. There are also considerable concerns regarding biosafety and with the potential for disrupting ecosystems and non-target organisms through horizontal gene transfer and ecological persistence. The positive effects of microbe-based control on public health demonstrated with the *Wolbachia* programs show that these successes should not be extended to other symbionts. The focus of translation should be on the characterization of the strain and the mechanism of action, contained ecological studies, semi-field validation, and assessment by regulatory agencies and the community, as well as post-release surveillance. The science of microbial ecology should be integrated to ensure that the laboratory phenotype is stable in the field.

13. Knowledge Gaps and Future Directions

There are several limitations to the predictability of the holobiont concept. First, many studies depend more on taxonomic descriptions than on experiments. Second, the environmental and microbial diversity of mosquito laboratory colonies pales in comparison to field populations. Third, strain variation can influence phenotype, yet most studies ignore absolute abundance and strain. Fourth, the interactions among bacteria, fungi, and resident viruses are rarely studied. Fifth, the complexity of the microbiome association with other variables can be further complicated by environmental factors. Research should shift to combination of field sampling and experimental studies. This should be coupled with multi-omics, high-resolution imaging, metabolomics, measurements of viral and host genomes. Viral signatures

may be discovered using machine learning. However, model-based interpretations should also be used to explain identified features. Other efforts aimed at integration and standardization should be used.

Integrative Synthesis

Distinctive characteristics of microbes are attributed to a variety of factors. Classification of microbes involves the genotype of the host and the relative abundance of the microbes. Shifts in microbes' diet and the temperature microbes experience play an important role as well. Instead of trying to erase all statistical noise, scientists should identify context that these host-microbe interactions occur in. These host-microbe interactions will require the collection of data concerning the larval and adult feeding habitats, nutrition, exposure to pesticides, blood meal source, type of mosquito, and type of virus. The role of microbiomes will have to be established. This will determine the best possible way to distinguish the role of the microbiome from the other factors. The mechanisms of interest will be determined by shifting from associative to perturbative. This can be done by isolating each of the candidate strains, controlled reconstruction of host-microbe defined communities, and measuring metabolite, host pathway and infection phenotype restoration by the addition of candidate components. The effect of competitive exclusion, dispersal, horizontal gene transfer, and seasonal bottlenecks will also have to be studied. The consideration of the introgression of symbionts is also essential. For deep sequencing to produce useful data for evolutionary studies, it needs to be done in the context of several independent simultaneous events to allow variability. Making public health methods available will require assessing the cost of the methods, the infection rate, and the community's acceptance of the methods, and the persistence of the benefits.

Another component of the research is designing the descriptive and functional taxonomy of the microbiome. Two populations of mosquitos can overlap in bacterial genera while differing in the production of metabolites/immune factors. Equally taxonomically distinct mosquito populations can overlap extensively in the production of metabolites/immune factors while having significant strain-level differences. To address these issues, research will incorporate analysis of the absolute microbiome together with metagenomics, metatranscriptomics, metabolomics, and trials on the integrity of the barrier and the activation of the immune system and viral replication. Integration across these will deepen our functional understanding of the microbiome, leading to greater reproducibility of studies by identifying stable mechanisms in the microbiome that are consistent in the face of variation in the microbial community. Fundamental improvements in reference materials, the inclusion of standard negative controls and mock communities, will also enhance the reproducibility of studies. In trying to answer the research questions, the importance of the biological level of causation should be kept in mind. For example, even with full-body sequencing, it may be difficult to find phenomena that are specific to the

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midgut, salivary glands, reproductive tissues, or the crop. Even with a massive depletion of viral RNA in the body, it would be unclear if mosquitoes can transmit the virus. Research should focus on the duration and the stages of infection and the spread and transmission of the virus from the mosquito saliva. These studies may determine if microbes limit access to saliva and impede the spread of the virus. Some microbes may even inhibit the first infection. Making use of microbiome-directed control technologies should aim to complement existing public health methods and systems. Though heavily controlling effects of microbes such as Wolbachia are feasible, other endosymbionts are a lot more complex and will require a lot more research to even evaluate for intentional release. The evaluation of the proposed technique should also consider genetic stability and the persistence of the system. Non-target disruption and reversibility should also be considered. Lastly, the method's evolutionary stability should also be evaluated. The biological effects of the technique should be also evaluated to determine if the goal is achieved. Integrated pest management techniques are almost always better than constantly developing new techniques. The biological, ecological, evolutionary and epidemiological research of holobiont will help estimate the disruption and effectiveness of microbial control outside a contained system. The primary mosquito colonies and community should be used as the research will help predict the duration of effectiveness and help evaluate the potential for disrupting microbial control. Research involving negative controls, analytical and sequencing codes with accompanying metadata for all manipulated isolates will help comparative research.

14. Conclusion

The reconstruction of vector competence in the mosquito holobiont gaps existing vector competence and integrates the mosquito and the microbiome as a unified system. Elements of the microbiome modulate mosquito immunity and metabolism, tissue barriers and feeding, survival of the mosquito and selection pressures on arboviruses. An example of a symbiont manipulation toward the control of a human disease vector is Wolbachia. Other relationships of bacteria, fungi and viruses are in preliminary stages of this process. The main obstacle for the next line of studies will be identifying the interactions that are effective, consistent, and transferable to other contexts. This will be resolved through the integration of multi-omics and long-term evolution studies. Given the adaptability of the microbiome, they must be considered in integrated control efforts.

Declarations:

Ethics approval and consent to participate

Consent for publication: All authors provided written informed consent to publish this study.

Availability of data and materials: No new data sets were generated or analysed during the preparation of this review article. All information discussed in this manuscript is derived from previously published studies, which are cited in the reference list.

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Authors Contributions

Kiran Bala Bhuyan developed the conceptual framework for the review and contributed to the scientific framework and critical revision of the manuscript. Jayaprakash Malik contributed to the literature review, interpretation of published findings, and manuscript drafting. Santhosh Goud contributed to the literature synthesis, manuscript preparation, organization of the content, and final revision. All authors read and approved the final version of the manuscript.

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