The Challenges of Microbial Control of Mosquito-Borne Diseases Due to the Gut Microbiome

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Mosquitoes are one of the deadliest animals on earth because of their ability to transmit a wide range of human pathogens. Traditional mosquito control methods use chemical insecticides, but with dwindling long-term effectiveness and negative effects on the environment, microbial forms of control have become common alternatives. The insecticide *Bacillus thuringiensis* subspecies *israelensis* (Bti) is the most popular of these alternatives, although it can also have direct effects on lowering environmental biodiversity and indirect effects on food-web relationships in the ecosystems where it is deployed. In addition, microbial control agents that impede pathogen development or transmission from mosquito to human are under investigation, including *Wolbachia* and *Asaia*, but unexpected interactions with mosquito gut bacteria can hinder their effectiveness. Improved characterization of mosquito gut bacterial communities is needed to determine the taxa that interfere with microbial controls and their effectiveness in wild populations. This mini-review briefly discusses relationships between mosquito gut bacteria and microbial forms of control, and the challenges in ensuring their success.

**Keywords:** 16S, *Asaia*, Bti, *Chromobacterium*, microbiome, mosquito, pathogen, *Wolbachia*

**INTRODUCTION**

Mosquitoes are major disease vectors causing more than 700,000 deaths and millions of new infections every year (World Health Organization, 2017). Chemical insecticides remain the most widely used form of mosquito control, but with a rise in resistant mosquito populations (Dai et al., 2015; Moyes et al., 2017; Li et al., 2018), contribution to population declines of off-target insects including bees, ladybugs, and wasps (Gossett, 2018), and dangers to human health (van den Berg et al., 2015), alternative approaches remain necessary. Since its commercialization in 1938, the insecticide *Bacillus thuringiensis* subspecies *israelensis* (Bti) has been a popular alternative to chemical insecticides and is the most common microbial form for controlling mosquitoes today (Sanahuja et al., 2011; Zhang et al., 2017). Though Bti largely overcomes the specific pitfalls of chemical treatments (Sanahuja et al., 2011), concerns remain over the direct effects of toxicity to off-target organisms that may lower environmental biodiversity, and the potentially detrimental indirect effects on both terrestrial and aquatic food-webs (as reviewed by Bruhl et al., 2020). With the ongoing widespread use of chemical insecticides and concerns lingering around long-term Bti use, other microbes have come to the forefront as potential control agents for mosquito-borne diseases.

Many proposed microbial control agents are symbiotic bacteria that have been exploited for their abilities to interfere with human pathogen viability within mosquitoes or reduce adult host
that are exploited for human pathogen control, such as the agents can be native endosymbionts of a target mosquito species which kill the vectors of disease transmission. Microbial control lifespan and reproductive abilities (Minard et al., 2013a; Huang Dacey and Chain Challenges Exploiting Mosquito Gut Bacteria possibly exposing mosquito larvae to low concentrations of toxin conditions, Bti toxins can persist for months after application, membrane, and lead to larval death. In certain environmental mosquito larvae. Enzymes present in the larval gut activate as three distinct crystals that become lethal once ingested by Bti produces four protoxins (Cry4A, Cry4B, Cry11A, and Cyt1A) BTI mosquito populations. the current limitations in characterizing gut microbiota of wild agents, the impact of gut bacteria on their efficacy, and some of pros and cons of Bti and three candidate microbial control et al., 2018; Tetreau, 2018), this mini-review focuses on the mosquito gut microbiome has shown to be important for interplay between the microbiome and the immune response, where different contexts can affect control success. For example, the mosquito gut microbiome has shown to be important for some microbial forms of control to be effective (Caccia et al., 2016), while at other times having little influence (Ramirez et al., 2014) or proving to be a hindrance to their efficacy (Patil et al., 2013; Hughes et al., 2014; Rossi et al., 2015; Zink et al., 2015). While microbial interactions within a mosquito can have important physiological repercussions on the host and on their susceptibility to pathogens (Guégan et al., 2018), the conditions under which they affect microbial control effectiveness are mostly unknown. Furthermore, as lab populations of mosquitoes can have distinct microbiomes compared to their wild counterparts (Hegde et al., 2018), such microbial interactions are difficult to predict when bringing a method of mosquito control from lab to field. While both direct and indirect effects of the mosquito gut microbiota can result in either positive or negative interactions with microbial forms of control, as reviewed elsewhere (Guégan et al., 2018; Tetreau, 2018), this mini-review focuses on the pros and cons of Bti and three candidate microbial control agents, the impact of gut bacteria on their efficacy, and some of the current limitations in characterizing gut microbiota of wild mosquito populations.

Bti

Bti produces four protoxins (Cry4A, Cry4B, Cry11A, and Cyt1A) as three distinct crystals that become lethal once ingested by mosquito larvae. Enzymes present in the larval gut activate these protoxins to a toxic state, form pores in the gut epithelial membrane, and lead to larval death. In certain environmental conditions, Bti toxins can persist for months after application, possibly exposing mosquito larvae to low concentrations of toxin for a prolonged period that could favor resistance development, but with the combination of all four toxins this is not a likely outcome (Bruhl et al., 2020). Bti tolerance in wild mosquito populations has only been reported twice, in a population of C. pipiens in Syracuse, New York (Paul et al., 2005) and an Aedes rusticus population in the French Rhône-Alpes (Boyer et al., 2012), with no further published reports indicating sustained selected resistance in these areas. Nevertheless, several investigations have been carried out to understand the basis of potential Bti tolerance development using lab-bred mosquitoes (Paris et al., 2012; Tetreau et al., 2013; Stalinski et al., 2014) with the causal mechanisms likely involving individual target-site modifications for each toxin.

There is some evidence, however, showing that the action of Bti is affected by mosquito gut bacteria. While there are conflicting results about the role that the midgut microbial community plays in the insecticidal activity and toxicity of Bt in lepidopterans (Broderick et al., 2006, 2009; Johnston and Crickmore, 2009; Raymond et al., 2010; Caccia et al., 2016), the effectiveness of Bti against mosquitoes has been shown to be enhanced by decreasing gut bacterial diversity before introduction of the insecticide. Populations of Anopheles stephensi with sterile guts show increased susceptibility to Bti compared to those colonized with bacteria, wherein the microaerophilic conditions of the normal larval gut might promote the degradation of Bti toxins via bacteria using them as a nitrogen source (Patil et al., 2013). Despite this hindrance by the gut bacterial community on Bti efficacy, the specific bacterial isolate that is responsible has yet to be identified. Conversely, Bti itself alters the microbiome of A. aegypti larvae upon introduction, resulting in lower bacterial diversity in those larvae most tolerant to the insecticide when compared to individuals unexposed to Bti (Tetreau et al., 2018). The presence of Bti might therefore negatively impact the protective action of symbiotic bacteria, for example by reducing the bacterium Acinetobacter, which may have antiviral properties (Lee et al., 2009) as it highly associates with A. aegypti (David et al., 2016) and Aedes albopictus (Minard et al., 2013b) in organs where viruses replicate (Crotti et al., 2009; Zouache et al., 2009). Though Bti is still considered a safer alternative to chemical insecticides, it is unclear if its continued release into the environment will result in persistence of mosquito populations with altered microbiomes that reduce the effectiveness of Bti or have reduced abilities to protect against other pathogens. Perhaps more concerning is the increasing evidence of both direct and indirect effects on off-target organisms that can lower ecosystem biodiversity and alter trophic food webs, highlighting the need for alternative mosquito control agents.

Wolbachia

Wolbachia is a genus of obligate intracellular bacteria found in many mosquito species. These bacteria can transmit vertically within an invertebrate population and horizontally within and across populations (Hughes et al., 2014). Wolbachia can also alter the fitness of infected insects (e.g., increase
adult mortality), inhabit insect reproductive organs to cause cytoplasmic incompatibility between sperm and egg, and inhabit the insect gut, which is a key environment for the replication and maturation of many human pathogens (Ricci et al., 2012; Dorigatti et al., 2018). Some natural strains of *Wolbachia* display inhibitory mechanisms against human pathogens such as arboviruses (Caragata et al., 2019), for example by diminishing the viral load of West Nile in *C. pipiens* and *Culex restuans* (Novakova et al., 2017). This makes *Wolbachia* a good microbial control agent candidate by means of targeting the pathogen as opposed to the mosquito itself, reducing off-target effects that negatively affect environmental biodiversity and food-web networks. In addition, *Wolbachia* has been found to have no negative impacts on the efficacy of Bti (Endersby and Hoffmann, 2013), therefore possibly allowing both control methods to be used simultaneously. While *Wolbachia* is widespread throughout many wild mosquito species of *Aedes*, *Culex*, *Coquillettidia*, and *Mansonia* (Ricci et al., 2012), lab strains of *Wolbachia* have been genetically manipulated for manual transinfection in mosquito species that do not typically host *Wolbachia*. *A. aegypti* is one of these species not naturally infected with *Wolbachia* but the introduction of this bacterium to the mosquito’s gut has inhibited host infection by *Plasmodium* parasites and dengue, chikungunya, West Nile, and yellow fever viruses (Moreira et al., 2009; Bourtzis et al., 2014; Jeffries and Walker, 2015). Viral inhibition by *Wolbachia* might be achieved through competition for cellular resources (Moreira et al., 2009) or by increasing expression of NADPH oxidase, which up-regulates reactive oxygen species and leads to host immune pathway activation (Pan et al., 2012). An increase in adult mortality due to *Wolbachia* infection may also contribute to lessening mosquito-borne virus transmission to humans, with mosquitoes dying younger thus not giving a virus enough time to replicate and migrate to the salivary glands for human inoculation (McMeniman et al., 2009). *Plasmodium* infection in *Anopheles* mosquitoes has also been suppressed by the introduction of *Wolbachia* lab strains (Bian et al., 2013), plausibly by the same mechanisms as for *A. aegypti* virus inhibition (Moreira et al., 2009; Pan et al., 2012).

The establishment of *Wolbachia* is, however, itself inhibited in the presence of certain commensal gut bacteria, limiting its effectiveness as a way to control the spread of mosquito-borne human pathogens. For example, the effectiveness of *Wolbachia* depends on the absence of *Asaia*, another prevalent bacterium naturally found in the guts of many mosquito species. *Asaia* and *Wolbachia* often compete with one another for resources that can lead to mutual exclusion, with wild strains of *Asaia* shown to inhibit the establishment of *Wolbachia* biologics via negative interference competition in *Anopheles* and *A. aegypti* mosquitoes (Hughes et al., 2014; Rossi et al., 2015). Likewise, the presence of native *Serratia* in the mosquito gut is also negatively correlated with the presence of *Wolbachia*, thus possibly impeding the establishment of *Wolbachia* (Zink et al., 2015). On the other hand, *Wolbachia*, which may suppress pathogen transmission to humans by infecting mosquito reproductive organs to cause cytoplasmic incompatibility, has not shown to have a major impact on the gut bacterial diversity of lab reared *A. aegypti* or *A. stephensi* (Chen et al., 2016; Audsley et al., 2017) or wild *A. aegypti* mosquitoes (Audsley et al., 2018), questioning its ability to impact host fitness in this regard. The mechanisms by which inhibitory gut bacteria may interfere with *Wolbachia* are not known, but their continued presence is possibly one of the reasons why some mosquito populations are unable to be stably infected with strains of *Wolbachia* (Hughes et al., 2014; Rossi et al., 2015; Zink et al., 2015) compared to others that are successful (Garcia et al., 2019; Nazmi et al., 2019). This variability should raise concerns about *Wolbachia* as a universally effective microbial control agent against mosquitoes.

### ASAIA

*Asaia* is another bacterial genus being considered for control applications for its ability to infect multiple host organs via mosquito hemolymph and its ability to establish itself in numerous natural mosquito populations via vertical and horizontal modes of transmission (Rami et al., 2018). Several important mosquitoes commonly host *Asaia* including *Anopheles gambiae, A. stephensi, A. albopictus, A. aegypti*, and most *C. pipiens* populations (Wilke and Marrelli, 2015). Some wild strains of *Asaia* have the ability to secrete proteins that are toxic to *Plasmodium* and limit the parasite’s reproduction in *Anopheles* vectors (Favia et al., 2007; Bongio and Lampe, 2015; Cappelli et al., 2019).

Unfortunately, common DNA sequencing methods are often unable to differentiate between different *Asaia* species within a bacterial community, limiting our ability to exploit the beneficial anti-pathogenic properties of a particular species within this genus. The identification of the specific *Asaia* strains that reduce mosquito competence of a human pathogen (e.g., by hindering *Plasmodium* reproduction) will enable development of microbial control agents with higher success rates against disease transmission, but might require a combination of sequencing techniques and biochemical tests for species level resolution and isolation (Rami et al., 2018). However, because *Asaia* competes with *Wolbachia* in the gut of target mosquitoes, there will likely be a loss of anti-pathogen activity in certain circumstances if exclusion of *Wolbachia* is unavoidable (Rossi et al., 2015), weakening its ability to prevent mosquito-borne disease transmission to humans since *Wolbachia* is widespread. As both *Asaia* and *Wolbachia* show potential as candidates for mosquito-borne disease control, it is important to keep in mind their exclusionary relationship, calling for methods of selectively inhibiting one or the other for example by genetic engineering.

### CHROMOBACTERIUM

A bacterial symbiont recently isolated from an *Aedes* population and being considered for mosquito-borne disease control is *Chromobacterium* sp. Panama (Csp_P). This bacterium can prevent dengue virus and *Plasmodium falciparum* infection in lab-reared *A. aegypti* and *A. gambiae*, respectively, by rapidly colonizing the mosquito gut, prompting expression of host immune factors, and maintaining an effective level of host
innate immunity (Ramírez et al., 2014). Csp_P also secretes an aminopeptidase that degrades the envelope protein of dengue virus that is required for attachment to host cells (Saraiya et al., 2018a) and romidepsin, a histone deacetylase inhibitor, that suppresses Plasmodium development in A. gambiae mosquitoes (Saraiya et al., 2018b). The entomopathogenic activity of Csp_P seems to be independent of the presence of a gut bacterial community, as rapid mortality is seen in lab-reared A. aegypti and A. gambiae with both aseptic and bacteria inhabited guts (Ramírez et al., 2014). As lab-raised mosquitoes have distinctly different gut bacterial communities than wild mosquitoes (Hegde et al., 2018), field studies are still needed to determine if Csp_P has any significant microbial interactions in wild target populations, and if it is able to be established in such populations with continued anti-malarial and anti-viral properties.

There are several other bacteria besides the ones mentioned that can inhibit transmission of human pathogens like La Crosse virus, dengue virus, and Plasmodium in mosquito vectors including Anopheles, Aedes, and Culex, but their lack of widespread presence throughout mosquito populations and the lack of knowledge regarding interference by other microbes has not been well established, limiting their potential at this point (Cirimotich et al., 2011; Joyce et al., 2011; Ramírez et al., 2012, 2014; Bando et al., 2013; Bahia et al., 2014; Dennison et al., 2016). Another challenge is that some control agents are species-specific and can even enhance vectorial capacity of other human pathogens, which is a problem for implementation in nature.

INADVERTENT EFFECTS OF MICROBIAL CONTROL IN WILD POPULATIONS

Microbial control agent candidates that display anti-transmission properties of a human pathogen in one mosquito species have sometimes been found to increase mosquito susceptibility to infection of the same or different human pathogen in another species. For example, although Wolbachia strains can suppress Plasmodium infection in Aedes and Anopheles, they can actually increase Plasmodium susceptibility in C. pipiens (Zele et al., 2014). Furthermore, while Wolbachia strains might be effective against Plasmodium in A. aegypti, they can increase susceptibility to dengue virus infection in these mosquitoes (King et al., 2018). Similarly, Bti-exposed lab strains of A. aegypti larvae bred to have low levels of resistance to Bti display a higher susceptibility to dengue virus as adults than individuals of this strain not exposed to the insecticide (Moltini-Conclois et al., 2018). Another promising microbial candidate displaying anti-malarial properties in Anopheles vectors is Serratia (Bando et al., 2013; Wang et al., 2017), but the presence of the bacterium promotes dengue and Chikungunya viral infections in Aedes mosquitoes (Apte-Deshpande et al., 2012, 2014; Wu et al., 2019). The introduction of a microbial control agent in the wild to manage the spread of one group of human pathogens must therefore not inadvertently get transmitted to the environment or to other mosquito species, or otherwise risk an inadvertent mosquito-borne disease outbreak.

CHALLENGES IN CHARACTERIZING MOSQUITO GUT BACTERIAL COMMUNITIES

To improve the chances of deploying successful microbial control agents to control mosquito-borne diseases, there needs to be concerted efforts to characterize the community of bacteria harbored by the guts of target mosquito populations. A major challenge is our ability to accurately assess the composition and diversity of microbial gut communities. These problems relate to the experimental conditions and methods used that might bias the distribution of bacteria observed, as well as the technical limitations of current sequencing and bioinformatics approaches.

The bacterial community composition of the mosquito gut is influenced by the water condition of breeding sites (Gimonneau et al., 2014; Muturi et al., 2018; Duguma et al., 2019), the developmental stage of the mosquito (Duguma et al., 2015; Receveur et al., 2018), and the mosquito species (Duguma et al., 2015; Muturi et al., 2016, 2017; Hegde et al., 2018; Thongsripong et al., 2018). The degree of influence that environmental microbes have on the colonization, succession, and temporal changes of bacteria in the mosquito gut remain unclear, but this is important to determine the long-term impact of microbials that alter microbiomes. So far, comparisons of gut bacteria among field-collected mosquito populations have revealed relatively little diversity at the phylum and family levels (Boissiere et al., 2012; Osei-Poku et al., 2012; Thongsripong et al., 2018). However, at lower levels of taxonomic classification gut bacterial diversity between mosquitoes increases significantly (Boissiere et al., 2012), some reports claiming up to a 90% difference in operational taxonomic units between two individual mosquitoes (Wang et al., 2011; Osei-Poku et al., 2012; Rosso et al., 2018). The inherent variation in biological and environmental factors affecting the mosquito microbiota continues to present challenges in predicting potential gut bacterial interactions with microbial control agents in nature.

While technological advances have enabled thorough investigations into the characterization of microbiomes, there still remains technical challenges to identify species and compare results across studies. Gut microbial communities are typically characterized using 16S ribosomal RNA (16S rRNA) gene sequencing, but inconsistencies in species profiling exist due to variations in experimental designs and analytical methods that introduce biases, preventing direct comparisons of microbial communities across studies (as reviewed by Pollock et al., 2018). For example, it has been shown that using different reference databases to classify sequences from the same dataset can result in substantial taxonomic differences for bacteria identified at low abundance (López-Garcia et al., 2018). Furthermore, the method employed for DNA extraction and the choice of target region within the 16S sequence also both affect the taxonomic recovery of microbes in general (Teng et al., 2018).

Some of the limitations of 16S sequencing can be addressed using whole metagenome sequencing because of the information acquired from other regions in bacterial genomes. While this can be more costly and challenging, metagenomics offers advantages
over 16S sequencing in its ability to profile bacterial genes and to both detect and identify a greater diversity of bacterial species (Ranjian et al., 2016). For example, whole metagenomics has uncovered for the first time a link between wild Anopheles albimanus microbiomes and resistance to the organophosphate insecticide fenitrothion (Dada et al., 2018). The combination of 16S and metagenomic sequencing has also been used to assess the influence of an Anopheles immune factor on the microbiota, revealing immune factor APL1 to have homeostatic control on the abundance of specific Cedecia and Klebsiella species in the gut (Miti et al., 2020). Similarly, the Caudal gene in D. melanogaster has a direct impact on the gut bacterial community but at a more general level, as this gene is responsible for repressing expression of antimicrobial peptide genes in the digestive tract (Ryu et al., 2008). Exploitation of mosquito immune responses to manipulate host gut bacterial makeup may, for example, aid in transfection success of microbial control agents by depleting inhibitory strains or by promoting dominance of natural symbionts known to inhibit human pathogens. As mosquito–transmitted pathogens continue to spread globally, we need the adoption of standardized robust techniques and analysis methods in mosquito microbiome research relating to microbial control agent development to permit cross-study comparisons and for the advancement of validating promising microbial controls in the field. This will facilitate selection of microbial agents with higher success rates of disease control by avoiding unwanted interactions with the mosquito gut microbiome.

**AUTHOR CONTRIBUTIONS**

DD conceived, designed, and wrote the manuscript. FC critically reviewed the manuscript. Both authors approved the final version of the manuscript.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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