EFFECTS OF ENTOMOPATHOGENIC *CHROMOBACTERIUM* (*CSP_P*) EXPOSURE ON THE MICROBIOTA AND FITNESS OF *ANOPHELES GAMBIAE*

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Abstract

Evidence that the mosquito's microbiota can alter *Plasmodium* susceptibility has led to interest in manipulating the mosquito microbiome to interfere with malaria transmission. Microbes that mosquitoes naturally harbor may modulate vectorial capacity by altering vector competence, daily survival, blood-feeding behavior or vector density. *Anopheles* exposed to *Chromobacterium (Csp_P)*, a bacterium isolated from field-caught *Aedes aegypti*, are more resistant to *Plasmodium* infection and are killed efficiently. This study evaluated the impact of *Csp_P* on fitness, multigenerational killing efficiency, and midgut microbiota composition in exposed *Anopheles gambiae*. To assess the effects on fitness parameters in exposed females, mosquitoes were fed a low (10^2 CFU/mL) or a high (10^7 CFU/mL) dose of *Csp_P* or *Pantoea spp.* (*P. sp.*) in an artificial nectar meal, and were subsequently offered a blood meal and allowed to oviposit. In a separate experiment, the offspring of females fed *Csp_P* (10^7 CFU/mL) were reared to measure transgenerational fitness effects. First generation offspring were exposed to *Csp_P* as either larvae or adults to test *Csp_P*'s multigenerational killing capacity. To measure the effect of *Csp_P* ingestion on the cultivable midgut microbiota, females were fed a high dose of either *Csp_P*, *Enterobacter sp. Zambia* (*Esp_Z*), or *P sp*., and, at 24 and 96 hours, individual midguts were dissected and plated on LB agar. Exposure to either dose of *Csp_P* or *P. sp.* did not alter the proportion ovipositing females or the number of eggs oviposited. Only adults fed *Csp_P* at the higher dose experienced reduced longevity. Maternal exposure to *Csp_P* significantly decreased survival, time to pupation, and survival of larval offspring; however, the life span and sex ratio of adult offspring was unaffected. When challenged with *Csp_P*, the F1 offspring of *Csp_P*-exposed females were killed as quickly as the F1 larvae of PBS-controls. Ingestion of *Csp_P* does not affect the total number or prevalence of the endogenous, cultivable bacteria. Overall, *Csp_P* (10^7 CFU/mL) attenuates the lifespan of exposed females without significantly

impacting fecundity, oviposition, or midgut microbiota composition. Furthermore, this bacterial strain perturbs the development and eclosion of first-generation offspring and maintains killing efficiency over at least two consecutive generations.

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Introduction

Malaria is a disease of significance to human health. Five species of *Plasmodium*, the protozoan parasite that causes malaria, are infectious to humans and are transmitted by *Anopheles* mosquitoes (Calderaro et al. 2013, Sinka et al. 2012). Each year, hundreds of thousands of deaths are attributable to infection with *P. falciparum*, and the majority of deaths occur in young children (Gulland 2012, Hay et al. 2010). Despite the investment in both human and mosquito-targeted interventions, billions of people worldwide are at risk of infection (Gulland 2012, Hay et al. 2010). Prophylactic and therapeutic anti-malarial drugs are available for human use; however, the evolution of drug resistant parasites and complications associated with drug distribution and their accessibility limit their efficacy (Takala-Harrison and Laufer 2015). Currently, only one malaria vaccine is licensed for human use. Although vaccination protects from severe clinical manifestations with *P. falciparum* malaria, the vaccine does not prevent infection long-term, which limits its potential applicability towards transmission interference (RTS,S Clinical Trials Partnership 2015). Parasites that differ genetically from the vaccine strain are not inhibited efficiently, further limiting vaccine efficacy (Neafsey et al. 2015).

The limited efficacy of human-directed control methods increases the reliance on vector-based control. Although chemical and biological insecticides drastically decrease susceptible mosquito populations, mosquitoes evolve resistance (Cisse et al. 2015, Gnanguenon et al. 2015, Paris et al. 2010). The degradation of insecticidal activity over time, the improper use of insecticide-treated bed nets, and implementation costs limit the effectiveness of chemical-based approaches (Chanda et al. 2015, Cisse et al. 2015, Haji et al. 2013). Bioinsecticides applied to the aquatic breeding sites lose mosquitocidal activity quickly, and weekly or monthly reapplications are needed for sufficient population depression (Fillinger et al. 2003, Goldman et al. 1986, Majambere et al. 2007, Paris et al. 2010, Paris 2011b). The persistent limitations of current

control strategies stimulate the innovation and implementation of new approaches. Recently, interest in exploiting the mosquito's microbiota as resource in malaria control emerged.

Endogenous midgut microorganisms play an integral role in life history of mosquitoes, including the regulation of pathogen susceptibility and transmissibility. Vectorial capacity, defined as the baseline transmission efficiency of a vectored pathogen, is described quantitatively by MacDonald's equation, (C= $\frac{ma^2VP^n}{-log_eP}$) (*m*=vector density related to the host population; *a*= the proportion of blood-feeding females in consideration of the length of the gonotrophic cycle; *V*= vector competence; *P*= mosquito daily survival; *n*= the extrinsic incubation period) (Reisen 1989). The microbiota can influence, either directly or indirectly, each vectorial capacity variable. Influence of the midgut microbiota on vector competence has been characterized best. Microorganisms that naturally colonize the midgut can alter *Plasmodium* susceptibility either by regulating immune responses or producing factors that interfere with the parasite's viability or infectivity (Bahia et al. 2014, Christophides et al. 2002, Cirimotich et al. 2011, Ramirez and Short et al. 2014, Ramphul et al. 2015, Rodrigues et al. 2010, Vlachou et al. 2005, Xi et al. 2008). The microbiota can shape vector population dynamics and the likelihood of vector-vertebrate host interactions. Entomopathogenic microorganisms limit malaria transmission by reducing vector density (Mohanty and Prakash 2000, Muspratt et al. 1963, Nnakumusana 1986, Prakash et al. 2010, Priyanka et al. 2001, Seye et al. 2009), fecundity (Garza-Hernandez et al. 2013, Nnakumusana 1986, Scholte et al. 2006), fertility (Nnakumusana 1986), and daily survival (Akhouayri et al. 2013, Bahia et al. 2014, Blanford et al. 2005, Gonzalez-Ceron et al. 2003, Ramirez and Short et al. 2014). In a laboratory setting, a *Chromobacterium* (*Csp_P*) isolated from *Aedes aegypti* (Ramirez et al. 2012) reduces *An. gambiae*'s susceptibility to *P. falciparum* and survival (Ramirez and Short et al. 2014). The multifactorial role of *Csp_P*, and other bacteria, in regulating *Plasmodium* transmission potentiates the introduction of microbiota-based malaria

control strategies. The process of microbiota acquisition and the roles of the microbiota in immune system regulation and malaria transmission are detailed below.

Acquisition and Composition of the Mosquito's Endogenous Microbiota

The mosquito's microbiota influences the life history of the mosquito, from development and reproductive capacity to nutrition and infection susceptibility (Bian et al. 2013, Blanford et al. 2005, Blanford et al. 2012, De Gaio et al. 2011, Dobson et al. 2002, Dong et al. 2009, Garza-Hernandez et al. 2013, Joshi et al. 2014, McMeniman et al. 2009, Minard et al. 2013, Nnakumusana 1986, Ondiaka et al. 2015, Scholte et al. 2006, Walker et al. 2011). The microbiota includes the non-pathogenic and pathogenic microorganisms found in association with the mosquito. As with vertebrates, a variety of microorganisms naturally inhabit the mosquito (Coon et al. 2014, Duguma et al. 2015, Gimonneau et al. 2014). Although the midgut microbiota is the most investigated tissue because of its interaction with potential pathogens, other tissues (the fat body, salivary glands, and reproductive organs) are colonized as well (Akhouyari et al. 2013, Damiani et al. 2010, Favia et al. 2007, Gimonneau et al. 2014, Gusmao et al. 2010, Lindh et al. 2008, Ricci et al. 2011a, Sharma et al. 2014, Wang et al. 2011). The reciprocal interactions of the midgut microbiota with the host and pathogen influence vector competence. Knowledge of how the microbiota is acquired and its composition offers insight into the mechanisms of pathogen interference and guidance in the design of future applications.

The mosquito's microbiota is acquired at several stages throughout the mosquito's lifespan. As larvae, mosquitoes are in an aquatic environment that hosts a diverse microbial community (Coon et al. 2014, Duguma et al. 2015, Gimonneau et al. 2014). The larvae filter-feed on organic matter, including bacteria and eukaryotic microorganisms. Ingestion of bacteria is essential for larval development (Chouaia et al. 2012, Coon et al. 2014, Mitraka et al. 2013, Wotton et al. 1997). In addition to ingestion of microorganisms naturally present in the water,

female mosquitoes can transmit bacteria, fungi, or yeasts vertically (Akhouayri et al. 2013, Bian et al. 2013, Damiani et al. 2010, Favia et al. 2007, Gusmao et al. 2010, Hughes et al. 2014, Lindh et al. 2008, Mitraka et al. 2013, Ricci et al. 2011a, Wang et al 2011). This intergenerational transmission can occur through infection of the egg cytoplasm (Werren 1997), colonization of the egg surface (Damiani et al. 2010, Favia et al. 2007), or inoculation of the breeding water at oviposition (Lindh et al 2008). Any microorganisms transferred to the breeding water can then be ingested once the larvae hatch.

When the larvae undergo metamorphosis into pupae, the midgut epithelium turns over, and a peritrophic matrix surrounds the sloughed midgut (Moll et al. 2001, Moncayo et al. 2005). The pupal stage does not feed, so no additional microorganisms are introduced at this point in the life cycle (Moll et al. 2001, Moncayo et al. 2005). At the transition from pupa to adult, a second peritrophic matrix may form around the first, and both are egested, leaving the adult gut essentially sterile (Moll et al. 2001, Moncayo et al. 2005). At emergence, the adult ingests water from the breeding site, and some of the imbibed bacteria and fungi colonize the adult gut (Pumpuni et al. 1996). The efficiency of the microorganisms' persistence from larva to adult, independent of ingestion at the adult stage (termed transstadial transmission), is unclear (Duguma et al. 2015, Favia et al. 2007, Gimonneau et al. 2014, Lindh et al. 2008, Mitraka et al. 2013, Pumpuni et al. 1996, Ricci et al. 2011, Seye et al. 2009). Although ingestion of larval water seems to be the most efficient mode of adult colonization, microorganisms can also be acquired from nectar (Alvarez-Perez et al. 2012, Pumpuni et al. 1996) and, more rarely, via sexual transmission (Damiani et al. 2008, Favia et al. 2007).

Over the past several years, a number of studies have evaluated the mosquito's endogenous bacterial population. Through the use of both cultivable and non-cultivable methods, such as metagenomic sequencing, the bacterial composition of a variety of mosquito vectors across the various life stages has been evaluated (Andrews et al. 2014, Apte-Deshpande et al. 2012, Boissiere et al. 2012, Chandel et al. 2013, Demaio et al. 1996, Djadid et al. 2011, Duguma

et al. 2015, Gimonneau et al. 2014, Ngwa et al. 2013, Osei-Poku et al. 2012, Pidiyar et al. 2004, Ramirez et al. 2012, Sharma et al. 2014, Terenius et al. 2008, Valiente Moro et al. 2013, Wang et al. 2011, Zouache et al. 2011). Despite the variability in bacterial composition among and within mosquito populations, several themes have emerged, including a "core microbiota," shared colonization patterns, and the presence of a dominant bacterial isolate.

Consistent identification of bacteria with particular characteristics hints at the existence of a core mosquito microbiota. In the midguts of *Aedes*, *Anopheles*, and *Culex* mosquitoes, Proteobacteria is the dominant phylum (Andrews et al. 2014, Apte-Deshpande et al. 2012, Boissiere et al. 2012, Chandel et al. 2013, Djadid et al. 2011, Duguma et al. 2015, Gimonneau et al. 2014,Osei-Poku et al. 2012, Pidiyar et al. 2004, Ramirez et al. 2012, Sharma et al. 2014, Valiente Moro et al. 2013, Wang et al. 2011, Zouache et al. 2011). Other dominant phyla include Bacteriodetes, Firmicutes, and Actinobacteria; however, the contribution of these bacteria and less common phyla to the overall midgut microbiota is more variable across vector species (Boissiere et al. 2012, Chandel et al. 2013, Osei-Poku et al. 2012, Ramirez et al. 2012, Sharma et al. 2014, Valiente Moro et al. 2013, Wang et al. 2011, Zouache et al. 2011). Within the dominant phyla, several genera have been repeatedly identified, including *Acinetobacter*, *Enterobacter*, *Bacillus*, *Serratia*, *Pseudomonas*, *Pantoea*, and *Asaia* (Andrews et al. 2014, Boissiere et al. 2012, Chandel et al. 2013, Chouaia et al. 2010, De Freece et al. 2014, Djadid et al. 2011, Gusmao et al. 2010, Minard et al. 2013, Osei-Poku et al. 2012, Pidiyar et al. 2004, Pumpuni et al. 1996, Ramirez et al. 2012, Straif et al. 1998, Terenius et al. 2008, Terenius et al. 2012). Several of the bacteria are found across mosquito genera and in both field-caught and laboratory populations (Boissiere et al. 2012, Duguma et al. 2015, Rani et al. 2009, Steyn et al. 2015, Wang et al. 2011). These commonalities in composition suggest that mosquito colonization may be semi-selective or may reflect an increased propensity for the mosquito to encounter these particular bacteria.

The composition studies also offer insight into the dynamics of microbiota acquisition. As filter feeders, the larvae ingest the microorganisms available in the aquatic environment.

Several studies show that the diversity in the water exceeds the diversity found in the larval gut (Boissiere et al. 2012, Coon et al. 2014, Duguma et al. 2015, Gimonneau et al. 2014, Wotton et al. 1997, Zouache et al. 2011). The larval habitat is a primary contributor to variability among and within vector species (Chavshin et al. 2012, Demaio et al. 1996, Duguma et al. 2015, Gimonneau et al. 2014, Moll et al. 2001, Moncayo et al. 2005, Ngwa et al 2013, Steyn et al. 2015, Tajedin et al. 2009, Wang et al. 2011).

When larvae develop into pupae and adults, the number of unique bacteria identified decreases, consistent with the physical turnover of the midgut at emergence (Chavshin et al. 2012, Demaio et al. 1996, Duguma et al. 2015, Gimonneau et al. 2014, Moll et al. 2001, Moncayo et al. 2005, Ngwa et al 2013, Steyn et al. 2015, Wang et al. 2011, Tajedin et al. 2009). Despite the proliferation of bacteria that occurs following a blood meal (Gusmao et al. 2010, Pumpuni et al. 1996), diversity decreases in the adult, and only a few bacterial genera remain associated with the adult gut (Terenius et al. 2012, Wang et al. 2011). The continued reduction in unique bacteria colonizing the gut at all life stages suggests that the midgut environment is semi-selective, with only a particular subset of bacteria capable of persisting throughout the mosquito's life span. Although a great deal of research is needed to elucidate the mechanisms determining microbiota composition, recent experiments have shown that both host and bacterial genetics can influence the capacity of a bacterium to colonize the adult midgut (Bando et al. 2013, Pei et al. 2015, Stathopoulos et al. 2014). For example, *Enterobacter* requires *waaL*, a gene necessary for proper LPS structure, for efficient colonization in the mosquito (Pei et al. 2015).

The majority of the variability is related to which constituents are dominant and which are minor. Typically, only one or two bacteria are dominant, whereas many others are present in much smaller numbers (Andrews et al. 2014, Boissiere et al. 2012, Chandler et al. 2015, Chouaia et al. 2010, Demaio et al. 1996, De Freece et al. 2014, Djadid et al. 2011, Minard et al. 2014, Ngwa et al. 2013, Osei-Poku et al. 2012, Pidiyar et al. 2004, Ramirez et al. 2012, Rani et al. 2009, Sharma et al. 2014, Straif et al. 1998, Terenius et al. 2008). The operational taxonomic units

(OTUs) defined by metagenomic sequencing methods reflect this observation. In a given sample, a single OTU contributes the majority of the reads, while unique, minor OTUs represent the bacteria present in low numbers (Boissiere et al. 2012, Ngwa et al. 2013, Wang et al. 2011). The dominant bacterium is typically a member of the "core microbiota," Gram-negative, and possessing general shared characteristics, such as catalase activity, osmotolerance, and the ability to colonize a microaerophilic environment (Alvarez-Perez et al. 2012, Boissiere et al. 2012, Chandel et al. 2013, Gimonneau et al. 2014, Ngwa et al. 2013, Wang et al. 2011). Several factors contribute to the variability with which a bacterium dominates the adult gut, including larval habitat (Boissiere et al. 2012, Chandel et al. 2013, Gimonneau et al. 2014), sex (Rani et al. 2009, Zouache et al. 2011, Valiente Moro et al. 2013), diet (Colman et al. 2012, Wang et al. 2011), and genetics (Bando et al. 2013, Pei et al. 2015, Stathopoulos et al. 2014).

Although less is known about the eukaryotic organisms associated with mosquitoes, similar patterns in colonization dynamics are observed. As observed for the bacteria, the population of eukaryotic microorganisms is more diverse in larvae than in adults, one isolate is dominant within an individual, and several genera are repeatedly identified (Chandler et al. 2015, Pereira et al. 2009; Lara da Costa and Cunha de Oliveira 1998; Frants and Mertvetsova 1986; Gusmao et al. 2010; Ignatova et al. 1996; Ricci et al. 2011b; Steyn et al. 2015, Tajedin et al. 2009). *Wickerhamomyces*, *Aspergillus*, *Saccharomyces*, and *Penicillium* are among the most commonly isolated eukaryotic genera (Chandler et al. 2015, Pereira et al. 2009; Lara da Costa and Cunha de Oliveira 1998; Frants and Mertvetsova 1986; Gusmao et al. 2010; Ignatova et al. 1996; Ricci et al. 2011b; Steyn et al. 2015, Tajedin et al. 2009). Additional studies are needed in the future to identify other members of the mosquito mycobiota and to dissect the factors contributing to colonization.

The Mosquito Innate Immune System and Interactions with the Endogenous Microbiota

The innate immune system is critical for mitigating pathogen-induced responses, and the effects of reciprocal microbiota-host interactions on immune signaling are important to dissect in their relevance to vectorial capacity. The mosquito's innate immune system consists of three main pathways: the Toll, immunodeficiency (IMD), and Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathways (Fragkoudis et al. 2009, Kingsolver et al. 2013, Levashina 2004). Each of the three main pathways follows a similar general path: Pathogen recognition receptors (PRRs) recognize pathogens via interactions with pathogen-associated molecular patterns (PAMPs); downstream of this pathogen recognition, an intracellular signal cascade is induced (Cirimotich et al. 2010). The intracellular cascade results in the nuclear translocation of transcription factors that regulate the expression of a variety of genes encoding PRRs, immune effectors, and proteins involved in immune regulation (Cirimotich et al. 2010).

Despite the cross-talk among these pathways, different pathways are activated preferentially in response to certain pathogens. The Toll pathway, analogous to the interleukin-1 receptor pathway in mammals (Barillas-Mury et al. 1996, Belvin and Anderson 1995), is most important in regulating responses to viruses (Dodson et al. 2014, Dong et al. 2012a, Sanders et al. 2005, Sim et al. 2013, Xi et al. 2008, Zambon et al. 2005), fungi (Bian et al. 2005, Dong et al. 2012a, Shin et al. 2005, Wang et al. 2015), Gram-positive bacteria (Barillas-Mury et al. 1996, Dimopoulos 1997, Dong et al. 2009, Michel et al. 2001, Nicolas et al. 1998, Warr et al. 2008), and the rodent malaria parasite *Plasmodium berghei* (Fraiture et al. 2009, Frolet et al. 2006, Garver et al. 2009, Mitri et al. 2009, Ramirez et al. 2014, Riehle et al. 2008). Melanization and phagocytosis, both of which are hemocyte-mediated (Kim et al. 2005, Ligoxygakis et al. 2002, Povelones et al. 2013, Ramirez et al. 2014, Wang et al. 2013a, Zou et al. 2008), are the primary

effector mechanisms promoted by the Toll pathway. The IMD pathway, analogous to the tumor necrosis pathway (Kleino et al. 2005), mainly responds to Gram-negative bacteria (Antonova et al. 2009, Bahia et al. 2014, Bian et al. 2010, Dong and Dimopoulos 2009, Georgel et al. 2001, Hoa and Zheng 2007, Kallio et al. 2005, Meister et al. 2009, Oliveira et al. 2011a, Sluss et al. 1996, Stathopoulos et al. 2014) and the human malaria parasite *Plasmodium falciparum* (Antonova et al. 2009, Garver et al. 2009, Garver et al. 2012, Garver et al. 2013, Meister et al. 2009). Among the main effector mechanisms, IMD regulates nitration and lysis via Rel2, an NF-kB-like transcription factor (Antonova et al. 2009, Dong and Dimopoulos 2009, Frolet et al. 2006, Garver et al. 2012, Garver et al. 2013, Lin et al. 2007, Luna et al. 2006, Meister et al. 2005, Mitri et al. 2009), and cellular responses such as cytoskeletal rearrangement and apoptosis through the Jun-terminal kinase (JNK) branch (Oliveira et al. 2012, Georgel et al. 2001, Leulier et al. 2000, Ramphul et al. 2015, Silverman et al. 2003). Although the JAK/STAT pathway is the most important in mediating anti-viral (Carissimo et al. 2015, Dong et al. 2012a, Dostert et al. 2005, Fragkoudis et al. 2008, Sim et al. 2013, Souza-Neto et al. 2009) and anti-fungal responses (Dong et al. 2012a), like the mammalian interferon pathway (Dupuis et al. 2003, Fu et al. 1992), this pathway also contributes to anti-*Plasmodium* (Bahia et al. 2011, Gupta et al. 2009) and anti-bacterial responses (Gupta et al. 2009, Oliveira et al. 2011a). In the case of each of these pathways, exposure to bacteria results in the translocation of primary transcription factors, although not all pathways contribute equally to this process (Barillas-Mury et al. 1996, Gupta et al. 2009, Hoa and Zheng 2007, Nicolas et al. 1998, Sluss et al. 1996).

The exogenous RNA interference (RNAi) pathway is involved in virus defense (Brackney et al. 2009; Campbell et al. 2008; Carissimo et al. 2015; Franz et al. 2006; Keene et al. 2004; McFarlane et al. 2014; Myles et al. 2008; Sanchez-Vargas et al. 2009; Sim et al. 2013). Although it is not directly involved in bacterial responses, this pathway intersects with the IMD and JAK/STAT pathways (Paradkar et al. 2012, Paradkar et al. 2014). In addition, the endogenous RNAi pathway is involved in the regulation of host mRNA expression, and therefore

the expression of genes important to bacterial colonization (Dennison et al. 2015, Osei-Amo et al. 2012, Zhang et al. 2013).

The mosquito's innate immune pathways are important in regulating the load and composition of the microbiota. RNAi-mediated silencing of the Toll and IMD pathway NF-kB-like transcription factors, Rel1 and Rel2, respectively; it also results in the proliferation of the endogenous microbiota (Dong et al. 2009, Meister et al. 2009, Yassine et al. 2014). Also, silencing of the complement-like protein TEP1 allows the bacteria to replicate (Dong et al. 2009). Both Rel1 and Rel2 control the expression of *TEP1* expression (Fraiture et al. 2009, Frolet et al. 2006, Garver et al. 2012, Garver et al. 2013, Riehle et al. 2008). Multiple PRRs, including AgDscam and PGRP-LC, also control the endogenous microbiota (Dong et al. 2006b, Dong et al. 2009, Dong and Dimopoulos 2009, Meister et al. 2009). In a study analyzing small nucleotide polymorphisms associated with *Serratia marcescens* colonization, Stathopoulos et al. (2014) made the interesting observation that certain AgDscam homologs are important in controlling proliferation once ingested.

Conversely, the bacteria present in the mosquito also regulate the expression of immunity-related genes. Xi et al. (2008) have shown that *Aedes aegypti* mosquitoes treated with antibiotics experience reduced expression of antimicrobial peptides (AMPs) when compared to mosquitoes with an intact microbiota. AMPs, which are controlled by both Rel1 and Rel2, inhibit viruses, fungi, bacteria, and *Plasmodium* through the disruption of the plasma membrane without affecting the cells of the mosquito (Antonova et al. 2009, Barillas-Mury et al. 1996, Boutros et al. 2002, De Gregorio et al. 2002, Dong et al. 2012a, Hoa and Zheng 2007, Lemaitre et al. 1996, Lin et al. 2007, Luna et al. 2006, Manfruelli et al. 1999, Meister et al. 2005, Shin et al. 2005, Xi et al. 2008). Other anti-bacterial effectors (e.g., PRRs, including AgDscam and PGRP-LC) and immune regulator genes are known to be up-regulated in bacteria-colonized mosquitoes (Dong et al. 2009). Also, genes involved in the melanization pathway, such as serine proteases and serine

protease inhibitors, are differentially regulated to control the endogenous microbiota (Dong et al. 2009).

Microbiota-mosquito immune interactions are further exemplified by the anti-bacterial immune responses that are induced following the proliferation of the microbiota after a blood meal. When female mosquitoes take a blood meal, the endogenous microbiota proliferates (Pumpuni et al. 1996, Gusmao et al. 2010); ingestion of blood, and more specifically heme, reduces the production of reactive oxygen species (ROS) via reduction in the expression of the heme peroxidase, IMPer (Oliveira et al. 2011a). This reduction in IMPer leads to a breakdown in the dual oxidase, DUOX, and the IMPer network that increases the permeability of this barrier to antimicrobial peptides (Kumar et al. 2010, Oliveira et al. 2011a). Although the peritrophic matrix, a physical barrier composed of chitin fibrils, chitin-binding domain proteins, and other components, forms around the blood bolus, some of the proliferating bacteria may be able to activate the mosquito's innate immune pathways, which are more sensitive to activation when ROS are down-regulated (Ha et al. 2009, Kumar et al. 2010, Molina-Cruz et al. 2008, Oliveira et al. 2011a).

Although little is known about the role of endogenous eukaryotic microorganisms in immune regulation, each of the pathways has been shown to be involved in the clearance of fungal pathogens (Bian et al. 2005, Dong et al. 2012a, Shin et al. 2005, Wang et al. 2015, Yassine and Kamareddine 2012). Presumably, the native eukaryotic organisms can regulate the innate immune pathways in a manner similar to the native bacteria; however, further research is required to investigate these relationships.

Overall, the mosquito's innate immune system is composed of three major pathways that are involved in the regulation of bacterial responses, and the endogenous microbiota influences the baseline expression of the mosquito's immune effectors. This reciprocal interaction is important to consider when evaluating the role exerted by bacteria on vector competence through immune regulation.

Influence of the Mosquito Microbiota on *Plasmodium* Vectorial Capacity

Plasmodium's Life Cycle in the Mosquito

In order for a mosquito to play a viable role in *Plasmodium*'s transmission cycle, the mosquito must be a competent vector. Vector competence is the ability of a mosquito to become infected with a pathogen, support the pathogen's life cycle, and transmit the pathogen to a susceptible vertebrate host (Christofferson and Mores 2011, Sardelis et al. 2001, Zhou et al. 2014). In the case of *Plasmodium*, the cycle begins with the mosquito feeds on an infected human and ingests blood cells containing the male and female gametocytes (Smith et al. 2014). Once inside the mosquito, the parasites undergo sexual replication to form a motile ookinete (Smith et al. 2014). At the ookinete stage, the parasite must overcome barriers, including the peritrophic matrix, digestive enzymes, immune effectors, blood meal components, and endogenous microorganisms, to invade the midgut columnar epithelial cells (Abraham and Jacobs-Lorena 2004, Han et al. 2000). This invasion process induces cellular stress and nitric oxide synthase (NOS), which may cause invaded cells to undergo apoptosis (Han et al. 2000, Kumar et al. 2010, Lim et al. 2005, Molina-Cruz et al. 2008, Molina-Cruz et al. 2013, Smith et al. 2012). The resulting damage may allow endogenous microorganisms to leak through and induce anti-bacterial responses, some of which overlap with and amplify the anti-*Plasmodium* responses (Christophides et al. 2002, Ramphul et al. 2015, Rodrigues et al. 2010, Vlachou et al. 2005). A subset of ookinetes that successfully invade the epithelial cells then traverse the invaded cells to reach the basal lamina, where they mature into oocyts (Smith et al. 2014). During cell traversal, the parasite is subject to additional immune responses, including melanization and other hemocyte-mediated effector responses (Aguilar et al. 2007, Baxter et al. 2007, Oliveira et al. 2012, Dong et al. 2006a, Dong et al. 2009, Fraiture et al. 2009, Frolet et al. 2006, Habtewold et al. 2008, Molina-Cruz et al. 2013, Povelones et al. 2009, Ramphul et al. 2015, Rodrigues et al.

2010, Riehle et al. 2008, Wang et al. 2013b). The few parasites that survive through to the oocycst stage reproduce asexually and release thousands of sporozoites (Smith et al. 2014). The sporozoites released into the hemolymph must evade additional immune challenges in order to invade the salivary glands (Bian et al. 2013, Habtewold et al. 2008). Once viable sporozoites reach the salivary glands and invade the salivary ducts, the mosquito is competent as a vector (Aly et al. 2009). The time from ingestion of a gametocyte-contaminated blood meal to transmissible sprozoites is the extrinsic incubation period (EIP), typically around 10-14 days (Aly et al. 2009). Most mosquitoes are not competent vectors for a given pathogen, because of the presence of numerous barriers to the infection; however, several *Anopheles* species are competent for the *Plasmodium* species infectious to humans (Sinka et al. 2012). Multiple biotic and abiotic factors contribute to a mosquito's ability to transmit the malaria parasite, including the midgut microbiota.

Vector Competence

The presence of bacteria in the midgut influences the vector competence of mosquitoes for transmitting *Plasmodium*. Depletion of the midgut bacteria with antibiotics increases the oocyst burden and the prevalence of multiple human and rodent malaria parasites in various *Anopheles* species (Beier et al. 1994, Dong et al. 2009, Gendrin et al. 2015, Noden et al. 2011, Sharma et al. 2013). In addition, reintroduction of live bacteria, particularly Gram-negative constituents, decreases the *Plasmodium* infection intensity in a dose-dependent manner (Dong et al. 2009, Gonzalez-Ceron et al. 2003). These observations indicate that the midgut bacteria limit the parasite's ability to infect the mosquito. On closer inspection, the presence of bacteria appears to alter the mosquito's vector competence for the malaria parasites through altered immune signaling or direct interactions with the parasite.

Immune Stimulation

Endogenous bacteria mediate both the basal and *Plasmodium*-induced immune responses of the mosquito, which alter the parasite's ability to complete its life cycle within the mosquito. The Toll and IMD pathways are particularly important for *P. berghei* and *P. falciparum* responses, respectively (Fraiture et al. 2009, Frolet et al. 2006, Garver et al. 2009, Garver et al. 2012, Garver et al. 2013, Meister et al. 2009, Mitri et al. 2009, Ramirez et al. 2014, Riehle et al. 2008). Immune genes involved in shared responses encode a variety of PRRs, effectors, and immune regulators, particularly those mediating melanization, lysis, and phagocytotic responses (An et al. 2013, Christophides et al. 2002, Dimopoulos et al. 1997, Dimopoulos et al. 2000, Dimopoulos et al. 2002, Eappen et al. 2013, Ramphul et al. 2015, Rodrigues et al. 2010, Vlachou et al. 2005). Overexpression of shared immune genes at the time of parasite exposure allows the mosquito to mount responses more quickly.

When the ookinete invades the midgut epithelium, the damage allows bacteria in the midgut to amplify the mosquito's immune responses that coincidently control *Plasmodium*. The peptidoglycan recognition protein, PGRP-LC, a PRR that acts as the transmembrane receptor of the IMD pathway, is involved in both anti-*Plasmodium* and antibacterial responses (Dong et al. 2009, Meister et al. 2009). Meister et al. reported that PGRP-LC is important in regulating endogenous and exogenous bacteria as well as immunity to *Plasmodium* (2009). Following RNAi-mediated silencing of PGRP-LC3, endogenous bacteria proliferate, and the mosquito becomes more susceptible to exogenous bacterial challenge and to *Plasmodium* (Meister et al. 2009). Treatment of mosquitoes with antibiotics prior to an infectious blood meal abrogates the role of PGRP-LC3-mediated protection against *Plasmodium* (Meister et al. 2009). The reliance on endogenous bacteria to induce the anti-parasitic responses suggests that the bacteria activate PGRP-LC3 more strongly than does the ookinete, and genes activated downstream of PGRP-LC are effective in controlling both the bacteria and *Plasmodium*. Also, when mosquitoes are infected with *P. berghei* after PGRP-LC3 silencing, the melanization of the ookinetes increases,

suggesting an increased reliance on Toll-mediated responses (Meister et al. 2009). Furthermore, the IMD pathway negatively regulates the melanization pathway (Frolet et al. 2006, Meister et al. 2009), suggesting that the presence of the endogenous bacteria can influence which effector response is preferentially activated following parasite invasion.

Other pathogen recognition receptors are likewise involved in bacteria-*Plasmodium* shared responses. Gram-negative binding proteins, particularly GNBPB4 (Warr et al. 2008); the fibronectin-related proteins, especially FBN9 (Dong et al. 2009, Dong and Dimopoulos 2009); and immunoglobulin super-family proteins, such as infection-response with immunoglobulin domain protein (IRID)-6 (Garver et al. 2008) and the alternatively spliced hypervariable immunoglobulin domain-encoding gene, AgDscam (Dong et al. 2006b, Dong et al 2012b), have been shown to be important in restricting both bacteria and *Plasmodium*.

Recent experiments also suggest that the presence of bacteria is critical for the induction of a "priming" response against *Plasmodium* in *An. gambiae*. Rodrigues et al. (2010) have hypothesized that parasite invasion allows bacteria-mediated activation of a hemocyte differentiation factor that induces the differentiation of hemocytes into granulocytes. The increase in granulocytic hemocytes protects against either a subsequent *Plasmodium* or exogenous bacterial challenge (Rodrigues et al. 2010). Also, the "priming" effect results in the increased expression of *TEP1* and *LRIM1*, both of which are critical in hemocyte-mediated responses against bacteria and *Plasmodium* (Rodrigues et al. 2010). Treatment of mosquitoes with antibiotic prior to the initial *Plasmodium* exposure does not result in the production of the hemocyte differentiation factor, and protective levels of TEP1 and LRIM1 are not induced (Rodrigues et al. 2010, Ramirez et al. 2015). Although these experiments further support the role of bacteria in the activation of anti-*Plasmodium* effectors, *An. albimanus* mosquitoes do not rely on the presence of bacteria to generate a "priming" response to *P. berghei* (Contreras-Garduno et al. 2015). These conflicting results concerning the involvement of the microbiota in regulating *Plasmodium*-

infection responses serve as a reminder that underlying species-specific differences in immunity play a critical role in controlling infection.

Re-introduction of Gram-negative bacteria isolates from field-collected mosquitoes can also alter the expression of genes that are important for *Plasmodium* defense. In addition to the general immune stimulation described above, exposure to *Wolbachia*, *Serratia marcescens*, *Enterobacter spp. Zambia* (*Esp_Z*), *Pantoea sp.,* or *Pseudomonas putida* increases the expression of antimicrobial peptides (Bahia et al. 2014; Cirimotich et al. 2011; Moreira et al. 2009; Ramirez and Short et al. 2014). The induction of AMPs may offer protection against *Plasmodium*; however, Gram-positive isolates, such as *Bacillus pumilus*, that do not enable a *Plasmodium*-protective phenotype also induce AMP expression (Cirimotich et al. 2011). Although the contribution of AMPs to *Plasmodium* defense is unclear, other immune factors that negatively affect *Plasmodium* development, including PGRP-LC, TEP1, Rel2, FBN9, and reactive oxygen species, are upregulated (Bian et al. 2013, Bahia et al. 2014, Hughes et al. 2011, Kambris et al. 2010, Moreira et al. 2009, Stathopoulos et al. 2014). Interestingly, natural variation within *Serratia marcescens* related to the *flhDC* plasmid influences the bacterium's ability to restrict *Plasmodium* infection, suggesting that the tripartite interactions can be quite specific (Bando et al. 2013).

Although the majority of overlapping *Plasmodium* and immune responses minimize *Plasmodium* infection, some bacteria induce responses that enhance vector competence. For example, CTL4 and CTLMA2 are over-expressed in Gram-negative or *P. berghei*-challenged mosquitoes (Osta et al. 2004, Povelones et al. 2013, Schnitger et al. 2009). The expression of these C-type lectins protects against *Escherichia coli* infection but enhances *P. berghei* infection through an inhibition of the melanization pathway (Osta et al. 2004, Povelones et al. 2013, Schnitger et al. 2009).

Direct Inhibition

In addition to indirectly inhibiting *Plasmodium* development, some bacteria interact directly with the parasite. Co-incubation of the sporognic stage of *Plasmodium* with certain Gram-negative bacteria inhibits ookinete formation or damages the parasite *in vitro* (Bahia et al. 2014, Cirimotich et al. 2011, Ramirez and Short et al. 2014). Such mosquito-independent inhibition is observed for bacteria that commonly colonize *Anopheles* mosquitoes, including *Comamonas sp.*, *Acinetobacter*, *Pseudomonas*, *Pantoea*, *Serratia*, *Enterobacter*, and *Elizabethkingia* (Bahia et al. 2014, Cirimotich et al. 2011, Ramirez and Short et al. 2014). The ability of bacteria that naturally associate with mosquitoes to impede *Plasmodium* competence may contribute to the natural variation in vector competence.

Bacteria directly impair *Plasmodium* development through species-specific mechanisms. The molecular mechanisms of parasite inhibition by bacteria with *in vitro* activity are beginning to be explored. Cirimotich et al. have demonstrated that an *Enterobacter* isolated from *Anopheles* in Zambia *Esp_Z*, produces hydrogen peroxide (2011). Introduction of anti-oxidants either *in vitro* or *in vivo* negates *Esp_Z*'s ability to block parasite development (Cirimotich et al. 2011). Other bacteria can secrete other protective factors that may be active against multiple parasite stages. For example, *Serratia marcescens* and *Chromobacterium spp. Panama* (*Csp_P*) inhibit both the asexual and sporogonic stages of *Plasmodium* when bacteria-free culture supernatant is tested (Bahia et al. 2014, Ramirez and Short et al. 2014). Further experiments are required to identify the molecules responsible for the inhibition and their mechanisms of action.

Other Variables

The microbiota can influence other factors that contribute to vectorial capacity including daily survival, vector density, and reproduction and blood feeding behavior. Currently, the entomopathogenic bacteria *Bacillus thuringiensis* and *B. sphaericus* are used to control larval populations (Barbosa et al. 2007, Fernandez-Luna et al. 2010, Futami et al. 2011, Joshi et al.

2014, Majambere et al. 2007, Paris et al. 2010, Singer 1973). In susceptible populations, exposure to these bacteria, or their derived toxins, decreases survival upward of 90% (Mohanty and Prakash 2000, Parakash et al. 2010, Seye et al. 2009). Reduced larval survival translates to fewer eclosed adult and decreases the risk of *Plasmodium* infection in treated areas (Geissbuhler et al. 2009, Karch et al. 1991, Kroeger et al. 1995, Kumar et al. 1994). Exposure to several entomopathogenic fungi can reduce the number of eggs a female produces (fecundity), further depleting vector density, and can suppress the propensity to blood feed (Garza-Hernandez et al. 2013, Nnakumusana 1986, Scholte et al. 2006). For example, infection with the entomopathogenic fungi *Beauveria bassiana* decreases the propensity to blood feed and reduces indirectly the number of gonotrophic cycles over a mosquito's lifetime (Blanford et al 2005, Darbo et al. 2012). In general, reducing the vector density limits the total number of blood-feeding females, which decreases the number of infectious bites (entomological inoculation rate).

Several other bacteria possess mosquitocidal properties. Although the majority of the mosquitocidal microorganisms characterized possess activity against larvae and pupae (Geetha et al. 2010, Geetha and Manonmari 2010, Geetha et al. 2011, Prabakaran et al. 2003, Ramirez and Short et al. 2014), several isolates also attenuate adult survival (Akhouaryi et al. 2013, Bahia et al. 2014, Gonzalez-Ceron et al. 2003, Ramirez and Short et al. 2014). The *Chromobacterium*, *Csp_P*, previously described that possesses *in vivo* anti-*Plasmodium* activity kills both the adult and larval stages of *Anopheles* mosquitoes (Ramirez and Short et al. 2014). Other bacteria commonly isolated from *Anopheles*, including *Serratia marcescens* and *Elizabethkingia*, decrease the longevity of colonized adults (Akhouaryi et al. 2013, Bahia et al. 2014, Gonzalez-Ceron et al. 2003). Also, bacteria-colonized mosquitoes die more quickly following a blood meal than do antibiotic-treated mosquitoes (Dong et al. 2009). Attenuating the lifespan of adult females may prohibit an infected female form surviving the length of the extrinsic incubation period as well as decrease the total number of mosquitoes.

In some cases, microorganisms can play a multifactorial role limiting vectorial capacity. For example, in addition to the observed effects on immature and adult longevity in *An. gambiae, Chromobacterium* (*Csp_P*) also reduces susceptibility to *P. falciparum* (Ramirez and Short et al. 2014). Evidence that *Csp_P* perturbs of vector density, daily survival, and vector competence makes *Csp_P* an attractive candidate to control malaria transmission.

Potential Application of *Chromobacterium* (*Csp_P*) in Malaria Control

The potential application of *Chromobacterium* (*Csp_P*) or *Csp_P*-derived factors in vector control necessitates further investigation of *Csp_P*-vector interactions. The aim of the project was to assess the effects of *Csp_P* exposure on fitness parameters and the microbiota composition of *An. gambiae* as well as the multigenerational killing efficiency of *Csp_P*. Evaluating direct and indirect fitness parameters following *Csp_P* exposure provides important insight into the feasibility of *Csp_P*-based control strategies since any *Csp_P*-conferred fitness advantages could negate the cost to survival. Additionally, exposure to *Csp_P* for two consecutive generations offers preliminary insights as to the rate at which resistance may evolve. Also, since *Csp_P* has been shown to produce anti-bacterial factors active against mosquito-isolated bacteria (Ramirez and Short et al. 2014), the influence of *Csp_P*, and two control bacteria, on the composition of the endogenous microbiota was assessed.

The results of these experiments suggest that *Csp_P* may impede vectorial capacity more broadly than the previous study indicated. Although ingestion of *Csp_P* does not alter the reproductive capacity of exposed females, maternal *Csp_P* exposure delays the development and reduces the survival and eclosion of first-generation offspring during the immature stages. The F1 larval and adult offspring of *Csp_P*-exposed females died as quickly as the F1 offspring of PBS-exposed females, suggesting that *Csp_P* maintains killing efficiency for at least two consecutive generations. Finally, the cultivable endogenous microbiota is not altered measurably following the *Csp_P* ingestion. Although additional studies are needed to assess the long-term effects of

multigenerational *Csp_P*-exposure on fitness under semi-field conditions and the potential for evolution of resistance, these results encourage further evaluation of *Csp_P* as means to control *Anopheles gambiae* and reduce *Plasmodium* transmission.

Materials and Methods

Mosquito Rearing

The Keele strain of *Anopheles gambiae* was reared at 27°C with 80% humidity with a 12-hour light/dark cycle. Unless otherwise noted, mosquitoes were maintained on a 10% sucrose solution.

Bacterial Cultures

Luria broth (LB), 5mL, was inoculated with 1µL of freezer stock of *Chromobacterium* (*Csp_P*), *Enterobacter* (*Esp_Z*), or *Pantoea* (*P. sp.*) and cultured overnight at 30°C with shaking. The overnight culture was the washed twice in 5mL of 1X phosphate buffer solution (PBS). After the second wash, the cultures were diluted to OD_{600}= 1.0 (±0.05) giving an approximate concentration of 10^8 colony forming units/mL (CFUs). To measure the concentration of bacteria fed *post hoc*, 50uL of serial dilutions (10^{-4}, 10^{-6}) of the $1OD_{600}$ culture were plated on LB agar then incubated at 30°C overnight before calculating CFUs (CFUs= $[(\frac{number\ of\ colonies\ X\ 10\text{^}-4}{o.o5mL}) + (\frac{number\ of\ colonies\ X10^{-6}}{0.05mL})]/2$).

Introduction of Bacteria in Sugar Meal

At 4-6 days post-emergence, adult females were placed into cups, provided 10% for at least 4 hours, and starved overnight prior to exposure. The morning of bacterial exposure, bacteria were added to a 1.5% sucrose solution with an intended final concentration of 10^2 CFU/mL or 10^7 CFU/mL. For all bacteria-feeding experiments, the same volume of 1X PBS was added to 1.5% as a negative control. A piece of filter paper was added to the 1mL of sucrose solution, and mosquitoes were allowed to feed for 24 hours. Following bacteria introduction in 1.5% sucrose, the mosquitoes were returned to 10% sucrose.

Blood Feeding

Blood meals were prepared by mixing 40% human red blood cells and 60% O+ human serum. Mosquitoes were offered a blood meal using membrane feeders as described previously (Xi et al. 2007). For all experiments, mosquitoes were starved 6-8 hours before being allowed to blood feed for one hour.

Fecundity and Oviposition

Six-to seven-day eclosed females were fed either a 10^2 or 10^7 dose of *Csp_P* or *P sp* (N≈25). Approximately 56 hours after exposure, each cup was provided a blood meal with one membrane feeder. At the end of the blood meal, the blood fed females were sorted from the non-fed. Three days later, blood fed females were placed into individual oviposition cups, a 50mL Falcon tube will 5-7.5mL of deionized tap water with a piece of filter paper placed at the bottom to collect eggs. After being allowed to oviposit for two days, the number of ovipositing females and the number of eggs oviposited were recorded. If females oviposited ten eggs or fewer their spermatheca was dissected to check insemination status. For spermathecae dissections, the spermathecae was first dissect in 30uL of 1X PBS then transferred to 10µL of Geimsa stain (diluted 1:100 in deionized water) and physically crushed onto a glass slide using ethanol-sterilized forceps, allowed to air dry, fixed with 50µL of methanol for 30 minutes, and rinsed with deionized water. After the slides with the fixed spermathecae air-dried, the slides were viewed using a light microscope at 10-40X. Only inseminated females were included in the oviposition and fecundity analyses.

Fertility and Offspring Development

Females, 6-7 days eclosed, were fed a 10^7 dose of *Csp_P* in cups (N≈30). Two days following exposure, the surviving mosquitoes for each treatment were transferred to a small cage

and were blood fed (3 membrane feeders per cage). Three days after the blood meal, two oviposition cups (50-mL of water with a piece of funneled filter paper) were placed in each cage. After being allowed to oviposit for two days, the eggs for each paper were counted. The egg-laden filter paper was placed in a 150mm X 25mm petri dish with 100mL of deionized tap water, and the eggs were allowed to hatch. The number of hatched larvae was recorded for two days. After emergence, the larvae were transferred to density-controlled trays (N=100) with 20 mg of larval food (liver powder, tropical fish flake food, and rabbit pellets mixed in a 2:1:1 ratio) and 1 pellet of cat food in 2L of deionized tap water. Throughout the experiment, 10.0 mg of food was added every other day. Water bacterial loads were measured throughout the experiment. Two water samples, 1mL each, were collected from the oviposition containers and at baseline, 4 days, and 8 days from the density-controlled trays. 50uL of non-diluted water samples and serial dilutions (10^{-2}, 10^{-4}) were plated on LB agar. The LB plates were incubated at 30°C and counted colonies at 18 and 42 hours to calculate the CFUs (CFUs=[$\frac{number\ of\ colonies \times 10\text{^}-2}{0.05mL}$+ $\frac{number\ of\ colonies\ X\ 10\text{^}-4}{o.o5mL}$]/2). The CFUs were transformed to log base 10 prior to data analysis. Survival was recorded at 4 days, 8 days, and each day once larvae began to pupate. The pupae were transferred to cups with 50mL of deionized tap water and placed in a small cage with 10% sucrose and allowed to emerge. The proportion of pupae that eclosed was recorded. The sex ratio (sex ratio= $\frac{number\ of\ males}{number\ of\ females}$) was calculated after all offspring eclosed as adults. Adult offspring were transferred to cups (N≈ 20-25) and used either for *Csp_P* exposure or survival bioassays. Survival of adult offspring was monitored daily until all mosquitoes died.

Csp_P *Susceptibility of Larval Offspring*

One to two days after the F1 larvae of *Csp_P* or PBS exposed females hatched, a subset of larvae were exposed to *Csp_P*. Larvae were transferred to a well of a 6-well plate (N=10) with

5 mL of sterile water and 2 mg of larval food and were exposed to a final concentration of 10^6 CFU/mL of *Csp_P* or an equal volume of 1X PBS. Survival was monitored daily for 3 days.

Csp_P *Susceptibility of Adult Offspring*

Three to five days after the F1 adult offspring eclosed, females were placed into cups (N≈25), starved overnight, and exposed to 10^7 CFU/mL *Csp_P* in 1.5% sucrose for 24 hours. After bacterial exposure in 1.5% sucrose, mosquitoes were provided 10% sucrose *ad libitum*. Survival was monitored until all mosquitoes died.

Microbiota Assays

At 6 days post-eclosion, adult females were placed in cups (N=30). Prior to bacterial exposure, individual midguts were dissected from mosquitoes after being starved overnight as baseline. For midgut dissections, each mosquito was surface sterilized in 70% ethanol for 30 seconds and rinsed in 1X PBS; then the midgut was dissected in 30 uL of 1X PBS, rinsed in 70% ethanol to surface sterilize the midguts, rinsed in 1X PBS, and stored in 120uL of 1X PBS. At 0 hours, mosquitoes were fed a 10^7 dose either *Csp_P*, *Esp_Z*, or *P sp*, or the same volume of 1X PBS. At 24 and 96 hours after exposure, individual midguts were dissected. For each replicate, six midguts per treatment per time point were homogenized and plated on LB agar as a serial dilution (ND, 10^{-2}, 10^{-4}) and incubated at 30°C. Colonies were counted at 18 and 42 hours after plating. The colony counts were then calculated as CFUs ($\frac{number\ of\ colonies \times dilution}{0.05mL}$ + 1) and log base 10 transformed prior to statistical analysis. Additionally, survival was monitored daily through 144 hours for the mosquito cohorts from which the dissected mosquitoes were taken.

Unique colonies, identified based on differential morphology, for each treatment and each time point were streaked to yield isolated, monoculture colonies. The 16S rDNA was amplified from each morphologically distinct colony using one of two methods. Direct amplification of 16S rDNA from the isolated bacterial colony was attempted first. Each unique

colony was suspended in 24uL of the appropriate PCR reagents (2.5uL 10X PCR buffer, 0.5 10mM of each primer, 0.1uL Platinum Taq Polymerase, 0.5 uL 10mM dNTPs, 0.75 uL 50mM $MgCl_2$) and the PCR was run using the 16S 27F (5'- AGA GTT TGA TCC TGG CTC AG -3') and 1492R (5'- ACG GyT ACC TTG TTA CGA CTT -3') primers under the following conditions: 94°C for 10 minutes, [94°C for 30 seconds, 55°C for 30 seconds 72°C for 90 seconds]X33 cycles, then 72°C for 10 minutes. Then 7 uL of PCR product was run on 1.0% agarose gel and visualized at 200ms exposure. The PCR samples for colonies with a 1465bp band were purified using the QIAquick PCR Purification kit (QIAGEN) and sent for sequencing with the 27F primer. The colonies that did not produce a band via the direct amplification method were cultured in 5mL of LB broth at 30°C shaking overnight. DNA was extracted from 1.5-mL of each unique colony culture using the genomic DNA isolation from Gram-positive protocol of the QIAamp DNA Mini kit (QIAGEN) with the following modifications: after re-suspension in lysozyme solution (200ug/mL) the samples were kept at 37°C overnight. The extracted gDNA from each unique colony (200ng) was then PCR amplified using the 16S 27F and 1492R primers under the same conditions as the direct amplification method, except the duration of the initial denaturing step at 94°C was 2 minutes rather than 10 minutes. The gDNA PCR amplification products were run on an agarose gel to confirm the 1465bp band, purified, and sequenced in the same manner as the direct amplification products. All unique colonies were PCR amplified and sequenced successfully using one of the two methods. Using the nucleotide BLAST from NCBI, then isolate colonies were matched to genus level. The match cut off applied was 90% coverage and 95% identity. To confirm the *Chromobacterium spp.*, *Enterobacter spp.*, and *Pantoea spp.* cultivated from the midguts was the fed isolate, 16S rDNA from a colony of each fed isolate was sequenced and used as a reference sequence.

Statistical Analysis

Data was analyzed using one-way ANOVA with Dunn's or Bonferroni's post-test, Kruskal-Wallis test with Bonferroni's post-test, Friedman test with Dunn's post-test, paired t-test, and pairwise Log-Rank (Mantel Cox) tests for survival analysis conducted in the GraphPad Prism statistical software package (Prism 5.05; GraphPad Software, Inc., San Diego, CA). For Figure 4A, analysis was performed using a Cox proportional hazards statistical analysis in R (R Foundation for Statistical Computing).

Results

Fitness Effects of *Csp_P* on Exposed Adult Females

Previous studies have shown that exposure of mosquitoes to a 10^8 CFU/mL dose of *Csp_P* robustly attenuates the survival of exposed adult females (Ramirez and Short et al. 2014). At this high dose, only a few females survived long enough to measure the fitness effects of exposure efficiently. Thus, in this experiment, two lower doses of *Csp_P*, 10^2 CFU/mL and 10^7 CFU/mL were used. To control for any non-specific effects of bacterial exposure on female fitness *Pantoea sp.*(*P sp.*), a Gram-negative bacterium isolated from field-collected *Anopheles* (Cirimotich et al. 2011), was fed at the same doses.

Females were fed either bacterium or PBS in 1.5% sucrose for 24 hours, blood fed two days later, and allowed to oviposit through five days after blood feeding to measure the number of egg-laying females, fecundity, and survival. Neither *Csp_P* nor *P. sp.* influenced the number of ovipositing females (Figure 1A; P= 0.9788) or fecundity (Figure 1B; P= 0.7205). Only ingestion of *Csp_P* at the higher dose significantly reduced longevity (Figure 1C; $P_{PBS\text{-}Psp10\text{^}2}$= 0.5771, $P_{PBS\text{-}Psp10\text{^}7}$= 0.8154, $P_{PBS\text{-}CspP10\text{^}2}$= 0.7046, $P_{PBS\text{-}CspP10\text{^}7}$ <0.0001).

Intergenerational Effects of *Csp_P* Exposure

Transgenerational Fitness Effects

To investigate whether *Csp_P* exposure transgenerationally affects mosquito fitness, the F1 progeny derived from either *Csp_P* (10^7 CFU/mL) or PBS challenged females were reared to monitor viability, development, and longevity. After allowing the *Csp_P*- or PBS-fed females to oviposit, their eggs were hatched. Exposure to *Csp_P* did not affect embryonic viability (Figure 2A, P= 0.4179). When the hatched F1 larvae were reared in density-controlled trays, the survival of the F1 larvae from *Csp_P*-exposed females was significantly decreased compared to the F1 larvae of PBS-exposed females (Figure 2B, P <0.0001). The divergence in larval survival

between these two groups mainly occurred during the third and fourth instars. Corresponding to this impairment in survival, the proportion of F1 larvae from *Csp_P*-challenged females that pupated was 32.3% lower (Figure 2C; P= 0.028, 95% CI 9.1-59.9%) and the proportion of F1 larvae from *Csp_P*-challenged females that eclosed to adults was 32.3% lower (Figure 2D; P= 0.0253, 95% CI 9.8-54.9%) than the F1 larvae from PBS-exposed females. Additionally, after adjusting for larval death, the median time to pupation was two days slower in the F1 larvae of females challenged with *Csp_P* (Figure 2E; P <0.0001).

The proportion of F1 pupae from *Csp_P*-exposed females that eclosed into adults was lower than the F1 pupae from PBS-exposed females by 11.5% (Figure 2F; P= 0.026, 95% CI 3.4-19.7%). Negative effects of maternal *Csp_P* exposure on the first-generation of offspring were limited to the immature stages. Maternal *Csp_P*-exposure affects neither longevity (Figure 2G; P= 0.7154) nor sex ratio (Figure 2H; P= 0.3447) of the F1 adult progeny.

To determine whether *Csp_P* is transferred vertically to F1 larvae or whether the bacterial load differs between the aquatic environments of the two groups of larvae, water samples were collected and cultured. Although breeding water bacterial load varied across time points (P< 0.05), the bacterial loads did not differ between groups at any of the time points (Figure 3; $P_{egg\ water}$ >0.05 $P_{Baseline}$ >0.05 P_{4Days} >0.05 P_{8Days} >0.05). In one of the three replicates, *Csp_P* was detected in the larval water of F1 offspring derived from females challenged with *Csp_P* at 4 days at loads of approximately 10^6 CFU/mL and decreased to less than 10^2 CFU/mL at 8 days (data not shown). Overall, the results from these experiments indicate that maternal *Csp_P* exposure negatively affects the immature stages of F1 progeny's development, particularly larvae, but does not significantly affect the F1 adult offspring.

Csp_P *Killing Capacity*

To evaluate *Csp_P*'s multigenerational killing capacity, the F1 larval and F1 adult offspring of *Csp_P*- and PBS-exposed females were exposed to *Csp_P*. Challenge of first instar larvae with *Csp_P* (10^6 CFU/mL) has been shown to cause significant mortality (Ramirez and Short et al. 2014). Here the F1 first instar larvae derived from females fed either *Csp_P* (10^7 CFU/mL) or PBS were challenged with *Csp_P* (10^6 CFU/mL) or PBS and their survival was followed for three days. F1 larvae challenged with *Csp_P*, regardless of the mother's *Csp_P* infections status, experienced significant mortality (Figure 4A; P= 1.81e-11). In support of the earlier experiment indicating that maternal *Csp_P*-exposure reduces F1 larval survival, F1 larvae derived from *Csp_P*-challenged mothers that were exposed to PBS as a negative control experienced impaired survival compared to PBS-challenged F1 larvae derived from PBS-exposed females (Figure 4A; P= 0.0344). However, no interaction exists between maternal and larval *Csp_P* exposure (Figure 4A; P= 0.6105), meaning that F1 larvae of *Csp_P*- or PBS-challenged females are susceptible, equally, to a 10^6 CFU/mL dose of *Csp_P*. Unlike the larval offspring, the F1 adult female progeny of *Csp_P*-challenged females died significantly faster following ingestion of *Csp_P* (10^7 CFU/mL) in an artificial nectar meal, than *Csp_P*-challenged F1 adult progeny of PBS-exposed females (Figure 4B; P= 0.0021). However, in one of these replicates median survival in the control group was lower.

Effects of *Csp_P* Exposure on the Midgut Microbiota

To determine whether *Csp_P* affects the load and composition of the midgut microbiota, the cultivable bacteria of individual midguts from females prior to bacterial exposure (baseline) and at 24 and 96 hours after ingestion of PBS, *Csp_P*, an *Enterobacter* (*Esp_Z*) isolated from *Anopheles* in Zambia (Cirimotich et al. 2011), or *P. sp.*. The dissection at the 24 hour time point was chosen due to evidence that *Csp_P* colonizes efficiently at 24 hours (Ramirez and Short et al. 2014); and the 96-hour time point coincides with the median survival of 10^7 CFU/mL *Csp_P*-fed

females (Figure 1C; Appendix 5). In this experiment, survival was attenuated significantly in *Csp_P*- ($P < 0.0001$) and *Esp_Z*- ($P = 0.0205$) exposed females (Figure 5); however, the relative survival at 4 days post-*Csp_P* exposure was 20.4% and median survival was not reached (Appendix 9).

The total number of cultivable bacteria for each group was assessed in reference to the baseline and the PBS control midgut bacterial loads. At 24 hours, the total number of cultivable bacteria in mosquitoes that fed on *Esp_Z* increased significantly compared to baseline mosquitoes ($P < 0.05$); but in relation to the PBS control, the load of cultivable bacteria did not differ across groups ($P > 0.05$) (Figure 6A). The bacterial load at 96 hours increased significantly for all groups, with the exception of *Csp_P*-fed, in reference to baseline ($P_{\text{Baseline- PBS,Esp\_Z, P. sp.}} < 0.0001$, $P_{\text{Baseline-Csp\_P}} > 0.05$), but the total number of bacteria did not increase compared to the 96-hour PBS control ($P_{\text{PBS-Csp\_P, Esp\_Z, P. sp.}} > 0.05$) (Figure 6A). Further, when only the endogenous bacteria (*Asaia*, *Elizabethkingia*, *Klebsiella*, and *Staphylococcus*) were used for the analysis, the same patterns were observed with the exception that the bacterial load in *Esp_Z*-fed females at 24 hours did not increase significantly relative to baseline ($P_{\text{Baseline-}Esp\_Z} > 0.05$) (Figure 6B).

The prevalence of the non-introduced, endogenous cultivable bacteria (*Asaia*, *Elizabethkingia*, *Klebsiella*, and *Staphylococcus*) did not differ significantly among the treatments relative to baseline or PBS (Figures 7A-D; $P_{\text{Baseline- PBS, }Csp\_P, Esp\_Z, P.sp.} > 0.05$, $P_{\text{PBS-}Csp\_P, Esp\_Z, P.sp.} > 0.05$). As expected, the prevalence of the introduced bacteria was higher in the correspondingly exposed group at 24 hours and absent from all other groups (Figures 7E-G; $P_{Esp\_Z} = 0.0080$, $P_{Csp\_P} = 0.0433$, $P_{P. sp.} = 0.0080$). The introduced bacteria were not found frequently at 96 hours, suggesting transient colonization (Figures 7E-G). Overall, feeding bacteria does not influence the composition of the cultivable midgut microbiota.

Discussion

Tripartite interactions between mosquito, microbiome, and pathogen have the potential to shape vectorial capacity. For instance, microbiota associated with the mosquito midgut may interfere with susceptibility to *Plasmodium* infection by altering immune signaling (Dong et al. 2009, Garver et al. 2008, Meister et al. 2009, Rodrigues et al. 2010, Warr et al. 2008) or producing factors that directly impair pathogen viability or infectivity (Bahia et al. 2014, Cirimotich et al. 2011, Ramirez and Short et al. 2014). Alternatively, the mosquito microbiome may disrupt vector-vertebrate host interactions by decreasing vector population size (Barbosa et al. 2007, Fernandez-Luna et al. 2010, Futami et al. 2011, Joshi et al. 2014), propensity to blood feed (Blanford et al. 2005, Darbo et al. 2012), or mosquito longevity (Akhouaryi et al. 2013, Bahia et al. 2014, Gonzalez-Ceron et al. 2003, Ramirez and Short et al. 2014). In a laboratory setting, the entomopathogenic *Chromobacterium* (*Csp_P*) has previously been shown to block *Plasmodium falciparum* infection and kill larval and adult *An. gambiae* (Ramirez and Short et al. 2014). This study further evaluated how *Csp_P* infection influences various life history traits of immature and adult *An. gambiae*. The direct and transgenerational impacts of *Csp_P* exposure on mosquito fitness, and the composition of cultivable midgut microbiota were tested to yield insights into the potential suitability of *Csp_P* as a biological agent for malaria control.

Csp_P and Female Fitness Parameters

Since entomopathogenic bacteria typically decrease the fecundity and proportion of egg-laying females (Garza-Hernandez et al. 2013, Joshi et al. 2014, Mmakumusana 1986, Scholte et al. 2006), *Csp_P* was anticipated to influence these fitness parameters negatively. Ingestion of either 10^2 or 10^7 CFU/mL *Csp_P*, or the control bacterium *P. sp.*, did not affect the proportion of female mosquitoes ovipositing (Figure 1A) or their fecundity (Figure 1B). Entomopathogens cited to interfere with reproduction typically induce immune responses (Dong et al. 2012, Hughes

et al. 2011), which may divert investment from egg production to anti-microbial responses (Reaney and Knell 2010, Rhen et al. 2006). Previously, it was shown the *Csp_P* (10^8 CFU/mL) does not activate *An. gambiae*'s IMD pathway 24 hours after infection (Rameriz and Short et al. 2014). The lack of such a *Csp_P* stimulated immune responses may therefore minimize reproductive costs of infection. Overall, *Csp_P* ingestion did not confer measurable reproductive costs or benefits.

Transgenerational Effects of *Csp_P* Exposure: Fitness Parameters

The F1 offspring derived from *Csp_P*- or PBS-challenged females were reared to evaluate transgenerational fitness effects. Pathogen exposure has been shown either to decrease (Nnakumusana 1986) or not to affect mosquito fertility (Darbo et al 2012, Linder and Promislow 2009). Although the viability of eggs laid by *Csp_P*-exposed females did not differ from those produced by PBS-exposed females (Figure 2A), a higher proportion of F1 progeny derived from *Csp_P* exposed females died prior to eclosion as adults (Figures 2B, D, F). Additionally, after accounting for larval death, maternal exposure to *Csp_P* was associated with the delayed pupation of F1 larvae (Figure 2E). These results suggest that the offspring of females exposed to *Csp_P*, in the immature stages, are not as healthy as the control progeny derived from PBS-exposed females.

Despite fitness deficits during the immature stages, maternal *Csp_P* exposure did not affect the longevity (Figure 2G) or sex ratio (Figure 2H) of F1adult offspring. Although *Csp_P* was detected in larval water in only one of the three replicates (data not shown), the role of *Csp_P* in directly mediating larval mortality and developmental delays cannot be excluded. In the future, plating larval cadavers to isolate *Csp_P* could substantiate or negate this bacterial strain's involvement in the fitness costs observed during the immature stages of development. Also, testing for the presence of *Csp_P* in F1 adult offspring using either culture dependent or

independent methods, would be beneficial to assess the potential for *Csp_P*'s intergenerational transmission. Since the transgenerational fitness effects were consistent across replicates regardless of *Csp_P* detection, other factors may likely contribute to these phenotypes.

Depletion of energy stores or interference with the transport of available nutrients to developing oocytes during vitellogenesis may explain the declined survival and delayed development of *Csp_P* F1 larvae. In other studies, pathogen infection has been shown to deplete energy stores, particularly lipids, in exposed mosquitoes (Oliveira et al. 2011b, Rivero et al. 2007). Yolk components, including maternally derived amino acids and lipids, are incorporated into the oocytes following a blood meal (Atella et al. 2006, Briegel et al. 2001, Hansen et al. 2014, Leyria et al. 2014). If *Csp_P* infection alters nutrient availability, composition, or transport to developing oocytes, then the F1 eggs of *Csp_P*-exposed females may have fewer nutritional reserves.

Since parental fecundity and F1 embryonic viability appear unaffected, the mechanism associated with delayed larval development appears not to affect the initial production of viable *An. gambiae* first instar stage larvae. Clifton and Norigea (2012) showed that undernourished female mosquitoes transfer fewer lipids to their offspring without affecting the proportion of mosquitoes ovipositing, although fecundity was marginally decreased by 10%. Unfortunately, the development of the hatched larvae was not followed (Clifton and Norigea 2012). For insects, most experiments that assess the transgenerational fitness costs of pathogen exposure stop following assessment of F1 embryonic fertility (Garza-Hernandez et al. 2013, Joshi et al. 2014, Mmakumusana 1986, Scholte et al. 2006, Shoemaker and Adamo 2007), so not much is known about offspring survival or developmental rate. Evidence from mammalian and avian systems, however, correlates maternal condition and offspring health (Coakley et al. 2014, Kallio et al. 2015). For example, in wild blue tits treated with malarone, an anti-malarial drug that decreases *Plasmodium* burden, fledging success increases (Knowles et al. 2009); in rat pups born to mother infected with *Mycoplasma arthridis*, neonatal survival decreases (Naot et al. 1978). However, the

potential differences in parental care after birth may confound the effect of resource investment during embryogenesis.

In these experiments, the F1 larvae derived from *Csp_P* exposed females were not affected until later larval instars, suggesting that metamorphosis may be dysregulated. Since nutritional status plays an integral role in metamorphosis regulation (Koyama et al. 2013, Layalle et al. 2008, Niwa and Niwa 2014, Telang et al. 2007, Telang et al. 2010), any changes to resource transfer during vitellogenesis may impact the development and survival of the resulting offspring. Lipids are of particular interest due to the link identified between neutral lipid storage in the larval midgut and metamorphic hormones, juvenile hormone and 20-ecdysone, signaling (Nishiura et al. 2007). However, amino acids may also be important nutritional signals, as they regulate the TOR pathway, which is coupled with developmental timing (Hansen et al. 2014, Koyama et al. 2013).

Additionally, studies indicate that metamorphosis and immune responses are interrelated processes, and that successful molts require competent immune responses (Altincicek and Vilcinskas 2008, Regan et al. 2013, Rus et al. 2013, Zhang and Palli 2009, Zhang et al. 2014). Since mosquito larvae utilize microorganisms as a nutrient resource during development, ingested bacteria or fungi may activate immune responses when the midgut tissue is turned over. Over-stimulation of immune responses, typically involving the melanization cascade, or an uncontrolled infection may abort a molt or result in death (Zhang et al. 2014). Often, the larval cadavers recovered from either *Csp_P* or PBS trays were melanized (personal observation). The bacterial loads in the larval water did not differ between the groups at any of the time points (Figure 3), suggesting the risk of bacteria-related death during development would be similar between the two groups in the absence of other differences. As such, the hypothesis that maternal *Csp_P*-exposure interferes with nutrient transfer during egg production causing transgenerational deficiencies in larval development and survival remains plausible.

Alternatively *Csp_P* may epigenetically alter the germline of exposed females, thus influencing gene expression in the resulting progeny. The epigenetic ensemble of *An. gambiae* was recently characterized and includes over 150 genes including histone modifying enzymes as well as a DNA methyltransferase (Jenkins and Muskavitch 2015). Although the transgenerational relevance of epigenetic changes is not well characterized, maternal life history has been shown to influence the epigenome and non-genetic inheritance of the offspring (Desai et al. 2015, Rechavi et al. 2014, Sinclair et al. 2007, Vangeel et al. 2015). For example, in sheep the preconception B vitamin and methionine levels altered the methylation pattern of pre-ovulatory oocytes (Sinclair et al. 2007). Even when the embryos were implanted in a sheep maintained on a normal diet, the offspring experienced altered immune responses, insulin resistance, and blood pressure elevation related to the dysregulated expression patterns of differentially methylated genes (Sinclair et al. 2007).

Additionally, exposure to microorganisms, including *Plasmodium* and *Wolbachia*, is known to alter the non-coding RNA profiles of mosquitoes (Dennison et al. 2015, Hussain et al. 2011, Mayoral et al. 2014, Osei-Amo et al. 2012, Zhang et al. 2013). Although any changes to non-coding RNA profiles are more likely to influence gene expression of the exposed females, non-coding RNA may play a transgenerational role (Rechavi et al. 2014, Sharma 2015, Yan 2014, Yuan et al. 2015). Transgenerational epigenetic changes that influence the expression of genes involved in metamorphosis, including cuticle proteins (Charles 2010, Farnesi et al. 2012), nutrient transporters (Atella et al. 2006, Hansen et al. 2014), and immune genes (Altincicek and Vilcinskas 2008, Regan et al. 2013, Rus et al. 2013, Zhang and Palli 2009, Zhang et al. 2014), or epigenetic regulating genes, such as DNA or histone modifying enzymes (Jenkins and Muskavitch 2015), could contribute to the reduced survival and delayed pupation following *Csp_P* exposure.

Despite the negative effects of maternal *Csp_P* exposure on the survival and the rate of development in the immature stages, the adult offspring were not impacted measurably. The F1

adult offspring did not differ in longevity (Figure 2G) or sex ratio (Figure 2H). The lack of a phenotype may support the hypothesis that F1 larvae of *Csp_P*-exposed females are undernourished. If the larvae acquire enough resources, though delayed, to progress through each of the molts, any previous deficits may be compensated for and leave the adult offspring relatively unaffected. Due to the limited number of F1 offspring reared to adulthood, the fecundity of F1 adult offspring was not measured; however, repeating these experiments to assess F1 reproductive fitness may offer valuable insight into long-term multigenerational fitness effects. Additionally, investigating whether all offspring are affected equally or if offspring of a few females experience delayed pupation and larval death may provide further insight into these phenotypes.

Transgenerational Effects of *Csp_P* Exposure: *Csp_P* Challenge

Exposing two consecutive mosquito generations to *Csp_P* did not diminish *Csp_P*'s killing efficiency. When the F1 larvae derived from *Csp_P*-exposed females or F1 controls derived from PBS-exposed females were challenged with a moderately lethal dose of *Csp_P*, median survival times did not significantly differ from one another (Figure 4A). When the F1 larvae were exposed to PBS as a negative control, the survival of F1 larvae from *Csp_P*-exposed females was lower than that of F1 larvae from PBS-exposed females (Figure 4A), reflective of the mortality observed in the larval development assay (Figure 2B).

Adult F1 female offspring from *Csp_P*-exposed females that were challenged with *Csp_P* (10^7 CFU/mL) died significantly faster than did female adult offspring of PBS-exposed females (Figure 4B). However, in one of the two replicates the control mosquitoes died more quickly. Given this observed variability, this experiment should be repeated in the future a third time to resolve the legitimacy of this phenotype. In addition, the low number of F1 adult females

that eclosed each replicate prevented exposure to PBS in parallel with F1 *Csp_P* challenge. These additional controls should also be included when this suite of experiments is replicated.

Retaining multigenerational killing capacity is encouraging; however, the selection pressure applied to the adults (F0) was low, 12.5-43.4% relative mortality at 96 hours after exposure (Appendix 1), and may not be predictive of long-term, multigenerational use. The bioinsecticides, *Bacillus thuringiensis*, *B. sphaericus*, and their derived toxins, kill over 90% of susceptible larvae (Goldman et al. 1986, Mittal et al. 1998, Paris et al. 2010, Paris et al. 2011, Wirth et al. 2000). Unfortunately, resistance to these mosquitocidal bacteria can be selected for in less than 15 generations (Goldman et al. 1986). In *Culex quinquefasciatus*, exposure of larvae to a concentration of *B. sphaericus* lethal to nearly 98% of mosquitoes within 48 hours, killed only 71.3% of mosquitoes within 48 hours the subsequent generation (Wirth et al. 2000). This observation suggests that exposure to a strongly selective dose of *Csp_P*, particularly as larvae, may potentiate the evolution of resistance at a faster pace than would ingestion of a moderately lethal dose in adults. Examining how larval exposure to *Csp_P* influences transstadial and intergenerational fitness parameters and the development of resistance to perturbations in mosquito lifespan is important for designing any larval stage-targeted *Csp_P*-based control. A selection experiment, exposing adults or larvae to *Csp_P* for multiple generations, may provide valuable insight into both the mechanisms of *Csp_P*-mediated mortality and potential for resistance to evolve.

Bacterial Exposure and the Midgut Microbiota

Since the composition of the midgut microbiota is known to influence vectorial capacity, the cultivable bacteria were measured following ingestion of known bacteria. The three bacterial isolates, *Csp_P*, *Esp_Z*, and *P. sp.*, were selected for this experiment; because they grow similarly at 30°C (data not shown), were isolated from field-caught mosquitoes (Cirimotich et al. 2011,

Ramirez et al. 2012) , and have all been shown to decrease vector competence of *An. gambiae* for *P. falciparum* (Bahia et al. 2014, Cirimotich et al. 2011, Ramirez and Short et al. 2014). Unlike the other two bacteria, *Csp_P* secretes anti-bacterial factors that are active against a broad range of bacteria naturally found in mosquitoes, and is not isolated commonly from *Anopheles* (Ramirez and Short et al. 2014). Due to previous evidence that *Csp_P* colonizes the *An. gambiae* gut by 24 hours (Ramirez and Short et al. 2014), prior to the dramatic decrease in survival, and that peak mortality occurs around 96 hours, these time points were chose to measure the cultivable midgut bacteria. Exposure to *Csp_P* was expected decrease the prevalence and load of the endogenous bacteria while *P. sp.* and *Esp_Z* were predicted not to affect on the midgut microbiota composition.

The total number of bacteria did not differ significantly from the PBS control at either time point for any of the bacteria (Figure 6A). Although not significant, when only endogenous bacteria (*Asaia*, *Elizabethkingia*, *Klebsiella*, and *Staphylococcus*) were included in the analysis, the total number of bacteria tended to be lower in the *Csp_P*- and *Esp_Z*-fed mosquitoes at 24 hours and in the *Csp_P*-fed individuals at 96 hours (Figure 6B). At 24 hours, the decrease in the load of endogenous bacteria at 24 hours likely relates to the decreasing trend in *Asaia* prevalence (Figure 7A) in parallel with the significant increase in prevalence of the fed bacteria (*Csp_P*, *Esp_Z*, *P. sp.*) (Figures 7E-G). Reciprocally, the prevalence of *Asaia* tended to increase at 96 hours and the fed bacteria prevalence decreased significantly (Figures 7A, E-G). The total midgut bacterial load for all groups, though not significant in *Csp_P*-exposed, was higher at 96 hours than prior to bacteria introduction (baseline) (Figure 6).

The increased bacterial load from baseline to 96 hours may reflect either the accumulation of bacteria over time, or the proliferation of endogenous bacteria due to the increased sucrose concentration from 1.5%, when the bacteria or PBS were introduced, to 10%, after the 24-hour bacterial or PBS exposure in our experimental protocol (Alvarez-Perez et al. 2012, Chouaia et al. 2010). Since bacteria associated with the mosquito midgut, such as *Asaia*,

proliferate in high sugar conditions (Alvarez-Perez et al. 2012, Chouaia et al. 2010), it is possible that *Asaia* proliferated more efficiently in the 10% sucrose environment than did the fed bacteria preventing their long-term colonization. In the future, maintaining the mosquitoes on a lower sucrose concentration may influence the colonizability of the fed bacteria.

The lack of evidence that *Csp_P* reduces the prevalence of the cultivable bacteria may be due to insufficient colonization or lack of signals to produce or secrete anti-bacterial factors. In *Chromobacterium violaceum* the production of chitinases, hydrogen peroxide resistance, and violacein rely on quorum sensing (de Oca-Mejia et al. 2015). If quorum sensing is required, *Csp_P*'s inability to colonize persistently, or proliferate to a critical population size, may prevent the production of anti-bacterial factors. If *Csp_P* was fed immediately after eclosion, when the gut relatively is sterile, colonization may occur more efficiently and alter the ability of other microorganisms to persist. Additionally, it would be interesting to see whether multigenerational *Csp_P* exposure affects microbiota composition.

Although *Csp_P* did not decrease the load or prevalence of the cultivable endogenous bacteria as predicted, exposure may influence the non-cultivable bacteria. Only two genera, *Asaia* and *Elizabethkingia*, were isolated regularly across all three replicates (Appendix 11). Although the bacterial diversity in lab-reared colonies is lower than in field-collected mosquitoes, non-cultivable bacteria most likely are present (Boissiere et al. 2012, Duguma et al. 2015, Rani et al. 2009, Steyn et al. 2015, Wang et al. 2011). To gain adequate insight into whether bacteria introduction can dysregulate the composition of an established microbiota, performing a metagenomic analysis of 16S rDNA sequences, in parallel with plating, would be beneficial.

A complication persistent across experiments was identifying an optimal dose. Previously, *Csp_P* killed approximately 90% of adults females fed a 10^8 CFU/mL dose within 48 hours (Ramirez and Short et al. 2014). During the exposed-female fitness bioassays, the 10^7 CFU/mL dose consistently caused moderate mortality (43.1-55%) at 96 hours (Figure 1C; Appendix 1); however, when the same dose was fed for the microbiota assays mortality at 96

hours was only 19-28% (Figure 5, Appendix 1). Before starting the microbiota assays, the decline in killing efficiency had been noted, and an attempt was made to determine whether a different dose might be more suitable. A range of doses from $1X10^6$-$5X10^9$ CFU/mL, at increments of half-a-log, were fed. Only the doses above $5X10^7$ CFU/mL resulted in significant mortality; and, consistent with the previous experiment, a 10^8 CFU/mL, or higher, dose killed at least 85% of mosquitoes died within 96 hours (Appendix 3). Doses in the 10^7-$5X10^5$ CFU/mL induced variable mortality (Appendices 1,3). Due to the past evidence of up to 70% killing efficiency (SvT-62, Appendix 1), the need for consistency across replicates, and the sample sizes required for the experiments, the 10^7 CFU/mL dose was chosen. The mean and median relative mortality at 96 hours across replicates for all experiments was 41.9% and 43.5%, respectively, and longevity was reduced significantly in each replicate (Appendix 1).

Multiple mosquito- and bacteria-related factors may contribute to the variation observed in *Csp_P* killing efficiency. Firstly, the dose fed was variable ($1.63X10^7$-$1.67X10^8$ CFU/mL) despite the intent of feeding *Csp_P* at $1X10^7$ CFU/mL (Appendix 1). The dose fed, within the intended 10^7 range, and relative survival (*Csp_P* survival/PBS survival) at 96 hours did not correlate (r^2= 0.01483 P= 0.5990, Appendix 2). The lack of correlation between the survival and dose fed indicates that other factors likely contribute to the variability observed. For example, the rearing conditions of exposed females, such as density and food availability, may have influenced the mosquitoes' susceptibility to *Csp_P* (Muturi et al. 2011, Muturi et al. 2012, Suwanchaichinda and Paskewitz 1998, Telang et al. 2012). Alternatively, differences in the *Csp_P* culture prior to washing, the amount of bacteria ingested, or the composition of the midgut microbiota may have been contributing factors. Although only correlational, during the exposed-female fitness assays (96 hour relative survival 50.3%; Appendix 5) *Serratia* was prevalent (39.4%, Appendix 4) while during the microbiota assays (96 hour relative survival 79.6%; Appendix 9) *Serratia* was absent from all groups (Appendix 4). *Serratia* possesses entomopathogenic activity against *Anopheles spp.* (Bahia et al. 2014, Gonzalez-Ceron et al. 2003), and the co-colonization of *Csp_P* and

Serratia may increase the observed mortality synergistically or additively. The threshold effect observed, i.e. doses over 10^8 CFU/mL killed nearly all and below 10^7 CFU/mL does not kill mosquitoes, suggests that either mosquitoes can control only *Csp_P* infection up to a critical number or *Csp_P* produces and secretes a lethal factor only after proliferating to a particular bacterial density.

Since the survival phenotype, and potentially the transgenerational fitness phenotypes, is dose-dependent, requiring ingestion of a starting concentration of 10^7 CFU/mL or higher, exposing mosquitoes to *Csp_P* in a more ecologically relevant setting may model the effects on mosquito life history more accurately. For example, in the laboratory the mosquitoes are starved overnight to ensure that mosquitoes feed on the bacteria to measure effects, but in a natural setting the mosquitoes may not ingest enough *Csp-P* to cause substantial mortality in exposed adults or the F1 progeny derived from challenged adults. In the future, experiments that introduce *Csp_P* into caged mosquito populations and evaluate fitness parameters under semi-field conditions will allow us to thoroughly assess the potential of this bacterial strain to control malaria transmission.

Conclusion

Overall, these experiments demonstrate that (i) ingestion of a moderately lethal dose of *Csp_P* negatively affects the condition of an exposed female's offspring without imparting measurable fitness benefits; (ii) exposure to *Csp_P* for two consecutive generations does not impact the killing efficiency of this bacterial strain; and (iii) introduction of bacteria to adult females does not dysregulate the endogenous cultivable midgut microbiota. The ability of *Csp_P* to impair the survival and development of F1 offspring derived from an exposed *Anopheles gambiae* female may increase the potential utility of *Csp_P* as an applied biopesticide for mosquito population reduction. Sugar baits could serve as a means of introducing high doses of

Csp_P into wild mosquito populations (Muller et al. 2010a, Muller et al. 2010b, Qualls et al. 2014, Stewart et al. 2013). The multifactorial features of *Csp_P* in decreasing vector density, daily survival, and vector competence for *P. falciparum*, without an identified fitness benefit, encourages the introduction of this bacterial strain to adult mosquitoes to control malaria transmission. To determine the feasibility of *Csp_P* to control larval populations, transstadial and intergenerational fitness experiments would need to be conducted after larval exposure. Additional laboratory and semi-field studies are essential to determine the long-term effects of *Csp_P* exposure on fitness parameters, *Plasmodium* susceptibility, mechanisms underlying direct and indirect mosquitocidal phenotypes, and the potential for the evolution of resistance. These experiments will provide a substantial foundation for informing the design and field deployment of any *Csp_P*-based mosquito control strategy.

Figure 1. Effect of *Csp_P* Exposure on Adult Female Fitness Parameters

All female mosquitoes were fed either a 10^2 or 10^7 CFU/mL dose of *Chromobacterium* (*Csp_P*) or *Pantoea sp.* (*P. sp.*) or 1X PBS in a 1.5% sucrose meal. Exposure to either a low or high dose of *Csp_P* or *P. sp.* did not alter oviposition (A; P= 0.9013) or fecundity (B; P= 0.7205). Each data point represents the proportion of ovipositing females (A) or the number of eggs per individual mosquito (B) for 3 or 4 biological replicates; the mean and error bars, representing the 95% confidence interval, are presented on the graph; total sample sizes: n_{PBS}= 56, $n_{P.\ sp.\ 10\^2}$= 49, $n_{Csp\_P\ 10\^7}$= 48, $n_{P.\ sp.\ 10\^7}$= 35, $n_{Csp\_P\ 10\^7}$= 50. Survival was monitored daily for ten days (C). Only the 10^7 CFU/mL dose of *Csp_P* significantly decreased survival ($P_{PBS\text{-}Psp10\^2}$= 0.5771, $P_{PBS\text{-}Psp10\^7}$= 0.8154, $P_{PBS\text{-}CspP10\^2}$= 0.7046, $P_{PBS\text{-}CspP10\&7}$ <0.0001). The data represents 3-4 biological replicate; total sample sizes: n_{PBS}= 231, $n_{P.\ sp.\ 10\^2}$= 208, $n_{Csp\_P\ 10\^2}$= 219, $n_{P.\ sp.\ 10\^7}$= 162, $n_{Csp\_P\ 10\^7}$= 271. A one-way ANOV (A), Kruskal-Wallis test (B), and pair-wise log-rank test of survival (C) was used to analyze the data.

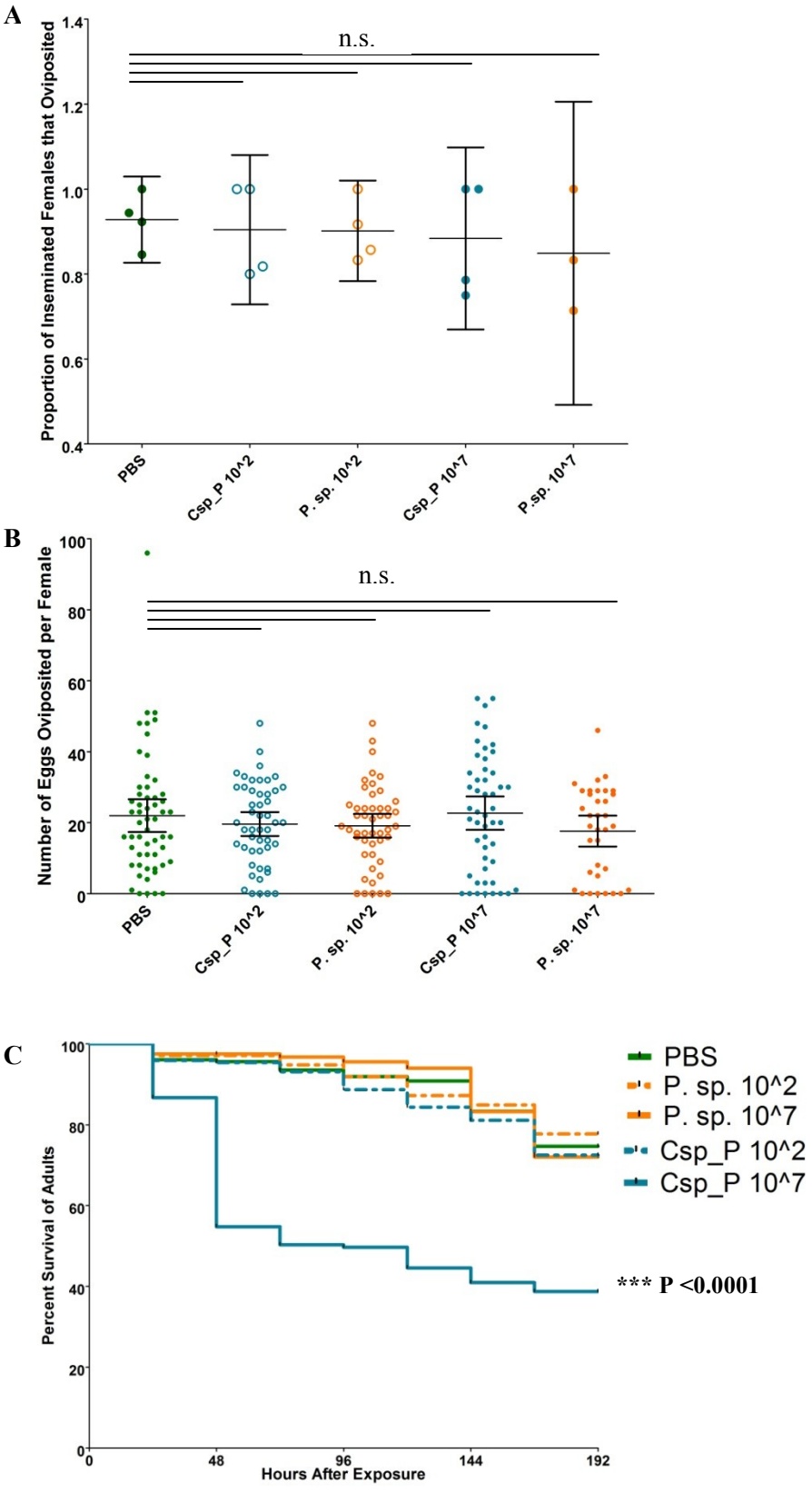
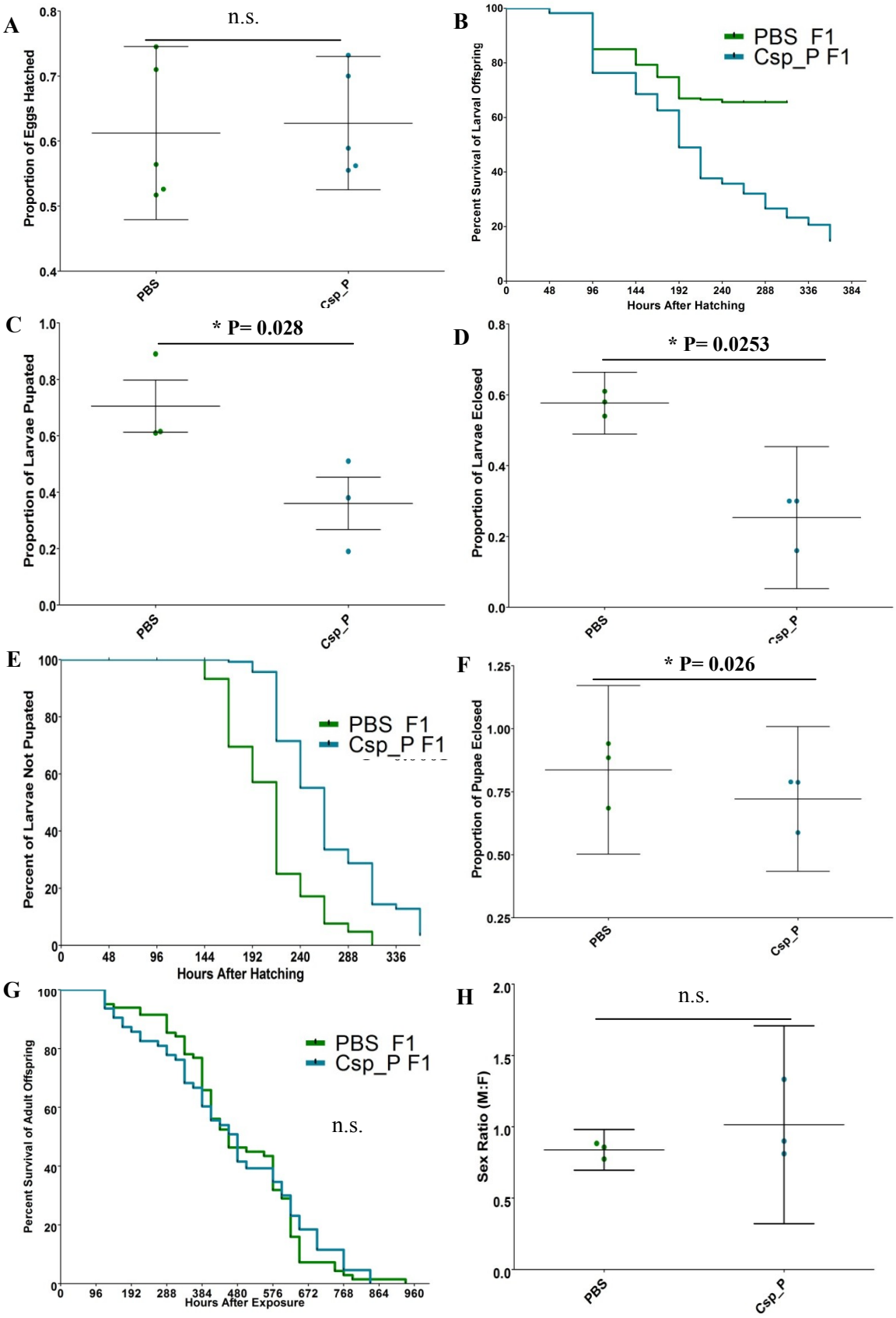

A
n.s.
Proportion of Inseminated Females that Oviposited
1.4
1.2
1.0
0.8
0.6
0.4
PBS
Csp_P 10^2
P. sp. 10^2
Csp_P 10^7
P. sp. 10^7
B
n.s.
Number of Eggs Oviposited per Female
100
80
60
40
20
0
PBS
Csp_P 10^2
P. sp. 10^2
Csp_P 10^7
P. sp. 10^7
C
Percent Survival of Adults
100
80
60
40
20
0
0
48
96
144
192
Hours After Exposure
PBS
P. sp. 10^2
P. sp. 10^7
Csp_P 10^2
Csp_P 10^7
*** P <0.0001

Figure 2. Intergenerational Fitness Effects of *Csp_P*

In all experiments female mosquitoes were allowed to feed on a 10^7 CFU/mL dose of *Csp_P* or an equal volume of PBS in a 1.5% sucrose meal, and then their F1 progeny were reared. Fertility (A) was not affected by maternal *Csp_P* exposure (P= 0.4179). The survival F1 larvae of *Csp_P*-exposed females was reduced significantly relative to F1 larvae of PBS control (B) (P <0.0001). Maternal *Csp_P* reduced the proportion of F1 larvae the pupated (C) (P= 0.028) and eclosed as adults (D) (P= 0.0253). Additionally, the median time to pupation was forty-eight hours longer in the offspring of *Csp_P*- exposed females (E) (P< 0.0001), and the number of *Csp_P* F1 pupae that eclosed as adults was decreased (F) (P= 0.026). The fitness deficits were restricted to the immature stages as neither adult offspring survival (G) (P= 0.7992) nor sex ratio (H) (P= 0.3482) were affected. For fertility (A), pupation (C), eclosion (D and F), and sex ratio (H) experiments, each data point represents the proportion for each of 3(C,D,F,H) or 5(A) biological replicates; total sample sizes: n_{PBS}(A)= 835, n_{Csp_P}(A)= 793, n_{PBS}(C,D)= 400, n_{Csp_P}(C,D)= 400, n_{PBS}(F)= 276, n_{Csp_P}(F)= 129, n_{PBS}(H)= 169, and n_{Csp_P}(H)= 167. The survival curves represent the average survival of 3 biological replicates (B,E,G); total sample sizes: n_{PBS}(B,E)= 400, n_{Csp_P}(B,E)= 400, n_{PBS}(G)= 82, and n_{Csp_P}(G)= 63. Paired t-tests (A,C,D,F,H) and pairwise log-rank survival analysis (B,E,G) were used to analyze data.


A
n.s.
Proportion of Eggs Hatched
PBS
Csp_P
B
PBS F1
Csp_P F1
Percent Survival of Larval Offspring
Hours After Hatching
C
* P= 0.028
Proportion of Larvae Pupated
D
* P= 0.0253
Proportion of Larvae Eclosed
E
PBS F1
Csp_P F1
Percent of Larvae Not Pupated
Hours After Hatching
F
* P= 0.026
Proportion of Pupae Eclosed
G
PBS F1
Csp_P F1
n.s.
Percent Survival of Adult Offspring
Hours After Exposure
H
n.s.
Sex Ratio (M:F)

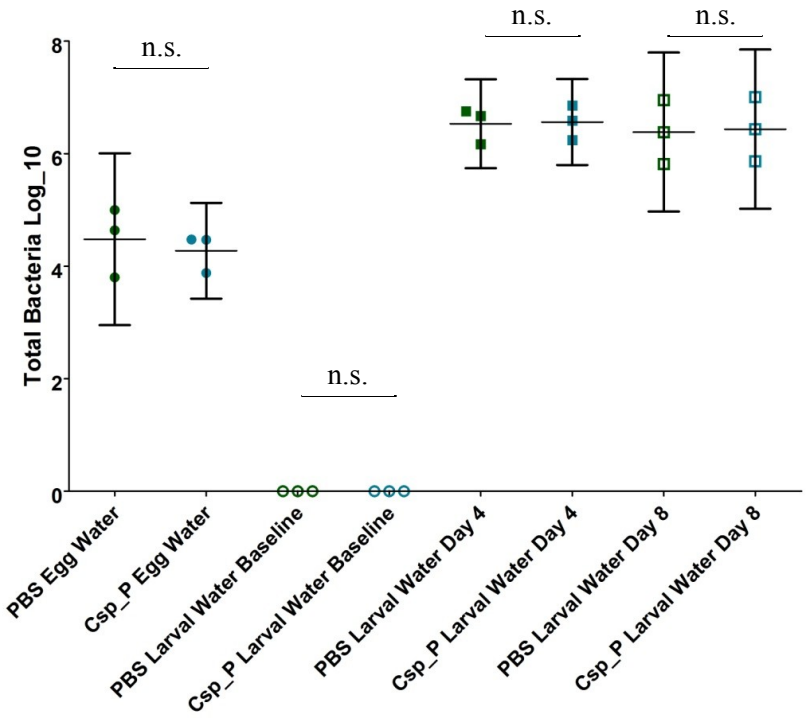
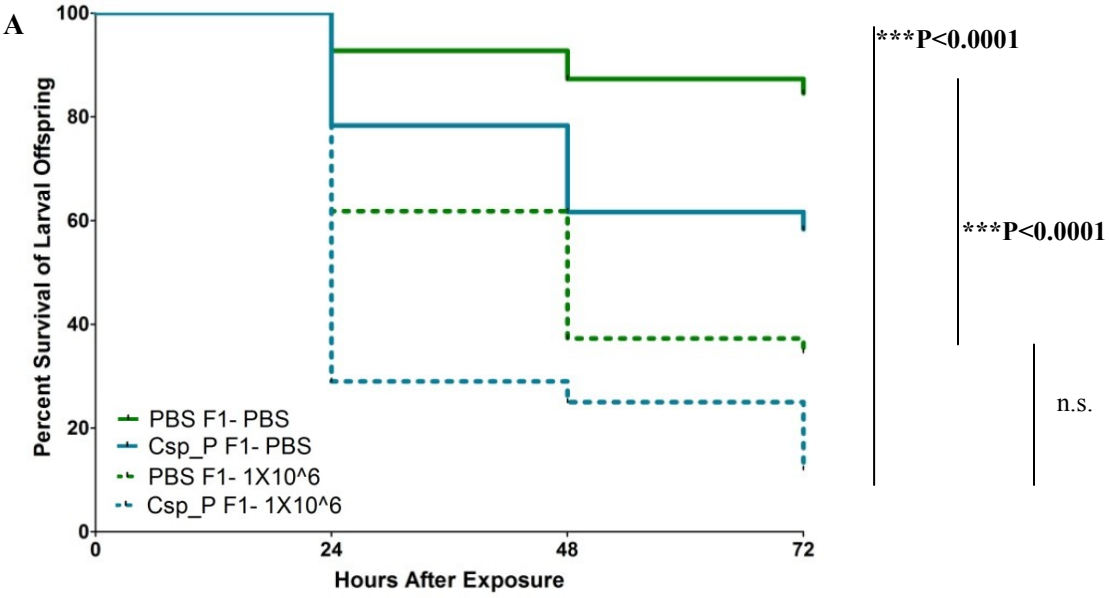


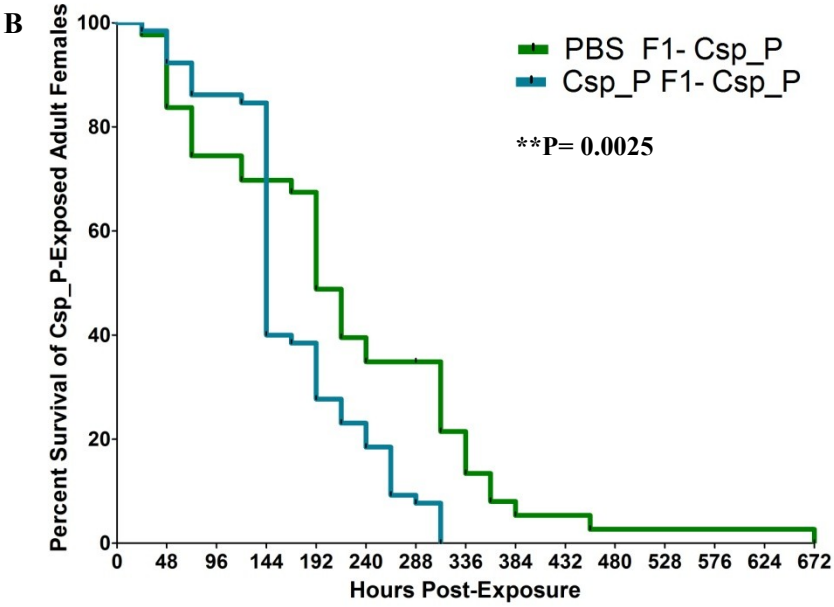
Figure 3. Bacterial Loads in the Water during Development of First-Generation Offspring of *Csp_P* Exposed Females

Two-1mL water samples were taken from the oviposition cups and from larval trays prior to adding food or larvae (baseline), and then again at 4 and 8 days after transfer of the larvae. Although the load of cultivable bacteria differed across time ($P < 0.0001$), the mean bacterial load did not differ between the two groups at any of the measured time points ($P > 0.05$). Each data point represents the average CFU of cultivable bacteria for each of the 3 biological replicate; error bars represent 95% confidence intervals. A one-way ANOVA with Bonferreni's multiple comparisons test was used to analyze the data.

Figure 4. Killing Efficiency of *Csp_P* in the First-Generation Offspring of *Csp_P* Exposed Females

For *Csp_P* susceptibility assays, F1 first instar larvae derived from either *Csp_P*- or PBS-exposed females were exposed to either PBS or 10^6 CFU/mL *Csp_P* and survival was monitored daily through 72 hours (A). Maternal exposure to *Csp_P* negatively affected survival of larvae in the presence of PBS (P= 0.0344) and larval *Csp_P* exposure reduced survival regardless of maternal exposure status (P= 1.81e-11); however, no interaction was detected between maternal and larval exposure (P= 0.6105), indicating both groups are susceptible equally to *Csp_P*. The F1 adult offspring derived from either *Csp_P*- or PBS-exposed females were exposed to *Csp_P*, 10^7 CFU/mL, in a 1.5% sucrose meal for 24 hours, and survival was monitored daily (B). The *Csp_P* F1 adults died significantly more quickly after exposure (P= 0.0025), suggesting increased susceptibility. Survival curves represent the average of 2 (B) or 4 (A) biological replicates; total samples sizes: $n_{PBS\text{-}PBS}$= 100, $n_{PBS\text{-}1X10\text{^}6}$= 110, $n_{Csp\_P\text{-}PBS}$= 90, $n_{Csp\_P\text{-}1X10\text{^}6}$= 110, n_{PBS}(B)= 43, and n_{Csp_P}(B)= 65. A cox proportional hazards statistical analysis (A) and pairwise log-rank survival analysis (B) were used to analyze the data.


A
Percent Survival of Larval Offspring
100
80
60
40
20
0
0
24
48
72
Hours After Exposure
PBS F1- PBS
Csp_P F1- PBS
PBS F1- 1X10^6
Csp_P F1- 1X10^6
***P<0.0001
***P<0.0001
n.s.


B
Percent Survival of Csp_P-Exposed Adult Females
100
80
60
40
20
0
0 48 96 144 192 240 288 336 384 432 480 528 576 624 672
Hours Post-Exposure
PBS F1- Csp_P
Csp_P F1- Csp_P
**P= 0.0025

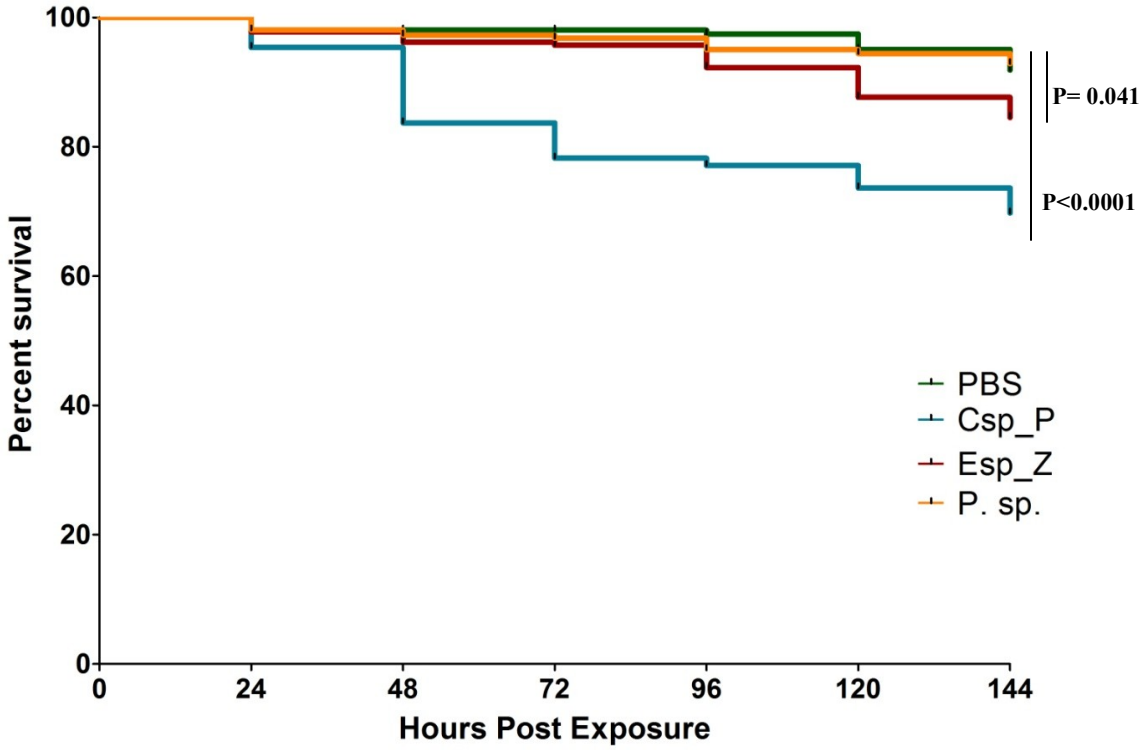


Figure 5. Survival of Bacteria-Exposed *Anopheles gambiae*

Female mosquitoes were fed a 1.5% sucrose meal with 10^7 CFU/mL of *Csp_P*, *Esp_Z*, or *P. sp.*, or an equal volume of 1X PBS for 24 hours. Exposure to *Csp_P* (P <0.0001) and *Esp_Z* (P= 0.0401) significantly reduced survival, and exposure to *P. sp.* had no impact on survival (P= 0.9223). The curves represent the average survival across 3 biological replicates; total sample size: n_{PBS}= 253, n_{Csp_P}= 365, n_{Esp_Z}= 264, and $n_{P.\ sp.}$= 256. Pairwise log-rank survival analysis was used to compare the survival effect of each bacterium relative to PBS.

Figure 6. Total Cultivable Midgut Bacteria in Bacteria-Exposed *Anopheles gambiae*

Seven- to- eight day eclosed female mosquitoes were fed a 1.5% sucrose meal with 10^7 CFU/mL of *Csp_P*, *Esp_Z*, or *P. sp.*, or an equal volume of 1X PBS for 24 hours. Individual midguts (N=6) were collected at baseline and for each treatment at 24 and 96 hours after introducing the bacteria. Compared to the baseline measurement, the average bacterial load in *Esp_Z* at 24 hours ($P < 0.05$), PBS, *Esp_Z*, and *P. sp.* at 96 hours ($P <0.001$) increases, but not in *Csp_P*-fed at 24 or 96 hours ($P >0.05$) (A). Relative to baseline, at 96 hours, the total number of endogenous bacteria (excludes cultivable fed bacteria) in PBS-, *P.sp.*- ($P <0.01$) and *Esp_Z*- ($P <0.001$) fed mosquitoes increases significantly but not in *Csp_P*-fed ($P >0.05$) (B). Bacteria introduction is not responsible for the increase in total bacteria, because the average total number of bacteria does not differ from PBS at the respective time points (A,B; $P >0.05$). Each data point represents the total cultivable bacteria (CFUs log bas 10 transformed) in an individual mosquito for three biological replicates; the mean and 95% confidence intervals are presented; total sample sizes: n=18 for each treatment at each time point (A,B). A one-way ANOVA with Bonferroni's multiple comparison test was used to analyzed the data.

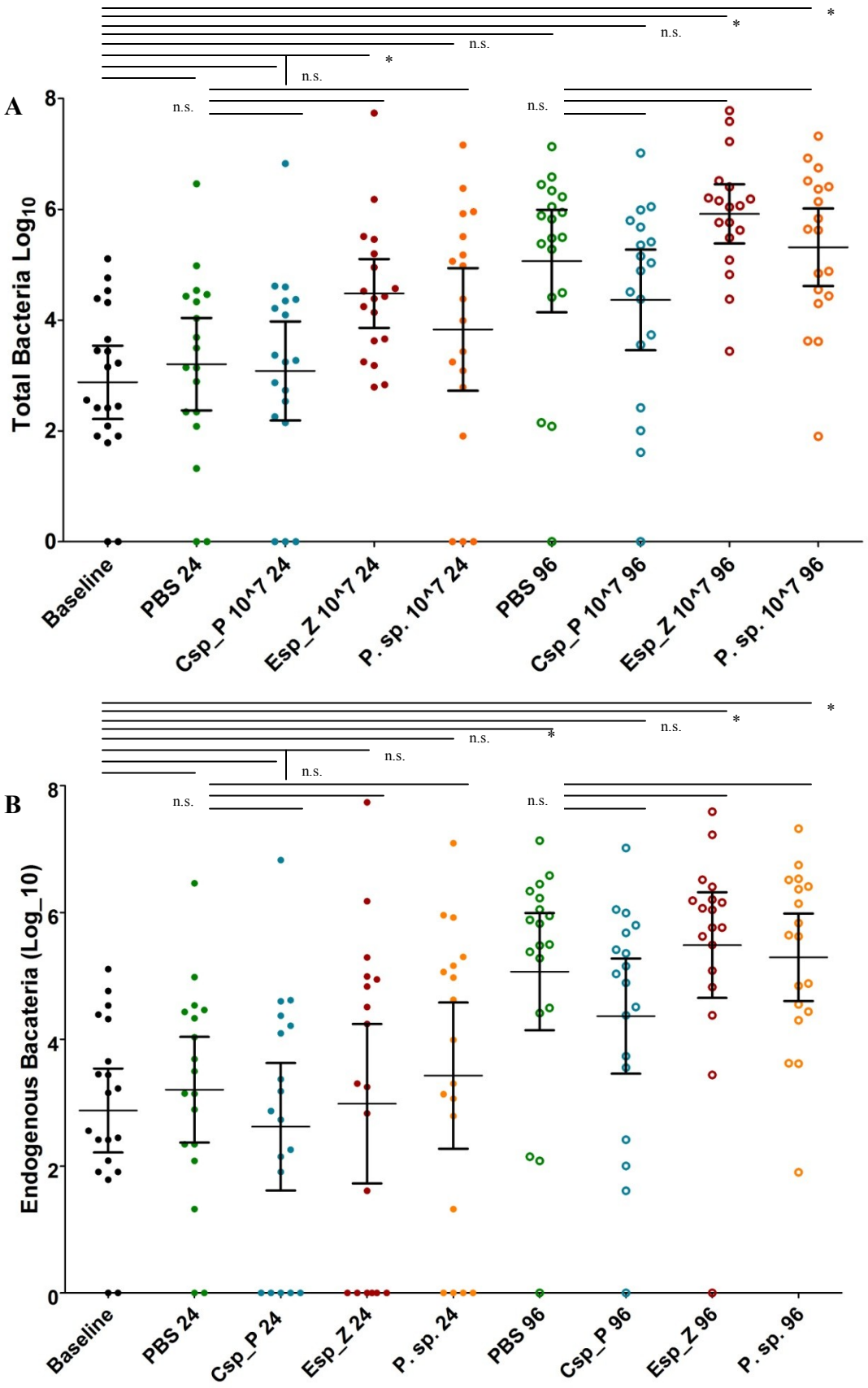
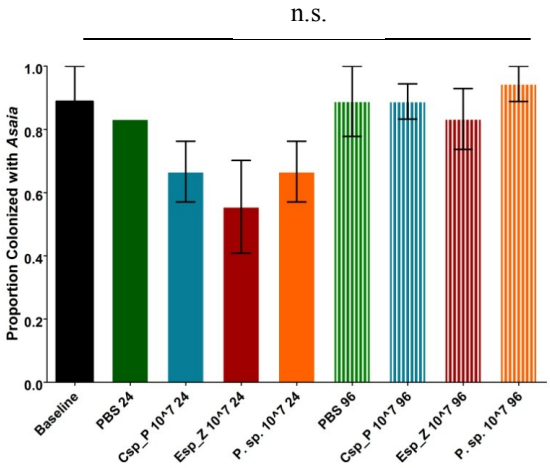
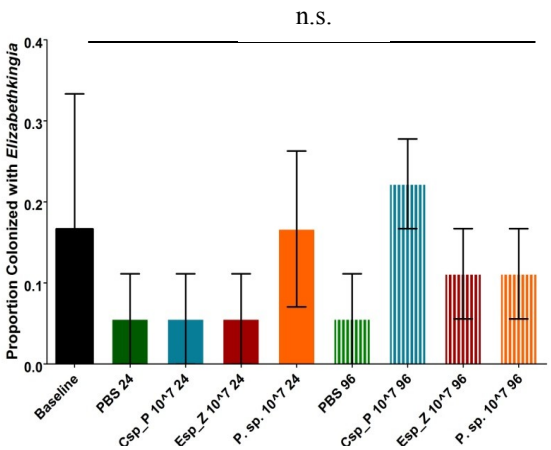
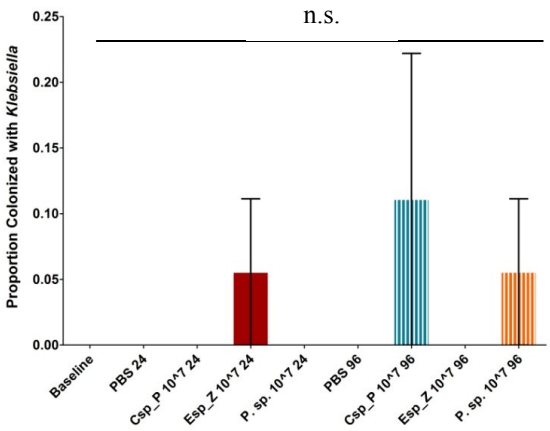

A
Total Bacteria Log10
0
2
4
6
8
n.s.
*
Baseline
PBS 24
Csp_P 10^7 24
Esp_Z 10^7 24
P. sp. 10^7 24
PBS 96
Csp_P 10^7 96
Esp_Z 10^7 96
P. sp. 10^7 96
B
Endogenous Bacateria (Log_10)
Baseline
PBS 24
Csp_P 24
Esp_Z 24
P. sp. 24
PBS 96
Csp_P 96
Esp_Z 96
P. sp. 96

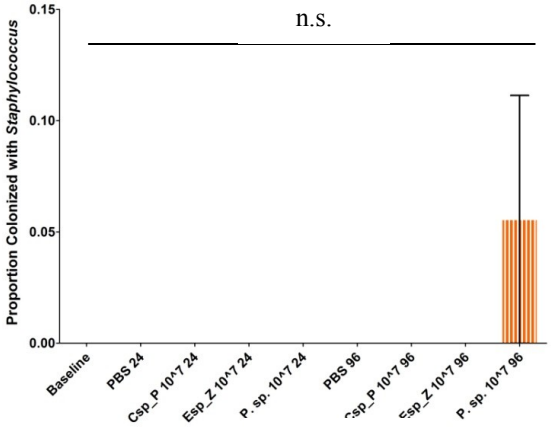
Figure 7. Prevalence of Cultivable Bacteria Species Identified in the Midgut of Bacteria-Exposed *Anopheles gambiae*

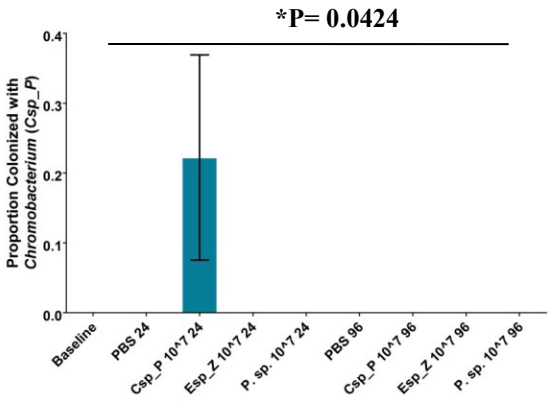
Seven- to- eight day eclosed female mosquitoes were fed a 1.5% sucrose meal with 10^7 CFU/mL of *Csp_P*, *Esp_Z*, or *P. sp.*, or an equal volume of 1X PBS for 24 hours. Individual midguts (N=6) were collected at baseline and for each treatment at 24 and 96 hours after introducing the bacteria. The prevalence for all colonies, including *Asaia* (A), *Elizabethkingia* (B), *Klebsiella* (C), *Staphylococcus* (D), *Csp_P* (E), *Esp_Z* (F), and *P. sp.* (G), was recorded. The prevalence of non-introduced bacteria (A-D) did not differ following exposure to the fed bacteria ($P > 0.05$). Introduction of bacteria did increase the prevalence of that bacterium in the respective treatment groups at 24 hours, *Csp_P* ($P = 0.0424$), *Esp_Z* ($P = 0.008$), and *P. sp.* ($P = 0.008$), but not at 96 hours ($P > 0.05$) (E-G). The bars represent the average prevalence of each bacterium across three biological replicates; the error bars included are 95% confidence intervals; total sample sizes: n=18 for each treatment at each time point. The Friedman test with Dunn's multiple comparison test was used to analyze the data.

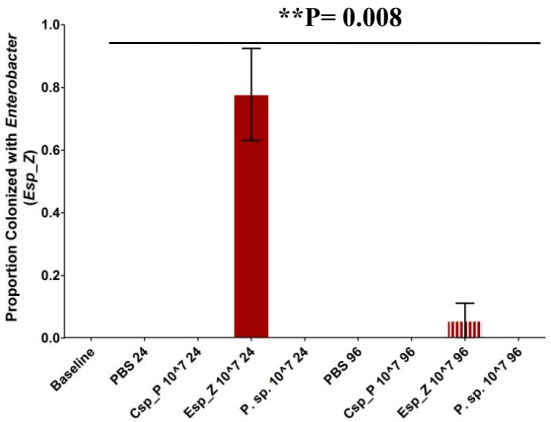

n.s.
Proportion Colonized with Asaia
1.0
0.8
0.6
0.4
0.2
0.0
Baseline
PBS 24
Csp_P 10^7 24
Esp_Z 10^7 24
P. sp. 10^7 24
PBS 96
Csp_P 10^7 96
Esp_Z 10^7 96
P. sp. 10^7 96

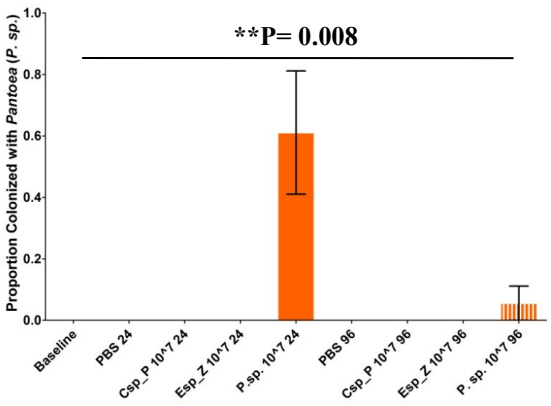

n.s.
Proportion Colonized with Elizabethkingia
0.4
0.3
0.2
0.1
0.0
Baseline
PBS 24
Csp_P 10^7 24
Esp_Z 10^7 24
P. sp. 10^7 24
PBS 96
Csp_P 10^7 96
Esp_Z 10^7 96
P. sp. 10^7 96


n.s.
Proportion Colonized with Klebsiella
0.25
0.20
0.15
0.10
0.05
0.00
Baseline
PBS 24
Csp_P 10^7 24
Esp_Z 10^7 24
P. sp. 10^7 24
PBS 96
Csp_P 10^7 96
Esp_Z 10^7 96
P. sp. 10^7 96


n.s.
Proportion Colonized with Staphylococcus
0.15
0.10
0.05
0.00
Baseline
PBS 24
Csp_P 10^7 24
Esp_Z 10^7 24
P. sp. 10^7 24
PBS 96
Csp_P 10^7 96
Esp_Z 10^7 96
P. sp. 10^7 96


*P= 0.0424
Proportion Colonized with Chromobacterium (Csp_P)
0.4
0.3
0.2
0.1
0.0
Baseline
PBS 24
Csp_P 10^7 24
Esp_Z 10^7 24
P. sp. 10^7 24
PBS 96
Csp_P 10^7 96
Esp_Z 10^7 96
P. sp. 10^7 96


**P= 0.008
Proportion Colonized with Enterobacter (Esp_Z)
1.0
0.8
0.6
0.4
0.2
0.0
Baseline
PBS 24
Csp_P 10^7 24
Esp_Z 10^7 24
P. sp. 10^7 24
PBS 96
Csp_P 10^7 96
Esp_Z 10^7 96
P. sp. 10^7 96


**P= 0.008
Proportion Colonized with Pantoea (P. sp.)
1.0
0.8
0.6
0.4
0.2
0.0
Baseline
PBS 24
Csp_P 10^7 24
Esp_Z 10^7 24
P. sp. 10^7 24
PBS 96
Csp_P 10^7 96
Esp_Z 10^7 96
P. sp. 10^7 96

Appendices

Supplementary Data

Raw Data Tables

1. Survival of *Csp_P*-Exposed Females for Each Replicate

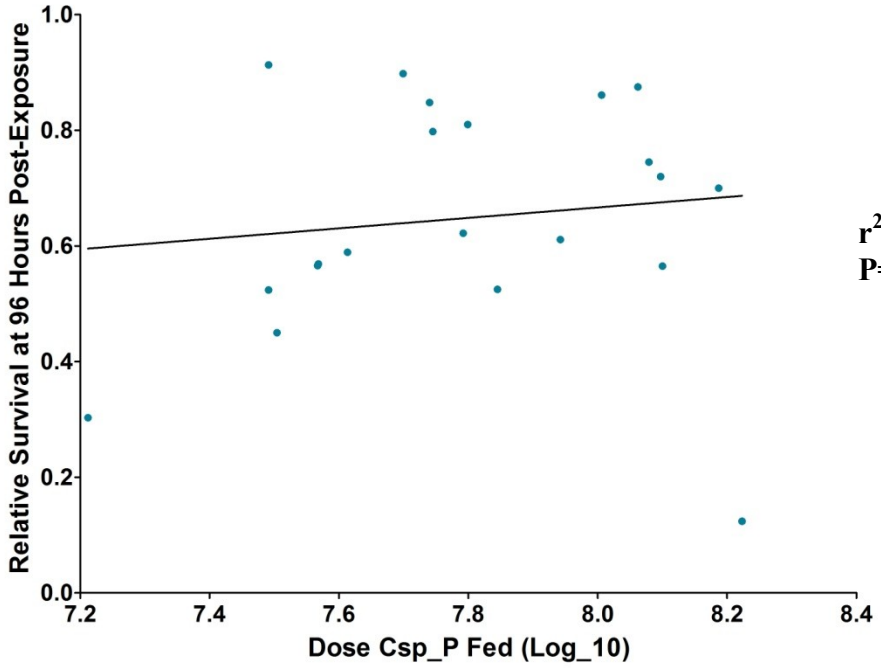
Experiment	Sample Size PBS	Sample Size *Csp_P*	Dose of *Csp_P* Fed (CFU/ mL)	Dose of *Csp_P* Fed (Log10)	Relative Survival in *Csp_P* Exposed at 96 Hours	Pairwise Log-Rank Test P-value
SvT-57 +	54	57	$3.19X10^7$	7.504	0.45	**<0.0001**
SvT-62	56	46	$1.63X10^7$	7.212	0.303	**<0.0001**
SvT-73 +	52	65	$3.70X10^7$	7.568	0.569	**<0.0001**
SvT-79 +	67	46	$7.00X10^7$	7.845	0.525	**<0.0001**
SvT-80 +	51	104	$3.10X10^7$	7.491	0.524	**<0.0001**
SvT-105 *	143	175	$6.30X10^7$	7.799	0.810	**<0.0001**
SvT-107 *	58	102	$1.25X10^8$	8.097	0.720	**0.0004**
SvT-109 *	56	86	$1.20X10^8$	8.079	0.745	**0.0064**
SvT-118	110	119	$1.67X10^8$	8.223	0.124	**<0.0001**
SvT-126	91	127	$1.54X10^8$	8.187	0.764	**<0.0001**
SvT-128	87	108	$1.26X10^8$	8.1	0.565	**<0.0001**
SvT-129	129	178	$1.15X10^8$	8.062	0.875	**0.0038**
SvT-131	125	196	$1.01X10^8$	8.006	0.861	**0.0024**
SvT-132	62	66	$8.75X10^7$	7.942	0.611	**<0.0001**
SvT-133	131	149	$5.56X10^7$	7.745	0.811	**<0.0001**
SvT-134	159	229	$3.69X10^7$	7.567	0.566	**<0.0001**
				Average Dose ^	6.37X10^7 CFU/mL	
				Range in Survival ^	(0.124, 0.875)	
				Average Survival ^	0.581	
				Median Survival ^	0.566	

+ Fecundity Bioassay Replicate

* Microbiota Assay Replicate

^Does not include microbiota assay replicates since blood feeding was not included in the protocol

2. Correlation of *Csp_P* Dose Fed and Survival



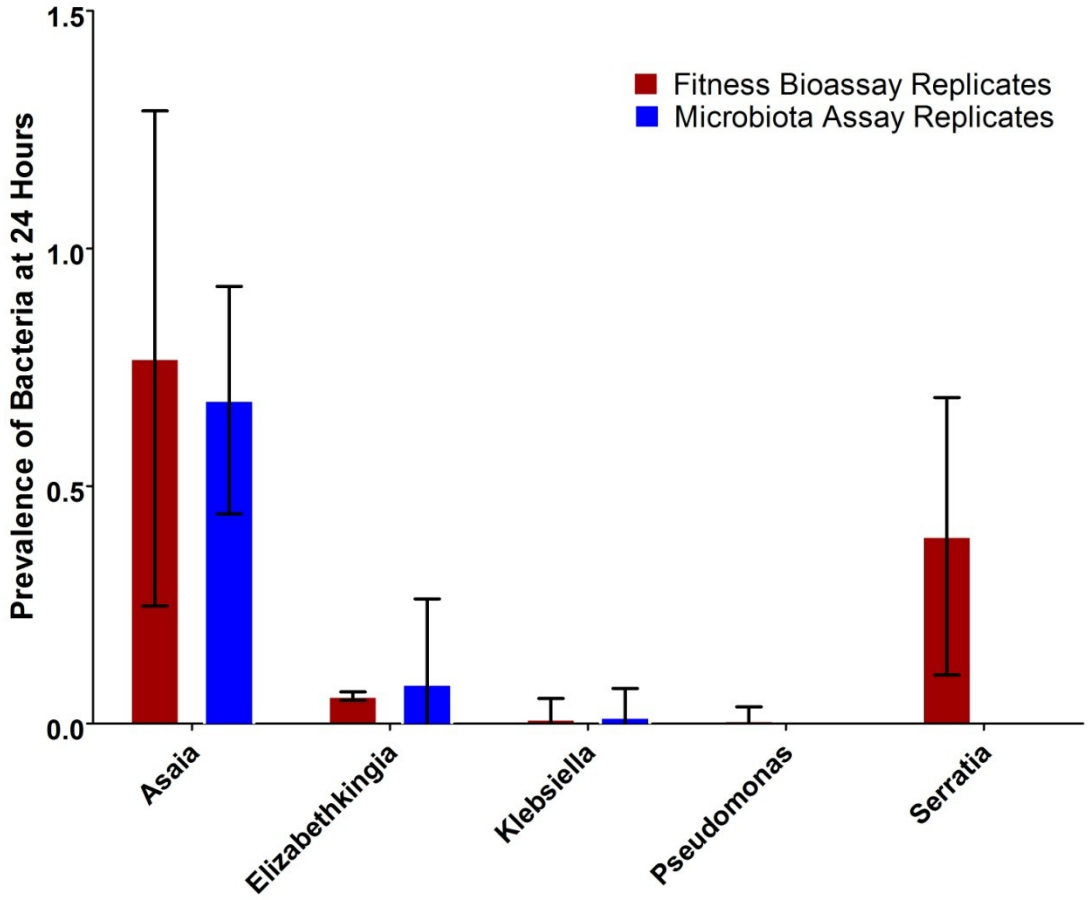
r^2= 0.01483
P= 0.5990

Best-fit values	
Slope	0.09020 ± 0.1687
Y-intercept when X=0.0	-0.05505 ± 1.316
X-intercept when Y=0.0	0.6103
1/slope	11.09
95% Confidence Intervals	
Slope	-0.2628 to 0.4433
Y-intercept when X=0.0	-2.810 to 2.700
X-intercept when Y=0.0	-infinity to 6.355
Goodness of Fit	
r^2	0.01483
Sy.x	0.2067
Is slope significantly non-zero?	
F	0.2860
DFn, DFd	1.000, 19.00
P value	0.5990
Deviation from zero?	Not Significant
Data	
Number of X values	21
Maximum number of Y replicates	1
Total number of values	21
Number of missing values	0

3. *Csp_P* Optimal Dose Survival Assays

Experiment	Sample Size PBS	Sample Size *Csp_P*	Intended Dose of *Csp_P* (CFU/mL)	Dose of *Csp_P* Fed (CFU/mL)	Relative Survival in *Csp_P* Exposed at 96 Hours	Pairwise Log-Rank Test P-value
SvT-87	42	36	5X10^9	1.75X10^10	0.00	**<0.0001**
SvT-87	42	44	1X10^9	3.5X10^9	0.095	**<0.0001**
SvT-87	42	51	5X10^8	1.75X10^9	0.165	**<0.0001**
SvT-87	42	46	5X10^7	2.75X10^8	0.822	**0.0135**
SvT-87	42	47	1X10^7	5.5X10^7	0.849	0.0523
SvT-87	42	41	5X10^6	2.75X10^7	0.947	0.2069
SvT-89	48	49	5X10^9	4.4X10^10	0.022	**<0.0001**
SvT-89	48	54	1X10^9	8.8X10^9	0.078	**<0.0001**
SvT-89	48	48	5X10^8	2.5X10^9	0.044	**<0.0001**
SvT-89	48	59	1X10^8	5X10^8	0.159	**<0.0001**
SvT-89	48	52	5X10^7	2.5X10^8	0.609	**<0.0001**
SvT-89	48	53	1X10^7	5X10^7	0.913	0.1472

4. Prevalence of Endogenous Midgut Bacteria



		Asaia	*Elizabethkingia*	*Klebsiella*	*Pseudomonas*	*Serratia*
Fecundity Bioassay Replicates	SvT-57	0.545	0.061	0.030	0.000	0.303
	SvT-73	0.961	0.059	0.000	0.020	0.353
	SvT-79	0.800	0.054	0.000	0.000	0.527
Microbiota Bioassay Replicates	SvT-105	0.625	0.125	0.000	0.000	0.000
	SvT-107	0.792	0.125	0.000	0.000	0.000
	SvT-109	0.625	0.000	0.042	0.000	0.000
	Mean	**0.7687**	**0.058**	**0.01**	**0.006667**	**0.3943**
	Difference	**-0.088**	**0.02533**	**0.004**	**-0.006667**	**-0.3943**
	95% CI	**-0.2870, 0.1110**	**-0.1737, 0.2243**	**-0.1950, 0.2030**	**-0.2057, 0.1923**	**-0.5933, -0.1953**

5. Figure 1

Figure 1A. Proportion of F0 Females that Oviposited					
	PBS	***Csp_P* 10^2**	***P. sp.* 10^2**	***Csp_P* 10^7**	**P. sp. 10^7**
SvT-57	0.944	1	1	1	-
SvT-73	0.846	0.8	0.857	1	0.833
SvT-79	0.923	1	0.833	0.75	1
SvT-80	1	0.818	0.917	0.786	0.714
Mean	**0.9283**	**0.9045**	**0.9018**	**0.884**	**0.849**
Lower 95% CI of mean	**0.8268**	**0.7286**	**0.7833**	**0.6696**	**0.4921**
Upper 95% CI of mean	**1.03**	**1.08**	**1.02**	**1.098**	**1.206**

Figure 1B. Number of Eggs Oviposited per F0 Female					
Individual	**PBS**	***Csp_P* 10^2**	***P. sp.* 10^2**	***Csp_P* 10^7**	***P. sp.* 10^7**
1	7	33	5	32	18
2	16	28	40	24	46
3	0	36	26	53	0
4	30	18	22	40	29
5	39	14	18	43	15
6	48	25	16	28	8
7	26	33	22	3	6
8	27	30	25	19	29
9	51	16	17	55	26
10	8	28	30	5	22
11	33	29	24	28	28
12	25	32	9	23	0
13	45	32	32	32	28
14	23	48	43	15	31
15	51	30	33	9	26
16	40	18	0	41	15
17	8	5	15	48	22
18	16	12	22	39	5
19	27	12	7	20	1
20	20	18	17	30	19
21	21	18	0	0	29
22	21	26	21	20	33
23	16	22	34	14	19
24	0	22	24	0	32
25	0	0	31	0	24
26	7	0	4	10	1
27	48	7	23	13	0
28	18	7	0	30	29
29	1	15	18	20	22
30	23	29	3	16	29
31	28	23	16	55	7
32	6	20	19	3	0
33	16	20	24	47	0
34	0	13	24	21	0
35	15	14	0	3	18
36	11	25	14	0	
37	96	15	19	34	
38	23	34	28	34	
39	4	8	17	29	

40	16	40	0	7	
41	11	20	24	0	
42	9	30	48	0	
43	23	4	11	24	
44	13	0	22	35	
45	28	32	29	0	
46	32	13	11	1	
47	14	6	15	30	
48	26	1	17	42	
49	13	0		22	
50	30			38	
51	25				
52	5				
53	8				
54	24				
55	49				
56	11				
Mean	**21.98**	**19.61**	**19.15**	**22.7**	**17.63**
Lower 95% CI of mean	**17.39**	**16.25**	**15.84**	**17.99**	**13.27**
Upper 95% CI of mean	**26.58**	**22.98**	**22.46**	**27.41**	**21.98**

Figure 1C. Percent Daily Survival After Bacterial Exposure					
Hours After Exposure	**PBS**	**P. sp. 10^2**	**Csp_P 10^2**	**P. sp. 10^7**	**Csp_P 10^7**
0	100	100	100	100	100
24	95.95	97.67	96.34	97.36	82.34
48	95.84	97.67	95.76	97.36	49.06
72	94.27	95.01	95.19	97.1	47.34
96	93.85	92.68	91.87	94.74	47.2
120	92.55	88.91	88.74	94.23	42.85
144	86.98	86.08	87.93	84.75	40.04
168	79.11	79.77	75.86	73.49	38.55
192	79.11	79.77	75.86	73.49	38.55
Median Survival		**Undefined**	**Undefined**	**Undefined**	**96**
Relative survival at 96 Hours		**98.7**	**97.9**	**100.9**	**50.3**

6. Figure 2

Figure 2A. Proportion of Eggs Hatched		
Experiment	***PBS***	***Csp_P***
SvT-98	0.71	0.7
SvT-126	0.517	0.589
SvT-128	0.564	0.555
SvT-129	0.526	0.562
SvT-131	0.745	0.732
Mean	**0.6124**	**0.6276**
Lower 95% CI	**0.4792**	**0.5252**
Upper 95% CI	**0.7456**	**0.73**

Figure 2B. Survival of F1 Larval Offspring		
Hours After Hatching	**PBS**	***Csp_P***
0	100	100
48	98.83	98.82
96	83.11	77.69
144	79.28	72.45
168	76.28	68.38
192	67.58	54.51
216	67.25	43.45
240	66.63	41.54
264	66.63	37.04
288	66.63	28.91
312	66.63	26.83
336	66.63	25.44
360	66.63	22.26
Median Survival	**Undefined**	**192**

Figure 2C. Proportion of F1 Larval that Pupated		
Experiment	**PBS**	***Csp_P***
SvT-98	0.615	0.19
SvT-126	0.89	0.51
SvT-134	0.61	0.38
Mean	**0.705**	**0.36**
Upper 95% CI	**0.307**	**-0.03978**
Lower 95% CI	**1.103**	**0.7598**
Mean Difference	**0.345**	
95% Confidence Interval	**0.09134-0.5987**	

Figure 2D. Proportion of F1 Larvae Eclosed		
Experiment	**PBS**	**Csp_P**
SvT-98	0.58	0.16
SvT-126	0.61	0.3
SvT-134	0.54	0.3
Mean	0.5767	0.2533
Upper 95% CI	0.4894	0.05254
Lower 95% CI	0.6639	0.4541
Mean Difference	0.3233	
95% Confidence Interval	0.09791 to 0.5488	

Figure 2E. Percentage of F1 Larvae not Pupated		
Hours After Hatching	**PBS**	***Csp_P***
0	100	100
48	100	100
96	100	100
144	100	100
168	83.02	99.41
192	58.41	96.02
216	21.11	71.99
240	7.048	60.33
264	2.532	34.57
288	0.7326	30.76
312	0	17.42
336	0	16.21
360	0	6.524
Median Time to Pupation	**216**	**264**

Figure 2F. Proportion of F1 Pupae that Eclosed		
Experiment	**PBS**	***Csp_P***
SvT-98	0.941	0.7875
SvT-126	0.685	0.588
SvT-134	0.885	0.789
Mean	**0.837**	**0.7215**
Upper 95% CI	**0.5027**	**0.4343**
Lower 95% CI	**1.171**	**1.009**
Mean Difference	**0.1155**	
95% Confidence Interval	**0.03373 to 0.1973**	

Figure 2G. Daily Survival of F1 Adults		
Hours After Eclosion	**PBS**	***Csp_P***
0	100	100
72	96.83	92.71
120	90.16	87.15
144	88.57	85.07
168	88.57	81.3
192	88.57	79.91
216	85.4	77.48
264	85.4	76.09
288	80.63	73.31
312	79.85	72.27
336	75.98	60.37
360	74.39	59.33
384	59.47	53.77
408	52.46	47.97
432	49.24	46.92
456	44.55	43.5
480	51.83	38.17
504	49.45	36.61
552	47.07	36.61
576	35.33	31.47
600	30.56	28.35
624	16.45	17.63
648	8.25	14.51
696	8.25	9.821
744	5.925	9.821
768	3.544	3.1
792	2.381	0
840	2.381	0
936	0	0
Median Survival	**456**	**468**

Figure 2H. Sex Ratio of F1 Adults		
Experiment	**PBS**	***Csp_P***
SvT-126	0.774	0.9
SvT-131	0.857	0.811
SvT-134	0.884	1.333
Mean	0.8383	1.015
Upper 95% CI	**0.6959**	**0.321**
Lower 95% CI	**0.9807**	**1.708**
Mean Difference	**-0.1763**	
95% Confidence Interval	**-0.8007 to 0.4480**	

7. Figure 3

Figure 3. Bacterial Load in the Aquatic Environment during Larval Development								
Experiment	**PBS Egg Water**	***Csp_P* Egg Water**	**PBS Larval Water Baseline**	***Csp_P* Larval Water Baseline**	**PBS Larval Water Day 4**	***Csp_P* Larval Water Day 4**	**PBS Larval Water Day 8**	***Csp_P* Larval Water Day 8**
SvT-98	3.8025	4.4765	0	0	6.169	6.243	6.955	7.006
SvT-126	5.0005	3.8795	0	0	6.676	6.59	5.8167	5.8665
SvT-134	4.64	4.4695	0	0	6.7547	6.8572	6.3858	6.43625
Mean	**4.481**	**4.275**	**0**	**0**	**6.533**	**6.563**	**6.386**	**6.436**
Upper 95% CI	**2.954**	**3.424**	**0**	**0**	**5.744**	**5.798**	**4.972**	**5.021**
Lower 95% CI	**6.008**	**5.126**	**0**	**0**	**7.323**	**7.328**	**7.8**	**7.852**
Mean Difference	**0.2058**		**0**		**-0.03017**		**-0.05042**	
95% Confidence Interval	**0.7814 to 1.193**		**-0.9873 to 0.9873**		**-1.017 to 0.9571**		**-1.038 to 0.9369**	

8. Figure 4

Figure 4A. Survival of F1 Larvae Exposed to *Csp_P*				
Hours After Exposure	**PBS F1-PBS**	***Csp_P* F1-PBS**	**PBS F1- *Csp_P* 10^6**	***Csp_P* F1- *Csp_P* 10^6**
0	100	100	100	100
24	93.54	78.13	61.46	29.17
48	87.29	59.17	37.08	18.33
72	83.54	57.92	33.75	10
Median Survival	**Undefined**	**Undefined**	**48**	**24**

Figure 4B. Survival of F1 Adults Exposed to *Csp_P*		
Hours After Exposure	**PBS**	***Csp_P***
0	100	100
24	97.92	98.94
48	84.86	91.25
72	76.14	81.86
120	71.79	79.08
144	71.79	48.23
168	69.61	47.16
192	52.2	38
216	43.49	33.1
240	39.14	28.19
264	39.14	27.13
288	39.14	25
312	28.27	16.67
336	21.75	2.778
360	17.4	0
384	15.23	0
408	10.88	0
456	8.696	0
552	4.348	0
792	0	0
Median Survival	**216**	**144**

9. Figure 5

Figure 5. Survival of Adults Exposed to Bacteria for Microbiota Assays				
Hours After Exposure	**PBS**	***Csp_P***	***Esp_Z***	***P. sp.***
0	100	100	100	100
24	98.1	95.32	97.92	98.2
48	98.1	83.31	95.88	97.34
72	98.1	76.9	95.11	97.18
96	97.15	77.34	90.54	94.81
120	94.58	71.75	83.37	93.77
144	90.09	67.69	78.09	90.95
168	90.09	67.69	78.09	90.95
Median Survival	**Undefined**	**Undefined**	**Undefined**	**Undefined**
Relative Survival at 96 Hours		**0.796**	**0.932**	**0.976**

10. Figure 6

Figure 6A. Total Bacteria Load Including Fed Bacteria									
	Baseline	**PBS 24**	***Csp_P* 24**	***Esp_Z* 24**	***P. sp.* 24**	**PBS 96**	***Csp_P* 96**	***Esp_Z* 96**	***P. sp.* 96**
SvT-105	1.908	0.000	4.348	7.738	3.246	6.228	5.155	5.488	5.624
SvT-105	2.088	3.140	0.000	4.139	5.176	5.946	5.356	7.784	6.367
SvT-105	3.225	4.432	2.149	4.388	3.087	5.824	4.892	5.763	6.924
SvT-105	4.763	3.146	2.533	5.197	1.908	5.279	7.017	4.823	3.623
SvT-105	3.156	2.083	3.243	4.524	4.386	5.884	5.414	6.187	4.845
SvT-105	3.652	1.322	0.000	3.659	4.981	4.413	6.047	7.225	5.644
SvT-105	0.000								
SvT-105	0.000								
SvT-107	2.415	4.463	4.617	2.833	5.960	6.338	5.678	6.205	4.550
SvT-107	4.319	3.497	4.373	3.248	5.920	6.584	5.033	5.085	4.300
SvT-107	2.417	4.536	3.369	6.179	5.063	5.380	4.378	6.156	6.748
SvT-107	1.908	4.982	3.274	5.458	0.000	6.449	5.991	6.518	6.515
SvT-107	1.785	3.688	4.213	5.510	5.508	5.494	3.734	5.763	6.139
SvT-107	2.557	0.000	4.095	4.245	3.433	5.480	4.510	5.624	5.834
SvT-109	4.390	4.031	2.258	4.429	2.790	2.083	3.554	3.439	6.409
SvT-109	3.450	2.344	6.828	4.571	0.000	0.000	2.417	6.406	7.322
SvT-109	3.438	4.331	0.000	3.181	0.000	2.149	5.799	7.589	3.616
SvT-109	5.108	2.34	2.870	2.792	3.991	7.133	1.613	6.066	4.881
SvT-109	4.533	6.461	2.733	4.953	7.161	6.047	2.004	4.378	4.438
SvT-109	2.447	2.893	4.599	3.626	6.380	4.496	0.000	6.042	1.903
Mean	**2.878**	**3.205**	**3.084**	**4.482**	**3.833**	**5.067**	**4.366**	**5.919**	**5.316**
Upper 95% CI	**2.216**	**2.371**	**2.190**	**3.860**	**2.726**	**4.142**	**3.457**	**5.386**	**4.615**
Lower 95% CI	**3.540**	**4.039**	**3.977**	**5.104**	**4.939**	**5.992**	**5.275**	**6.452**	**6.016**
Mean Difference Baseline		**-0.327**	**-0.205**	**-1.604**	**-0.955**	**-2.189**	**-1.488**	**-3.041**	**-2.438**
Lower 95% CI		**-1.911**	**-1.789**	**-3.187**	**-2.538**	**-3.772**	**-3.072**	**-4.624**	**-4.021**
Upper 95% CI		**1.256**	**1.378**	**-0.0205**	**0.629**	**-0.606**	**0.095**	**-1.458**	**-0.854**
Mean Difference PBS			**0.122**	**-1.277**	**-0.627**		**0.701**	**-0.852**	**-0.249**
95% Confidence Interval			**-1.503, 1.746**	**-2.901, 0.348**	**-2.252, 0.997**		**-0.924, 2.325**	**-2.476, 0.722**	**-1.873, 1.376**

Figure 6B. Total Bacterial Load of Endogenous Bacteria									
	Baseline	**PBS 24**	***Csp_P* 24**	***Esp_Z* 24**	***P. sp.* 24**	**PBS 96**	***Csp_P* 96**	***Esp_Z* 96**	***P. sp.* 96**
SvT-105	1.908	0.000	0.000	7.738	0.000	6.228	5.155	5.488	5.624
SvT-105	2.088	3.140	0.000	0.000	5.161	5.946	5.356	0.000	6.367
SvT-105	3.225	4.432	2.149	0.000	3.065	5.824	4.892	5.763	6.531
SvT-105	4.763	3.146	1.908	4.832	1.322	5.279	7.017	4.823	3.623
SvT-105	3.156	2.083	3.182	4.509	3.301	5.884	5.414	6.187	4.845
SvT-105	3.652	1.322	0.000	0.000	4.975	4.413	6.047	7.225	5.644
SvT-105	0.000								
SvT-105	0.000								
SvT-107	4.390	4.031	2.258	0.000	2.79	2.083	3.554	3.439	6.409
SvT-107	3.450	2.344	6.828	0.000	0.000	0.000	2.417	6.406	7.322
SvT-107	3.438	4.331	0.000	0.000	0.000	2.149	5.799	7.589	3.616
SvT-107	5.108	2.344	2.87	1.613	3.991	7.133	1.613	6.066	4.881
SvT-107	4.533	6.461	2.733	4.945	7.093	6.047	2.004	4.378	4.438
SvT-107	2.447	2.893	4.599	3.301	5.301	4.496	0.000	6.042	1.903
SvT-109	2.415	4.463	4.617	2.833	5.958	6.338	5.678	6.205	4.550
SvT-109	4.319	3.497	4.373	3.248	5.920	6.584	5.033	5.085	4.300
SvT-109	2.416	4.536	3.369	6.179	5.063	5.38	4.378	6.156	6.748
SvT-109	1.908	4.982	0.000	4.991	0.000	6.449	5.991	6.518	6.515
SvT-109	1.785	3.688	4.213	5.292	4.623	5.494	3.734	5.763	6.139
SvT-109	2.557	0.000	4.095	4.242	3.134	5.480	4.510	5.624	5.834
Mean	**2.878**	**3.205**	**2.622**	**2.985**	**3.428**	**5.067**	**4.366**	**5.487**	**5.294**
Upper 95% CI	**2.216**	**2.371**	**1.616**	**1.726**	**2.274**	**4.142**	**3.457**	**4.653**	**4.605**
Lower 95% CI	**3.540**	**4.039**	**3.628**	**4.243**	**4.581**	**5.992**	**5.275**	**6.320**	**5.983**
Mean Difference Baseline		**-0.3272**	**0.2561**	**-0.1066**	**-0.5496**	**-2.189**	**-1.488**	**-2.609**	**-2.416**
Lower 95% CI		**-2.141**	**-1.558**	**-1.92**	**-2.363**	**-4.003**	**-3.302**	**-4.422**	**-4.229**
Upper 95% CI		**1.486**	**2.07**	**1.707**	**1.264**	**-0.3753**	**0.3253**	**-0.7949**	**-0.6022**
Mean Difference PBS			**0.5833**	**0.2206**	**-0.2224**		**0.7006**	**-0.4196**	**-0.2269**
95% Confidence Interval			**-1.277, 2.444**	**-1.64, 2.081**	**-2.083, 1.638**		**-1.16, 2.561**	**-2.28, 1.441**	**-2.088, 1.634**

11. Figure 7

Figure 7A. Prevalence of *Asaia* in Bacteria-Exposed Females									
Experiment	**Baseline**	**PBS 24**	***Csp_P* 24**	***Esp_Z* 24**	***P. sp.* 24**	**PBS 96**	***Csp_P* 96**	***Esp_Z* 96**	***Psp sp.* 96**
SvT-105	1.000	0.833	0.667	0.500	0.500	0.667	0.833	0.833	0.833
SvT-107	1.000	0.833	0.833	0.833	0.667	1.000	1.000	1.000	1.000
SvT-109	0.667	0.833	0.500	0.333	0.833	1.000	0.833	0.667	1.000
Mean	**0.889**	**0.833**	**0.667**	**0.555**	**0.667**	**0.889**	**0.889**	**0.833**	**0.944**
Lower 95% CI	**0.411**	**0.833**	**0.253**	**-0.077**	**0.253**	**0.411**	**0.649**	**0.420**	**0.705**
Upper 95% CI	**1.367**	**0.833**	**1.080**	**1.188**	**1.080**	**1.367**	**1.128**	**1.247**	**1.184**

Figure 7B. Prevalence of *Elizabethkingia* in Bacteria-Exposed Females									
Experiment	**Baseline**	**PBS 24**	***Csp_P* 24**	***Esp_Z* 24**	***P. sp.* 24**	**PBS 96**	***Csp_P* 96**	***Esp_Z* 96**	***P. sp.* 96**
SvT-105	0.000	0.167	0.167	0.000	0.167	0.167	0.167	0.167	0.167
SvT-107	0.500	0.000	0.000	0.167	0.333	0.000	0.333	0.000	0.167
SvT-109	0.000	0.000	0.000	0.000	0.000	0.000	0.167	0.167	0.000
Mean	**0.167**	**0.056**	**0.056**	**0.056**	**0.167**	**0.056**	**0.222**	**0.111**	**0.111**
Lower 95% CI	**-0.550**	**-0.184**	**-0.184**	**-0.184**	**-0.247**	**-0.184**	**-0.016**	**-0.128**	**-0.128**
Upper 95% CI	**0.884**	**0.295**	**0.295**	**0.295**	**0.580**	**0.295**	**0.460**	**0.351**	**0.351**

Figure 7C. Prevalence of *Klebsiella* in Bacteria-Exposed Females									
Experiment	**Baseline**	**PBS 24**	***Csp_P 24***	***Esp_Z 24***	***P. sp. 24***	**PBS 96**	***Csp_P 96***	***Esp_Z 96***	***P. sp. 96***
SvT-105	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
SvT-107	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
SvT-109	0.000	0.000	0.000	0.167	0.000	0.000	0.333	0.000	0.167
Mean	**0.000**	**0.000**	**0.000**	**0.056**	**0.000**	**0.000**	**0.111**	**0.000**	**0.056**
Lower 95% CI	**0.000**	**0.000**	**0.000**	**-0.184**	**0.000**	**0.000**	**-0.367**	**0.000**	**-0.184**
Upper 95% CI	**0.000**	**0.000**	**0.000**	**0.295**	**0.000**	**0.000**	**0.589**	**0.000**	**0.295**

Figure 7D. Prevalence of *Staphylococcus* in Bacteria-Exposed Females									
Experiment	**Baseline**	**PBS 24**	***Csp_P* 24**	***Esp_Z* 24**	***P. sp.* 24**	**PBS *96***	***Csp_P* 96**	***Esp_Z* 96**	***P. sp.* 96**
SvT-105	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.167
SvT-107	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
SvT-109	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Mean	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.056**
Lower 95% CI	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**-0.184**
Upper 95% CI	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.295**

Figure 7E. Prevalence of *Csp_P* in Bacteria-Exposed Females									
Experiment	**Baseline**	**PBS 24**	***Csp_P* 24**	***Esp_Z* 24**	***P. sp.* 24**	**PBS *96***	***Csp_P* 96**	***Esp_Z* 96**	***P. sp.* 96**
SvT-105	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
SvT-107	0.000	0.000	0.167	0.000	0.000	0.000	0.000	0.000	0.000
SvT-109	0.000	0.000	0.500	0.000	0.000	0.000	0.000	0.000	0.000
Mean	**0.000**	**0.000**	**0.222**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
Lower 95% CI	**0.000**	**0.000**	**-0.410**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**
Upper 95% CI	**0.000**	**0.000**	**0.855**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**	**0.000**

Figure 7F. Prevalence of *Esp_Z* in Bacteria-Exposed Females									
Experiment	**Baseline**	**PBS 24**	***Csp_P* 24**	***Esp_Z* 24**	***P. sp.* 24**	**PBS *96***	***Csp_P* 96**	***Esp_Z* 96**	***P. sp.* 96**
SvT-105	0.000	0.000	0.000	1.000	0.000	0.000	0.000	0.000	0.000
SvT-107	0.000	0.000	0.000	0.500	0.000	0.000	0.000	0.000	0.000
SvT-109	0.000	0.000	0.000	0.833	0.000	0.000	0.000	0.167	0.000
Mean	**0.000**	**0.000**	**0.000**	**0.778**	**0.000**	**0.000**	**0.000**	**0.056**	**0.000**
Lower 95% CI	**0.000**	**0.000**	**0.000**	**0.145**	**0.000**	**0.000**	**0.000**	**-0.184**	**0.000**
Upper 95% CI	**0.000**	**0.000**	**0.000**	**1.410**	**0.000**	**0.000**	**0.000**	**0.295**	**0.000**

Figure 7G. Prevalence of *P. sp.* in Bacteria-Exposed Females									
Experiment	**Baseline**	**PBS 24**	***Csp_P* 24**	***Esp_Z* 24**	***P. sp.* 24**	**PBS *96***	***Csp_P* 96**	***Esp_Z* 96**	***P. sp.* 96**
SvT-105	0.000	0.000	0.000	0.000	0.333	0.000	0.000	0.000	0.000
SvT-107	0.000	0.000	0.000	0.000	0.500	0.000	0.000	0.000	0.000
SvT-109	0.000	0.000	0.000	0.000	1.000	0.000	0.000	0.000	0.167
Mean	**0.000**	**0.000**	**0.000**	**0.000**	**0.611**	**0.000**	**0.000**	**0.000**	**0.056**
Lower 95% CI	**0.000**	**0.000**	**0.000**	**0.000**	**-0.251**	**0.000**	**0.000**	**0.000**	**-0.184**
Upper 95% CI	**0.000**	**0.000**	**0.000**	**0.000**	**1.473**	**0.000**	**0.000**	**0.000**	**0.295**

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Curriculum Vitae

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Education

Johns Hopkins Bloomberg School of Public Health, Baltimore, MD 2014-2016
ScM in Molecular Microbiology and Immunology, May 2016

Iowa State University, Ames, IA 2010-2014
B.S. in Animal Ecology, with honors, *magna cum laude*, 2014
Minors in Emerging Global Diseases and Microbiology
B.S. in Biology, *magna cum laude*, 2014

Research Experience

Department of Molecular Microbiology and Immunology, Johns Hopkins Bloomberg School of Public Health, Baltimore, MD
Masters Research Nov 2014- May 2016
Research Advisor: Dr. George Dimopoulos
- Thesis: Effects of Entomopathogenic *Chromobacterium* (*Csp_P*) Exposure of the Microbiota and Fitness of *Anopheles gambiae*

Department of Animal Science, Iowa State University, Ames, IA
Research Associate I June- Aug 2014
Undergraduate Research Assistant Apr- May 2014
Independent Study Research Jan- May 2014
Research Advisor: Dr. Susan Carpenter
- Construct chimeric infectious clones of porcine reproductive and respiratory syndrome virus (PRRSV) to interrogate the importance of structural gene mutations in recrudescence

Department of Entomology, Iowa State University, Ames, IA
Undergraduate Research Assistant June 2013- Feb 2014
Research Supervisor: Mr. Eric Clifton, M.S.
- Field study of the impact of insecticide seed treatments on the ecology of agricultural pests such as soybean aphids and soybean nematodes
- Laboratory study to evaluate how field-isolated entomopathogenic fungi affects waxmoth larval development

Palm Beach Zoo, West Palm Beach, FL
Conservation and Research Intern May- Aug 2012
Honors Thesis Research
Research Advisors: Dr. Stephanie Allard (Palm Beach Zoo) and Dr. Stephen Dinsmore (Iowa State)
- Behavioral study on the impact of altered food presentation on the activity levels and feeding posture repertoire of captive red-ruffed lemurs
- Development of rapid and in-depth animal welfare surveys

International Student Volunteers, Waterberg, South Africa

Volunteer Research Assistant June-July 2011

- Ecological assessment of Lapalala Game Reserve

Department of Animal Science, Iowa State University, Ames, IA

Honors Research Jan- May 2011

Research Advisor: Dr. Don Beitz

- Investigation of how acid treatment of cattle feed impacts digestibility

Honors and Awards

Gamma Sigma Delta Honor Society of Agriculture, 2013

Beta Beta Beta Biological Honors Society, 2011

Presentations

van Tol, S., S. M. Short, G. Dimopoulos. *Impact of Non-lethal Exposure to an Entomopathogenic Chromobacterium (Csp_P) on Anopheles gambiae Microbiota and Fitness.* American Society of Tropical Medicine and Hygiene, Philadelphia, PA, 26 October, 2015. Poster.

van Tol, S., S. M. Short, G. Dimopoulos. *Impact of Non-lethal Exposure to an Entomopathogenic Chromobacterium (Csp_P) on Anopheles gambiae Microbiota and Fitness.* The Future of Malaria: A Young Scientists' Meeting, Rockville, MD, 23 October, 2015. Poster.

Evans, A.B., S. Van Tol, K.S. Dorman, S. Carpenter. *In vivo variation in the envelope proteins of porcine reproductive and respiratory syndrome virus contributes to immune escape and recrudescence.* American Society for Virology Annual Meeting, London, Ontario Canada, July 2015. Oral.

Evans, A.B., S. Van Tol, K.S. Dorman, S. Carpenter. *In vivo variation in the envelope proteins of porcine reproductive and respiratory syndrome virus contributes to immune escape and recrudescence.* All Iowa Virology Symposium, Iowa City, IA, March 2015. Oral.

van Tol, S. *Improving Animal Welfare: Altering Feeding Presentation Encourages Feeding Posture Variation and Increase Activity Levels of Captive Lemurs*. Honors Program Alumni Research Forum, Ames, IA, April 2013. Poster.

Professional Organizations

American Society for Microbiology, 2014-Present

American Society of Tropical Medicine and Hygiene, 2015-present