Assessment of the diversity and roles of bacterial symbionts in fruit fly development and response to biological control

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DECLARATION BY THE CANDIDATE

I, JOSEPH GICHUHI, declare that the work presented in this PhD thesis is my own work, that it has not been submitted for any degree or examination at any other University and that all the sources I have used or cited have been acknowledged by complete reference.

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DEDICATION

This work is dedicated to my parents, siblings, family and friends who have always believed in me and supported my endeavors. God bless you all.

PREFACE

This work was carried out by J. Gichuhi under supervision of Prof. J. Van den Berg, IPM program, Unit for Environmental Sciences and Management, North-West University, Dr. S. Ekesi and Dr. F. Khamis of the Plant Health Department and Dr. J. Herren of the Human Health Department of the International Centre of Insect Physiology and Ecology (icipe). The institution uses the lowercase 'icipe' as a brand name, and is referenced as so throughout the thesis. This thesis is submitted in fulfillment for the award of the degree of Doctor of Philosophy in *Environmental Science* of the North-West University.

The thesis is written in an article format style. Chapter 1 presents the introduction followed by a literature review in Chapter 2. Findings of the study are presented in the format of manuscripts for publication in Chapters 3 to 5. Lastly, Chapter 6 presents a general discussion, conclusions and recommendations from the study. Chapters 1, 2 and 6 are authored in the NWU Harvard, Reference Style of the Faculty of Law and APA, published by the Library Services of the NWU. The three manuscripts for publications that were developed from this work are each formatted according to author requirements of the respective target journals as follows:

Chapter	Journal	Status
3	INSECTS (Multidisciplinary Digital Publishing Institute)	Published
4	Applied Entomology (Wiley Online Library)	Prepared
5	INSECTS (Multidisciplinary Digital Publishing Institute)	Prepared

The author instructions for the Insects Journal and the Journal of Applied Entomology are

provided in Appendices A and B respectively. A declaration of language editing is also provided in appendix C.

As an additional requirement by the North-West University, the author contributions for each article/manuscript are described in Table A, together with each author's consent to use these manuscripts as part of this thesis.

Table A: Author contributions and consent for use of manuscripts for this thesis

Author	Article/ manuscript	Contribution	Consent*
J Gichuhi	1-3	Principle investigator: Responsible for study design, sampling, conducting experiments, analysis and interpretation of data as well as writing of the articles and thesis	[signature]
J vd Berg	1-3	Promoter: Supervised the design and execution of the study including interpretation of findings as well as intellectual input during writing of the articles and thesis.	[signature]
J Herren	1-3	Co-promoter: Supervised the design and execution of the study including interpretation of findings as well as intellectual input during writing of the articles and thesis.	[signature]
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S Ekesi	1-3	Co-promoter: Supervised the design and execution of the study including interpretation of findings as well as intellectual input during writing of the articles and thesis.	[signature]

*I declare that the stated contributions are accurate and that I consent to the use of this manuscript/ article as part of the thesis of Mr. J Gichuhi.

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ABSTRACT

Tephritid fruit flies are among the most destructive pest species of fruits and vegetables in many regions of the world. Apart from high losses in yield, tephritid fruit fly pests pose great socioeconomic and ecological challenges as well as demand effective measures to curb infestation which can be costly. Among currently used management options are the use of chemical insecticides, behavioral, genetic, cultural and biological approaches. However, no single method or combination of control strategies as used in integrated pest management programmes may be infallible to various constraints. It is therefore necessary to broaden the scope of plausible methods of addressing integrated pest management of tephritid fruit flies. This study examined the bacteria associated with the oriental fruit fly, *Bactrocera dorsalis* (Hendel) (Diptera: Tephritidae) particularly in the African region where this invasive pest has established, with a view of identifying the roles of these bacteria in regards to development of the fly and its biological control. Specimens of this pest were collected from various locations in Africa and screened for the endosymbiotic bacteria, *Wolbachia.* More specimens from Kenya were screened using a high throughput sequencing approach to explicate the gut microbiome associated with this fly. A technique to remove all bacteria from the flies and reintroduce single bacterial isolates back was used to study the roles of individual bacterial isolates during early developmental stages of the fly, and later on to test effects of such bacteria when the flies are exposed to the entomopathogenic fungus, *Metarhizium anisopliae* ICIPE 69, that has been commercialized in Kenya as a biological control agent for this pest. A low prevalence of *Wolbachia* that did not strictly associate with maternal haplotypes of *B. dorsalis* was detected in the African populations. A diverse composition of gut bacterial communities mostly in the phyla Proteobacteria, Firmicutes, Actinobacteria, and Bacteroidetes was observed in *B. dorsalis* specimens from Kenya. The recorded compositions suggested a strong effect of diet and environment on the microbiome structure of this fruit fly. A potential entomopathogen, *Serratia,* was identified among the bacterial communities of this host. In addition, it was observed that the absence of bacteria in this host negatively impacted development of the embryo and larval stages. A strain of *Lactococcus lactis* was also observed to diminish survival of this pest, when challenged with the entomopathogenic fungus, *M. anisopliae* ICIPE 69. These findings present useful insights in the biology of this fly as mediated by associated bacteria which may inform pest management options such as selection of probiotics in mass rearing strategies, as well as potential candidates for exploration as bacterial entomopathogens.

Key words: Tephritid fruit fly, *Bactrocera dorsalis*, bacterial symbionts, gut bacteria, biological control, endosymbionts, fruit fly development

CHAPTER ONE

1. Introduction

1.1 Background of the study

Fruit flies of the family Tephritidae are globally considered a menace to fruit and vegetable production with some pest species posing high phytosanitary threats, attacking a wide range of crops, degrading the quality of produce and often resulting in reduced yields in absence of effective control measures. Infestations have been recorded in South East Asia, Australia, Africa as well as in South and North America (Lux *et al*., 2003; Wan *et al*., 2011; Wan *et al*., 2012; USDA-APHIS, 2014; Manrakhan *et al*.,2015; Wei *et al*., 2017; Nugnes *et al*., 2018). Some species like *Bactrocera dorsalis* are highly invasive with projections for future global distribution indicating a wider occupancy within the tropic, sub-tropic and temperate regions (Stephens *et al*., 2007, De Meyer *et al*., 2010). In Africa, tephritid pest populations consist of both native species and exotic species that have established following introduction events (De Meyer *et al*., 2012). The majority of the native species that attack commercially grown fruit crops belong to two genera, *Ceratitis* (95 species) and *Dacus* (195 species) (White and Goodger, 2009) whereas a few other species belong to the genera *Trithithrum* and *Bactrocera* (De Meyer *et al*., 2012). Exotic pest species that have invaded and established in this region include the oriental fruit fly, *Bactrocera dorsalis* (Hendel), the Solanum fruit fly, *Bactrocera latrifrons* (Hendel), the melon fly, *Zeugodacus* (formerly *Bactrocera*/*Dacus) curcubitae* (Coquillett) and the peach fruit fly *Bactrocera zonata* (Saunders) (Lux *et* al., 2003; Drew *et al*., 2005; De Meyer *et al*., 2012; De Meyer et al., 2015). Both native and invasive fruit fly pest species in Africa cause considerable damage to cultivated fruit and vegetables with the native species

alone estimated to cause average yield losses of up to 40% for mango (*Mangifera indica*) and 53% for vegetables such as pumpkin (*Cucurbita pepo*) and tomatoes (*Solanum lycopersicum*) (Ekesi *et al.,* 2016). In many African countries, direct crop losses together with the cost of control measures as well as indirect losses attributed to quarantine restrictions often run into millions of dollars per annum (Ekesi *et al*., 2016). Infestation of produce with fruit fly pests is a therefore a constraint to fruit and vegetable production for both subsistence and commercial producers alike, in the Afrotropical region.

Many countries have imposed quarantine restrictions on the import of fruit and vegetable produce from countries where species of quarantine importance have been reported to occur. In some extreme cases, importation of produce from countries infested with certain fruit fly species has been banned (Mumford, 2002; USDA-APHIS, 2008; Otieno, 2011; Jose *et al*., 2013; Ekesi *et al.,* 2014; Ekesi *et al*., 2016). However, a more common measure is a requirement that fruit and vegetable produce be subjected to prescribed quarantine treatments before or during export. Such treatments include hot water treatment (Sharp and Martinez, 1990), low temperature storage or more commonly shipping/transportation (Jessup and Baheer, 1990; Manrakhan and Grout, 2010; Grout *et al.,* 2011) and ionizing radiation (Hallman, 1999).

Several approaches to the control of tephritid fruit flies have been attempted in the past including the use of pesticides such as malathion and spinosad (Vargas *et al*., 2015). The latter has been widely adopted as a more effective substitution for malathion which was previously commonly used for fruit fly control (Hafsi *et al*., 2015; Braham *et al*., 2007; Manrakhan *et al*., 2013). However, some fruit fly species such as the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae), *Z. curcubitae* (De Meyer *et al*.,

2015), the olive fruit fly, *Bactrocera oleae* (Rossi) (Diptera: Tephritidae) and *B. dorsalis* have been reported to show resistance to these insecticides including spinosad (Magana *et* al., 2007; Hsu *et al*., 2012; Kakani *et al*., 2010; Hsu and Feng, 2006). This raises concern that resistance may not only spread geographically but also that other species may evolve resistance. In addition, the widespread use of chemical insecticides has been associated with various risks to non-target arthropod species, as well as threats to human health and pollution of the environment (Forget, 1993; Igbedioh, 1991). For this reason, alternative and safer methods that alleviate over-reliance on chemical pesticides have over the past decades been developed and adopted in integrated pest management (IPM) programmes.

Among these strategies is the use of entomopathogenic fungi such as *Metarhizium anisopliae* (Garcia *et al*., 1985; Goble, 2009), *Isaria fumosorosea* (Carneiro and Salles, 1994), *Aspergillus ochraeus* (Castillo *et al*., 2000), *Beauveria bassiana* (De La Rosa *et al*., 2002; Goble, 2009), *Lecanicillium (*formerly *Verticillium*) *lecanii* (Veroniki *et al*., 2005), *Beauveria brongniartii*, *Mucor hiemalis*, *Penicillium aurantiogriseum* and *P. chrysogenum* (Konstantopoulou and Mazomenos, 2005).

Entomopathogenic nematodes have also been evaluated as a possible control strategy for fruit flies. Some nematode species such as *Steinernema riobrave*, *Heterorhabditis bacteriophora*, *H. zealandica*, *S. feltiae, S. khoisanae* and *S. carpocapsae* have been shown to have good potential (Patterson and Lacey, 1999; Malan and Manrakhan, 2009; Soliman *et al*., 2014).

Similarly, entomopathogenic viruses such as the Queensland fruit fly virus (Bashiruddin,

1988) that infects *Bactrocera tyroni* (Froggatt) also presents potential for control for these pests.

In addition, several parasitoids that parasitize eggs, larval and pupal stages of different fruit fly species have been discovered and are being used in management of these pests. Most of these parasitoids are hymenopteran (Costa *et al*., 2009). Documentation of indigenous fruit fly parasitoids began as early as 1912 in Africa, Australia and Hawaii (Silvestri, 1914a, b). Africa has a high diversity of indigenous parasitoids, often achieving parasitism rates from 2.4% to as high as 83% in different tephritid fruit fly species (Vayssières *et al*., 2012; Mkize *et al*., 2008). Successful classical biological control of fruit fly species using exotic parasitoid species has been reported in Africa, for instance using the parasitoid *Psyttalia fletcheri* (Silvestri) (Hymenoptera: Braconidae) for the management of *Z. curcubitae* and *Fopius arisanus* (Sonan) (Hymenoptera: Braconidae) for the management of *B. zonata* on the Reunion Island (Sonan) (Rousse *et al*., 2006; Quilici *et al*., 2004). Exotic parasitoids have been shown to achieve parasitism rates of up to 80% (Quilici *et al*., 2008). In 2006, the egg parasitoid *F. arisanus* and the larva parasitoid *Diaschamimorpha longicaudata (*Ashmead) (Hymenoptera: Braconidae) were imported into the International Centre for Insect Pest and Ecology (icipe) quarantine facility and have since been released in various countries in Africa achieving up to 40% parasitism rates (Mohamed *et al.,* 2016).

The responses of various insect pest species to IPM strategies can be influenced by the diverse and complex interactions between the insect pests and their associated microbiota. Microbes have multiple roles that mediate the interactions between phytophagous insects, their host plants and natural enemies (Janson *et al*., 2008; Ferrari

et al., 2011; Frago *et al*., 2012; Herren and Lemaitre, 2012; Biere and Bennet, 2013; Su *et al*., 2013; Douglas, 2016). Some microbes have been reported to confer parasitism resistance to their insect hosts (Oliver *et al*., 2003; 2005; 2010; Ferrari *et al*., 2004; von Burg *et al*., 2008, Vorburger *et al*., 2010), against pathogenic nematodes (Damodaram *et al.,* 2010), against pathogenic fungi and bacteria (Currie *et al*., 1999*;* Ferrari *et al*., 2004; Kaltenpoth *et al*., 2005; 2010; Scarborough *et al*., 2005) and also against predators (Kellner *et al*., 1996; 2003; Piel *et al*., 2004).

The proposed study will enumerate the endosymbionts and other symbiotic bacteria associated with *B. dorsalis* populations in Kenya and asses how their symbiotic interactions affect biological control of this pest.

1.2 Problem statement

Numerous tephritid fruit fly species infest fruit and vegetable crops that are grown for subsistence use by African farmers. Subsistence producers of affected crops are the most challenged in terms of food security due to direct crop losses and because they often have very little to invest in crop production and protection. In order to minimize crop loss, subsistence farmers frequently harvest their produce before it is mature and ripe, therefore settling for poor quality and poor nutrition rather than risking large crop losses.

Similarly, infestation by tephritid fruit fly pests largely affects commercial scale fruit and vegetable farming in many African countries. Mainly, commercial production incurs extra costs associated with control of pests as well as with quarantine treatment of produce in order to comply with export market standards.

The threat of fruit fly introduction into new regions and subsequent severe socioeconomic

consequences that may follow resulted in countries such as Japan, USA, New Zealand and Chile not to allow the importation of fresh fruit and vegetables from countries where these pests are endemic or have been introduced (FAO, 2015).

The cost of production including costs associated with pest management and phytosanitary treatments, crop losses and loss of revenue from damaged products that fail to enter the lucrative international markets due to quarantine measures against fruit fly infestation have negatively impacted fruit and vegetable production, especially in Africa (Badii *et al.,* 2015).

The widespread use of chemical insecticides to control tephritid fruit flies and other crop pests poses great risks to the environment and non-target organisms (Igbedioh, 1991). There is also growing concern over the impacts of chemical insecticide use on human health. Human exposure to insecticides has been associated with an elevated rate of chronic diseases including several types of cancer, reproductive disorders, birth defects and neurodegenerative disorders among others (Mostafalou and Abdollahi, 2013). This association has been a strong factor in consumer influence that has led to increasing preference for organic produce over conventionally produced food that is grown with chemical insecticides (Yiridoe *et al*., 2005; Moser *et al*., 2011; Bilal *et al*., 2015; Ditlevsen *et al*., 2015; Nandi *et al*., 2017; Khan *et al*., 2019; Wang *et al*., 2019). In addition, over reliance on chemical pesticides in plant protection is problematic because of tendencies of pest species to develop resistance.

Biological control agents are a key component of IPM programmes. However, facultative endosymbionts in various insect taxa have been reported to protect their hosts from

natural enemies, including predators, parasitoids, fungal pathogens and viruses (Haine, 2008). Such symbiotic interactions pose cryptic challenges to the development, implementation and sustainability of the use of biological control agents in management of tephritid fruit flies.

Some endosymbionts have been found to enhance the survival of their insect host especially in challenging environments, for example, through provision of essential nutrients (Oliver *et al*., 2010). This greatly enhances the ability of pest species to survive through different seasons usually by attacking alternative crops. Indeed, the fruit fly species *B. oleae* has been found to benefit from a bacterial symbiont that allows it to infest and survive in unripe olives laden with a phenolic glycoside which is produced by the plant as a defence mechanism against pests (Ben-Yosef *et al*., 2015). This suggests that similar symbiotic relationships could also be present in other tephritid fruit fly species. This possibility compounds management of tephritid fruit flies, especially so for most cultural control strategies.

Currently, not much data are available on the possible myriad of effects that endosymbiotic bacteria may have on tephritid fruit fly hosts. The complex interactions between endosymbionts and their respective host pest species potentially influences pest persistence and distribution. These interactions have not been studied in the past and remain unaccounted for in management strategies.

1.3 Justification

The development and implementation of effective IPM strategies for fruit flies is a positive measure to reduce dependence on chemical insecticides. Implementation of such IPM

programs will synergistically accommodate fruit and vegetable market trends that are shifting towards produce grown without the use of pesticides. Although successes in the development of IPM programs for fruit fly control have been reported, their effective and sustainable implementation is challenged by some of the effects that may result from interactions between symbiotic microbiota and the pest. For example, it has been reported that some endosymbiotic bacteria confer protection to their hosts against natural enemies such as entomopathogens, nematodes and predators.

Previous studies have detected several endosymbiotic bacteria such as *Wolbachia* and "*Candidatus Erwinia dacicola"* in some fruit fly species (Arthofer *et al*., 2009; Estes *et al*., 2009; Martinez *et al*., 2012; Morrow *et al*., 2014; Morrow *et al*., 2015;). The possibility of infection with other endosymbionts such as *Spiroplasma*, *Arsenophonus*, *Sodalis*, *Cardinium, Hamiltonella* and *Rickettsia* in fruit flies can also not be ruled out. Some of the latter endosymbionts have also been reported to protect their hosts against entomopathogenic fungi (Lukasik *et al*., 2012) other pathogens (Hendry *et al*., 2014) and against parasitic hymenopterans (Rothacher *et al.,* 2016). In this regard, proactive studies of the interactions between endosymbionts found in fruit flies and the biological control agents used in the management of fruit flies are required.

In addition, endosymbiotic relationships may provide opportunities for development of novel control methods for fruit fly pests. Control methods can be derived from symbiotic manipulations such as cytoplasmic incompatibility between symbiont-infected and uninfected hosts or incompatibility between infected insect hosts (Riegler and Stauffer 2002; Zabalou *et al*., 2009; Harris *et al*., 2010) and male killing (Hurst *et al*., 1994; Darby *et al*., 2010; Cheng *et al*., 2016; Harumoto and Lemaitre, 2018;).

Studies on the symbiont diversity and their host population dynamics are therefore necessary to facilitate development of symbiont-based methods for management of tephritid fruit flies. This has for instance been demonstrated in *C. capitata,* where a *Wolbachia* endosymbiont induces reproductive incompatibility between infected males and uninfected females. This has been suggested as a viable means of inducing sterility in field populations, a technique referred to as the Incompatible Insect Technique (Zabalou *et al*., 2009).

Evaluation of symbiont-pest interactions may potentiate exploitation of symbionts in pest management, which would contribute to effective management of fruit flies and ultimately synergize current measures of mitigating against losses due to fruit fly infestation for both subsistence and commercial producers, as well as the larger agriculture-dependent economies.

1.4 Research questions

The following research questions were addressed during this study:

1. Which endosymbionts occur in domesticated and wild populations of *B. dorsalis*?
2. What are the dynamics of *B. dorsalis* population invasions and infection patterns of the predominant endosymbiont species?
3. Which bacterial symbionts are associated with different Kenyan populations of *B. dorsalis*?
4. What is the impact of bacterial symbionts on the development, life history traits of *B. dorsalis*?
5. How do bacterial symbionts affect the use of the entomopathogenic fungus

Metarhizium anisopliae as a biological control agent of *B. dorsalis*?

1.5 Objectives

1.5.1 Main objective

The main objective of this study was to determine the role of bacterial symbionts in the metabolism, life history traits and response of *B. dorsalis* to biological control agents.

1.5.2 Specific objectives

i. To explore the diversity of endosymbionts in both wild and domesticated populations of *B. dorsalis*

ii. To assess the dynamics of host population invasion and infection patterns of the predominant endosymbiont species in *B. dorsalis*

iii. To determine the diversity of bacterial symbionts from several *B. dorsalis* populations from Kenya

iv. To determine the roles of bacterial symbionts on the development and life history traits of *B. dorsalis*

v. To determine whether bacterial symbionts influence the response of *B. dorsalis* to biological control using the entomopathogenic fungus, *M. anisopliae.*

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CHAPTER TWO

2. Literature review

2.1 Tephritid fruit flies

There are approximately 4,000 known species in the family Tephritidae and approximately 200 of these species have been reported to be pests of various fruit and vegetable crops (White and Elson-Harris, 1992; Ansari *et al*., 2012).

2.2 Distribution

Tephritidae are widely distributed globally in temperate, tropical and subtropical regions (Headrick and Goeden, 1998). In Africa, most pest fruit fly species belong to the genera *Bactrocera*, *Ceratitis*, *Dacus* and *Trirhithrum* (De Meyer *et al*., 2012). In sub-Saharan Africa, there are several native tephritid fruit fly species of the genera *Ceratitis* and *Dacus*. These include the mango (*Mangifera indica)* pests: *Ceratitis cosyra* (Walker), *C. rosa* (Karsch), *C. capitata* (Wiedemann), *C. quinaria* (Bezzi), *C. anonae* (Graham) and *C. fasciventris* (Bezzi) that are known to cause up to 70% losses in yield (Lux *et al*., 2003). Other indigenous species include *C. rubivora* (Coquillett), *C. punctata* (Wiedemann), *C. discussa* (Munro), *C. ditissima* (Munro), *C. pedestris* (Bezzi), *D. bivittatus* (Bogot), *D. lounsburyii* (Coquillett), *D. ciliatus* (Loew), *D. punctifrons* (Wiedeman), *D. frontalis* (Becker) and *D. vertebratus* (Bezzi) among others (Badii *et al*., 2015).

Several introductions and establishments of exotic fruit fly species have occurred into Africa over the years. These include *B. zonata* (Saunders) which was introduced into Egypt during 1999 (De Meyer *et al*., 2012), *B. dorsalis* (previously *B. invadens*) which was first detected in Kenya in 2003 (Drew *et al*., 2005) after which it rapidly invaded other

African countries. *B. latifrons* (Hendel) which primarily attacks Solanaceae crop species and also causes damage to tomato (*Lycopersicon esculentum*) (De Meyer *et al*., 2012) was detected in Tanzania during 2006 (Mwatawala *et al*., 2009). It is not clear when *Zeugodacus curcubitae,* which attacks plants in the Caricaceae, Cucurbitaceae, Moraceae and Solanaceae families (Mcquate and Teruya, 2015), was introduced into Africa (De Meyer *et al.,* 2015).

2.3 Damage caused by fruit flies

Female fruit flies directly damage fruit by making punctures on the fruit skin during oviposition where they deposit their eggs underneath the fruit skin. During oviposition, some bacteria from the fruit fly intestinal flora gets introduced along with the eggs, which subsequently act on surrounding fruit tissues causing them to rot (Badii *et al*., 2015). This rotting process softens fruit tissues in time for hatching larvae, making it easier for the larvae to feed inside the fruit. Other microbes also enter the fruit through the oviposition punctures and thrive in the decaying fruit tissue (Badii *et al*., 2015). A significant proportion of the fruit pulp is consumed as the larvae develop into the second and third instars.

2.4 Economic impact of fruit fly pests

Tephritid fruit flies are considered the most destructive pests of fruit and vegetable crops throughout the world (Jose *et al*., 2013). Many export markets for horticultural products have imposed bans on produce from African countries where fruit fly infestations are rife (Ekesi *et al*., 2016). For example, importations of mango, cucurbits (Cucurbitaceae) citrus (Rutaceae) and avocado (*Persea americana*) produce from East African countries to South Africa, the Seychelles, Mauritius and the USA have been banned and high rejection rates of produce are experienced in the European Union markets (Badii *et al*., 2015,

Europhyt, 2017). During 2008, South Africa imposed quarantine restrictions on produce infested with *B. dorsalis*, a measure that diminished Kenya's export market for avocadoes and also affected Mozambique's export capacity to its main trade partner, South Africa (Jose *et al*., 2013). These losses negatively affect the income, nutrition, food security and livelihoods of many subsistence and small-scale producers of these crops in developing countries and continue to undermine the Millennium Development Goal of eradication of poverty and hunger in Africa (UN DESA, 2016).

2.5 Management strategies for fruit flies

2.5.1 Sterile Insect Technique

This strategy involves artificial reproductive sterilization of the males of the pest species. This is most commonly accomplished through irradiation using beams of electrons, X-rays or gamma rays from a Caesium 137 or a Cobalt 60 source (Robinson, 2005). These males are mass reared and released within a target region where they are expected to out-compete wild males in mating with fertile wild females hence, reducing the number of viable offspring produced. This method was successfully used to eradicate *Z. cucurbitae* from Okinawa, Japan (Yosiaki *et al*., 2003) and to suppress populations of *C. capitata* from infested regions of South Mexico (Hendrichs and Hendrichs, 1998; Hendrichs *et al*., 2002) and from Chile in 1995 (SAG, 1996). Various efforts have been made to evaluate the implementation of this technique to control fruit flies in Africa (Ogaugwu, 2007; Barnes *et al*., 2015).

The implementation of the Sterile Insect Technique (SIT) against *C. capitata* in South Africa has been faced with various challenges (Barnes, 2007) including variation in

climate, with some regions having much more favourable conditions for development of the pest and therefore the programme has greater success in some regions than in others (Barnes *et al*., 2015). In regions where the programme has gained success, significant reductions in production costs associated with pest management were reported. For example, in the Hex River Valley, in only three years after the start of the program, the cost of management of this species was reduced by 67% from approximately US$500,000 per annum. Rejection rates due to fruit fly infestation of exported grapes destined for the USA were also reduced by approximately 50% (Barnes *et al*., 2002).

2.5.2 Attract and kill method

2.5.2.1 Parapheromones

Parapheromones are commonly used in the Male Annihilation Technique (MAT) for fruit fly control, a strategy aimed at reducing male fruit flies to such low numbers that very few or no females will find a male to mate with. These lures are designed to be highly species specific and efficient and capable of attracting males over long distances. Parapheromones applicable in MAT for control of fruit flies include Methyl eugenol (ME) (benzene, 1, 2-dmethoxy-4-2-propenyl), Cuelure (CUE) (4-(p-hydroxyphenyl-2-butanone acetate) and raspberry ketone (RK) (4-(p-hydroxyphenyl)-2-butanone) (Vargas *et al*., 2010). Others such as Trimedlure (TML) (tert-butyl-4-5-chloro-2-methylcyclohexane-1-carboxylate), Terpinyl acetate (TA) (alpha, alpha-4- trimethyl-3-cyclohexene-1-methanol), Vertlure (VL) (methyl-4-hydroxybenzoate) as well as ME are used for monitoring of fruit flies (Badii *et al*., 2015).

2.5.2.2 Food baits and chemical control

Hydrolysed proteins, yeast products and ammonium derivatives are used as food baits to attract especially female fruit flies that require a protein meal before oviposition in the Bait Application Technique (BAT). A killing agent is incorporated into the food bait, for instance spinosad, which is used as a killing agent in the commercial bait GF120 (Vayssières *et al*., 2009). The use of insecticides for control of fruit flies has been one of the most widely used strategies, especially the use of the organophosphate, malathion (Manrakhan *et al*., 2013). The efficacy of malathion against fruit flies has been reported to decline, prompting the use of more effective alternatives such as spinosad (Braham *et al*., 2007; Hafsi *et al*., 2015; Manrakhan *et al*., 2013). However, chemical control faces the challenge of resistance development (Hsu *et al*., 2012). Insecticides also pose risks to non-target species and pollute the environment if applied in non-restricted volumes and in a manner that does not restrict their dispersal (Igbedioh, 1991; Forget, 1993).

2.5.3 Cultural control

Cultural control measures are aimed at disrupting the reproductive cycles of fruit fly pest species (Badii *et al*., 2015, Sarwar, 2015). Orchard sanitation and crop hygiene are accomplished through measures such as regular collection and destruction of dropped fruits which have been found to have higher infestation densities than fruits on the plant (Rwomushana, 2008; Xia *et al*., 2018). Destruction can be accomplished by burying fruit deep in the ground, crushing, and exposing fruits to sunlight for several days in air tight polythene bags (Badii *et al*., 2015; Ullah *et al*., 2015; Khan *et al*., 2017). Other measures include avoidance of planting of other crop species that may also be infested by the same

fruit fly species nearby and avoiding planting of the same crop varieties that have different growth cycles. In some cases, harvest can be done early for fruits that are attacked when ripe, so that ripening does not occur on the plant (Ekesi and Billah, 2007; Sarwar, 2015). This strategy may however also have negative effects on fruit quality since early harvesting may affect fruit flavours (Kader, 2008).

2.5.4 Physical fruit protection

This is an effective but laborious method for protection of fruits against fruit flies. Netting or bagging (Allwood, 1997) of developing fruits on the plant before pest attack shields the fruit from contact with flies and also with other predators such as birds. Physical barriers however are not only useful in keeping of fruit flies, but also in preservation of post-harvest quality of fruits (Sharma *et al*., 2014; Xia *et al*., 2019).

2.5.5 Biological control

Various natural enemies and entomopathogens of fruit fly pest species have been identified and are harnessed in biological control strategies. Entomopathogenic fungi such as *Metarhizium anisopliae* (Garcia *et al*., 1985), *Isaria fumosorosea* (Carneiro and Salles, 1994), *Aspergillus ochraeus* (Castillo *et al*., 2000), *Beauveria bassiana* (De La Rosa *et al*., 2002), *Lecanicillium* (formerly *Verticillium*) *lecanii* (Veroniki *et al*., 2005), *B. brongniartii*, *Mucor hiemalis*, *Penicillium aurantiogriseum* and *P. chrysogenum* (Konstantopoulou and Mazomenos, 2005) have been reported to have activity against various fruit fly species. These biological pesticides are used to treat soils in orchards where they are active against larval and pupal stages of fruit flies. These fungi can also be formulated as granules that are easy to disperse and mix with soil (Ouna, 2010) or in

auto-dissemination devices that target adult flies to reduce their fertility and fecundity (Ouna, 2010). Similarly, entomopathogenic viruses capable of infecting fruit flies, such as the Queensland fruit fly virus, an icosahedral single stranded RNA virus suggested to belong to the family: Picornaviridae (Bashiruddin, 1988) also present a viable mechanism for control for this pest.

Entomopathogenic nematodes such as *Heterorhabditis bacteriophora*, *Steinernema riobrave, S. feltiae and S*. *carpocapsae* have been shown to have good potential (Patterson and Lacey, 1999; Malan and Manrakhan, 2009; Soliman *et al*., 2014) against tephritid fruit fly species such as *B. zonata*, *C. capitata* and *Rhagoletis indifferens*. The possible wide scale implementation of this strategy has not been reported yet.

Several hundred hymenopteran parasitoid species have been recorded as tephritid fruit fly parasitoids. These are classified into Braconidae, Figitidae, Eulophidae, Pteromalidae and Diapriidae families (Ovruski *et al*., 2000). Among them, *Fopius arisanus* (Sonan) (Hymenoptera: Braconidae) has successfully been used to suppress *B. dorsalis* populations in French Polynesia. Based on this success it can also be used as a model for introduction into other infested areas (Vargas *et al*., 2007). The larval parasitoid *Diachasmimorpha longicaudata* (Ashmead) (Hymenoptera: Braconidae), was also used successfully to suppress populations of the Caribbean fruit fly (*Anastrepha suspensa* (Loew) (Diptera: Tephritidae) (Sivinski *et al*., 1996). Similarly, the generalist pupal parasitoid *Muscidifurax raptor* (Girauld and Saunders) (Hymenoptera: Pteromalidae) has been shown to have good potential for control of *C. capitata* (Kapongo *et al.,* 2007) and therefore the possibility for use against more fruit fly species. Effective programs for fruit fly control using parasitoid species involve mass rearing and release of these parasitoids

in infested areas.

Other natural enemies such as predators can also be used in management of fruit fly infestation. The presence of the African Weaver Ant, *Oecophylla longinoda* (Latreille) (Hymenoptera: Formicidae) on fruit trees has for example been shown to deter fruit fly oviposition (Van Mele *et al*., 2007). Similarly, the big headed ant, *Pheidole megacephala* (Fabricius) (Hymenoptera: Formicidae) has been shown to have a role in ecological management of citrus orchards infested with *C. capitata,* among other citrus pest (Bownes *et al*., 2014).

2.6 Integrated Pest Management

The use of insecticides as primary control method for fruit fly pests is fallible to the emergence of insecticide resistance (Jin *et* al., 2011). In addition, it is difficult to target cryptic life stages of fruit flies that develop inside fruit tissue where they do not come into contact with topical pesticide applications (Korir *et al*., 2015). Owing to the high economic value of the fruit and vegetable production industry (Schreinemachers *et al*., 2018) and the low threshold for damage by fruit fly species (Jin *et al*., 2011), it is necessary to use integrated pest management (IPM) strategies to suppress pest populations. IPM improves market opportunities for the produce (Jin *et al.,* 2011) as well as alleviates over-reliance on chemical insecticides and reduces the associated unintended effects of insecticides on the environment and on non-target species (Brethour *et al*., 2001).

IPM strategies are increasingly gaining reputation for their effectiveness in control of tephritid fruit flies in Africa (Ekesi *et al*., 2011). In Africa for example, the International Centre for Insect Pest and Ecology (icipe) through the African Fruit Fly Program (AFFP)

has fronted the use of IPM strategies in management of fruit flies that infest mango (Muriithi *et al.*, 2016). This strategy includes five components: use of food baits, use of fungal based biopesticides, MAT, orchard sanitation and the mass rearing and release of the two hymenopteran parasitoid species (*F. arisanus* and *D. longicaudata*) (Muriithi *et al.*, 2016). This strategy has been reported to be widely adopted by Kenyan growers, ostensibly due to its success in improving yields (Korir *et al*., 2015). Indeed, dissemination and adoption of this strategy has been shown to significantly improve mango yield and income while reducing mango yield losses due to fruit fly infestation by an average of 19% (Muriithi *et al*., 2016). In addition, it has been estimated that approximately US $20 is generated for every US $1 invested in fruit fly IPM research and interventions in Kenya alone (Kassie *et al*., 2016). IPM technologies and innovations that have been tested and proven effective under field conditions (Ekesi *et al*., 2016) are applied in many African countries to address the fruit fly problem (Rwomushana and Tanga, 2016).

2.7 Fruit fly symbionts

Symbiotic relationships between insects and microbes play important roles in evolution and up to 20% of all insect species are known to depend on endosymbiotic bacteria for their development and survival (Takiya *et al*., 2006; Moran *et al*., 2008; Engel and Moran, 2013; Su *et al*., 2013; Douglas, 2016; Chen *et al*., 2017). Microbes that form symbiotic relationships with insect hosts are generally termed 'symbionts' although those that have evolved the ability to live inside the cells of the host rather than on tissue surfaces are referred to as endosymbionts. In the Tephritidae, two main endosymbionts have been reported to associate with various fly species. "*Candidatus Erwinina dacicolae"*, a non-pathogenic endosymbiont was reported in *B. oleae* (Estes *et al*., 2009). So far, *Wolbachia*

has been the most reported species that infect tephritid fruit flies (Table 1).

Table 1. Species of tephritid fruit fly in which *Wolbachia* infections have been reported.

Species	**Reference**
Anastrepha fraterculus (Wiedemann)	(Coscrato *et al*., 2009)
A. ludens (Loew)	(Coscrato *et al*., 2009)
A. obliqua (Macquart)	(Coscrato *et al*., 2009)
A. striata (Schiner)	(Coscrato *et al*., 2009)
A. serpentina (Wiedemann)	(Coscrato *et al*., 2009)
Bactrocera ascita (Hardy)	(Jamnongluk *et al*., 2002)
B. caudata (Fabricius)	(Kittayapong *et al*., 2000)
B. correcta (Bezzi)	(Kittayapong *et al*., 2000)
B. diversa (Coquillett)	(Kittayapong *et al*., 2000)
B. dorsalis (Hendel)	(Sun *et al*., 2007)
B. modica (Hardy)	(Kittayapong *et al*., 2000)
B. pyrifoliae (Drew and Hancock)	(Kittayapong *et al*., 2000)
B. tau (Walker)	(Kittayapong *et al*., 2000)
Ceratitis capitata (Wiedemann)	(Rocha *et al*., 2005),
Dacus destillatorius (Bezzi)	(Kittayapong *et al*., 2000)
Rhagoletis cerasi (Lonicera)	(Riegler and Stauffer 2002)
R. cingulata (Loew)	(Schuler *et al*., 2009)
R. pomonella (Walsh)	(Schuler *et al*., 2011)
Zeugodacus curcubitae (Coquillett)	(Kittayapong *et al*., 2000)

Endosymbionts produce a wide range of effects in their insect hosts. These effects include manipulation of host reproduction and at least one strain of *Wolbachia* has been found to cause cytoplasmic incompatibility (CI) in the European cherry fruit fly, *R. cerasi* (Riegler and Stauffer, 2002), as well as in experimental hosts, *C. capitata* (Zabalou *et al*., 2009)

and *B. oleae* (Apostolaki *et al*., 2011). Cytoplasmic incompatibility results in embryonic mortality when an uninfected female or female infected with a different strain of the CI-causing endosymbiont mates with an infected male. This is a potential avenue for control of insect pest species such as tephritid fruit flies that naturally harbour such endosymbionts. The strategy that has been proposed is referred to as the incompatible insect technique (IIT) (Zabalou *et al*., 2009).

Another important role played by some endosymbiont species is protection of their insect host from pathogens and natural enemies. Particularly, *Spiroplasma* and *Rickettsia* have been shown to protect aphid hosts from infection with entomopathogenic fungi (Lukasik *et al*., 2012), an effect that is likely to be produced even in other insect hosts.

Co-transmission of certain species of endosymbionts in an insect pest can also confer additive protective effects to the host insect from natural enemies, as is the case for *Spiroplasma* (strain MSRO) co-occurrence with *Wolbachia* (strain *w*Mel), which together confer protection against the parasitic wasp *Leptopilina heterotoma* (Thomson) (Hymenoptera: Eucoilidae) (Xie *et al*., 2014).

Some endosymbionts also enhance survival of their insect hosts by helping them counter host plant defence mechanisms. This has been observed for the Enterobacteriaceae "*Candidatus Erwinia dacicola"* that counters the effects of oleuropein, a phenolic glycoside in unripe olives thus enabling the olive fruit fly *B. oleae* larvae to survive in the unripe fruit (Ben-Yosef *et al*., 2015).

However, not all instances of endosymbiont-host interaction may be beneficial to the host insect. For example, infection of the two-spot ladybird *Adalia bipunctata* (L.) (Coleoptera:

Coccinellidae) with male-killing Rickettsia has been shown to be detrimental to the insect, decreasing its longevity and fecundity (Hurst *et al*., 1994). Similar effects are highly likely in other insect-endosymbiont interactions and are worth investigation among tephritid fruit flies for exploitation in IIT.

2.8 Bacterial symbionts in *B. dorsalis*

The invasive *B. dorsalis* is among the most studied tephritid fruit fly species, especially regarding its associated bacterial symbionts. Members of the phyla Proteobacteria, Firmicutes, Bacteroidetes and Actinobacteria have so far been the most frequently identified bacteria associated with this pest (Wang *et al*., 2011; Shi *et al*., 2012; Wang *et al*., 2013, Andongma *et al*., 2015; Liu *et al*., 2016; Gujjar *et al*., 2017; Khaeso *et al*., 2017; Bai *et al*., 2018; Liu *et al*., 2018; Akami *et al*., 2019). However, little is known of which communities of bacteria associate with populations of *B. dorsalis* in Africa. In addition, the nature of host-symbiont relationships for majority of fruit fly species remains poorly studied.

Proactive studies on the nature of interactions that endosymbionts, as well as surface microbes that often associate with tephritid fruit flies in less defined symbiotic relationships, will unravel new possibilities in pest management. Findings on these interactions will be useful in realizing the possibility of novel strategies such as IIT as well as inform the intersection of symbionts with currently applied fruit fly pest control strategies. This study will therefore assess the diversity of bacterial endosymbionts and other gut bacteria that associate with the oriental fruit fly, *Bactrocera dorsalis* and investigate the dynamics of these associations and their implications on the host populations over time in several locations in Africa. In addition, the study will investigate

some roles of *B. dorsalis* associated bacteria in the development of the fly as well as in the response of this pest to biological control. Understanding the symbiont-host interactions in this pest will provide insight and potentiate the exploitation of bacterial symbionts in management strategies.

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CHAPTER THREE

3. Unexpected diversity of *Wolbachia* associated with *Bactrocera dorsalis* (Diptera: Tephritidae) in Africa

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3.1 Abstract

Bactrocera dorsalis (Hendel) is an important pest of fruit in many countries worldwide. In Africa, this pest has spread rapidly and has become widely established since the first invasion report in 2003. *Wolbachia* is a vertically transmitted endosymbiont that can significantly influence aspects of the biology and particularly reproduction of its host. In this study, we screened *B. dorsalis* specimens collected from several locations in Africa between 2005 and 2017 for *Wolbachia* using a PCR-based assay to target the *Wolbachia* surface protein (*wsp*), *16S rRNA* gene and the MLST genes for *Wolbachia.* Out of 357 individuals tested, 10 were positive for *Wolbachia* using the *wsp* marker. We identified four strains of *Wolbachia* infecting two *B. dorsalis* mitochondrial haplotypes. We found no strict association between the infecting strain and host haplotype, with one strain being present in two different host haplotypes. All the detected strains belonged to Super Group B *Wolbachia* and did not match any strains reported previously in *B. dorsalis* in Asia. These findings indicate that diverse *Wolbachia* infections are present in invasive populations of *B. dorsalis*.

Keywords: *Wolbachia*; *Bactrocera dorsalis*; Oriental fruit fly; *Wolbachia* surface protein; mitochondrial COI haplotype

3.2 Introduction

Bactrocera dorsalis (Hendel) (Diptera: Tephritidae) is amongst the most serious pests of cultivated fruits across Asia and Africa owing to its high adaptation, polyphagy, fecundity and the extent to which it causes yield and revenue losses [1]. First reported in Africa in 2003, this pest has rapidly spread and established in most African countries often displacing the native *Ceratitis cosyra* (Walker) (Diptera: Tephritidae) as the primary fruit fly pest of mango [2,3]. The invasion of Africa by this pest had major consequences for fruit production, causing major losses in yield [4] as well as revenues [5].

Wolbachia is an intracellular bacterial parasite known to infect many arthropod species [6–8]. *Wolbachia* are maternally-transmitted in the egg cytoplasm and therefore have evolved a number of reproductive manipulations to increase the fitness of *Wolbachia*-infected matrilines. In many cases, *Wolbachia* cause cytoplasmic incompatibility between uninfected females and infected males. This ability to cause cytoplasmic incompatibility can result in *Wolbachia*-infected lineages rapidly increasing in frequency in a host population. The release of *Wolbachia*-infected incompatible males is potentially a very effective mechanism for decreasing pest insect populations (incompatible insect technique, IIT) [9]. IIT may have a number of benefits relative to the sterile insect technique (SIT) because radiation is not required. Notably, a symbiont-based pest management technique utilizing a *Wolbachia* strain that causes cytoplasmic incompatibility in fruit flies has been demonstrated in the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) [10] and evaluated for the olive fruit fly *Bactrocera oleae* (Rossi) (Diptera: Tephritidae) [11]. In addition, *Wolbachia*-induced cytoplasmic incompatibility can be used to spread symbionts and transgenes through

target insect populations, which could be useful for controlling pests and blocking the capacity of vectors to transmit diseases [12–15]. Some *Wolbachia* strains have also been found to modify their host's susceptibility to parasitoids [16] and therefore knowledge of *Wolbachia* infection status can be of relevance to optimizing integrated pest control strategies employing parasitoid wasps.

Wolbachia is a diverse bacterial clade that have been broadly categorized into several super groups. Currently, there are at least 16 recognized super groups, designated A-F and H-Q [17–26]. Some strains show strong associations with certain host species, while others infect more than one host species and observations of multiple infections of the same species or even the same individuals are commonly reported [27–33]. This pattern indicates that over evolutionary timescales, horizontal transmission of *Wolbachia* is commonplace [34]. At the population level, *Wolbachia* are transmitted vertically and since mitochondria are co-inherited, this can establish a linkage disequilibrium between *Wolbachia* and the host mitochondrial haplotype [35].

In the Tephritidae family, several studies have detected *Wolbachia* strains in the genera *Rhagoletis* [28,36,37], *Anastrepha* [31,38–42], *Ceratitis* [9,43,44] *Dacus* [45–47] and *Bactrocera* [45–50]. In *B. dorsalis*, *Wolbachia* has been reported at low prevalence in populations from China [49] and Thailand [48,50]. The objective of the current study was to investigate the presence and diversity of *Wolbachia* strains in *B. dorsalis* populations in Africa and to evaluate the infection patterns of *Wolbachia* and associations with mtDNA haplotypes in populations of this host sampled between 2005 and 2017.

3.3 Materials and Methods

3.3.1 Insect collection

Bactrocera dorsalis male flies were collected using methyl eugenol traps from mango farms in 2017 from Mwanza (2° 43' 01.3" S 33° 01' 20.4" E) and Morogoro (06° 57' 38.5" S 037° 31' 59.1" E) in Tanzania, Bunamwaya (0° 16' 17.8752" N 32° 33' 25.6284" E) in Uganda and Kassala (15° 28' 39.1728" N, 36° 21' 57.9204" E), Gezira (14° 36' 29.4" N 33° 47' 27.5" E) and Singa (12° 47' 46.6" N 33° 11' 51.5" E) in Sudan. *Bactrocera dorsalis* female flies were retrieved from infested mango fruit collected from mango farms in 2017 from Nguruman (01 48' 32"S 036 03' 35"E), Kitui (01°21'S, 38°00'E), Muranga (0°42'50.0"S 37°07'03.4"E) and Embu (0°28'56.6"S 37°34'55.5"E) in Kenya. Infested fruit were dissected to enable third-instar larvae to emerge and pupate in fine sterile sand. Pupae were sieved from the sand and maintained in ventilated perspex cages until eclosion. All samples were stored in absolute ethanol at -20°C.

3.3.2 DNA extraction

DNA was extracted from each individual using the ISOLATE II Genomic DNA Kit (Bioline, London, UK). Voucher specimens collected between 2005 and 2009 in African sites as well as in Sri Lanka from an earlier study of *B. dorsalis* were obtained from the molecular biology laboratory at the International Centre of Insect Physiology and Ecology, *icipe* [51,52].

3.3.3 *Wolbachia* screening and host mitochondria amplification

Wolbachia infections were initially screened by PCR using the *wsp* primers 81F and 691R [53] and subsequently all positives were screened using the *16S rRNA* primers for *Wolbachia pipientis* [54] and the *Wolbachia* MLST gene primer sets [55]. Reactions were

set up in total volumes of 10µl each, containing 5 × MyTaq reaction buffer (5 mM dNTPs, 15 mM MgCl2, stabilizers and enhancers) (Bioline, London, UK), 2 µM of each primer, 0.25 mM MgCl2 (Thermo Fischer Scientific, Massachusetts, USA), 0.125 µl MyTaq DNA polymerase (Bioline, London, UK) and 7.5 ng/µl of DNA template. These reactions were set up in a Master cycler Nexus gradient thermo-cycler (Thermo Fischer Scientific, Massachusetts, USA). Cycling conditions for the *16S rRNA* primers included an initial denaturation for 2 min at 95 °C, followed by 30 cycles of 1 min at 95 °C, 1 min at 52 °C and 1 min at 72 °C, then a final elongation step of 10 min at 72 °C. For the MLST and *WSP* primers, an initial denaturation for 2 min at 94 °C was used followed by 40 cycles of denaturation of 30 s at 94 °C, 45 s at annealing temperature (55 °C –*wsp*, 54 °C –*hcpA*, *gatB*, *ftsZ*, *coxA* and 59 °C –*fbpA*), 1 min 30 s at 72°C followed by a final extension step of 10 min at 72 °C. Host mitochondrial DNA was amplified by PCR in similar reaction volumes and cycling conditions as the *wsp* and MLST genes, using the primer LCO1490 and HCO2198 [56] at an annealing temperature of 50.6 °C. PCR products were run through 1% agarose gel electrophoresis and visualized by ethidium bromide staining and UV trans-illumination. Direct sequencing was done for all host COI and *Wolbachia* positive samples. Sequencing was carried out in both directions (F and R) for *Wolbachia* and host COI.

3.3.4 Sequence analysis

Wolbachia sequences and representative host haplotype sequences were submitted to the GenBank (accession numbers given in Table 1 and Figure 3). Sequence alignments were performed using Clustal W in Geneious 8.1.9 software (www.geneious.com) [57]. Phylogenetic trees were constructed by the maximum-likelihood method with the Tamura-

Nei model in Geneious 8.1.9 software. Support for tree topology was assessed by bootstrap resampling. Haplotype maps were generated using median-joining network algorithm in the population analysis with reticulate trees (popART) software (http://popart.otago.ac.nz) [58][59]. Allelic profiles for *wsp* and MLST sequences obtained were inferred using the *Wolbachia* MLST database (https://pubmlst.org) [55].

3.4 Results

3.4.1 *Wolbachia* prevalence

Using a PCR-based assay to amplify the *Wolbachia wsp* gene, we found that out of the 357 individuals tested, 10 were positive for *Wolbachia* (Table 1). These included 6 samples collected between 2005 and 2009 from African sites and 2 collected in Sri Lanka in 2007. In the samples collected in 2017, only 2 were found positive, which corresponds to an overall *Wolbachia* prevalence of 3.6% in the period between 2005 and 2009 and 1.1% in 2017 for the sampled African populations of *B. dorsalis*. Two sites, Muranga and Nguruman, had *Wolbachia* positives in the 2005 to 2009 sample set but not in the 2017 set, whereas Kitui had no positives in the period between 2005 and 2009 but one positive in 2017.

For a total of 6 samples (Ng13, Ki1, Mu2, Tzc13, Tg6 and Sl11), we amplified the *Wolbachia coxA* gene in addition to *wsp,* whereas in fewer samples (Ki1, Ng13, Mu2 and Sl11) other MLST genes were amplified and sequenced (Table 1). A full MLST profile and 16SrDNA was achieved for one sample (Tzc13), which had identical allelic profiles in their *wsp* hypervariable regions and *coxA* locus to strains in the *Wolbachia* pubMLST database, however, none of Tzc13's other loci were identical to those in previously

characterized *Wolbachia* strains (Table S1). The rest of the positive samples had incomplete profiles and partial similarities to those in the database.

Table 1. *Wolbachia* screening of sampled populations using different markers. Initial screening was carried out using the *wsp* gene assay (denoted in red) and positives were subsequently also screened using 16S and MLST gene assays (denoted in blue). Numbers represent the number of samples for which there was successful gene amplification, and year indicates when samples were collected.

Locality	Speci-men code	Year	Sex / (n)	*wsp* +	16S +	*coxA* +	*fbpA* +	*gatB* +	*hcpA* +	*ftsZ* +
Nguruman, Kenya	Ng	2008	m (15)	1	-	1	1	-	-	-
Nguruman, Kenya	Ng*	2017	f (15)	-	-	-	-	-	-	-
Kitui, Kenya	Ki-h	2005	m (15)	-	-	-	-	-	-	-
Kitui, Kenya	Ki	2017	f (15)	1	-	1	-	1	-	-
Muranga, Kenya	Mu	2005	m (15)	1	-	1	1	-	-	-
Muranga, Kenya	Mu*	2017	f (15)	-	-	-	-	-	-	-
Embu, Kenya	Em	2017	f (15)	-	-	-	-	-	-	-
Dar es Salaam, Tanz.	Tz	2009	m (15)	-	-	-	-	-	-	-
Mwanza, Tanzania	Tz-ab	2017	m (30)	-	-	-	-	-	-	-
Morogoro, Tanzania	Tz-c	2017	m (15)	1	1	1	1	1	1	1
Kawanda, Uganda	Ug	2007	m (15)	-	-	-	-	-	-	-
Bunamwaya, Ugan.	Ug-b	2017	m (30)	-	-	-	-	-	-	-
Khartoum, Sudan	Su	2007	m (15)	1	-	-	-	-	-	-
Kassala, Sudan	Su-a	2017	m (15)	-	-	-	-	-	-	-
Gezira, Sudan	Su-b	2017	m (15)	-	-	-	-	-	-	-
Singa, Sudan	Su-c	2017	m (15)	-	-	-	-	-	-	-
Zaria, Nigeria	Zr	2005	m (15)	-	-	-	-	-	-	-
Monts Kouffe, Benin	Be	2009	m (15)	1	-	-	-	-	-	-
Lome, Togo	Tg	2009	m (15)	1	-	1	-	-	-	-
UBG, Ghana	Gh	2009	m (15)	1	-	-	-	-	-	-
Ibadan, Nigeria	Ib	2009	m (15)	-		-	-	-	-	-
Ranbukpitiya, Sri Lanka	Sl	2007	m (15)	2	-	1	-	-	-	-

3.4.2 Phylogenetic reconstruction for the detected *Wolbachia*

To investigate the phylogenetic relationship between the detected *Wolbachia,* we constructed a phylogenetic tree with 41 sequences available in GenBank. We used *wsp* sequences for 5 African samples and 1 Sri Lankan sample that were supported by at least an additional gene. None of these *Wolbachia* were identical to previously detected *Wolbachia* strains in *B. dorsalis* or other *Bactrocera* species. The *wsp* sequences clustered together with other *Wolbachia* strains in super group B (Figure 1). Two samples collected in Kenya in the period between 2005 and 2009 (Ng3 and Mu2) were found to have identical *wsp* sequences, whereas all other samples had *wsp* gene sequences that were unique.

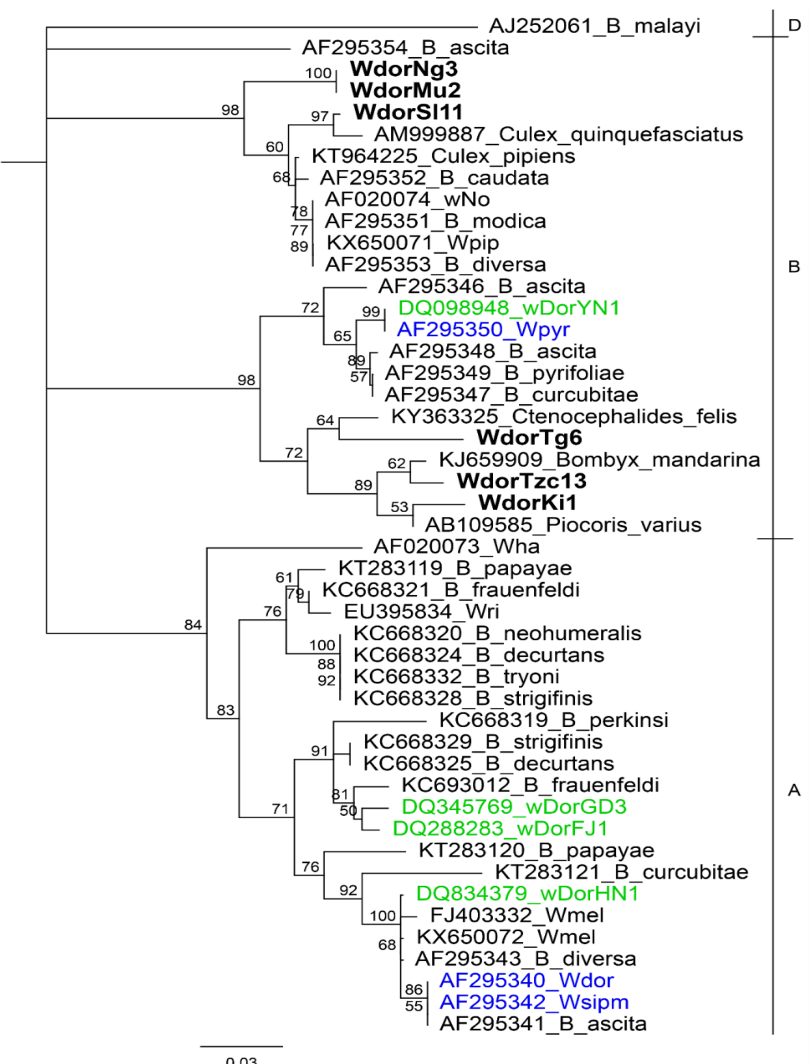

Figure 1. Neighbour joining tree based on *Wolbachia* surface protein (*wsp*) gene sequences of *Wolbachia* detected from *B. dorsalis* in this study (in bold), from *B. dorsalis* in China (labelled in blue), from *B. dorsalis* in Thailand (labelled in green) and from other *Bactrocera* species. Sequences from closest homology matches to *Wolbachia* detected in ths study are also included. Other common *Wolbachia* strains (wMel, wRi, wHa, wNo and wPip) are also included. *Wsp* sequence from *Wolbachia* endosymbiont of *Brugia malayi* is included as an outgroup. Sequences are labelled with genbank accession numbers followed by strain name or host organism, or strain name for sequences from this study. *Wolbachia* super groups are indicated in higher case letters on the right.

Among the MLST genes, the *coxA* gene had the highest number of amplified sequences. The phylogenetic relationships between strains as inferred by *coxA* gene sequence was not fully identical to that inferred by the *wsp* gene. Notably, the *coxA* gene sequence for

Sl11 clustered with *Wolbachia* group A. However, it was also observed that samples Ng3 and Mu2, which had identical *wsp* sequences also had identical *coxA* sequences.

All loci except fbpA supported the infection in Tzc13 as a super groupB *Wolbachia*. Mu2 and Ng13 that were found to be identical using wsp and coxA were also found identical and confirmed to be super group B using the fbpA gene. Similarly, the fbpA locus also agreed with both wsp and coxA for Ki1 as a super group B strain (Figure S1). Unlike the wsp gene sequence, the sequence of the hcpA gene of SL11 also clustered it with super group A *Wolbachia*, as was the case with coxA marker.

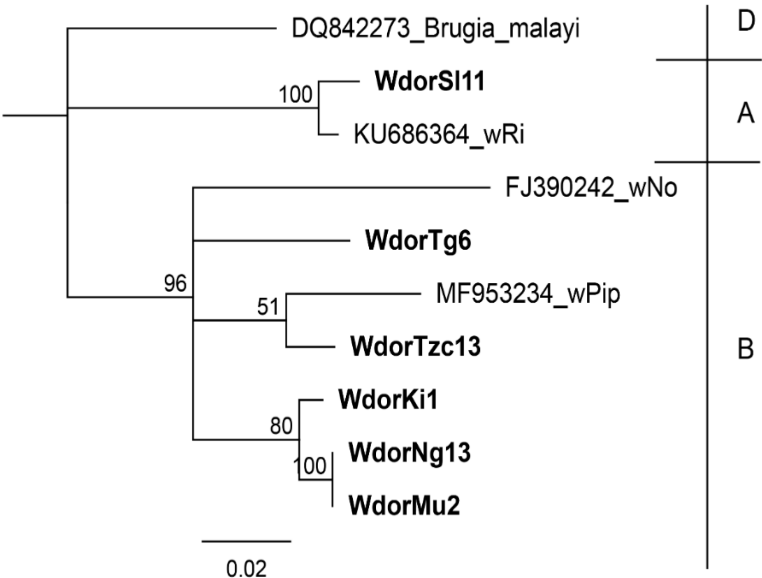

Figure 2. Neighbour joining tree based on the cytochrome c oxidase subunit I (*coxA*) gene sequences from this study. The detected *Wolbachia* are denoted by W followed by the host denoted as dor (*B. dorsalis*), population (Tg-Togo, Tzc-Tanzania, Ki-Kitui, Sl-Sri Lanka, Ng- Nguruman and Mu- Muranga) and population sample ID. Bootsrap values are indicated above branches. Sequences from other common *Wolbachia* strains (wPip, wNo and wRi) are included, while a sequence from *Wolbachia* endosymbiont of *Brugia malayi* is included as an outgroup. *Wolbachia* super groups are indicated in higher case letters on the right. Branches with bootsrap support lower than 50% are collapsed.

3.4.3 *Wolbachia* infection vs host mitochondrial haplotypes

The samples collected in 2017 clustered into 7 mitochondrial *COI* haplotypes (Hap1-Hap7), whereas samples collected between 2005 and 2009 clustered into five of the aforementioned haplotypes (with the exception of Hap5 and 7) and an additional 12 smaller haplotypes (Figure 3). The *COI* gene sequences of the *Wolbachia*-infected *B. dorsalis* from African populations indicated that all were either Hap1 (H-NG13, Ki1 and Tzc13) or Hap2 (H-Mu2 and H-Tg6). The infected Sri Lankan sample (Sl11) did not cluster into any of the 7 major and 12 minor mitochondrial *COI* haplotypes known from Africa. The two samples that had identical *wsp* and *coxA* gene sequences (Ng3 and Mu2) were found to have different mitochondrial *COI* haplotypes.

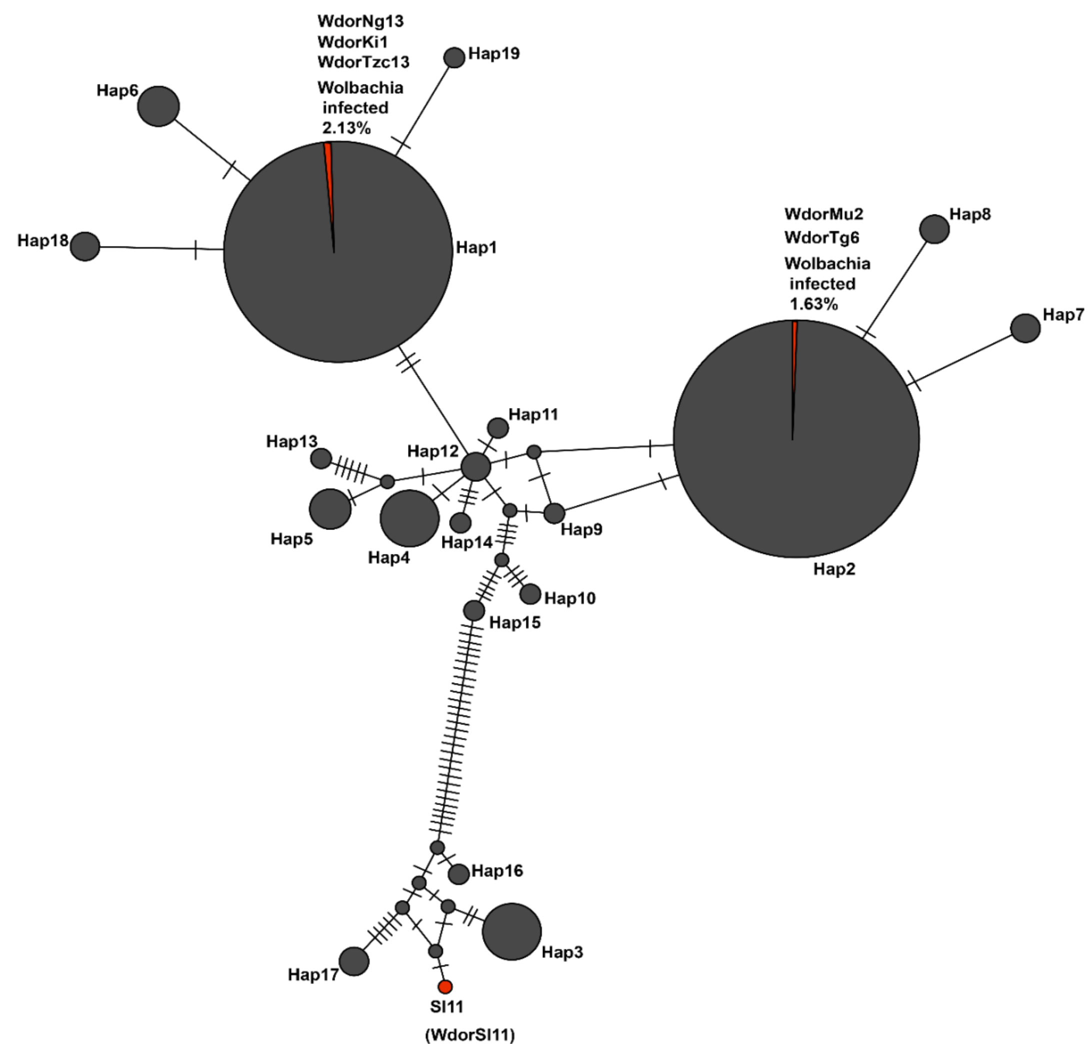

Figure 3. Haplotype map of *Bactrocera dorsalis* mtCOI sequences from African populations. Node size is proportional to number of samples while mutations are represented as hatchmarks. Proportions of *Wolbachia* infected samples (H-Ng13, Ki1 and TZc13 in Haplotype1 and H-Mu2 and H-Tg6 in Haplotype 2) in their respective haplotypes are shaded in red. Sequences of represented haplotypes are accessible at Genbank using the accessions MK314052-MK31452 for Haplotypes: 3, 2, 4, 6, 7, 5 and 1 respectively, JQ692656, JQ692727, JQ692777, JQ692863, JQ692731, JQ692684, JQ692812, JQ692698, JQ692723, JQ692816, JQ692816 and JQ692691 for haplotypes 8-19 respectively. The infected Sri Lanka sample (labelled Sl11, Genbank accesion: JQ692764) is not numbered to distinguish it from haplotypes detected in Africa.

3.4.4 *B. dorsalis* population structure dynamics

In sites that were sampled in both the 2005 to 2009 period and 2017, the most dominant haplotype in the 2005 to 2009 population was also dominant in the 2017 populations (Figure 4), inferring minimal change in the population structures as inferred by mitochondrial haplotype.

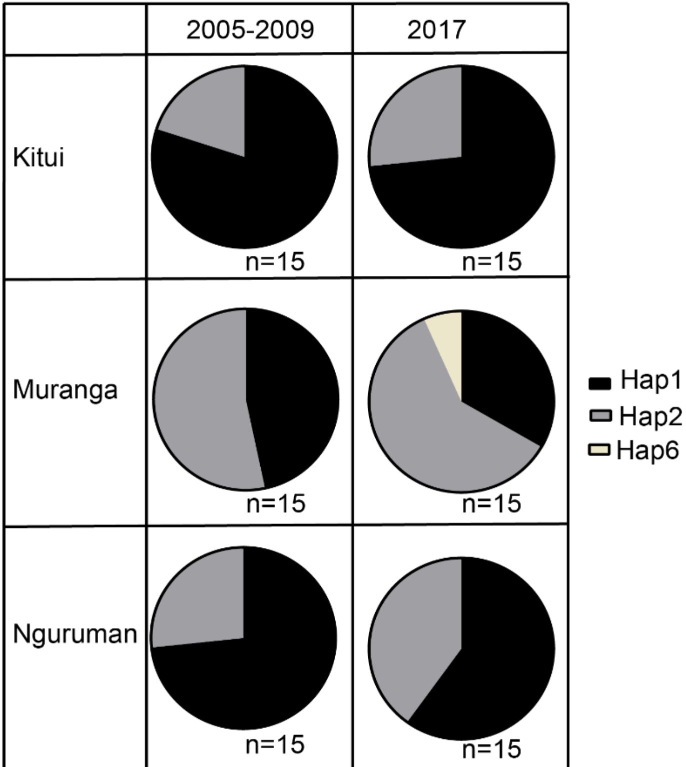

Figure 4. Haplotype compositions in sites sampled during the period between 2005 to 2009 (left column) and in 2017 (right column).

3.5 Discussion

We investigated *Wolbachia* infections in *B. dorsalis* in Africa and one location outside of Africa. *Wolbachia* sequences were detected in 10 samples. Overall this indicates a low rate of *Wolbachia* infection (2.3%) across the African populations, although infection rates appear to be marginally higher than found in Asia [48,49]. It is notable that a higher prevalence of *Wolbachia* infection was recorded in Sri Lanka, which is within the native

range for this species. Based on their *wsp* and *coxA* sequence, four distinct variants of *Wolbachia*: WdorTg6, WdorTzc13, WdorKi1 and WdorNg3/WdorMu2 were detected in the African populations of *B. dorsalis*. A fifth variant, WdorSl11, was detected in one of the Sri Lankan samples. All of these variants were distinct from those previously recorded in *B. dorsalis* [48,49] and therefore suggest that this species has a high diversity of low prevalence *Wolbachia*.

The WdorNg3/WdorMu2 variant was detected in two individuals from two different sampling sites, Nguruman and Muranga, which are within geographically separated agroecological zones in Kenya. It was notable that this variant was recorded in two different mitchondrial haplotypes (Hap1 and Hap2). *Wolbachia* strains transmitted from an infected female to her offspring (vertical transmission) tend to associate strictly with the same host haplotypes while strains transmitted horizontally (from one individual to another unrelated individual) do not. Altogether, the non-concordance with mitochondrial DNA, high diveristy and low prevalence, suggest that *Wolbachia* strains infecting African populations of *B. dorsalis* may have undergone horizontal transmission. The low prevalence of the detected *Wolbachia* in few individuals of *B. dorsalis* is therefore more indicative of transient infections that could have spilled over from an unknown source. In four out of the 10 *wsp* positive samples, we were not able to amplify any of the MLST or the *16S rRNA* gene, which could be a sign of a partial transfer of genes (more common than the transfer of a full *Wolbachia* genome). This phenomenon has been observed in previous studies [45] where *Wolbachia* pseudogenes have been detected in other tephritid fruit flies. These four could therefore either be remnants of past infections and

subsequent horizontal gene transfer or may be spillover from an unknown source. Future research should monitor vertical transmission rates and tissue localization patterns of *Wolbachia* to determine the nature of these infections.

With the exeption of one locus in Tzc13 (*fbpA*), all the *Wolbachia* variants detected among the African popualtions clustered with super group B *Wolbachia*, which differs from the majority of super group A strains as found in *B. dorsalis* species in China [49] and Thailand [48]. Similarly, it was observed that WdorSl11 clearly segregated as super group A *Wolbachia* using the *coxA* and *hcpA* markers, although it clustered together with super group B strains using the *wsp* marker. This suggests possible recombination between an A and B group *Wolbachia*, an observation that has been reported in previous studies [60,61]. However, sequencing results of the *16S rRNA*, *gatB* and *ftsZ* loci of SL11 (data not shown) showed a close interfering secondary chromatogram in few segments, suggesting that the sample is likely to have been infected by two *Wolbachia* strains (A and B group).

In addition, none of the detetcted *Wolbachia* had complete identity of allellic profiles to *Wolbachia* strains in the *Wolbachia* MLST database. Full length sequences of each of the MLST and *wsp* marker could not be retrieved for all the samples and this limitied our ability to confirm their designation as novel strain types through allelic profiles.

Some strains of *Wolbachia* are known to cause male-killing and in insect populations with these strains, infected male specimens are generally not observed or observed only very

rarely. To avoid bias against detection of male-killing *Wolbachia*, female specimens are generally screened. *Wolbachia* are also known to reach high densities in insect ovaries and therefore may be easier to detect in females. The most widely used collection method for tephtritid fruit flies involves attraction by male-specific sex pheremones. Therefore the majority of the samples we screened, in particluar from the 2005 to 2009 collections, were male. It is possible that if female flies had been screened, a higher *Wolbachia* prevalence rate might have been recorded. However, it is noteworthy that the female samples from the 2017 collections did not have a higher rate of *Wolbachia* infection. The *Wolbachia* variants that were found in males (WdorTg6, WdorTzc13, WdorNg3/WdorMu2 and WdorSl11) are unlikely to have a male-killing phenotype, whereas we cannot rule out the possibility that WdorKi1, which was detected in a female fly, causes male-killing.

The population structure of *B. dorsalis* in the African region was observed to be largely unchanged between the 2005 to 2009 and 2017 samples, with two major mtCOI haplotypes (Hap1 and Hap2) dominanting in both [52]. This suggests that based on mitochondrial DNA diversity, the current population structure has been largely unchanged since the initial *B. dorsalis* invasion in the Eastern Africa region and that no significant new reintroductions with new mtCOI haplotypes have occurred in this region since the first invasion. To confirm this, however, it would be nessesary to use nuclear DNA markers as mitochondrial DNA introgression could have occurred. The two main haplotypes, Hap1 and Hap2, in addition to Hap3, Hap4 and Hap7 were observed to be fairly distributed across the East and West of Africa. Only two haplotypes: Hap5 and Hap6 were observed in Eastern Africa only while the rest of the smaller mtCOI haplotypes (Hap8 to Hap18)

were observed in West Africa, particularly in Nigeria, except for Hap8 that was observed exclusively in Benin. Previous microsatelite genotyping data also revealed differences between the Eastern Africa populations with that of Nigeria, which were more closely allied to the diverse Sri Lankan populations of *B. dorsalis* [52]. It has been suggested that contemporary geneflow may have contributed to this diversity in West Africa [52].

3.6 Conclusions

We detected four different *Wolbachia* variants in African populations of the oriental fruit fly. Analysis of the host mtCOI haplotypes did not reveal a link between a particular *Wolbachia* variant and host haplotypes. Only the two dominant haplotypes were found to be infected with *Wolbachia* in Africa. These *Wolbachias* should be investigated for their capacity to manipulate host reproduction and to confer hosts with differential suseptibility to parasitoids and pathogens. A comprehensive understanding of the role of *Wolbachia* in this species could improve effectiveness of integrated control strategies and eventually play a role in the sustainable pest management of *B. dorsalis*.

3.7 Acknowledgments

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3.9 Supplementary material

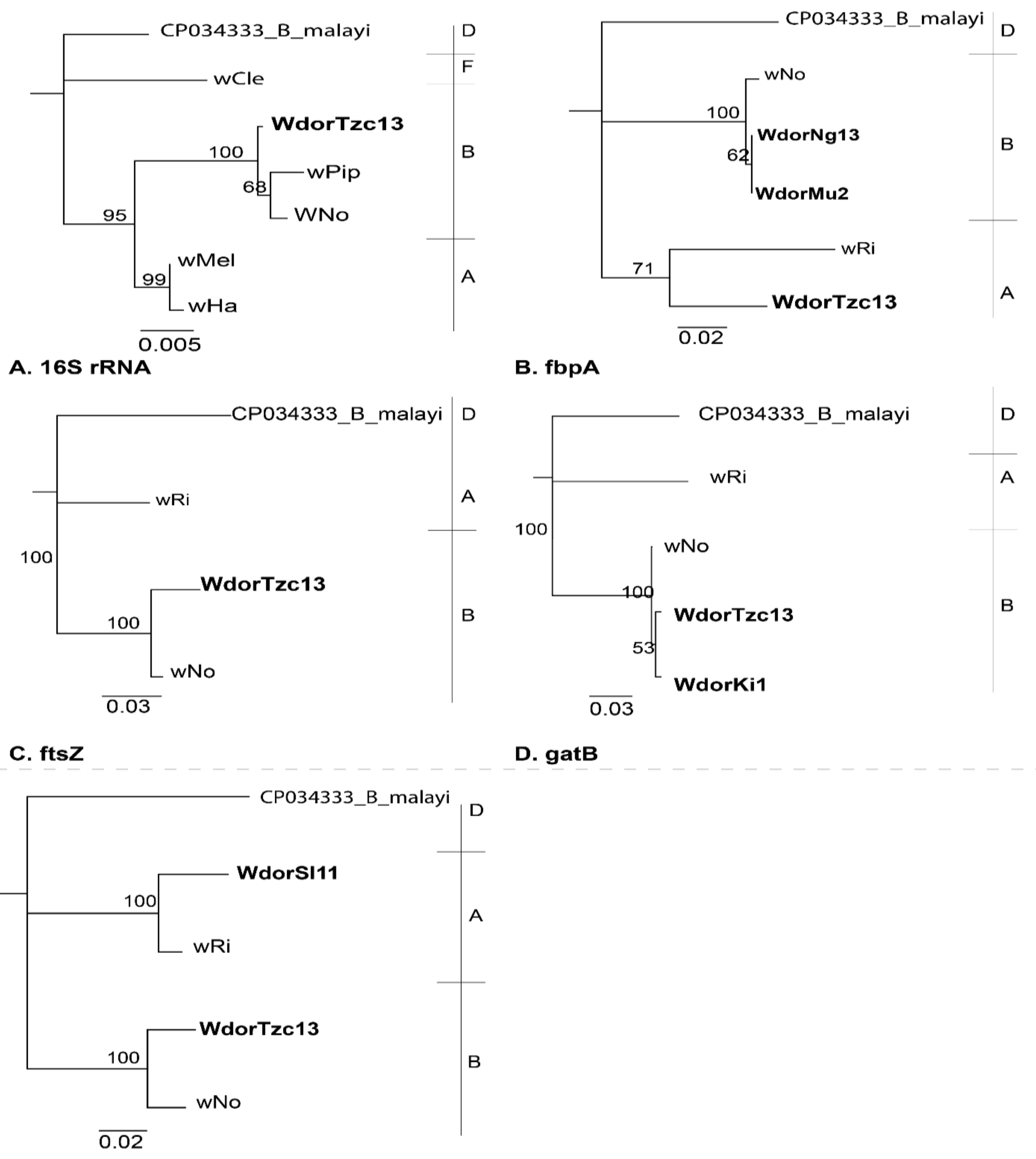

Figure S1. Neighbour joining trees showing relationship between *Wolbachia* in *B. dorsalis* (in bold) to strains in known super groups. Trees were generated from sequences of *16S rRNA* (A), *fbpA* (B), *ftsZ* (C), *gatB* (D) and *hcpA* (E) genes. Sequences of *Wolbachia* endosymbiont of *Brugia malayi* are included as out-groups in all the trees and respective super groups are indicated to the right of each tree. Bootstrap values are indicated above the branches.

Table S1. *Wolbachia* MLST and *wsp* gene allelic profiles. Exact matches to alleles present in the database are shown whereas instances where no exact matches were found are represented as N. Dashes represent the genes for each strain that could not be amplified. GenBank accession numbers for respective sequences are shown in brackets.

Strain	MLST typing allele numbers					*WSP* typing allele numbers				
	gatB	***coxA***	***hcpA***	***ftsZ***	**fpbA**	***wsp***	**HVR1**	**HVR2**	**HVR3**	**HVR4**
WdorNg13	-	N (MK314069)	-	-	N (MK875805)	N (MK314063)	264	N	3	239
WdorMu2	-	N (MK314068)	-	-	N (MK875804)	N (MK314062)	264	N	3	239
WdorKi1	N (MK875808)	224 (MK314067)	-	-	-	N (MK314064)	N	N	N	N
WdorTzc13	N (MK875807)	73 (MK314070)	N (MK875809)	N (MK875815)	N (MK875806)	N (MK314064)	148	141	239	102
WdorSu6	-	-	-	-	-	N (MK875814)	N	N	N	N
WdorBe3	-	-	-	-	-	N (MK875811)	10	8	N	N
WdorGh4	-	-	-	-	-	N (MK875812)	N	N	N	N
WdorTg6	-	N (MK314071)	-	-	-	N (MK314065)	N	N	N	N
WdorSl6	-	-	-	-	-	N (MK875813)	N	12	21	90
WdorSl11	-	15 (MK314072)	N (MK875810)	-	-	N (MK314066)	N	8	N	N

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CHAPTER FOUR

4. Diversity of gut bacterial communities in *Bactrocera dorsalis*-possible shifts with diet and environment

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4.2 Abstract

Bacterial communities associate with different insect hosts establishing complex communities within the host's gut environment. In the oriental fruit fly, *Bactrocera dorsalis* (Hendel) (Diptera: Tephritidae), bacterial communities have been shown to have several physiological and behavioral consequences. We examined the structure of bacterial communities in adults and larvae of *B. dorsalis* derived from several wild populations in Kenya, as well as a laboratory-reared population using high throughput sequencing of the bacterial 16S rRNA gene. Proteobacteria and Firmicutes were the most dominant phyla among adult specimens, while in larval specimens, Actinobacteria in addition to the two aforementioned bacterial phyla were the most dominant. Only one genus, *Bacillus,* among the detected genera was common to both life stages. A unique bacterial composition was observed among larval specimens from the laboratory-reared colony, indicating a composition specifically adapted to the artificial rearing diets. Some field derived specimens were observed to have relatively unique compositions for each site despite being derived from a common mango variety, suggesting an environmental influence in proliferation of gut bacteria in this fly. These findings are relevant to the ecology of this pest and provide opportunity for exploitation of bacterial symbionts and communities in integrative strategies with currently available pest management options.

Keywords: gut bacteria, *Bactrocera dorsalis*, 16S r RNA gene, bacterial symbionts

4.3 Introduction

The Oriental fruit fly, *Bactrocera dorsalis* (Hendel) (Diptera: Tephritidae) is among the most important quarantine pests globally (Hsu, Feng, Wu, Hsu, and Feng, 2004). Since the first invasion report in Africa (Lux, Copeland, White, Manrakhan, and Billah, 2003), the pest has established in many parts of the continent, primarily infesting mango (*Mangifera indica)* among other crops (Drew, Tsuruta, and White, 2005; Ekesi, Nderitu, and Rwomushana, 2006; Mwatawala, De Meyer, Makundi, and Maerere, 2006; Correia, Rego, and Olmi, 2008; Rwomushana, Ekesi, Gordon, and Ogol, 2008). African countries where this pest occurs, have recurrently faced export quarantine restrictions from lucrative markets on their produce, resulting in substantial economic losses (Badii, Billah, Afreh-Numah, Obeng-Ofori and Nyarko, 2015; Ekesi, de Meyer, Mohamed, Virgilio, and Borgemeister, 2016). Integrated pest management programmes have been shown to have greater efficacy in management of this pest (Verghese, Sreedevi, and Nagaraju, 2006; Kibira et al., 2015; Korir et al., 2015; Muriithi et al., 2016; Nyang'au et al., 2017), although management is generally constrained by the relatively quick succession of stadia in the fly's life cycle.

As with other insects and animals, the biology of this pest is greatly influenced by bacterial symbionts residing in the tissues and gut of the host (Gould et al., 2018). Notably, a few studies have identified the endosymbiotic bacteria, *Wolbachia* in populations of *B. dorsalis* (Kittayapong, Milne, Tigvattananont, and Baimai, 2000; Jamnongluk, Kittayapong, Baimai, and O'Neill, 2002; Sun, Cui, and Li, 2007; Gichuhi, Khamis, Van den Berg, Ekesi and Herren, 2019). *Wolbachia* is a known "master manipulator" of insect host reproductive traits. However, more research to elucidate specific consequences of the

presence of *Wolbachia* in *B. dorsalis* still needs to be done. Similarly, gut bacteria have been shown to directly affect the nutrient ingestion and foraging behavior of *B. dorsalis* (Akami et al., 2019). Associations between different bacterial symbionts and life stages have been made in this pest (Zhao et al., 2018), suggesting the likelihood that symbionts play a role in the varying nutritional requirements of the host at each life stage. Some bacterial symbionts have been found to influence the developmental time, morphological parameters and survival of the oriental fruit fly (Khaeso et al., 2017). This pest also benefits from the ability of some of its gut symbionts to break down toxicants, which has been linked to the genesis of insecticide resistance in the host species (Cheng et al., 2017; Pietri and Liang, 2018). Commensal bacteria have also been found to influence mate-selection behavior in *B. dorsalis* (Damodaram, Ayyasamy, and Kempraj, 2016).

Characterization of the gut bacterial communities of *B. dorsalis* has been done using both culture-based and Next Generation Sequencing techniques (Wang, Jin, and Zhang, 2011; Shi, Wang, and Zhang, 2012; Wang et al., 2013; Pramanik, Al-Mahin, Khan, and Miah, 2014; Yao et al., 2015; Andongma *et al.,*2015; Damodaram et al., 2016; Liu et al., 2016; Yong, Song, Chua, and Lim, 2016; Cheng et al., 2017; Gujjar, Govindan, Verghese, Subramanian, and More, 2017; Khaeso *et al*., 2017; Bai et al., 2018; Liu et al., 2018; Zhao et al., 2018; Akami et al., 2019). In most of these studies, bacteria belonging to four phyla: Firmicutes, Proteobacteria, Bacteroidetes and Actinobacteria have by far been the most detected in the gut of this fly, and therefore it is possible that these bacteria form strong symbiotic relationships with this host.

Symbiont transmission in various hosts is a major determinant of perpetuation of symbiotic relationships (Bright and Bulgheresi, 2010). Although different bacterial

symbionts have been detected in *B. dorsalis*, elaborate transmission mechanisms have not been elucidated for all of them, which may be partly due to the dynamic environments encountered by each of the fly's life stages as well as the foraging behavior of the pest. Mechanisms for vertical and horizontal transmission of *Citrobacter freundii* in this pest have been demonstrated (Guo et al., 2017). It has been suggested that generally, female fruit flies introduce some of their gut bacteria in fruit during oviposition which helps in softening and rotting of the food and in turn these bacteria are taken up by developing larvae (Badii et al., 2015).

The composition of bacterial communities in insects is determined by various factors including environmental habitat, diet, developmental stage and host phylogeny (Yun et al., 2014), This study attempts to identify gut bacterial symbionts associated with *B. dorsalis* and evaluate the influence of geographical environments as well as dietary sources in the composition of the gut symbiont communities.

4.4 Materials and methods

4.4.1 Insect collection

Infested Kent variety mangoes were collected from 3 randomly selected mango farms per region in different agro-ecological zones in Kenya between 2016 and 2017 (Figure 1).

Mangoes were properly washed in water, dissected and placed on sterile sand in ventilated cages for third instar larvae to emerge and burrow to pupate in sand. Pupae were retrieved from sand though sieving in distilled water then maintained in sterile Petri dishes in ventilated Perspex cages at 27 °C and 60% humidity until emergence. Several third instar larvae were directly retrieved from the infested mangoes for DNA extraction. A few adult and larval samples for the study were obtained from the International Centre

of Insect Physiology and Ecology (icipe) fruit fly laboratory colonies. The sampled colony was derived from infested mango collected from different farms across Kenya and maintained for more than 40 generations in the laboratory at 27 °C and 60% relative humidity. Adult flies were fed on a diet consisting of 3 parts sugar and 1-part enzymatic yeast hydrolysate ultrapure (USB Corporation, Cleveland, Ohio, USA), and water on pumice granules. For each generation, fresh mango domes were used as oviposition receptacles, from which embryos were washed in distilled water before inoculation on larval rearing diets (Chang, 2009). Same age and generation of laboratory reared flies were used for this study.

4.4.2 DNA extraction

A total of five adult specimens and 4 larval specimens from each of the four of the sampled regions were selected randomly for DNA extraction. One-day old adult flies were surface sterilized in 70% ethanol, in 5% v/v sodium hypochlorite solution followed by 3 washes in sterile phosphate buffered saline (PBS) for 3 minutes in each solution. Fly guts were dissected in PBS solution under a Leica EZ4D stereomicroscope (Leica Microsystems Limited, Switzerland). Similarly, third instar larvae retrieved directly from the infested mangoes were surface sterilized and their guts dissected out in PBS solution.

DNA was extracted using the ISOLATE II Genomic DNA Kit (Bioline, London, UK) according to the manufacturer's instructions. DNA extracted from gut samples was submitted for high throughput sequencing, targeting the v4 region of the bacterial 16s rRNA gene using the Illumina Miseq platform. Larval DNA was sequenced at the Centre for Integrated Genomics, University of Lausanne, Switzerland and adult DNA at Macrogen Europe Laboratory, the Netherlands. Adult and larval sequence sets were therefore

analyzed separately.

4.4.3 *16S rRNA* gene sequence analysis

Sequence reads were checked for quality using FastQC v 0.11.28 (Andrews, 2010) and pre-processed to remove adapters and sequencing primers using Cutadapt v1.18 (Marcel, 2011). Forward and reverse reads were imported into the QIIME2-2018.11 (Boylen et al., 2018). The deblur plugin (Amir et al., 2017) was used to further filter the reads based on per base quality scores, merge the paired-end-reads and cluster reads into operational taxonomic units (OTUs).

A total of 638,815 sequence reads from adult specimens and 56,425 from larval specimens were retained after removal of spurious reads and all reads shorter than 240 and 272 nucleotides in length respectively were subjected to further analysis. These sequences clustered into 235 OTUs (adult) and 402 OTUs (larval). Of these, 50 OTUs (adult) and 94 OTUs (larval) survived low count and interquartile range-based variance filtering to eliminate OTUs that could arise from sequencing errors and contamination. Taxonomic assignment was done using the blast classifier against the Silva132 reference database (Quast et al. 2013) at a 99% identity cut-off. OTU prevalence and variance-based filtering as well as alpha and beta diversity measures were applied to the data in the Microbial Analyst Marker Data Profiling (Dhariwal et al. 2017). Shannon diversity indices were applied along with Mann-Whitney and analysis of variance statistics in profiling alpha diversity between sets of samples. Beta diversity was evaluated using Bray-Curtis and unweighted Unifrac distances. Significance testing was done using analysis of group similarities (ANOSIM) and non-metric multidimensional scaling (NMDS) used for ordination. The differential gene expression analysis based on the negative

binomial distribution (DESeq 2) (Love, Huber, & Anders, 2014) was used to evaluate differential abundance of bacterial genera reads between sample groups.

4.5 Results

4.5.1 Species richness

Adult and larval specimens derived from the laboratory-reared colony were observed to have a higher species richness and abundance relative to the majority of the specimens retrieved from different mango growing regions in Kenya (Figure 2a and b). Adult specimens from Makueni had the most similar profile of species richness and abundance among adult samples from the same region, whereas in larvae, the most similar profiles were recorded among specimens from the laboratory-reared colony. Larval specimens from field sites notably had much lower species richness than the laboratory reared larvae.

4.5.2 Bacterial taxa

Bacteria belonging to 5 phyla were detected in adult *B. dorsalis* samples, whereas in larvae, the detected bacteria clustered in 3 phyla (Figures 3a and 3b). Adults from Embu and Nguruman were dominated by Firmicutes whereas those from Makueni and the icipe colony were dominated by Proteobacteria. Variations between sites were observed among adult samples from Kitui and Muranga and generally bacteria in the phyla: Bacteroidetes, Chlamydiae and Actinobacteria were recorded less frequently among all the samples. In contrast, Actinobacteria were more conspicuous in larval samples from Makueni and occurred variably in samples from other sites. Larval samples from Muranga were dominated by Proteobacteria, whereas varying proportions of this phylum were in the rest of the larval samples. Notably, larvae from the icipe colony had almost equal

proportions of bacteria out of the three phyla: Proteobacteria, Firmicutes and Actinobacteria, which were detected in larvae.

At the genus level, the collective composition of bacteria detected in adult specimens was quite different from that of larval samples with the exception of *Bacillus* spp. which were recorded in both life stages (Figures 4a and 4b). In almost all adult samples from sites other than Embu, a distinct presence of *Enterobacter* spp. was recorded often occurring together with *Klebsiella* spp. in samples from Makueni and the Laboratory reared colony. In the Embu and Nguruman adult set of samples, a high dominance of *Lactococcus* spp. was recorded whereas the Kitui and Muranga set of adult samples had a pronounced presence of a number of other bacterial genera besides the aforementioned dominant ones. Among larvae, *Providencia* spp. were recorded to dominate in samples from Muranga and one from Embu whereas *Lactobacillus* spp. were present in high proportions in the rest of the samples from Embu. An almost consistent composition of bacterial genera was recorded among the larvae from the laboratory reared colony.

4.5.3 Alpha diversity

No significant differences were recorded in the bacterial composition in adult *B. dorsalis* collected from different sites. However, some statistically significant differences were recorded in the bacterial composition between larval samples collected from different sites (figure 5).

4.5.5 Beta diversity

To assess the amount of variation in bacterial composition between samples from different sites, NMDS ordination of Bray-Curtis dissimilarity was done. Significant

dissimilarity was observed among both adult and larval samples derived from different locations (figure 6).

4.5.6 Differential abundance of bacterial OTUs

Using DESeq2 at an adjusted p-value cut off of 0.05, three genera of bacteria: *Enterobacter* (p-value=3.44E-37, FDR=5.50E-36) *Klebsiella* (p-value=3.11E-17, FDR=2.48E-16) and *Serratia* (p-value=6.48E-13, FDR=3.45E-12) were found to occur in significantly different abundances among adult samples from the different sites, whereas in the larvae samples, *Lactobacillus* was significantly more abundant (p-value=0.002, FDR=0.031) in Embu samples than in other sampled sites (figure 7).

4.6 Discussion

We evaluated the diversity of bacterial communities residing in the guts of adult and larval stages of *B. dorsalis* and observed a generally higher bacterial abundance in adult specimens. Having examined newly eclosed adult flies that could not acquire more bacteria through foraging, the observed site-specific compositions are likely to represent carry-over bacteria across developmental stages. The higher abundance in adult specimens suggests that adult flies are able to accommodate much higher densities of bacteria in their gut than larvae can, which could be as a result of extensive structural modifications that occur in the alimentary canal as larvae transition through pupal stage to adult stage and therefore the adult fly may have a much higher gut surface area to support higher gut bacterial densities than larvae. In addition, the passage rate of food content in larvae is considerably much faster than in adults, which may also contribute to lower bacterial densities in larvae than in adult flies. Nutritional requirements and metabolism of the fly is expected to change across the fly's life cycle and this may also

contribute to differences in both species richness and type of bacteria present in the different life stages. In addition, bacteria belonging to the Enterobacteriaceae family have been suggested to interfere with proliferation of other bacterial types in the guts of the Mediterranean fruit fly, *Ceratitis capitata* (Behar, Jurkevitch and Yuval, 2008). In adult samples from Makueni and icipe as well as larval samples 1, 2 and 3 from Muranga and larval sample 2 from Embu, a dominance of Enterobacteriaceae was recorded, suggesting that in *B. dorsalis* as well, dominance of Enterobacteriaceae interferes with proliferation of other types of bacteria, therefore contributing to the observed bacterial community structure.

A predominance of Proteobacteria and Firmicutes was observed among adult samples whereas these two bacterial phyla in addition to Actinobacteria were observed among the sequenced larval samples. The general reduction in Actinobacteria proportions in adult samples shows a possibility that bacterial symbionts in this phylum may play a greater role in the biology of the fly at lower life stages than at adult stage. This observation has been made previously in laboratory and field populations of *B. dorsalis* (Zhao et al., 2018). However, possibly due to diet, host plant and environmental parameters, the diversity of bacterial phyla and components observed in this study is relatively low when compared to other similar studies elsewhere. For instance, >10 bacterial phyla were detected across life stages of *B. dorsalis* derived from populations in China (Zhao et al., 2018). However, no previous recording of Chlamydiae has been reported in *B. dorsalis*. Altogether, the common phyla to this study with previous studies suggest a characteristic microbial fauna in *B. dorsalis*.

Three bacterial genera: *Enterobacter*, *Klebsiella* and *Lactococcus* were most dominant

among adult samples, whereas *Providencia*, *Cutibacteria*, *Lactobacillus* and *Pantoea* were highly abundant in larval samples. Most of these bacterial genera have previously been reported among gut bacterial communities of *B. dorsalis* (Wang et al., 2011; Shi et al., 2012; Gujjar et al., 2017; Khaeso et al., 2017; Liu et al., 2016; Bai et al., 2018; Liu et al., 2018; Akami et al., 2019). It is noteworthy that sampling of adult and larval samples in this study was carried out a year apart, and therefore the recording of no common bacterial genera between the adult and larval specimens, other than *Bacillus*, does not necessarily infer that trans-stadial transmission of other *B. dorsalis* gut bacteria does not occur in the sampled sites.

A significant difference in bacterial composition was recorded between the laboratory-reared larval samples and those from field sites, outlining a possible strong effect of diet in gut bacterial composition in *B. dorsalis*. Larvae from all field collections fed on similar nutrient compositions in the Kent variety mango, whereas the laboratory colony fed on the regular rearing diet for *B. dorsalis* (Chang, 2009). The inclusion of streptomycin in the rearing diets of fruit flies also impacts the bacterial composition of such flies. Nevertheless, apart from the direct effect of diet on gut bacterial composition, abiotic factors may also contribute to these differences. This is likely because wild derived insects are subject to highly variable environmental conditions, whereas laboratory derived samples are reared under controlled conditions. Indeed, the role of the environment in determining the composition of gut bacterial communities is well separated from the influence of diet as observed for the genera *Enterobacter, Klebsiella*, *Serratia* and *Lactobacillus* that were differentially abundant of in field sites rather than in the laboratory-reared specimens.

A potentially entomopathogenic bacteria: *Serratia* was identified in a few samples from two sites and appeared to promote proliferation of other uncommon bacteria in one of the samples in which it was highly abundant (Kitui 3). It is possible that proliferation of these uncommon bacteria was an opportunistic consequence of the pathogenesis of *Serratia*. Such a microbe could be useful in development of bacterial -based entomopathogenic strategies for management of the oriental fruit fly.

This study outlines the expected diversity of bacterial communities in the guts of *B. dorsalis* flies from different sites in Kenya and the possible influences that diet and environment have in the structure of these bacterial communities. Understanding bacterial symbiont structures and dynamics may lead to exploitation of the pest's symbiont elements in pest management strategies such as incorporation of useful symbionts in sterile insect technique programmes and application of entomopathogenic microbes in pest population suppression.

4.7 Conflicts of Interest

The authors declare no conflict of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

4.8 Author Contribution

F.M.K, S.E. and J.K.H. conceptualized the study; JG carried out the investigation, data analysis and original draft preparation. F.M.K., S.E., J.K.H and J.V.B. supervised the design and implementation of the study. All authors reviewed and edited the final draft. S.E acquired funds for the study.

4.9 Data availability

All sequence reads from this study are publicly available at the Sequence Read Archive (SRA) under the BioProject: PRJNA545161.

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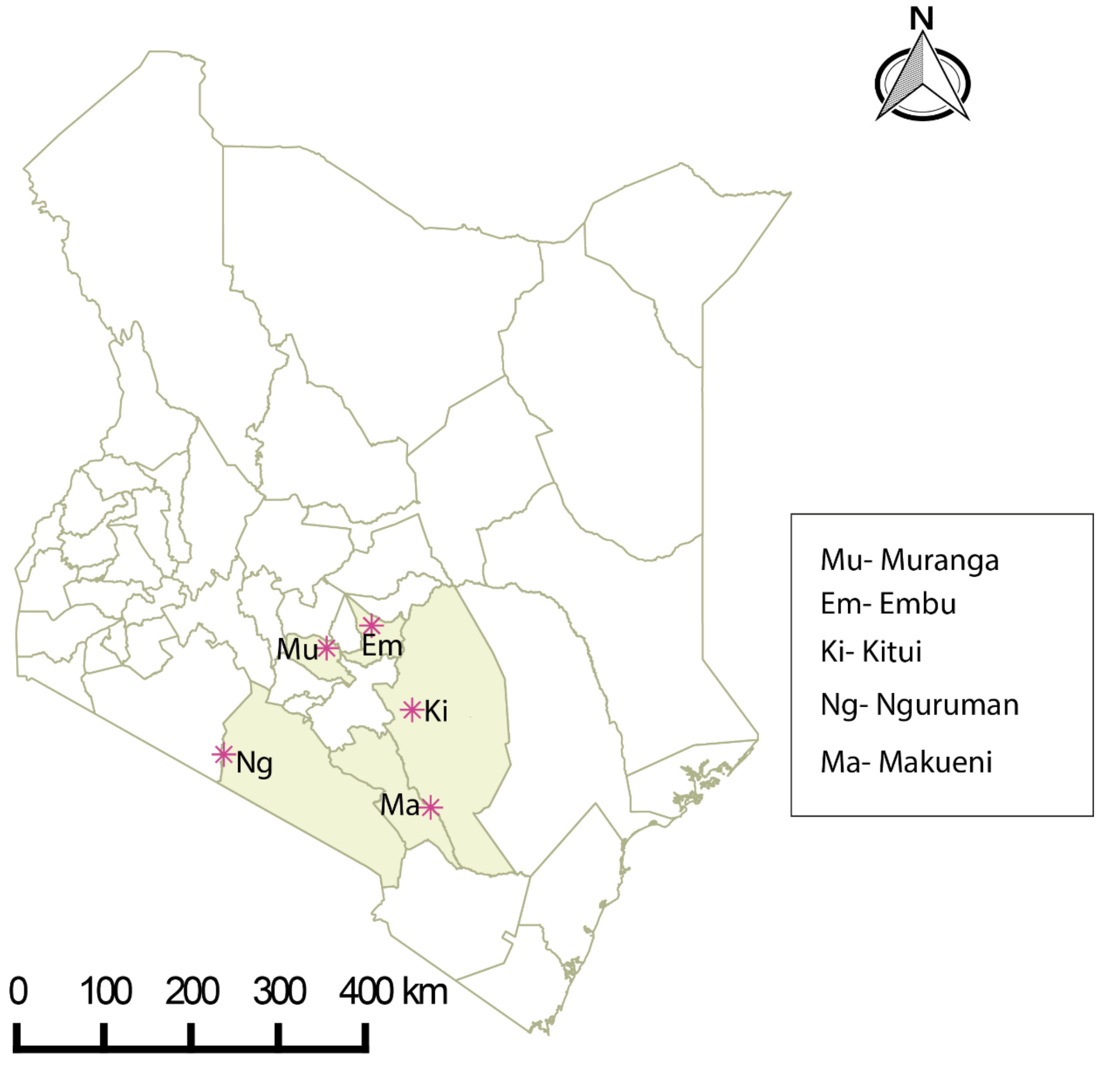

Figure 1 Map of Kenya showing field sites where *B. dorsalis* specimens were collected.

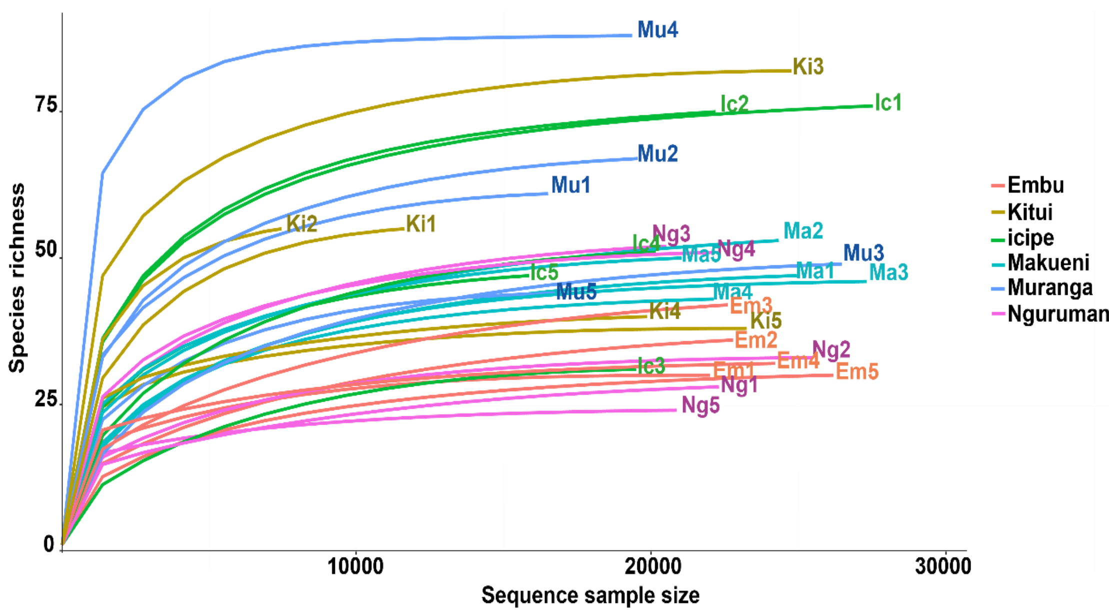

Figure 2a Rarefaction curves showing the relative species richness and abundance in adult specimens of *B. dorsalis* from different mango growing regions and from a laboratory colony. Each specimen is labelled with a site abbreviation followed by per site specimen identifier.

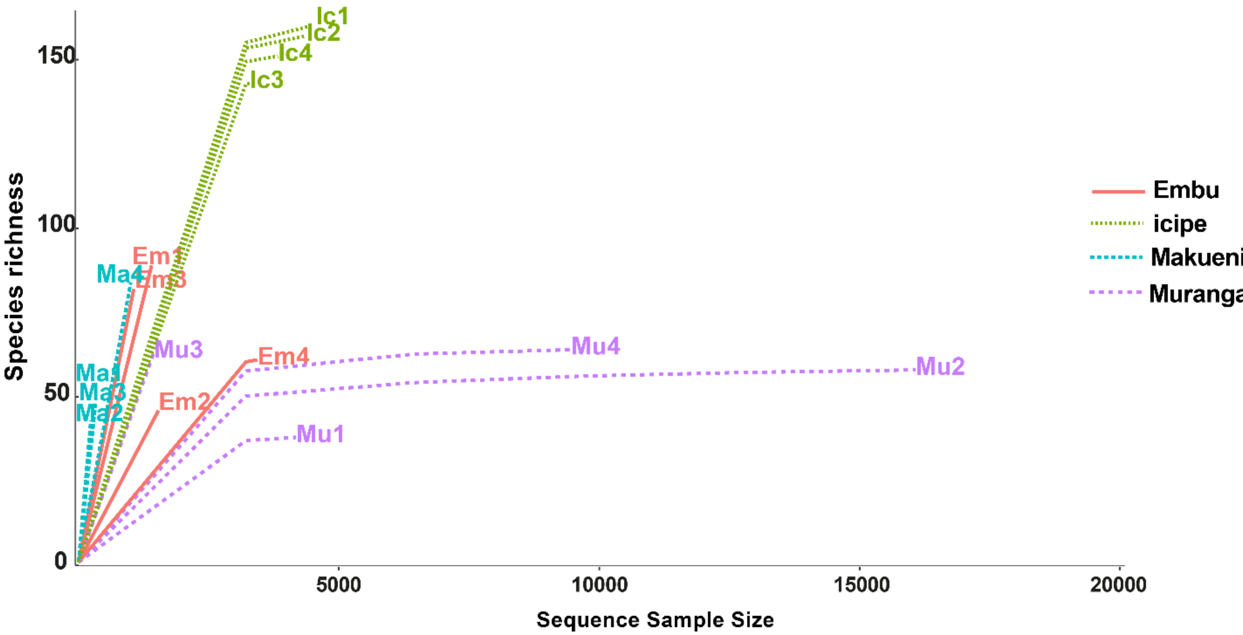

Figure 2b Rarefaction curves showing the relative species richness and abundance in larval specimens of *B. dorsalis* from different mango growing regions and from a laboratory colony. Each specimen is labelled with a site abbreviation followed by per site specimen identifier.

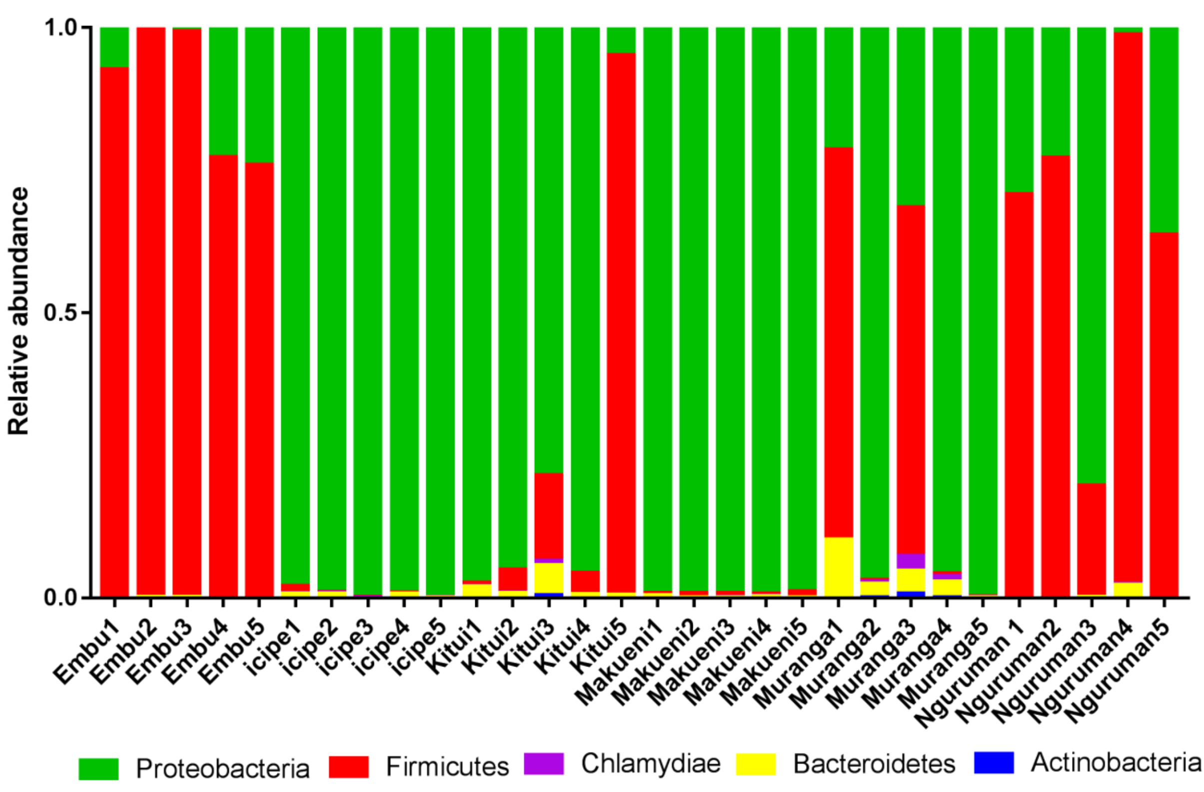

Figure 3a Relative abundance of bacterial phyla detected in *B. dorsalis* adult specimens retrieved from infested mango fruit from different mango farming regions in Kenya and from a laboratory reared colony.

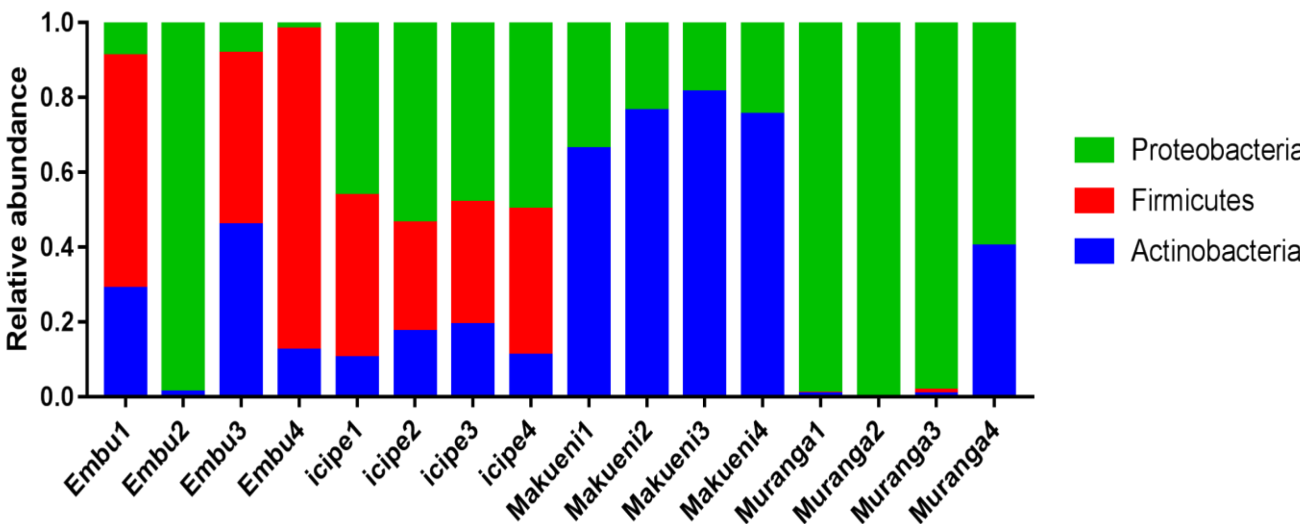

Figure 3b Relative abundance of bacterial phyla detected in *B. dorsalis* larvae specimens retrieved from infested mango fruit from different mango farming regions in Kenya and from a laboratory reared colony.

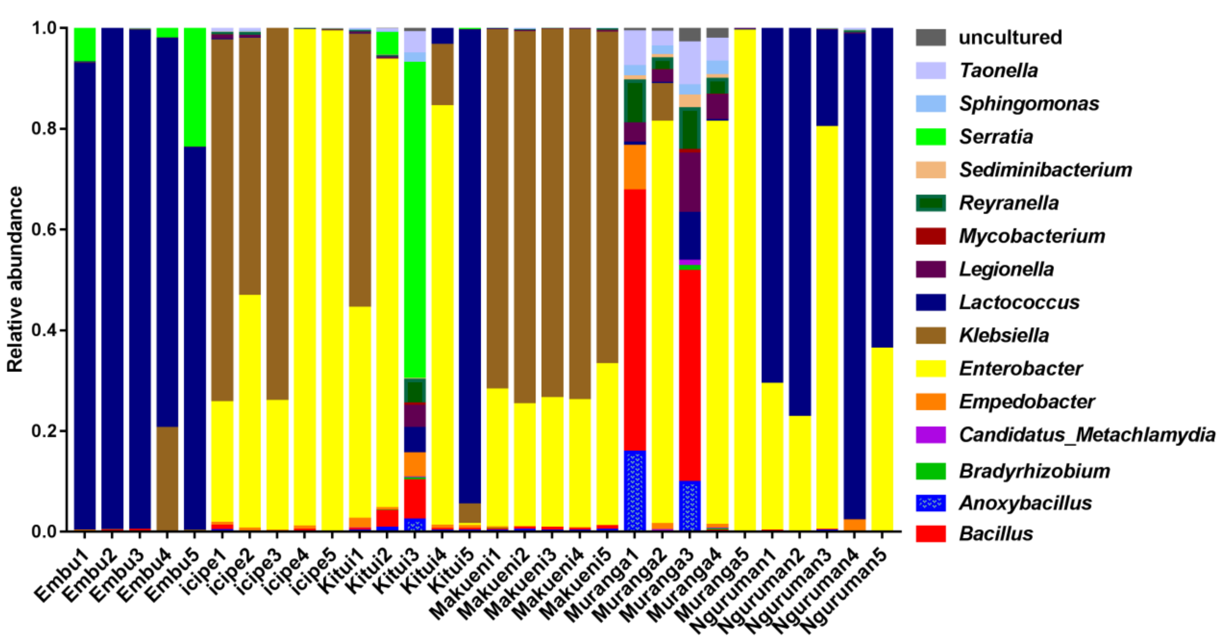

Figure 4a Relative abundance of bacterial genera in adult samples of *B. dorsalis* collected from different sites.

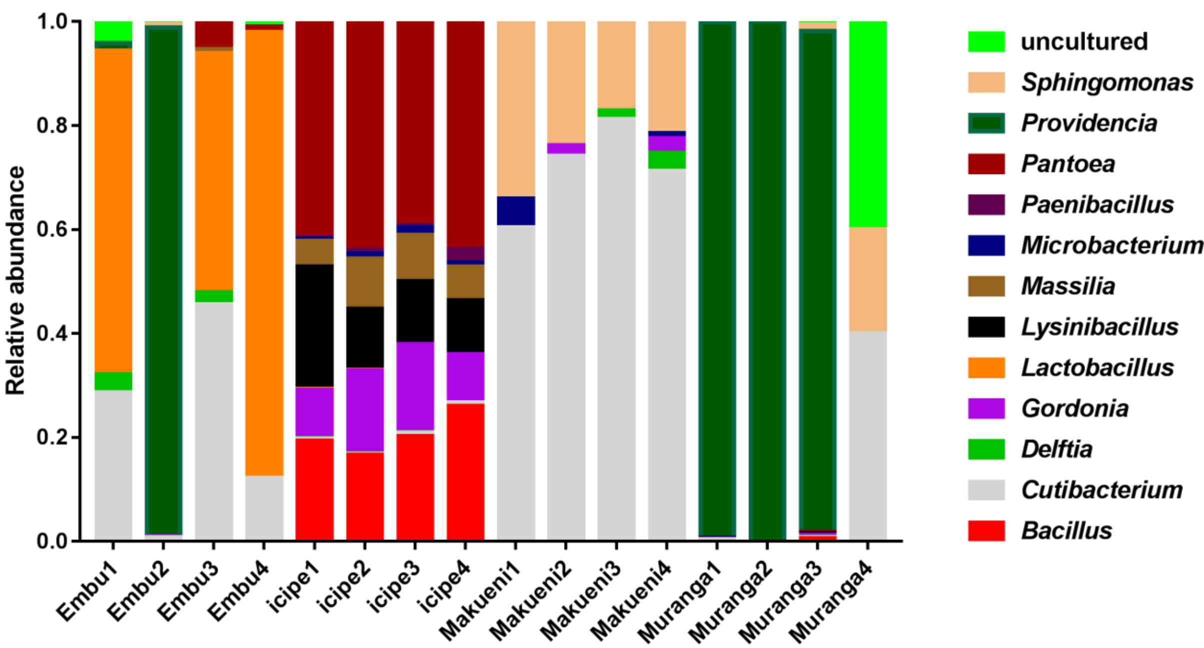

Figure 4b Relative abundance of bacterial genera in larvae samples of *B. dorsalis* collected from different sites.

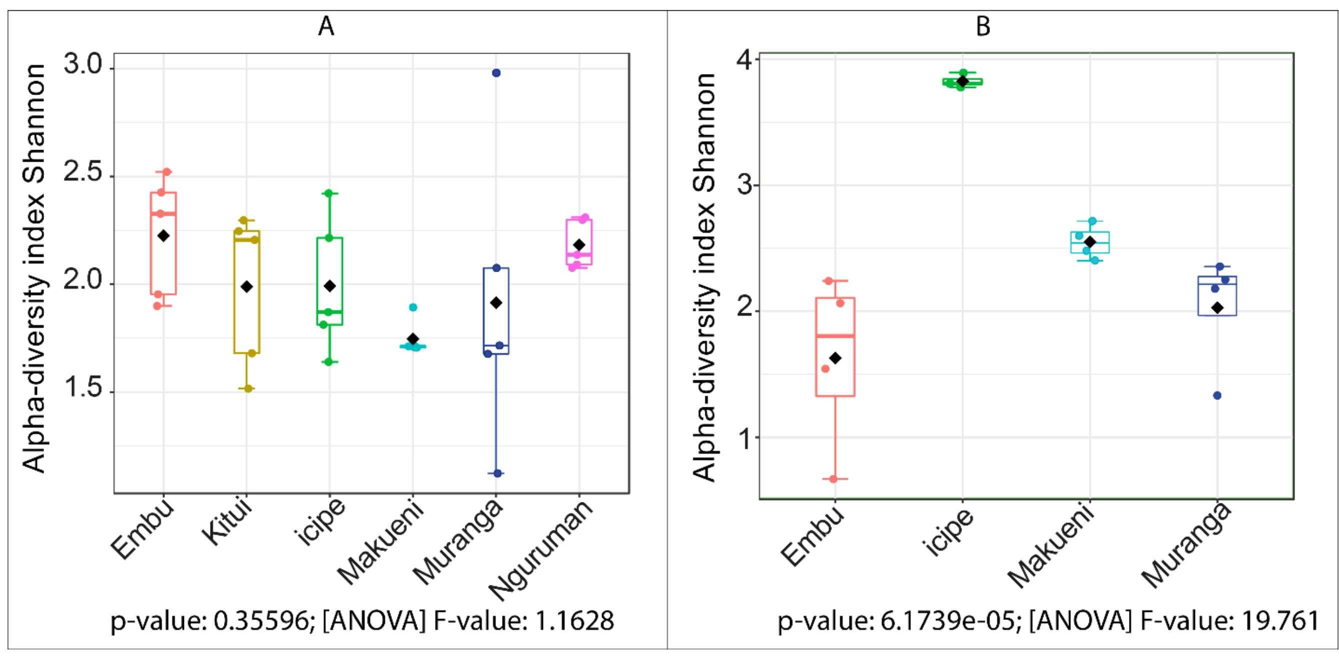

Figure 5 Alpha diversity between A) adult and B) larvae of *B. dorsalis* specimens collected from different sites.

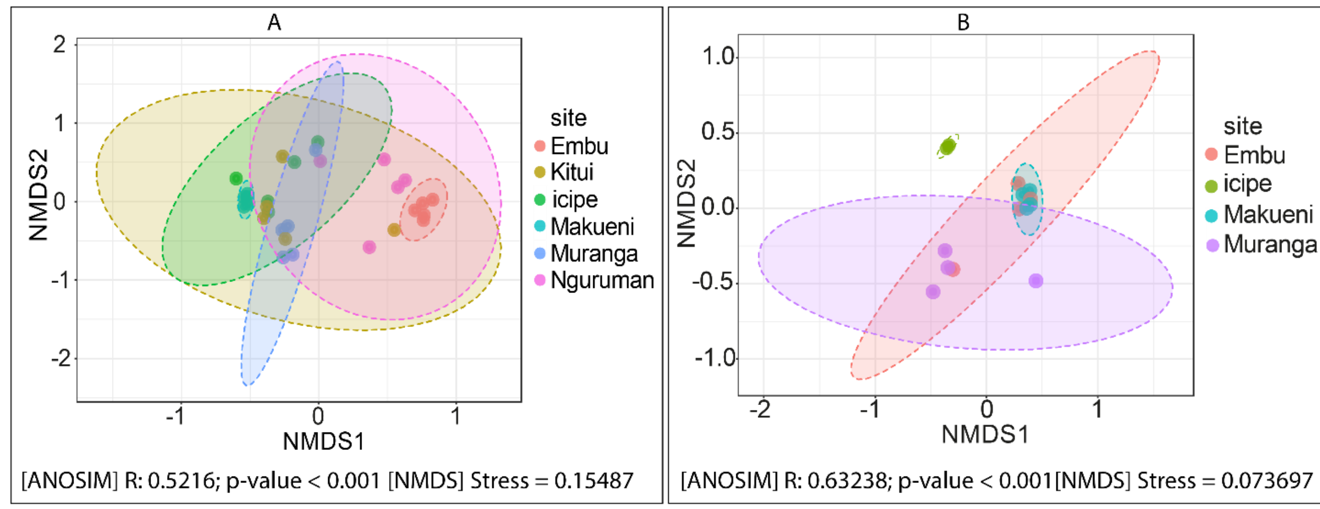

Figure 6 NMDS ordination of Bray-Curtis distances between A. adult specimens from different sites and B. larvae specimens from different sites.

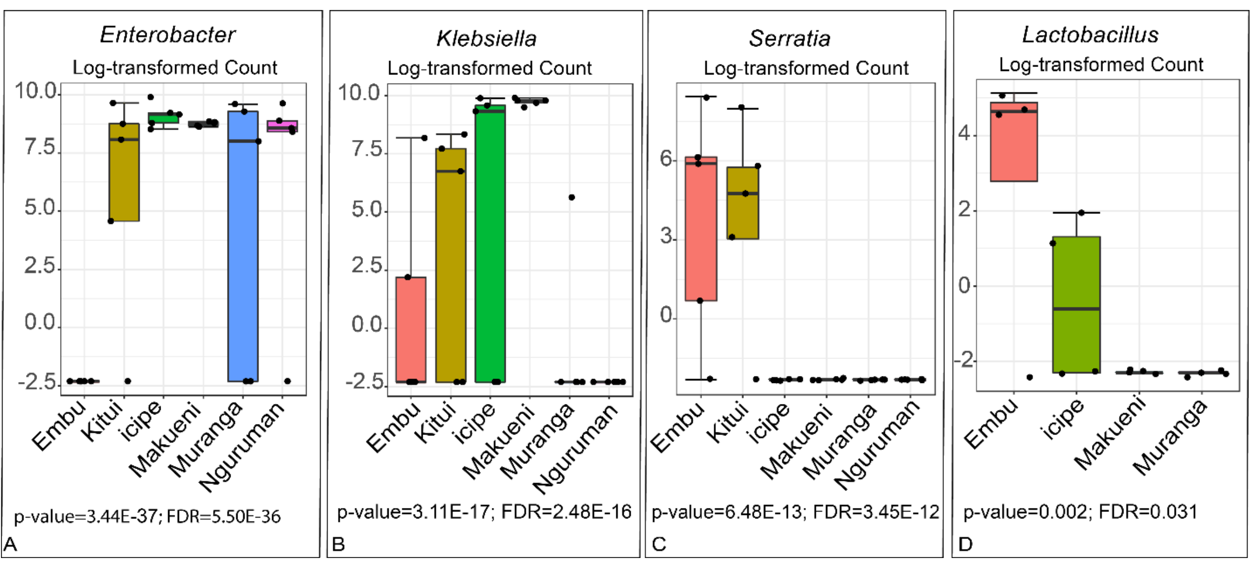

Figure 7 Differential abundance of bacterial genera between adult samples (A-C) from different sites and larval samples (D) from different sites.

CHAPTER 5

5. Gut bacteria modulate early development of the oriental fruit fly and its response to the entomopathogenic fungus, *Metarhizium anisopliae*

Prepared for submission to **INSECTS**

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5.1 Abstract

Interactions between insects and bacteria are associated with evolutionary, physiological and ecological consequences for the host. A wide range of bacterial communities associate with the oriental fruit fly, *Bactrocera dorsalis* (Diptera: Tephritidae) (Hendel), an important pest of cultivated fruit in most regions across the globe. We evaluated the roles of individual bacterial isolates in the development of axenic *B. dorsalis* fly lines and their responses to the entomopathogenic fungus, *Metarhizium anisopliae*. Embryonic development was recorded to be fastest in flies with an intact gut microbiome and slowest in germ-free fly lines. Similarly, a significantly lower larval development period was also recorded in flies with an intact gut microbiome. A strain of *Lactococcus lactis* was observed to reduce the survival of adult *B. dorsalis* challenged with a standard dosage of *M. anisopliae* conidia whereas *Providencia alcalifaciens* was observed to improve survival of the flies under the same conditions. These findings present a plausible rationale for selection and use of probiotics in insect diets used in sterile insect technique (SIT) programmes, as well as integrated pest management programs which combine SIT with entomopathogenic fungi.

Keywords: *Bactrocera dorsalis*, *Metarhizium anisopliae*, gut bacteria, SIT

5.2 Introduction

Bactrocera dorsalis is a widespread pest of cultivated fruit and has established itself in various locations across Asia, Africa, and been intercepted in North America and recently in Europe [1–8]. Infestation with this pest has high implications for production, trade and socio-economic aspects of affected countries and the pest is considered as a high risk quarantine pest [9–12].

Effective management of *B. dorsalis* typically involves combination of several methods in integrated pest management (IPM) programs [13]. Methods that avoid over reliance on chemical insecticides are prompted by safety to the environment and to non-target species, which may include useful natural enemies of the pest such as parasitic species [14]. Some common methods include maintaining orchard sanitation, release of parasitoid wasps, application of entomopathogenic biopesticides, mass trapping, male annihilation, protein baiting and mass release of irradiated males in sterile insect technique (SIT) programs [13,15–19]. The possibility of development of an endosymbiont-based technique for suppressing fruit fly populations has been demonstrated in other tephritid fruit fly species [20,21].

The association between insects and bacteria in symbiotic relationships has great implications on the physiology, ecology and evolution of the host [22]. For this reason, a lot of attention is being given to understanding symbiont-host interactions in diverse groups of insects. For a serious pest like *B. dorsalis*, identification of bacterial communities that associate with this host would provide useful cues for how this microbiome could be exploited for control of this pest.

Numerous studies have reported the diversity and described the microbiome structure of

this pest's gut [23–32]. Some specific roles of bacterial isolates from *B. dorsalis* have been reported. These include the host's foraging behavior and nutrient ingestion [27], selection of mates [33], development, survival and morphology [25] and responses to insecticide [34].

This study investigates the effects of individual bacterial isolates on the early stages of development of *B. dorsalis* as well as the implications of rearing flies with single bacterial isolates on the survival of adult flies exposed to entomopathogenic fungus.

5.3 Material and methods

5.3.1 Bacterial isolation

Infested mango fruit collected from farms in Embu (S 0° 28' 56.6" E 37° 34' 55.5"), Muranga (S 0° 42' 50.0" E 37° 07' 03.4"), Nguruman (S 01 48' 32" E 036 03' 35") and Kitui (S 01 °21' E 38° 00'), in Kenya, were washed in distilled water, dissected and placed on sterile sand in ventilated cages for third-instar larvae to burrow and pupate in sand. Puparia were retrieved from sand through sieving and maintained in sterile Petri dishes in ventilated Perspex cages until eclosion. A proportion of third stage larvae were directly retrieved from the fruit for gut dissection. A pool of guts from two larvae and two newly emerged adult flies per sampled region were dissected in sterile phosphate buffered saline (PBS) after surface sterilization of the specimens. The selected larvae and adult flies were surface sterilized in 70% ethanol, 5% v/v sodium hypochlorite solution followed by three washes in distilled water for 3 min in each solution. Dissected guts were homogenized using pestles in 1 ml microfuge tubes containing 300 µl PBS. Five µl of the fourth serial dilution of each homogenate was inoculated under aerobic conditions on brain heart infusion (BHI) solid media using a spread plate technique[35] and incubated

at 37 °C for 14 hours. Representative colony forming units (CFUs) were selected based on morphology and were clonally propagated up to four times to ensure purity on BHI agar plates. Pure isolates of the propagated bacteria were cryopreserved in 60% glycerol stocks at -80 °C.

5.3.2 Bacterial isolate identification

Pure cultures were sub-cultured in BHI broth and incubated at 37 °C for 16 hours on a shaking platform at 300 revolutions per minute (rpm). Bacterial cells were harvested from media then washed three times in PBS by centrifugation at 10000 rpm for 10 min, each time discarding the supernatant.

DNA was extracted from these bacterial cells using the ISOLATE II Genomic DNA Kit (Bioline, LondoUK). The V1-V3 region of the bacterial 16S rRNA gene was amplified through PCR using the 28F (5'-GAGTTTGATCNTGGCTCAG-3') and 519R (5'-GTNTTACNGCGGCKGCTG-3') primers. Reactions were set up in total volumes of 10 µl each, containing 5 × MyTaq reaction buffer (5 mM dNTPs, 15 mM MgCl2, stabilizers and enhancers) (Bioline, London, UK), 2 µM of each primer, 0.25 mM MgCl2 (Thermo Fischer Scientific, Massachusetts, USA), 0.125 µl MyTaq DNA polymerase (Bioline, London, UK), and 7.5 ng/µl of DNA template. These reactions were set up in a Master cycler Nexus gradient thermo-cycler (Thermo Fischer Scientific, Massachusetts, USA). Cycling conditions included an initial denaturation for 2 min at 95 °C, followed by 35 cycles of 30 s at 95 °C, 40 s at 54 °C and 1 min at 72 °C, then a final elongation step of 10 min at 72 °C. PCR products were run through 1% agarose gel electrophoresis and visualized by ethidium bromide staining and UV trans-illumination. Direct sequencing in both forward and reverse directions was done for all amplified samples. Homology searches using

BLAST against the 16S ribosomal RNA sequence database at the National Center for Biotechnology Information (NCBI) were done to infer identity and similarity of isolates to subject sequences in the database. All sequences were deposited in GenBank.

5.3.3 Generation of axenic lines

Bactrocera dorsalis embryos were collected from gravid females from the icipe *B. dorsalis* laboratory reared colony using perforated mango domes. The colony was established by rearing *B. dorsalis* from infested mango collected from different farms across Kenya and maintained for more than 40 generations in the laboratory at 27 °C and 60% relative humidity. Adult flies were fed on a diet consisting of 3 parts sugar and 1 part enzymatic yeast hydrolysate ultrapure (USB Corporation, Cleveland, Ohio, USA), and water on pumice granules. For each generation, fresh mango domes were used as oviposition receptacles, from which embryos were washed in distilled water before inoculation on larval rearing diet (Chang, 2009).

Embryos were surface sterilized in 70% ethanol for 5 min, then dechorionated in 7% v/v sodium hypochlorite solution for 3 min in a fine mesh (Nitex Nylon100 um) basket. Dechorionated embryos were then rinsed three times in distilled water for 5 min each then flooded with absolute ethanol. Subsequent procedures were carried out in a sterile laminar flow hood. Using a fine camel hair brush, embryos from the bottom of the basket were transferred and spread out on 2 cm x 2 cm x 4 mm sponge cloth immersed in larval rearing diet [36] in flat base 30 mm x 100 mm cylindrical test tubes. Approximately 100 embryos were placed in each tube. Axenic control lines were derived at this step by plugging cotton wool up to 3 cm from the top of the tube.

5.3.4 Generation of mono-association lines

An inoculum of 50 µl of 1 X 10^4 cfu /ml of each bacterial isolate was introduced in triplicate per experiment directly onto the embryos before plugging the tubes with cotton wool.

5.3.5 Rearing and quality checking of fly lines

All tubes were maintained at 27 °C and 70% humidity in a germ-free environment. A control group with an intact microbiome (whose embryos were not dechorionated) was included in triplicate in each experiment. To compensate for the diminishing diet due to feeding by developing larvae, 200 µl of larval rearing diet was added to each tube every 24 h after hatching. When larvae were fully developed to the mature third instar, they moved upwards inside the tubes to burrow into the cotton wool plugs. Cotton wool plugs with puparia were submerged in autoclaved distilled water at room temperature and carefully pulled to free puparia. The retrieved puparia were dried on sterile paper towel and maintained on sterile Petri dishes placed in sterilized ventilated perspex cages until eclosion.

To quality check axenic lines, random third stage larvae were retrieved from axenic control tubes per experiment and homogenized in 50 µl PBS. 5 µl of this homogenate was plated on nutrient agar plates and incubated at 37 °C and checked for bacterial growth after 15 h.

5.3.6 Effects of gut bacteria on development of immature stages

Tubes with embryos were monitored daily and the time taken to record at least 10 first instar larvae in each tube recorded as duration to hatching. Similarly, the time taken from hatching to the recording of the first 10 puparia on the cotton wool plugs were recorded

for each tube as the larval stage duration. Measurements of 1-day old puparia weight, dorsal to ventral length as well as width of the sixth segment were also recorded. Weights were recorded in triplicates of 20 puparia from every fly line, whereas length and width measurements were recorded from 20 puparia per fly line.

5.3.7 Effects of gut bacteria on survival of mature stages exposed to *Metarhizium anisopliae*

Metarhizium anisopliae ICIPE69 conidia were obtained with permission from the International Centre for Insect Physiology and Ecology (icipe) germplasm. Newly emerged adult flies from each of fly line were exposed to 0.3 g of dry spores of *M. anisopliae* for a duration of 1 min. Exposure of each fly line was done in triplicate consisting of 20 flies per replicate, in a contamination device made from a 50 ml falcon tube lined with velvet cloth. Exposed flies were released into 10 cm x 10 cm x 10 cm ventilated cages and maintained on adult *B. dorsalis* rearing diet [37] and sterile water saturated on cotton wool. A control set derived from unexposed flies was included in each treatment. The treatments were composed of a total of 14 fly lines composed of 12 fly lines each mono-inoculated with one of 12 different gut bacterial isolates, a control with an intact gut microbiome and a control reared in germ-free conditions. The daily survival of flies in each treatment were recorded until the last fly died. Survival of control sets that were not exposed to the fungus from each fly line was monitored daily for 30 days.

5.3.8 Data analysis

All data from the development of embryos and larvae as well as from puparia measurements were analyzed using one-way analysis of variance (ANOVA) after satisfying ANOVA assumptions. Normality of data was confirmed using the Shapiro-Wilk test. All analyses were conducted in the R statistical software [38].

Survival curves for adult flies exposed to *M. anisopliae* were generated using the Kaplan-Meier method in the Graph Pad Prism software, version 7.00 for Windows (www.graphpad.com). Differences in survival between flies from the different fly lines were assessed using the Mantel-Cox log rank tests in Graph Pad Prism software version 7.00.

5.4 Results

5.4.1 Isolation and identification of cultivable bacteria

A total of 12 unique bacterial isolates were identified from the *B. dorsalis* gut cultures through sequencing and homology searches using BLAST [39]. These included 7 isolates in the phylum Proteobacteria and five isolates in the phylum Firmicutes (Table 1).

Table1. Description of identified bacterial isolates and their GenBank accession numbers.

Isolate	Accession	Phylum	Family	Closest match	% Identity
BD1	MK968291	Proteobacteria	Enterobacteriaceae	*Citrobacter freundii*	100
BD2	MK968292	Proteobacteria	Enterobacteriaceae	*Enterobacter tabaci*	100
BD3	MK968293	Proteobacteria	Enterobacteriaceae	*Enterobacter cloacae*	100
BD4	MK968294	Proteobacteria	Enterobacteriaceae	*Klebsiella oxytoca*	100
BD5	MK968295	Firmicutes	Streptococcaceae	*Lactococcus lactis*	100
BD6	MK968296	Firmicutes	Streptococcaceae	*Lactococcus lactis*	99.8
BD7	MK968297	Firmicutes	Streptococcaceae	*Lactococcus lactis*	99.8
BD8	MK968298	Firmicutes	Streptococcaceae	*Lactococcus lactis*	100
BD9	MK968299	Firmicutes	Streptococcaceae	*Lactococcus lactis*	100
BD10	MK968300	Proteobacteria	Enterobacteriaceae	*Providencia alcalifaciens*	100
BD11	MK968301	Proteobacteria	Enterobacteriaceae	*Providencia rettgeri*	100
BD12	MK968302	Proteobacteria	Enterobacteriaceae	*Enterobacter asburiae*	100

5.4.2 Influence of bacteria on embryo and larval development

The 12 bacterial isolates were used to generate mono-association *B. dorsalis* lines through inoculation in germ free embryos. To assess effects of these bacteria on *B. dorsalis* development, we monitored the duration taken by each fly line for embryos to hatch as well as the duration of the larval stages. Eggs from fly lines with an intact microbiome (hereafter referred to as Ut-control (untreated control)) were recorded to take the shortest duration to hatch ($F= 6.272$, $df =13$, $P < 0.001$) whereas the longest duration was recorded for the germ-free line, hereafter referred to as (axenic). The *L. lactis* BD5, *L. lactis* BD8 and *E. asburiae* did not vary significantly from the Ut-control (figure 1a).

The shortest duration of larval stage was recorded for the Ut-control while the *L. lactis* BD9 line had the longest larval stage duration (F =5.11, df =13, P < 0.0001) (figure 1b).

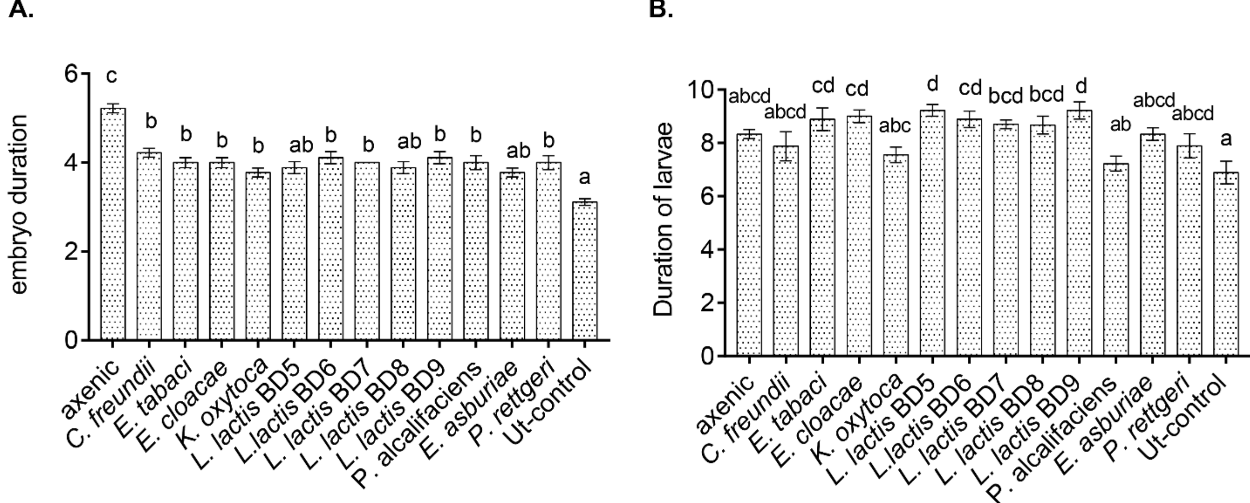

Figure 1. Mean duration between A) bacteria inoculation and emergence of first instar larvae and B) emergence of first instar larvae to pupation. Means with different superscripts are significantly different (Tukey's p= 0.05).

5.4.3 Puparia metrics from different fly lines

The *L. lactis* BD9 fly line had the highest mean values for puparia length compared to the rest of the lines (F =3.096, df = 13, P < 0.0001) (figure 2a). None of the fly lines exhibited a significant variation in width of puparia from the other fly lines (F =1.709, df = 14, P > 0.05) (figure 2b). However, significant differences were recorded when comparing mean weights of puparia between the fly lines with the highest mean weight of 20 puparia recorded for the *L. lactis* BD7 fly line and the lowest for the Ut-control (F = 426.8, df = 13, P < 0.001) (figure 2c).

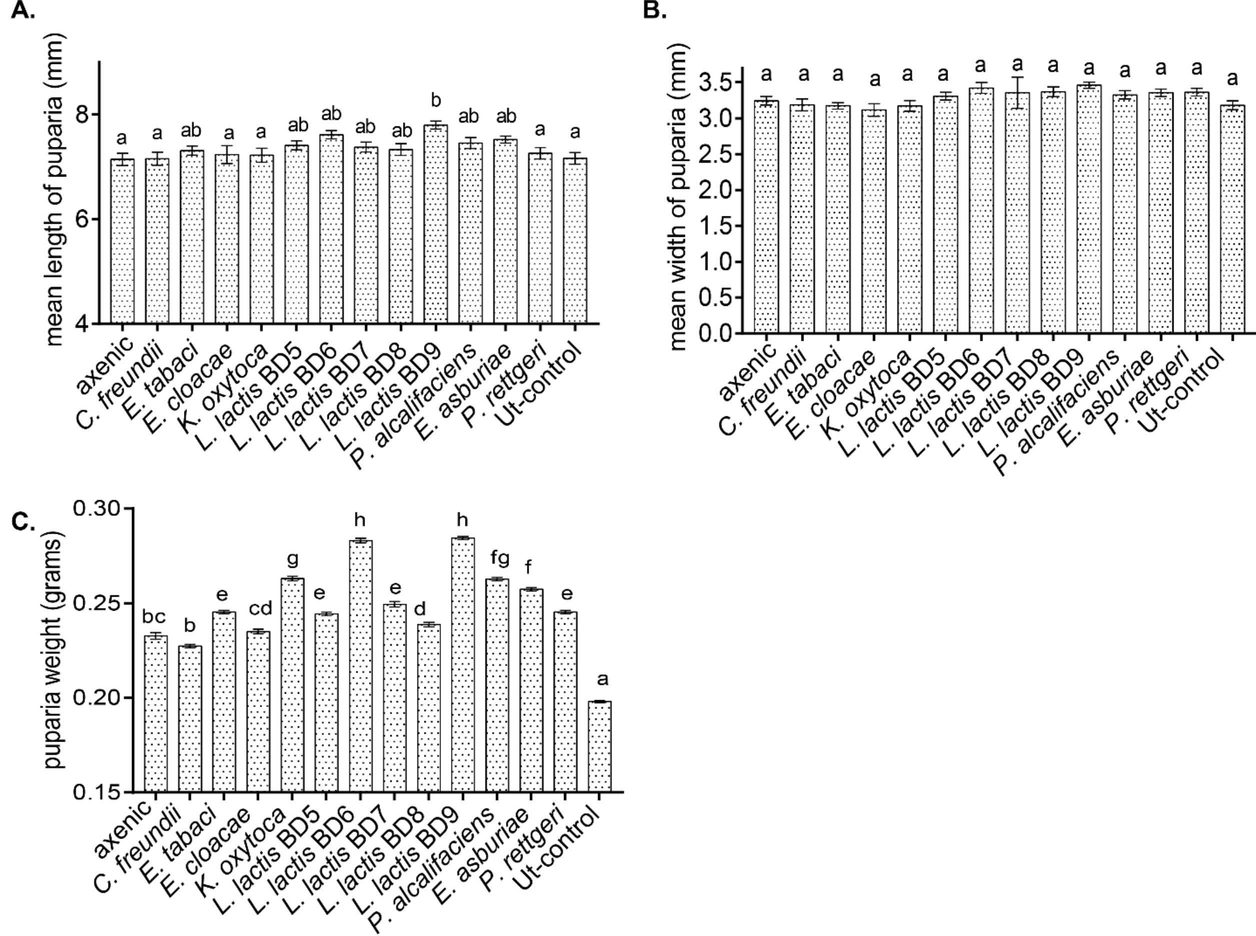

Figure 2. Comparison of puparia parameters among fly lines a) mean (± SE) lengths of puparia b) mean (± SE) width of puparia and c) mean (± SE) weights of sets of 20 puparia. Means with different superscripts are significantly different (Tukey's p= 0.05).

5.4.4 Survival of fly lines post-exposure to *M. anisopliae*

The survival rates of *B. dorsalis* from the different fly lines was monitored daily after exposure to *M. anisopliae*. All individuals in the unexposed control sets from all fly lines (unexposed to fungus) survived until day 30 when the experiment was terminated and therefore no correction was made in the survival analysis for treatments. The survival rate of each fly line was preliminarily compared to that of the axenic and the Ut-control lines before grouping the fly lines in groups of five, inclusive of the axenic and Ut-controls in each group, based on minimum deviation from these controls. No significant variations

in survival were recorded among the control lines (axenic and Ut-control) and the *C. freundii*, *E. tabaci* and *E. cloacae* lines (Log rank Mantel-Cox χ^2=2.78, df=4, P value=0.596) (fig. 3a) and also amongst the control lines and the *K. oxytoca*, *L.lactis* BD5 and *L. lactis* BD6 lines (Log rank Mantel-Cox χ^2=7.36, df=4, P value=0.118) (fig 3b). However, significant differences in survival were recorded among the controls and the *P. alcalifaciens*, *E. asburiae* and *P. rettgeri* lines (Log rank Mantel-Cox χ^2=11.79, df=4, P value 0.019) (fig. 3c) as well as among the controls and *L. lacti*s BD7, *L. lactis* BD8 and *L. lactis* BD8 lines (Log rank Mantel-Cox χ^2=16.18, df=4, P value 0.002) (Fig. 3d).

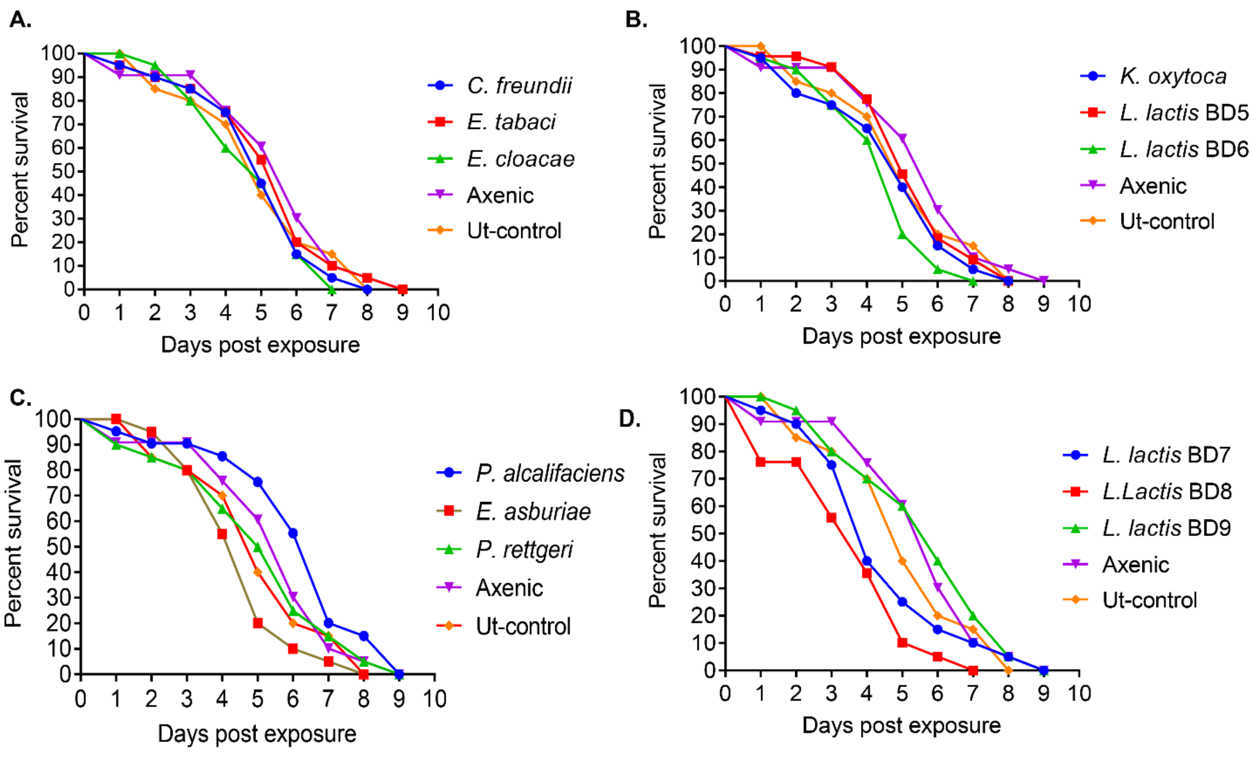

Figure 3. Comparison of survival after exposure to *M. anisploiae* between axenic and Ut-controls with A) *C. freundii*, *E. tabaci* and *E. cloacae* lines, B) *K. oxytoca*, *L. lactis* BD5 and *L. lactis* BD6 lines, C) *P. alcalifaciens*, *E. asburiae* and *P. rettgeri* lines and D) *L. lactis* BD7, *L. lactis* BD8 and *L. lactis* BD9 lines.

5.5 Discussion

Enterobacteriaceae have been commonly identified in the gut microbiomes of *B. dorsalis* [27,31]. Similarly, Streptococcaceae have also been found to associate commonly with *B. dorsalis*, more specifically bacteria in the *Lactococcus* genus [25–27]. The 12 isolated bacteria species all belonged to either of these two families. All the isolated bacteria have been reported as members of the gut microbiota among different populations of *B. dorsalis* [26,32,40,41].

In this study, cultivable bacteria were isolated using non-selective enriched media under aerobic conditions. It is possible that more isolates would have been obtained if both aerobic and anaerobic conditions had been employed. However, for practicality of possible application of the identified bacteria in mass-rearing techniques, non-fastidious micro-organisms that would easily proliferate in aerobic fly-rearing conditions would be ideal and therefore we used only aerobic conditions for isolation. Similarly, various *B. dorsalis* gut bacteria are reported to be transferred vertically from mother to offspring through contamination of the egg surfaces [41]. During oviposition, some bacteria from the gut coats the protective outer layer (chorion) of the egg. These bacteria may get ingested together with the chorion by emerging larvae [42] or may proliferate in the fruit causing rot and in turn get ingested by the feeding larvae [43]. In order to study effects of removal of bacteria from insects, many studies typically use broad-spectrum antibiotics. It is arguable that the use of antibiotics may engender various ecological problems, especially related to resistance. However, antibiotics may have detrimental effects on host organisms, for instance the interference of the protein synthesis mechanisms of the host by the broad spectrum antibiotic tetracycline [44]. In addition, dechorionation of embryos

followed by rearing on germ-free diets has been observed to be more effective in eliminating bacteria from insect eggs than the use of antibiotics [44]. For this reason, we adopted a dechorionation approach to generate axenic flies for mono-association experiments. In all quality checks of bacterial growth from third stage larvae of the axenic line, none of the plates recorded bacterial growth, inferring a strong elimination effect of the dechorionation technique. However, the technique also suffers from inducing mortality in a substantial proportion of dechorionated embryos.

The absence of bacteria in the axenic fly line was recorded to delay embryo development significantly compared to lines with individual bacterial isolates as well as the Ut-control with an intact microbiome. This indicates that the presence of bacteria promotes embryonic development and the effect is stronger when the combinations of inherent host gut symbionts are present than when a single bacterial species dominates.

Larval development was observed to be quicker in flies with an intact microbiome and slowest in fly lines with *L. lactis* BD5 and *L. lactis* BD. Previous observations on *B. dorsalis* indicated that *L. lactis* prolongs larval development when supplemented in the diets of non-axenic flies [25]. This therefore suggests that in *B. dorsalis*, some strains of *L. lactis* may be more detrimental than beneficial, even though its association with this host is more often than not by chance and could therefore play beneficial roles in the host.

Subtle effects of bacterial isolates on puparia size and weight were observed although there were no significant differences in widths of puparia among all fly lines indicating that this parameter may not be useful for testing variables in *B. dorsalis.* Lower puparia weight in the Ut- control than in lines with re-introduced bacteria supports a previous report which demonstrated that supplementing larval diets with certain gut bacteria results in a

significant increase in puparia weight [25] in *B. dorsalis* as well as in the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann)(Diptera: Tephritidae) [45]. Increased pupal weight due to addition of probiotics has been associated with increased adult size and mating success of males [45].

We recorded significant differences in survival of *B. dorsalis* flies after exposure to the entomopathogenic fungus, *M. anisopliae.* Notably, the *P. alcalifaciens* fly line showed a comparatively higher survival while the *L. lactis* BD8 line exhibited poor survival relative to control treatments and other lines. This suggests that *P. alcalifaciens* possibly modulates the fly's immunity in a manner that enhances its survival under conditions of challenge with entomopathogenic fungi. Repression of entomopathogenic infections due to gut bacteria has been reported in the onion fly, *Delia antiqua* (Meigen, 1826) (Diptera: Anthomyiidae) [46]. This observation therefore outlines a major benefit for the host that could be a factor that selects for frequent association of *B. dorsalis* with such symbionts. Similar findings where bacterial symbionts enable *B. dorsalis* flies to recover from effects of irradiation in SIT have been reported [32]. In contrast, *L. lactis* BD8 could be pathogenic to *B. dorsalis* and the synergism between this isolate and *M. anisopliae* resulted in poor survival of exposed flies. Similar observations on *L. lactis* and its potential pathogenic effects in *B. dorsalis* were reported from supplementation of larval rearing diet with *L. lactis* [25].

This study has identified a strong effect of gut bacteria during early development of *B. dorsalis* and outlined two bacterial isolates that modulate the survival of this pest when challenged with entomopathogenic fungi. The individual influence of these two isolates on the development of immature stages of *B. dorsalis* is comparably similar at the embryo

and larval stages whereas in the case of pupal weight, *P. alcalifaciens* significantly promoted higher weight and is therefore associated with improved adult performance [44] compared to *L. lactis* BD8. These findings are relevant to pest management and can be used to select probiotics for use in the artificial diets that are used to rear insects that are released as part of SIT programs. These findings are also relevant in IPM where SIT and the use of entomopathogenic fungi are combined.

5.6 Author Contributions

Conceptualization: F.M.K, S.E. and J.K.H.; Investigation, data analysis and original draft preparation: J.G.; Supervision: F.M.K., S.E., J.K.H and J.V.B.; Writing-review and editing of final draft: all authors; Funding acquisition: S.E.

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5.9 Conflicts of Interest

The authors declare no conflict of interest. The funders had no role in the design of the study, in the collection, analyses, or interpretation of data, in the writing of the manuscript, or in the decision to publish the results.

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CHAPTER SIX
6 General discussion, conclusion and recommendation

6.1 General discussion and conclusion

Various challenges posed by establishment of invasive species have far reaching effects across wider geographical regions. In Africa, invasive insect pest species continue to be a threat to agricultural production, which is by far one of the most relied upon economic activities. The invasion of Africa by the oriental fruit fly, *B. dorsalis,* in 2003 and its subsequent rapid spread and establishment in many countries across the continent is a classic example of the risk the continent faces owing to its suitable conditions for many kinds of invasive pests to thrive(Lux *et al*., 2003; Drew *et al.,* 2005; Mwatawala *et al*., 2006; Correia *et al*., 2008; Khamis *et al*., 2009; Jose *et al*., 2013; Manrakhan *et al*., 2015). *Bactrocera dorsalis* is a high-risk quarantine pest and has attracted imposition of quarantine restrictions on fresh host fruit, from many countries where the pest is present (USDA-APHIS, 2008; Badii *et al*., 2015; FAO, 2015; Ekesi *et al.,* 2016; Europhyt, 2017). At international level, such restrictions translate into high economic losses due to loss of lucrative markets. Implementation of quarantine measures is also costly and not all producers may have access to infrastructure needed for implementation of quarantine treatments. At a local scale, fruit fly infestation diminishes yield and in turn food supplies as well as revenue for farmers. The problem is also magnified by the presence of many other tephritid fruit fly pest species across agricultural zones. It is appreciable that globally, factors such as preference for organic and chemical pesticide-free produce are influencing the adoption of alternative ways to with deal fruit fly pests. Sound pest management strategies are therefore geared towards minimizing direct application of chemical insecticides onto fruit and vegetables. A combination of various cultural,

biological, behavioural, genetic and chemical methods in integrated pest management (IPM) programmes have been shown to have higher efficiency in controlling most tephritid fruit flies (Verghese *et al*., 2006; Ekesi *et al*., 2011, Kibira *et al*., 2014; Muriithi *et al.*, 2016; Nyang'au *et al*., 2017). Indeed, some studies in Kenya have shown notable willingness from mango growers to adopt IPM programmes in management of tephritid fruit flies (Korir *et al*., 2015).

However, prominently lacking in the currently used pest management methods are strategies that rely on microbial associations with tephritid fruit flies. In other arthropods, including the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) (Diptera: Tephritidae) and the olive fruit fly, *Bactrocera oleae* (Rossi) (Diptera: Tephritidae), the possibility to exploit *Wolbachia*, a bacterial symbiont that alters the host's reproductive compatibility to an uninfected mate, to suppress pest populations, has been demonstrated (Apostolaki *et al*., 2011; Zabalou *et al*., 2004, Zabalou *et al*., 2009). To explore this possibility in the invasive *B. dorsalis* species, the first part of this study sought to establish whether the endosymbiont *Wolbachia* is accommodated in *B. dorsalis* populations in Africa and if so, whether the association is stable and finally if any reproductive manipulations are possible in this pest. To achieve this, a molecular detection assay was used to screen for *Wolbachia* from *B. dorsalis* representative specimens collected from distinct populations in different countries in Africa. Also, in order to assess the infection patterns of *Wolbachia* and the dynamics of *B. dorsalis* populations in Africa, we screened for *Wolbachia* from population sets collected shortly after discovery of invasion, between 2005 and 2009 and compared these to samples collected in 2017. The following were the main findings:

- Out of 357 screened specimens, only 10 were positive for *Wolbachia.* Of these, two were

from the 2017 sample set, 6 from the 2005-2009 set and 2 were from a representative set from a Sri Lankan population included in this work for comparison. For the African samples, this number of positives corresponds to a *Wolbachia* prevalence of 3.6% in the populations sampled between 2005 and 2009, and 1.1% in the 2017 populations. This low prevalence of infection is within range of *Wolbachia* detected in *B. dorsalis* populations in China (0.7-3%) and Thailand (1.15%) as reported in previous studies (Sun *et al.,* 2007; Kittayapong *et al*., 2000). These findings are therefore indicative of a low threshold for accommodation of *Wolbachia* in natural populations of *B. dorsalis,* irrespective of their geographical location. In addition, none of the *Wolbachia* detected among African populations was identical to those from the Sri Lankan population or to strains reported elsewhere. This suggests that the infection in African populations is most likely to have been acquired within unique ecological niches in the continent in the course of invasion and establishment.

- Two sites, Muranga and Nguruman, at which *Wolbachia* infection was detected in the 2005-2009 sample set did not have *Wolbachia* infection in the 2017 set. This could be as a result of loss of infection over time within the populations at these sites. However, we cannot rule out the possibility of detecting infection in these sites if extensive sampling were to be carried out. These two sites are geographically distinct from each other but within proximal range of each other within Kenya and since an identical *Wolbachia* strain was recorded in these two sites, these two sites were most likely invaded by the same population of the pest. This suggests that *Wolbachia* infections could in future be used as a tool to derive the migratory patterns of invasive species over defined geographical areas.

- *Wolbachia* infection in the African *B. dorsalis* populations did not associate strictly with maternally transmitted mitochondrial cytochrome oxidase I gene of the host. This infers that there is a high rate of horizontal transfer of the detected *Wolbachia* strains among populations of *B. dorsalis* in Africa. Putting into account that strict maternal transmission often leads to high prevalence of *Wolbachia* in a population, the low prevalence of detected *Wolbachia* also suggests a horizontal mode of transmission. A similar scenario has been reported in the African region in a population of mosquitoes (Niang *et al*., 2018).
- There have been minimal changes in the population structure of *B. dorsalis* in the Eastern Africa region where sites that were sampled during 2005-2009 were also sampled in 2017. This therefore infers that subsequent invasion events since 2003 have not occurred in this region, or if there have been any reintroductions, they have occurred from the same source population. Relating this to infection status, a lower *Wolbachia* infection rate was recorded in the 2017 collected *B. dorsalis* samples than in the 2005-2009 set of samples. This is a likely suggestion that should all *Wolbachia* infections have originated from the invasion source, then minimal re-introduction events have taken place and only few of the original *Wolbachia* infecting the invading population have persisted in the pest over time. However, the *Wolbachia* strains recorded in the 2017 specimens were not identical to those recorded in the 2005-2009 specimens. As such, these infections could have been acquired over time as the pest interacts with other arthropods species and therefore the lower infection status cannot conclusively be related to occurrence of reintroduction events. This finding reveals that assessment of pest population dynamics over time using molecular markers is a robust tool for monitoring the spread and establishment of important pest species.

- Although the low prevalence of *Wolbachia* detected in *B. dorsalis* populations prohibited further experimentation through rearing of naturally infected flies, we could not rule out the possibility that the two strains that were detected in female samples during 2017 could have a male killing phenotype in this host. Male killing is among other phenotypes that *Wolbachia* is known to confer to arthropod hosts (Werren *et al*., 2008). *Wolbachia* in male insect hosts is usually at a dead end and hence not transmissible, and, based on this, many studies prefer to screen only female specimens (Hart, 1995). However as demonstrated in this study, where voucher specimens are to be screened for *Wolbachia* infection, it is a good strategy to include both male and female specimens in order to infer or rule out characteristics such as male killing in situations where this cannot be done through experimentation.

Apart from the possible manipulations exerted by endosymbionts on insect hosts that could be exploited in pest management, understanding the compositions of bacterial communities in a host can provide a standpoint of exploitation of bacterial associations to alter some aspects of the host's biology that are influenced by such bacteria. In view of pest management in tephritid fruit flies, gut bacterial communities that improve the host's fitness can be exploited by supplementing such bacteria in rearing diets of flies for mass release in SIT programs (Augustinos *et al.,* 2015, Kyritsis *et al*., 2017, Khaeso *et al*., 2017). However, numerous studies characterizing the compositions of gut symbionts in *B. dorsalis* have reported huge variations in community structure in different geographical locations (Wang *et al*., 2011; Shi *et al.,* 2012; Wang *et al*., 2013; Pramanik *et* al., 2014; Damodaram *et al*., 2016; Liu *et al*., 2016; Yao *et al*., 2015; Andongma *et al.,*2016; Yong *et al.,* 2016; Cheng *et al*., 2017; Gujjar *et al*., 2017; Khaeso *et al*., 2017; Bai *et al*., 2018;

Liu *et al*., 2018; Zhao *et al*., 2018; Akami *et al*., 2019). The second part of the study therefore sought to outline the gut bacterial community structure for *B. dorsalis* populations in the African region and investigate whether the structure is influenced by geographical location as well as diet. A high throughput sequencing approach targeted at the bacterial 16S rRNA gene was used to identify all bacteria in gut samples of adult and larva samples derived from several mango growing regions in Kenya as well as from a laboratory reared colony at the International Centre of Insect Physiology and Ecology (icipe). The following were the main findings:

- At the phylum level, outstanding differences in the predominant bacteria were observed in different sampling sites, with adult flies from Embu and Nguruman being dominated by Firmicutes, those from Kitui Makueni and icipe by Proteobacteria, whereas those from Muranga had mixed proportions of Firmicutes, Proteobacteria and Bacteroidetes. In larvae, samples from the icipe colony and Embu had mixed proportions of Proteobacteria, Firmicutes and Actinobacteria whereas those from Makueni and Muranga were dominated by Actinobacteria and Proteobacteria respectively. All the Phyla recorded coincide with the most reported bacterial Phyla in this pest in other geographical locations out of Africa (Wang *et al*., 2011; Shi *et al.,* 2012; Wang *et al*., 2013; Pramanik *et* al., 2014; Liu *et al*., 2016; Yao *et al*., 2015; Andongma *et al.,*2016; Yong *et al.,* 2016; Cheng *et al*., 2017; Gujjar *et al*., 2017; Khaeso *et al*., 2017; Bai *et al*., 2018; Liu *et al*., 2018).
- The Phylum Chlamydiae was identified in some adult *B. dorsalis* specimens. This was an unusual finding which to the best of our knowledge has not been reported among *B. dorsalis* microbiota before. However, it is not unusual for members of this phylum to be found in an arthropod species, as some bacteria in this phylum has been recorded in

other insects (Thao *et al.,* 2003).

- The most dominant genera detected in adult fruit fly samples were *Lactococcus, Enterobacter* and *Klebsiella,* whereas among larvae, *Lactobacillus*, *Pantoea Cutibacterium* and *Providencia* were dominant. Only one bacterial genus, *Bacillus,* was detected in both adult and larval specimens. It is not unusual to record such a varied gut bacterial community between life stages of *B. dorsalis* as similar findings have been reported before (Andongma *et al.,* 2015). These shifts in gut bacteria are likely to occur due to variations in the differences in diet and micro-environments that are experienced at these two life stages.
- There was no significant difference in species richness of bacteria among adult flies from the different locations. However, samples of larvae from the different sites differed significantly.
- Three bacterial genera were differentially abundant in adult samples among the sampled sites: *Enterobacter*, *Klebsiella* and *Serratia* whereas in larvae, *Lactobacillus* was significantly more abundant in Embu samples than in other sampled sites. This suggests that some bacterial species are likely to play stage-specific roles and could therefore explain shifts in bacterial communities between premature and mature stages of *B. dorsalis* as recorded in this study and as reported previously (Andongma *et al*., 2015). In addition, differential abundance of *Lactobacillus* in only one field site despite a common diet and common life stage among field sites suggests an environmental influence in gut bacterial composition (Yun *et al.,* 2014).
- A potentially entomopathogenic bacteria, *Serratia* was detected in some of the *B. dorsalis* samples. Some bacterial species in the genus *Serratia* have been described to produce

toxins than are capable of killing insect hosts (Dodd *et al.,* 2006; Tambong *et al*., 2016; Pineda-Castellanos *et al.*, 2015; Ruiu, 2015: Zhou *et al*., 2016). Such entomopathogenic bacteria can be integrated in insect pest management strategies as has been demonstrated for control of other insect species (Jackson *et al.*, 1992; Prischmann, 2008; Kavitha, 2010; Ruiu, 2015, Hurst, 2000; Mampallil *et al*., 2017). Detection of *Serratia* in *B. dorsalis* is therefore interesting in the sense that since the bacteria can be accommodated in this pest, development of management strategies exploiting this entomopathogen would therefore not face challenges associated with non-accommodation of the bacteria in this host.

In order to exploit gut bacterial symbionts in management strategies for tephritid fruit flies, it is imperative that the targeted symbionts be easy to cultivate and maintain under standard conditions used in rearing of the targeted fruit fly species. Having outlined the possible bacterial communities in *B. dorsalis*, the third part of this study investigated the cultivable bacterial communities from this pest and utilized an axenic fly approach to evaluate the effects of the isolated bacteria on the early development of *B. dorsalis,* as well as how such fly lines survive after exposure to entomopathogenic fungi. The following were the main findings:

- A total of 12 bacterial isolates classified into the phyla Firmicutes and Proteobacteria were identified using a culture-dependent and molecular approach. These 12 include: *Citrobacter freundii, Enterobacter tabaci, Enterobacter cloacae, Klebsiella oxytoca, Providencia alcalifaciens*, *Providencia rettgeri, Enterobacter asburiae* and 5 strains of *Lactococcus lactis.*
- Flies with an intact microbiome exhibited the shortest duration to hatch, whereas the

longest duration was observed in the germ-free line. This is an interesting finding that indicates that bacteria play a direct or cryptic role in the transition from the embryonic stage to larval stage of development in *B. dorsalis.* It has been suggested that during oviposition, some gut bacteria are passed on and coat the chorion of fruit fly eggs (Behar *et al.,* 2008). Even though it is hard to conclude whether this transfer of bacteria is deliberate, it is apparent from the findings of this study that the bacteria which are passed on have a role in embryo transition to larvae, in addition to suggested roles such as aid in fruit rotting and hence nutritional provisioning for the emerging and developing larvae (Behar *et al.,* 2008). We propose that such passed gut bacteria do take part in breakdown of structural components of the embryo's chorion possibly through production of proteolytic enzymes and thus speed up the hatching process. However, this proposition will need experimental investigation employing gene expression profile analysis between germ free and bacteria-laden embryos as a means to proof of concept.

- Similarly, flies with an intact microbiome had the fastest development time at larval stage whereas the axenic line took significantly longer along with majority of the other fly lines except the *P. alcalifaciens* and *K. oxytoca* lines. This finding indicates that there is a complex interplay between different members of the *B. dorsalis* associated bacterial communities and the host, which influences the rate of development of immature stages of this pest. There is therefore no evidence from this study to suggest that a probiotic can be selected for larval rearing diets in SIT programmes based on the rationale of reduced larval development time.
- Having an intact microbiome, reintroduced bacteria, or none at all, did not seem to associate with any reduction or increase in pupal widths. We therefore propose that pupal

widths are not ideal parameters for testing the effects of bacterial roles in development of *B. dorsalis*, which may also apply to other tephritid fruit fly species.

- Differences were observed in mean weights of puparia between the fly lines with the highest mean observed in the *L. lactis* BD7 fly line and the lowest in the Ut-control. This is a likely indication that presence of an intact bacteriome in this fly increases the competition for nutrient utilization between the host and the gut bacteria as opposed to the presence of only individual bacterial isolates. However, *L. lactis* has been reported to delay larval development in *B. dorsalis* (Khaeso *et al*., 2017) and is therefore in agreement with the findings of this study. The prolonged larval duration in the *L. lactis* BD7 fly line could have provided larvae with more time to accumulate and store nutrients which resulted in higher mean pupal weights.
- After exposure to entomopathogenic fungi, significant differences in survival were observed among germ-free fly lines, fly lines with an intact microbiome and the *P. alcalifaciens* line as well as the *L. lactis* BD8 line. Survival curves among these isolates suggest an enhanced survival in the *P. alcalifaciens* fly line and diminished survival in the *L. lactis* BD8 fly line. This finding agrees with that of a previous study which suggested that *L. lactis* exhibits pathogenic effects in *B. dorsalis* (Khaeso *et al.,* 2017) and therefore synergism between this isolate and *Metarhizium anisopliae* icipe 69 may have resulted in the significantly lower survival recorded for this fly line. On the other hand, the *P. alcalifaciens* fly line exhibited a higher survival rate in comparison to the other fly lines, suggesting a protective effect of this bacteria on the host against the entomopathogenic fungus. However, this bacterium has been described to have pathogenic effects in the Mexican fruit fly, *Anastrepha ludens* (Loew) (Rangel *et al*., 2013) and therefore the

increased survival observed in *B. dorsalis* seems to correspond to activation of systemic immunity by the bacteria that offers some degree of protection from the entomopathogenic fungus.

6.2 Recommendations and future research needs

The key findings of this study indicate that *B. dorsalis* populations can accommodate potentially useful bacteria that could in future be utilized in symbiont-based pest management strategies. Towards this end, the following research needs should be addressed:

- *B. dorsalis* can accommodate diverse *Wolbachia* strains. Screening of *B. dorsalis* from more diverse agro-ecological zones and more populations within the African continent is recommended as a means of identifying stable and frequently associated *Wolbachia* strains in the region.
- The true origin of *Wolbachia* in *B. dorsalis* remains elusive. It is recommended that when possible, screening of *Wolbachia* in this pest should be carried out hand in hand with screening of other arthropods that interact with *B. dorsalis* flies, for example, parasitoid wasps, that should be sampled from within the same ecological niches.
- The non-concordance with host mtCOI haplotypes of the detected *Wolbachia* suggests high rates of horizontal transmission. It is recommended that vertical transmission monitoring be done in infected sub-populations of this fly species.
- A possible environmental and dietary shift in gut microbiota of *B. dorsalis* was identified. It is recommended that high throughput sequencing approaches be used to further explore variations that could be seasonal or due to host plant utilization.

- Survival of *B. dorsalis* flies exposed to entomopathogenic fungi appears to be constrained by species of *L. lactis*. It is recommended that before selection of *L. lactis* strains as possible probiotics for SIT, proper characterization of the benefits of such a selection be first demonstrated through sufficient experimentation. In addition, in this study, *P. alcalifaciens* prolonged the survival of *B. dorsalis* exposed to entomopathogenic fungi. This bacterium should be evaluated as a candidate probiotic where SIT is combined with biocontrol using entomopathogenic fungi in order to enhance fitness of sterile *B. dorsalis* males.

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Appendix A

Instructions for Authors (excerpt)-Multidisciplinary Digital Publishing Institute

- **Research manuscripts** should comprise:
    - Front matter: Title, Author list, Affiliations, Abstract, Keywords
    - Research manuscript sections: Introduction, Materials and Methods, Results, Discussion, Conclusions (optional).
    - Back matter: Supplementary Materials, Acknowledgments, Author Contributions, Conflicts of Interest, References.

Front Matter

These sections should appear in all manuscript types

- **Title:** The title of your manuscript should be concise, specific and relevant.
- **Author List and Affiliations:** Authors' full first and last names must be provided.
- **Abstract:** The abstract should be a total of about 200 words maximum. The abstract should be a single paragraph and should follow the style of structured abstracts, but without headings
- **Keywords:** Three to ten pertinent keywords need to be added after the abstract.
- **Introduction:** The introduction should briefly place the study in a broad context and highlight why it is important. It should define the purpose of the work and its significance, including specific hypotheses being tested.
- **Materials and Methods:** They should be described with sufficient detail to allow others to replicate and build on published results. New methods and protocols should be described in detail while well-established methods can be briefly described and appropriately cited. Give the name and version of any software used and make clear whether computer code used is available.
- **Results:** Provide a concise and precise description of the experimental results, their interpretation as well as the experimental conclusions that can be drawn.
- **Discussion:** Authors should discuss the results and how they can be interpreted in perspective of previous studies and of the working hypotheses. The findings and their implications should be discussed in the broadest context possible and limitations of the work highlighted. Future research directions may also be mentioned.
- **Conclusions:** This section is mandatory, and should provide readers with a brief summary of the main achievements/results of your work.
- **Supplementary Materials:** Describe any supplementary material published online alongside the manuscript (figure, tables, video, spreadsheets, etc.). Please indicate the name and title of each element as follows Figure S1: title, Table S1: title, etc.
- **Acknowledgments:** All sources of funding of the study should be disclosed.

- **Author Contributions:** For research articles with several authors, a short paragraph specifying their individual contributions must be provided.
- **Conflicts of Interest:** Authors must identify and declare any personal circumstances or interest that may be perceived as inappropriately influencing the representation or interpretation of reported research results. If there is no conflict of interest, please state "The authors declare no conflict of interest."
- **References:** References must be numbered in order of appearance in the text (including table captions and figure legends) and listed individually at the end of the manuscript.

  In the text, reference numbers should be placed in square brackets [ ], and placed before the punctuation; for example [1], [1–3] or [1,3]. For embedded citations in the text with pagination, use both parentheses and brackets to indicate the reference number and page numbers; for example [5] (p. 10). or [6] (pp. 101–105).

  References should be described as follows, depending on the type of work:

Journal Articles:
1. Author 1, A.B.; Author 2, C.D. Title of the article. *Abbreviated Journal Name* **Year**, *Volume*, page range. Available online: URL (accessed on Day Month Year).

Books and Book Chapters:
2. Author 1, A.; Author 2, B. *Book Title*, 3rd ed.; Publisher: Publisher Location, Country, Year; pp. 154–196.
3. Author 1, A.; Author 2, B. Title of the chapter. In *Book Title*, 2nd ed.; Editor 1, A., Editor 2, B., Eds.; Publisher: Publisher Location, Country, Year; Volume 3, pp. 154–196.

Unpublished work, submitted work, personal communication:
4. Author 1, A.B.; Author 2, C. Title of Unpublished Work. status (unpublished; manuscript in preparation).
5. Author 1, A.B.; Author 2, C. Title of Unpublished Work. *Abbreviated Journal Name* stage of publication (under review; accepted; in press).
6. Author 1, A.B. (University, City, State, Country); Author 2, C. (Institute, City, State, Country). Personal communication, Year.

Conference Proceedings:
7. Author 1, A.B.; Author 2, C.D.; Author 3, E.F. Title of Presentation. In *Title of the Collected Work* (if available), Proceedings of the Name of the Conference, Location of Conference, Country, Date of Conference; Editor 1, Editor 2, Eds. (if available); Publisher: City, Country, Year (if available); Abstract Number (optional), Pagination (optional).

Thesis:
8. Author 1, A.B. Title of Thesis. Level of Thesis, Degree-Granting University, Location of University, Date of Completion.

Websites:
9. Title of Site. Available online: URL (accessed on Day Month Year).

Unlike published works, websites may change over time or disappear, so we encourage you create an archive of the cited website using a service such as WebCite. Archived websites should be cited using the link provided as follows:
10. Title of Site. URL (archived on Day Month Year).

Appendix B

Instructions for Authors (excerpt)-Journal of Applied Entomology

Parts of the Manuscript

The manuscript should be submitted in separate files: Title page, main text file; figures; tables.

Title Page

The title page should contain:

i. The full names of the authors;
ii. The author's institutional affiliations where the work was conducted;
iii. Acknowledgements (including funding information).
iv.The present address of any author, if different from where the work was carried out, should be supplied in a footnote.

Main Text File

For short communications and original articles the main text should be presented in the following order:

i. Abstract and key words;
ii. Main text; including Introduction, Materials and Methods, Results, and Discussion;
iii. Conflict of Interest Statement;
iv. Author Contribution;
v. Data Availability Statement;
vi. References;
vii. Figure legends;

Abstract

Please provide an abstract of no more than 300 words containing the major keywords.

Main Text

The main text should consist of the following sections: Introduction, Materials and Methods, Results, and Discussion.

The journal uses British spelling; however, authors may submit using either option, as spelling of accepted papers is converted during the production process.

Author Contribution

This section is mandatory and should follow the example below:Author 1, author 2 and author 3 conceived research.

- Author 2 and author 3 conducted experiments.
- Author 4 and author 5 contributed material.
- Author 3 analysed data and conducted statistical analyses.
- Author 2 and author 3 wrote the manuscript.

- Author 1 and author 4 secured funding.
- All authors read and approved the manuscript.

References

References should be prepared according to the Publication Manual of the American Psychological Association (6th edition). This means in text citations should follow the author-date method whereby the author's last name and the year of publication for the source should appear in the text, for example, (Jones, 1998). The use of et al is determined by the number of authors and whether it is the first time a reference has been cited in the paper:

- articles with one or two authors include all names in every in-text citation;
- articles with three, four, or five authors include all names in the first in-text citation but are abbreviated to the first author name plus et al. upon subsequent citations;
- articles with six or more authors are abbreviated to the first author name plus et al. for all in-text citations.

The complete reference list should appear alphabetically by name at the end of the paper. A sample of the most common entries in reference lists appears below. Please note that a DOI should be provided for all references where available. Please note that for journal articles, issue numbers are not included unless each issue in the volume begins with page one.

Journal article

Morton, A. & Garcia-del-Pino, F. (2017). Laboratory and field evaluation of entomopathogenic nematodes for control of *Agriotes obscurus* (L.) (Coleoptera: Elateridae). *Journal of Applied Entomology, 141*, 241-246.

Estes, R. E., Tinsley, N. A. & Gray, M. E. (2016). Evaluation of soil-applied insecticides with Bt maize for managing corn rootworm larval injury. *Journal of Applied Entomology, 140*, 19-27. DOI: 10.1111/jen.12233

Book

Gullan, P. J. & Cranston, P. S. (2005). *The insects - an outline of entomology*. Oxford: Blackwell Publishing.

Chapter in an Edited Book

Kiss, J., Komaromi, J., Bayar, K., Edwards, C. R. & Hatala-Zseller, I. (2005). Western corn rootworm (*Diabrotica firgifera virgifera LeConte*) amid crop rotation systems in Europe. In S. Vidal, U. Kuhlmann & R. Edwards (Eds.), *Western corn rootworm: ecology and management* (pp. 189–220). Wallingford, United Kingdom: CABI Publisher.

Unpublished Work

Unpublished work should only be cited when it has been accepted for publication, and then together with the name of the journal in which it has been accepted.

Ciosi, M., Toepfer, S., Li, H., Haye, T., Kuhlmann, U., Wang, H., Siegfried, B. & Guillemaud, T. (2008). European populations of Diabrotica virgifera virgifera are resistant to aldrin, but not to methyl-parathion. *Journal of Applied Entomology* (in press).

Websites

Estes, R. (2004). *2004 Evaluations of Rootworm Control Products*. [Text article]. Retrieved from http://bulletin.ipm.illinois.edu/print.php?id=182

Using the DOI (Digital Object Identifier) Number

Nestel D., Papadopoulos N. T. & Miranda Chueca M. A. (2008). Current advances in the study of the ecology of fruit flies from Europe, Africa and the Middle East. *Journal of Applied Entomology*, DOI: 10.1111/j.1439-0418.2008.01378.x

Tables

Tables should be self-contained and complement, not duplicate, information contained in the text.

Figure Legends

Legends should be concise but comprehensive – the figure and its legend must be understandable without reference to the text. Include definitions of any symbols used and define/explain all abbreviations and units of measurement.

Appendix C

Declaration of language editing

Language editing statement

To whom this may concern,

I, Prof. Johnnie Van den Berg, hereby declare that the thesis titled: “Assessment of the diversity and roles of bacterial symbionts in fruit fly development and response to biological control” by J Gichuhi has been edited for language correctness and spelling by some of the supervisors. No changes were made to the academic content or structure of this work.

J van den Berg

30 May 2019

Prof. Johnnie Van den Berg — Date