Dynamics of the Queensland Fruit Fly microbiome under changes in host environment

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Bachelor of Pharmacy (Hons.)

Master of Science in Applied Microbiology and Biotechnology

A thesis submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

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October 17, 2019

Table of Contents

Chapter 3: Fruit host-dependent fungal communities in the microbiome of wild Queensland fruit fly larvae

Chapter 4: Gut microbiome of the Queensland fruit fly during metamorphosis

Chapter 5: Artificial larval diet modulates the microbiome of the Queensland Fruit fly during domestication process

Chapter 6: General Discussion

Summary

Tephritid fruit flies are the most economically damaging insect pests of fruit and vegetables globally. Their biology is intimately linked to their microbiome. However, the complexities of the tephritid microbiomes remain poorly understood, largely because of the technical limitations of culture-dependent methods. To address this knowledge gap, the present study comprehensively characterized the bacterial and fungal microbiome of the Queensland fruit fly, *Bactrocera tryoni* (Froggatt) (aka: 'Qfly'), using Next-Generation Sequencing (NGS) to analyse both 16s rRNA and ITS amplicons on the Illumina MiSeq platform. Qfly is a highly polyphagous species that is Australia's most widespread and economically important fruit fly pest of horticulture.

This thesis explores the microbial communities in wild Qfly larvae and their relationship to their host fruits. Qfly larvae from fruit infested in nature are found to harbour a diverse array of bacteria and fungi. The fungal microbiome, most of which comprises previously undescribed yeasts and yeast like fungi, closely reflects that of the host fruit indicating horizontal transfer as a dominant influence, although there are also some differences that suggest a closer association between larvae and some components of the fungal microbiome. In contrast, the larval bacterial microbiome is related less to the host fruit microbiome, likely reflecting vertical transfer during egg laying.

The culture-independent approach enabled assessment of gut microbiome communities across all developmental stages in the wild-type flies prior to the domestication process. This comparative analysis between larvae, pupae and the adult gut microbiome revealed diverse microbial communities of bacteria and fungi in the larvae and adults. However, different bacterial and fungal taxa are abundant in the larvae and adult gut, which is likely related to differences in their nutritional biology. This thesis also includes the first comparative study of how artificial larval diets affect microbial communities across all developmental stages in the Qfly during the domestication process. The taxonomic profile of microbiome was different between carrot and the gel diet reared Qfly across all developmental stages whereby species richness was significantly higher in Qfly reared on the gel-based diet. Qfly reared on the gel diet reared scored higher in quality metrics than did those reared on the carrot diet at generation 5.

Overall, my thesis provides valuable insights for understanding tephritid microbiomes and related ecology, in particular the Qfly's ability to infest a vast diversity of fruit types, and as well as for laboratory and factory-scale rearing. This knowledge may enable manipulation of the gut bacteria and fungi to improve the quality of artificially reared Qfly, and may also provide useful starting points for the development of pest management solutions.

Keywords: *B. tryoni,* Gut microbiome, Next generation sequencing, Domestication, Metamorphosis, bacteria, yeast, Microbial ecology

Statement of Originality

I would like to clarify that this thesis, entitled "**Dynamics of the Queensland Fruit Fly microbiome under changes in host environment"** contains all of my original research work that I conducted during my PhD candidature and has not previously been submitted or part of this thesis not being used for any other degree or diploma in any other universities; this complete thesis is only being submitted to Macquarie University.

I would like to certify that to my best knowledge and belief, the thesis contains no material previously published or written by another person except any help and assistance that I received in my research work have been acknowledged appropriately.

I would also certify that all literature and other information sources used in this thesis also are acknowledged accordingly. This thesis did not need any ethical approval by Macquarie University as organism that was studied is an insect, Queensland fruit fly, *Bactrocera tryoni*.

Rajib Majumder
October 16, 2019

Acknowledgements

Praises be to the God Shiva almighty!

Firstly, I would like to thank to my principal supervisor Professor Philip W. Taylor for his endless support towards my end of PhD degree. I have truly blessed being a research student under your supervision. Your kindness and care for a student like me, always feel confident and special. Thank you so much Professor, you are the best.

I would like to express my deepest sense of gratitude to my adjunct supervisor Dr. Toni A. Chapman for her continuous support, advice and affection throughout my PhD candidature. Being a supportive supervisor, she has been always next to me to provides her maximum support, I am so grateful to you. I feel so lucky that I have you as my supervisor. I express my very special appreciation and thanks to my associate supervisor Dr. Brodie Sutcliffe for her friendly guidance, extended discussion and contributions for the progress on my project. Still can remember our successful discussions and endless meetings on microbiome analysis. I have learned a lot from you. Thank you so much for being there for me when I need your help the most. You are an amazing person.

I would like to acknowledge Dr Badrula Alam, Dr. Ania Deutscher, Dr. Bishwo Mainali, Dr. Ronald Lee and Dr Md Mohosinul Haque for their generous supports and collaborative efforts that facilitated my PhD journey. I also express my cordial gratitude to all my fruit fly lab mates especially Dr. Vivian Méndez, Dr. Soo Jean Park, Dr. Jeanneth Perez, Dr. Tahereh Moadeli, Jess Inskeep, Urvashi Lallu, Jamil Hossain Biswas, Sushil Gaire, Jacqui Delahunt Smoleniec and Khandaker Asif Ahmed for their generous cooperation in setting up different experiments and in maintaining a friendly research environment. They are lovely, caring, and supportive lab mates every research student wishes for.

A very special thanks to Salah Mohammad Adnan, as a brother, friend and colleague, your endless support helped me a lot throughout my candidature. Sayeeda Tasneem Chowdhury, best friend with kindness and pure heart, I always admire your encouragement and inspiration. Thank you so much Tasneem always being there for me. Sayed Mahbub Morshed, a true brother and best buddy, thank you so much for your advices, inspiration and

support. I am very lucky to get you beside me in my worst. I also thank all my friends and relatives for their supports and appreciation throughout my PhD candidature.

Finally, I would like to immensely appreciate the endless support and love my father, Mr. Harendra Majumder and my lovely, mother Mrs. Joyanti Majumder provided me throughout my life. I am always blessed to have you guys in my life. Without you two, my life is completely empty. Whatever I have achieved throughout my career, it's all because of your love and inspiration. Ma and Baba, I love you a lot and will be always in my prayers. A very special thanks to my only younger brother Rupak Majumder, who takeover all my responsibilities back in Bangladesh and take care of my whole family. I am proud of you brother. A lot of love to my grandma for being always there with our family and your support.

I would like to dedicate my PhD thesis to my respected father "Mr. Harendra Majumder"

I love you Baba

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Chapter 1 **"**General introduction"
I, RM conceived and wrote this chapter (90%).
Reviewing, editing and improvements were provided by BS, TAC and PWT (10%).

Chapter 2 "Next-Generation Sequencing reveals relationship between the larval microbiome and food substrate in the polyphagous Queensland fruit fly" has been published in *Scientific Reports*

- This chapter was conceptualised and designed by myself (90%) under the supervision of TAC (10%)
- Collection of infected fruits was arranged by myself, however the actual collection was carried out by our collaborators. The processing of samples was carried out by myself (100%).
- COI PCR analysis and identification was completed by myself (100%).
- All 16sDNA sequencing (Sanger sequencing and next generation sequencing including DNA extraction and PCR) was sent to a commercial service provider (The Australian Genome Research Facility Ltd - AGRF).
- All bioinformatics and statistical data analysis was performed by myself (70%), was formatted under the direct teaching and supervision of BS (30%).
- The manuscript was written by myself (80%) with contributions from BS, with reviewing, editing and improvements provided by BS, PWT and TAC (jointly 20%).

Chapter 3 "Fruit host-dependent fungal communities in the microbiome of wild Queensland fruit fly larvae" has been formatted and submitted to *Frontiers in Microbiology (Fungi and their interaction)* (Currently under review)

- This chapter was conceptualised and designed by myself (90%) under the supervision of TAC (10%)
- Collection of infected fruits was arranged by myself, however the actual collection was carried out by our collaborators. The processing of samples was carried out by myself (100%).
- COI PCR analysis and identification were completed by myself (100%).
- All 16sDNA sequencing (Sanger sequencing and next generation sequencing including DNA extraction and PCR) was sent to a commercial service provider (The Australian Genome Research Facility Ltd - AGRF).
- All bioinformatics and statistical data analysis was performed by myself (90%), with the help and guidance of BS (10%)
- The manuscript was written by myself (80%) with contributions from BS, with reviewing, editing and improvements provided by BS, PWT and TAC (jointly 20%).

Chapter 4 "Gut microbiome of the Queensland fruit fly during metamorphosis" has been formatted in accordance with style of *Frontiers in Microbiology*

- This chapter was conceptualised and designed by myself (90%) under the supervision of TAC (10%)
- Collection of infected fruits was arranged by myself, however the actual collection was carried out by our collaborators. Colony setup, sample collection, sterilization, gut dissection and sample processing were carried out by myself (100%).
- COI PCR analysis and identification were completed by myself (100%).
- All 16sDNA sequencing (Sanger sequencing and next generation sequencing including DNA extraction and PCR) was sent to a commercial service provider (The Australian Genome Research Facility Ltd - AGRF).
- All bioinformatics and statistical data analysis was performed by myself (90%) with the help and guidance of BS (10%).

- The manuscript was written by myself (80%) with contributions from BS, with reviewing, editing and improvements provided by BS, PWT and TC (jointly 20%).

Chapter 5 "Artificial larval diet modulates the microbiome of the Queensland Fruit fly during domestication process" has been formatted in accordance with style of *Frontiers in Microbiology*

- This chapter was conceptualised and designed by myself (90%) under the supervision of TAC (10%)
- Collection of infected fruits was arranged by myself, however the actual collection was carried out by our collaborators. Qfly domestication and rearing (from G0 to G5), sample collection, sterilization, gut dissection and sample processing were carried out by myself (100%).
- Qfly fitness traits and behaviour experiments were performed by myself (75%) with help and assistance from SMA (25%).
- All 16sDNA sequencing (Next generation sequencing including DNA extraction and PCR) was sent to a commercial service provider (The Australian Genome Research Facility Ltd - AGRF).
- All bioinformatics and statistical data analysis (microbiome) was performed by myself (90%) with the guidance from BS (10%).
- Data analysis of the fitness and behaviour experiments was performed by myself (80%) and SMA (20%) provided guidance and assistance in performing the statistical analyses.
- The manuscript was written by myself (80%) with contributions from SMA and BCD, with reviewing, editing and improvements provided by BCD, BS, TAC and PWT (jointly 20%).

Chapter 6 "Discussion"

I, RM conceived and wrote this chapter (90%).

Reviewing, editing and improvements were provided by BS, TAC and PWT (10%).

Chapter One

General Introduction

1.1 Queensland fruit fly, *Bactrocera tryoni*

Tephritid fruit flies are the most economically damaging insect pests in many tropical, subtropical and temperate regions around the world (Christenson and Foote, 1960; Hendrichs et al., 2015). Some particularly damaging species are polyphagous, and thus affect production of a wide range of fruit and vegetable crops (Bateman, 1972; Fletcher, 1987). In Australia, the most destructive fruit fly is the Queensland fruit fly ('Qfly'), *Bactrocera tryoni* (Froggatt) (Drew et al., 1982; Clarke and Dominiak, 2010; Fanson et al., 2014). While native to Australia, originally this species was predominately restricted to tropical regions. However, Qfly has now invaded southern areas of eastern Australia, New Caledonia, French Polynesia, Pitcairn Islands and Cook Islands (http://www.spc.int/Pacifly/), infesting more than 100 types of crops across these regions (Drew, 1989; Hancock et al., 2000; Sutherst et al., 2000; Sultana et al., 2017). Recent outbreaks have been reported in Tasmania, (e.g., Flinders Island; https://www.dpipwe.ras.gov.au.) and in New Zealand (Biosecurity New Zealand, 2019; https://www.biosecurity.govt.nz) and "fruit fly free" horticultural regions of South Australia and Tasmania are increasingly under threat of Qfly invasion (figure 1) (Sutherst et al., 2000; Sultana et al., 2017).

Like other holometabolous insects, the Qfly goes through a complete metamorphosis process during its lifecycle. This includes four major life stages: egg, three larval instars, pupa, and adult (Anderson, 1962; 1963a; b). The eggs are oviposited by adult females under the skin of fruits, and it is the larvae, which develop within the fruits, that cause crop destruction. Following larval development (7-8 days), third instar larvae jump from the fruit to the ground. These larvae burrow 2-3 cm underground, where pupation occurs in the soil. Under suitable conditions, the Qfly pupal stage lasts 8-10 days before an adult fly emerges. At maturity, the adult fly is approximately 7 mm long, deep reddish brown in colour and has a bright yellow humeral callus (figure 2) (Hancock et al., 2000). In the wild, Qfly takes 11-12 days to become sexually mature after emergence (Bateman, 1972; Tychsen, 1977; Meats, 1981; Fletcher, 1987). In general, Qfly completes all developmental stages in 3-4 four weeks, and it is estimated to complete around 8 to 10 generations in a year in some regions. However, in temperate regions it may only complete 3-4 generations per year (Bateman, 1972; Meats, 1981)

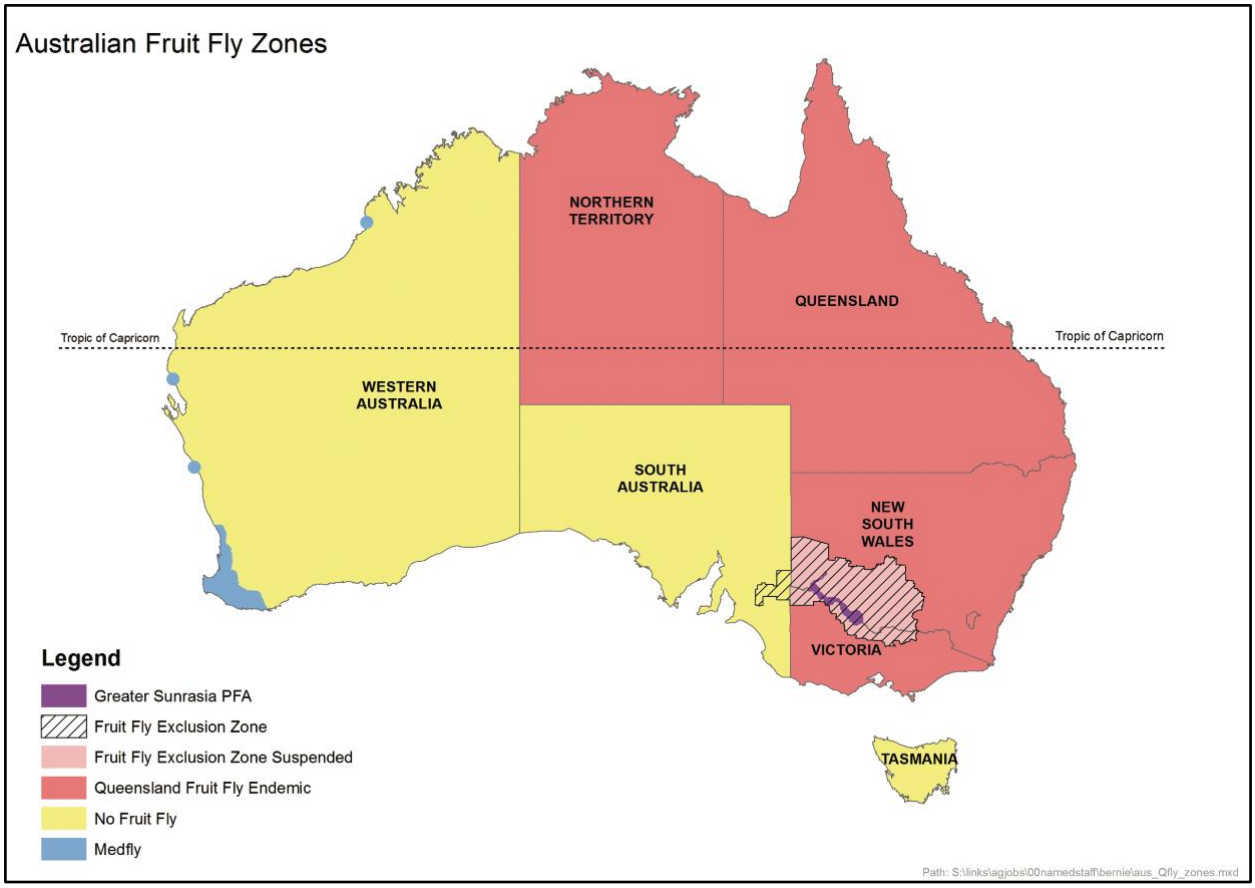

Figure 1 | Distribution of the states in Australia where the Queensland fruit fly *B. tryoni* are observed (Dominiak and Mapson, 2017).

1.2 Pest management and Sterile Insect Technique (SIT)

Cultivated crops which are infested by Qfly are prohibited, through strict quarantine restrictions, from entering domestic and international markets (Clarke et al., 2005; Australia, 2011). As a polyphagous herbivore which infests an extensive range of host vegetables and fruits, this species causes significant losses to Australia's $9 billion-plus per annum horticulture industries (Reynolds et al., 2017). In Australia, approximately $128.7 million were spent between July 2003 and June 2008 on fruit fly monitoring and pest management activities (Plant Health Australia, 2011).

In the past, farmers have largely depended on traditional pest management techniques, for example, pesticides (e.g. dimethoate and fenthion) and repellents (an alternative form of pesticides including tallow, coal-tar, wood-tar, creosote, carbolic acid and vinegar) to control

Qfly and protect cops (Bateman et al., 1966; Jessup et al., 2007; Dominiak and Ekman, 2013; Suckling et al., 2016; Stringer et al., 2017). Other pest control techniques include lure and kill, primarily with protein bait sprays (Lloyd et al., 2003; Balagawi et al., 2014), male annihilation technique (MAT) using cue-lure as a potent male attractant ((Monro and Richardson, 1969; Domimak et al., 2003), natural enemies as biological control agents (Snowball et al., 1962a, b; Snowball, 1966; Ero et al., 2011), sterile insect technique (SIT) (Monro, 1961; Monro and Osborn, 1967; Bateman, 1991; Meats et al., 2003) and fruit destruction (Domimak et al., 2003). The traditional approaches, however, have numerous costs and drawbacks associated with them. For example, pesticides are harmful to the environment and the native flora/fauna. Recently, the production, supply, and use of two major pesticides, Dimethoate and Fenthion, have been greatly restricted by the Australian Pesticides and Veterinary Medicines Authority (APVMA) (Reynolds et al., 2017). Among all mentioned pest management techniques above, SIT is one of the most promising sustainable approaches to control Qfly.

Photo: Australian Horticultural Exporter's association

Figure 2 | Queensland fruit fly (Female), *Bactrocera tryoni* (Froggatt) (aka: "Qfly")

In SIT, flies are reared in a factory, sterilized with irradiation and then released. Sterile male Qfly mate with wild females in the field, inducing reproductive failure (Knipling, 1955; Benelli et al., 2014; Stringer et al., 2017). In North and Central America, SIT has been used successfully to eradicate screwworm (Krafsur et al., 1987; Vargas-Terán et al., 2005).

Further, this technique has successfully supressed or inhibited populations of several fruit flies globally, including Mediterranean fruit fly, or 'Medfly' *Ceratitis capitata* (Enkerlin et al., 2017), Mexican fruit fly *Anastrepha ludens* (Orozco-Dávila et al., 2015), Melon fly *Zeugodacus cucurbitae* (previously *Bacterocera cucurbitae*) in Japan and Oriental fruit fly *B. dorsalis* (Vargas et al., 2010). SIT has been also used to suppress Qfly populations in Australia (Monro, 1961; Monro and Osborn, 1967; Dominiak and Ekman, 2013; Stringer et al., 2017).

SIT was first proposed as a Qfly control strategy in the 1960s, and in 1989, this technique was used successfully to eradicate Qfly in western Australia (Sproul et al., 1992). Despite these numerous successes, however, there is room for improvement of SIT. In some studies, high mortality rates are reported for sterile male flies released into the field, leading to poor SIT performance. This issue is thought to be the result of low-quality sterile male flies being released. Quality control assays with minimum performance criteria have been established to standardise the fitness of SIT flies. In order to meet these parameters, a number of steps have been identified for optimisation, including domestication, diet, fitness of the male files, irradiation dose, handling and transport stress, release methods and field conditions (Chambers, 1977; Enkerlin, 2007; Meats and Edgerton, 2008; Collins et al., 2009; Dominiak et al., 2011).

1.3 Domestication and diet

Insects are commonly reared under artificial conditions, associated with a controlled environment, for many generations. Over generations, insects adapt to the laboratory environment (‘domestication’) (Pérez et al., 2018). There are a number of notable differences between the artificial environment and nature (e.g. temperature fluctuations, disease exposure, variety in diet etc). This results in quantifiable differences between populations of wild and domesticated insects (Chambers, 1977; Cayol et al., 2000; Hoffmann et al., 2001). In particular, domestication can affect insect fitness, mating success, development, and reproduction (Pité, 2000; Diamantidis et al., 2011). However, SIT, and insect research, depends on the domestication of large numbers of insects in controlled, and artificial rearing conditions (Hoffmann and Ross, 2018). Paradoxically, the success of SIT depends on the ability of domesticated insects to survive, mate, and mate in nature, thus motivating an optimisation of the domestication processes.

Previous studies observed that the domestication of tephritid fruit flies has a significant effect on major life history traits, environmental tolerance, and sexual performance (Pérez et al., 2018). Specifically, domesticated flies may have faster development, maturing at much younger ages than wild type flies (Mangan, 1997; Miyatake, 1998; Eberhard, 1999; Briceño and Eberhard, 2002; Meats et al., 2004; Schutze et al., 2015), increased fecundity (Liedo and Carey, 1996; Diamantidis et al., 2011), decreased lifespan (Hernández et al., 2009; Diamantidis et al., 2011), modified diurnal patterns of sexual activity (Moreno et al., 1991), a reduced ability to evade predators (Hendrichs et al., 2007), and lower sexual competitiveness (Rull et al., 2005; Pereira et al., 2007). Moreover, genetic variation has been found to decline in different laboratory populations of the Qfly (Gilchrist et al., 2012).

Diet has been observed to influence the biology and physiology of domesticated fruit flies. For example, diet composition has been shown to affect lifespan, reproductive rate, mating effort, success in both pre- and post-copulatory competition and immune function of the Qfly (Pérez-Staples et al., 2007; Fanson et al., 2009; Fanson and Taylor, 2012). Further, lab-adapted females can become more efficient in converting consumed protein into eggs, when compared with wild females (Bravo and Zucoloto, 1998; Meats et al., 2004). These findings suggest a complex relationship between adaptation to the laboratory, nutrition, and the expression of life-history traits in lab-adapted colonies. As the larval diet is considered to be a crucial component of mass rearing of tephritid flies, significant effort has been invested in the optimisation of larval diet formulations. For several decades, Qfly have been reared using a traditional solid diet containing biological bulking agents such as wheat meal, dehydrated carrot, or lucerne chaff (e.g. Finney, 1956; Steiner, 1966; Meats et al., 2004; Dominiak et al., 2014; Fanson et al., 2014). Most small and moderate-scale rearing uses a carrot-based media, and the recent larger-scale rearing uses a lucerne chaff-based diet (Jessup, 1999; Chang and Cohen, 2009; Fanson et al., 2014; Dominiak and Fanson, 2017; Mainali et al., 2019). Although this diet has a number of benefits, being readily available and economical, as well as being easy to prepare, it has also a number of drawbacks. These drawbacks include that ingredients are difficult and costly to store and handle, and for disposal. Further, variation in the quality of bulking agents can lead to variability in the quality of produced flies (Chang and Cohen, 2009; Dominiak and Fanson, 2017; Mainali et al., 2019). An alternative liquid diet circumvents these drawbacks (Chang et al., 2006; Chang,

2009; Ekesi et al., 2014), however, it relies on physical support substrates to maintain a physical consistency for consumption (Mainali et al., 2019).

Recently, a gel-based artificial larval diet was developed to increase productivity and improve the quality of the Qfly. This diet showed great potential for mass rearing of quality sterile Qfly (Moadeli et al., 2017; Moadeli et al., 2018a; Moadeli et al., 2018b; c). The gel diet formulation contains sugar, yeasts, and wheat germ oil as sources of amino acids, carbohydrate, minerals, sterols, vitamins, and fatty acids. In a study comparing traditional diets (both solid and liquid) with this gel-based larval diet, it has the advantage of physical consistency, as well as better performance outcomes on all quality control parameters for domesticated Qfly (Mainali et al., 2019). However, the performance of this diet on newly introduced wild-type populations, which are in an early stage of the domestication process, is unknown.

1.4 Microbiome of the Qfly

In insects, the gut microbiome has significant influence on health and homeostasis (Dillon and Dillon, 2004; Engel and Moran, 2013). The existence of a symbiotic relationship between tephritid fruit flies and their microbiome has been known for more than 100 years (Petri, 1909; Petri, 1910). Fruit flies have established symbiotic associations with a variety of bacterial and fungal microorganisms (Petri, 1909; Mazzon et al., 2008; Andongma et al., 2015; Morrow et al., 2015; Hadapad et al., 2016). The microorganisms inhabiting the gut of insects may be either harmful or beneficial to their hosts. For example, a beneficial relationship is observed in insects that depend on their gut microorganisms to supply beneficial nutrients and undertake metabolic activities. This situation commonly occurs when the diet of the host insect is plant material, including feeders of plant sap ((Fukatsu and Hosokawa, 2002), wood (Warnecke et al., 2007; Hongoh et al., 2008) and fruit (Petri, 1910). Conversely, a harmful relationship can exist between insects and pathogenic microbes. For example, the bacteria *Providencia*, a gram-negative opportunistic, non-spore forming pathogen (Galac and Lazzaro, 2011) and the fungi *Metarhizium anisopliae* are known insect pathogens (Lu et al., 2015).

As a whole, microbial communities in the gut of insects are known to play a complex role in the health and physiology of insect hosts. These communities enhance food

adaptability (Tsuchida et al., 2008), nutrition (Warnecke et al., 2007), protection against parasites via competition or immune priming through colonization resistance (Vollaard and Clasener, 1994; Koch and Schmid-Hempel, 2011), increase immunity (reviewed in Broderick and Lemaitre, 2012) and toxin degradation (Kikuchi et al., 2012). On the other hand, they can have deleterious effect such as an increase in susceptibility to toxins (Broderick et al., 2006) and enhanced attraction of predators (Leroy et al., 2011).

1.4.1 The bacterial microbiome of the Qfly

Bacterial populations associated with fruit flies play a role in detoxification, immune response, sexual behaviour, reproduction, survival and carbohydrate metabolism (Engel and Moran, 2013; Miller, 2013; Ben-Yosef et al., 2015; Liu et al., 2016; Ras et al., 2017). Specifically, bacteria protect fruit flies by degrading a variety of insecticides (Boush and Matsumura, 1967), by preventing the establishment or proliferation of pathogenic bacteria (Behar et al., 2008a) and enhancing copulatory activity in males and egg production by females (Ben-Yosef et al., 2008). Despite this, most research on the tephritid fruit fly bacterial microbiome has been conducted on the nutritional benefits of these communities. For example, bacterial populations have been found to supply nutrients through the hydrolysis of proteins, synthesis of amino acids and nitrogen fixation (Marchini et al., 2002; Dillon and Dillon, 2004; Behar et al., 2005). Specific examples include studies of *B. oleae* and its associated gut bacteria, which play a vital role in the digestion of green olive, specifically protein hydrolysis (Pavlidi et al., 2017), and in the medfly, where some of its bacterial microbiota are known to be diazotrophic (nitrogen fixers) and pectinolytic (hydrolysers of pectin substances in plants), providing biologically available nitrogen to the host, and accelerating fruit decay, respectively (Behar et al., 2005; 2008a).

Taxonomically, the most abundant bacterial families in fruit flies are associated with two bacterial phyla of Proteobacteria and Firmicutes. Additionally, many of the gut bacteria identified to be beneficial belong to the Enterobacteriaceae family (*Enterobacter, Klebsiella*, and *Pectobacterium* species) (Ras et al., 2017). For example, *Candidatus* Erwinia dacicola has been identified as providing essential amino acids to *B. oleae* larvae, enabling them to develop in unripe olives that contain oleuropein, which inhibits development of other insects (Ben-Yosef et al., 2015). *Candidatus* Erwinia dacicola also increases reproduction in *B. oleae* (Ben-Yosef et al., 2014).

The majority of studies investigating fruit fly gut bacterial communities have focused on the adults (Wang et al., 2011; Aharon et al., 2013; Andongma et al., 2015; Ben-Yosef et al., 2015; Morrow et al., 2015; Yong et al., 2017b; Ventura et al., 2018; Woruba, 2018), although some have also been conducted on larvae (Aharon et al., 2013; Andongma et al., 2015; Ben-Yosef et al., 2015; Yong et al., 2017b; Deutscher et al., 2018; Ventura et al., 2018; Majumder et al., 2019). Bacterial communities in larvae might be influenced by host fruits. These bacteria vertically transmitted from their mother Qfly to larvae during oviposition in infested fruits (Deutscher et al., 2018; Majumder et al., 2019). Previous investigations of the adult and larval stages in parallel did not find any major variation in bacterial classes and families present (Aharon et al., 2013; Andongma et al., 2015). Based on previous studies that have used culture dependent and culture independent techniques, the bacterial family Enterobacteriaceae is considered to be dominant and commonly found in the majority of fruit flies, including *Bactrocera* spp (Fitt and O'Brien, 1985; Lloyd et al., 1986; Capuzzo et al., 2005; Sacchetti et al., 2008; Tsuchida et al., 2008; Thaochan et al., 2010; Estes et al., 2012; Reddy et al., 2014; Wang et al., 2014b; Morrow et al., 2015; Thaochan et al., 2015; Yong et al., 2017a), *C. capitata* (Marchini et al., 2002; Behar et al., 2005; Behar et al., 2008a; Behar et al., 2008b; Ben Ami et al., 2010; Aharon et al., 2013; Morrow et al., 2015) and *Anastrepha* spp. (Kuzina et al., 2001; Ventura et al., 2018). Enterobacteriaceae dominant as the bacteria vertically transferred from adult tephritid females to eggs during oviposition, and then to larvae (Courtice, 1984; Sacchetti et al., 2008; Lauzon et al., 2009; Estes et al., 2012; Aharon et al., 2013). The bacterial family Pseudomonaceae and Acetobacteraceae are two major bacterial families associated with the phylum Proteobacteria. *Pseudomonas* belong to the family of Pseudomonaceae commonly found and abundant as the stable community within the gut of *C. capitata*. However, *Pseudomonas aeruginosa* has been identified as a cause of reduced longevity in *C. capitata* (Behar et al., 2008a). On the other hand, *Acetobacter tropicalis* associated with Acetobacteraceae were detected as a major symbiont in *B. oleae* via a specific end-point PCR but was not found during culture independent technique of 16S rRNA gene amplicon next generation sequencing (NGS) studies (Ben Ami et al., 2010; Ben-Yosef et al., 2014). Except for *A. tropicalis* in *B. oleae,* till now Acetobacteraceae was observed only at minimal levels in adults of other fruit flies (Yong et al., 2017a; Woruba, 2018). Bacterial microbial communities from the Firmicutes are commonly present in the adults of *Bactrocera* spp. For example, *Bacillales* has been identified in *B. zonata* (Reddy et al., 2014), and in *B. oleae* (Estes et al., 2012), and bacteria of the order Lactobacillales have been identified in Qfly (Thaochan et al., 2010; Morrow et al., 2015; Thaochan et al., 2015),

B. minax (Wang et al., 2014a), *B. cacuminata* (Thaochan et al., 2015), *B. neohumeralis* (Morrow et al., 2015), *B. oleae* (Estes et al., 2012) and *B. dorsalis* (Andongma et al., 2015). Firmicutes have not frequently been reported for *C. capitata*, although *Leuconostoc* were recently detected in *C. capitata* (Malacrinò et al., 2018). Lactobacillales tend to be more common in laboratory-reared than field collected *Bactrocera* spp. flies (Morrow et al., 2015).

Few studies have investigated the bacterial communities present in *Bactrocera* species through the process of metamorphosis (Andongma et al., 2015; Yong et al., 2017a). Metamorphosis is a conspicuous and abrupt transformation process in which an insect undergoes a complex remodelling of both of its external and internal morphology, partitioning different developmental stages (Truman and Riddiford, 1999; Grimaldi et al., 2005; Johnston and Rolff, 2015). In Qfly, no complete study of the gut microbiome (both bacteria and fungi) has been performed during metamorphosis of wild type or domesticated flies. Only two studies demonstrated that the composition of bacterial communities is known to be less complex in larval and pupal stages and this gradually increases during adult maturation in some fruit flies (Yong et al., 2017c; Zhao et al., 2018). Despite clear evidence that the microbiome is a major mediator of fitness in fruit flies, substantial knowledge gaps still remain in the physiological and ecological diversity of the Qfly gut microbial community (Thaochan et al., 2010; Morrow et al., 2015; Deutscher et al., 2018; Woruba, 2018; Majumder et al., 2019). General descriptions are available of the bacteria associated with domesticated Qfly larvae (Deutscher et al., 2018), pupae (Fitt and O'Brien, 1985) and adult flies (Thaochan et al., 2010; Morrow et al., 2015; Woruba, 2018), however, a number of key questions remain. For example, how do Qfly larvae acquire their microbial community? What role do fruit hosts play in the establishment of the Qfly larvae microbiome in nature? and which transmission processes (i.e., horizontal or vertical transfer) predominate in shaping Qfly microbiomes through development?

1.4.2 Fungal microbiome of the Qfly

As previously described for bacteria, some fungi form beneficial relationships with insects (Gonzalez, 2014). Some yeasts play a role in food selection for insects, even before the establishment of an association (Stefanini, 2018). The mechanism for this has been demonstrated in numerous beetle species, and involves the production of volatile fermentation products by yeasts, which act as olfactory stimuli to insects (Nout and Bartelt,

1998; Gillott, 2005; Ganter, 2006). Multifaceted relationships have been found between *Drosophila* and yeasts. Yeast not only help the insect to find suitable food, but the presence of yeast in the larval diet boosts larval development (Becher et al., 2012). Fungi and yeast-like fungi have been found to supply organic nitrogen, essential vitamins (thiamin and riboflavin) and lipids to the larvae, and in one instance were shown to improve *Drosophila* larvae health when growth impairment occurred due to sunlight exposure (Bruins et al., 1991). There are also a number of fascinating instances in which insects cultivate fungal gardens, practicing a process called fungal horticulture. For example, Attini ants and Brazilian stingless bees cultivate fungi within their nests (Menezes et al., 2015; Yun et al., 2018), while Ambrosia beetles (Platypodinae, Coleoptera) excavate tunnels in trees to grow their fungal gardens. These insects use the cultivated fungi (a form of vegetative mycelium) as their primary food source (Currie et al., 1999). These fungal gardens also include yeasts (*Candida kashinagacola*, *Ambrosiozyma clade*) (Suh et al., 2013) and represent a source of essential nutrients, such as nitrogen, for Ambrosia beetles (Martin, 1987). Furthermore, yeasts are also beneficial for honeybees, being abundant in nectar and bee bread (a mixture of the honey and bee secretions which provides a rich source of protein) (Sammataro and Yoder, 2011), and are known to affect honeybee fitness (Gilliam and Prest, 1972; Gilliam, 1979; Sandhu and Waraich, 1985).

In addition to nutrition, yeasts play an important broader role in insect behaviour and physiology. For example, yeasts can regulate interactions among insect species. Beekeepers often use newly harvested or after-wintered beehives with sugar supplemented with baker's yeast to support bee development (Stefanini, 2018). Significant amounts of yeasts (*Starmerella meliponinorum*) are present in the bread produced by the stingless meliponine bees, suggesting that they can subsist on this substrate (Teixeira et al., 2003). Indeed, the presence of yeasts (*Saccharomyces* spp.) in bread increases its attractiveness to honeybees (Pain and Maugenet, 1966). Interestingly, significant amounts of yeasts have been detected in healthy adults of stingless bees (*Tetragonisca angustula*) (Teixeira et al., 2003). As a courtship ritual, males of *D. subobscura* give the females a nuptial gift, and the presence of yeasts in the nuptial gift makes the female more fecund (Steele, 1986). Even during oviposition, yeasts play a relevant role, with many *Drosophila* species' females preferring to lay the eggs in substrates containing yeasts (Oakeshott et al., 1989).

Recently, the identification of antagonistic interactions between fungal pathogens and garden yeasts of the leaf-cutting ant *Atta texana*, suggested that insects may exploit yeasts to control diseases (Rodrigues et al., 2009). Various polyphagous insect taxa have close relationships with gut fungal communities, including Lepidoptera, Chrysomelidae (Coleoptera), Curculionidae (Coleoptera), plant-galling Cecidomyiidae (Diptera) and all plant-feeding hemipteran families (Bissett, 1988; Six, 2003; Janson et al., 2008). In phytophagous insects, however, fungal communities are generally not as dominant as bacterial communities in the gut microbiome (Fermaud and Menn, 1989), and relatively few studies have been conducted on this group.

Yeast and yeast-like fungi are known to have active roles in the ecology of tephritid fruit flies (Piper et al., 2017; Malacrinò et al., 2018). More than 700 species of fungi have been identified as entomopathogenic (Hajek and St. Leger, 1994). For example, *Metarhizium anisopliae* fungus is pathogenic to *Drosophila* (Lu et al., 2015) and *Metarhizium anisopliae* (Metschnikoff) Sorokin and *Beauveria bassiana* (Balsamo) Vuillemin (*Deuteromycotina Hyphomycetes*) are pathogenic to adults and pupae of the Mediterranean fruit fly (Lacey et al., 2001; Quesada-Moraga et al., 2006; Almeida et al., 2007; Ortu et al., 2009). Furthermore, *Paecilomyces fumosoroseus* (Wize) Brown and Smith (Deuteromycotina, Hyphomycetes) has been found to reduce fecundity and fertility of the Mediterranean fruit fly (Castillo et al., 2000). Conversely, inactivated yeasts have been applied successfully to fruit fly artificial diets (Cohen, 2015) and are commonly used as fruit fly baits (Bortoli et al., 2016). Yeasts are an essential source of nutrients, and are commonly used in the diets of larvae and adults in the mass rearing of the fruit flies in laboratory and factory facilities. Yeast supplements are routinely provided as a source of nutrients including amino acids (protein), carbohydrates and micronutrients including vitamins, minerals and cholesterol (Nestel et al., 2004; Fanson and Taylor, 2012; Nash and Chapman, 2014; Moadeli et al., 2018a). Cultivable yeasts have been identified in Qfly (Deutscher et al., 2016) and the total gut fungal microbiome has been investigated in wild *B. oleae* (Malacrinò et al., 2015). Developing a better understanding of the fungal microbiome of Qfly may be important in developing strategies for optimising the quality of flies produced during mass rearing. To date, the two studies that have investigated the fungal microbiome of Qfly both used traditional culture-dependent methods to isolate some fungal strains (Deutscher et al., 2016; Piper et al., 2017). These methods, however, are known to have serious technical limitations. The use of advanced next-generation sequencing

has revolutionised microbial community studies but has yet to be applied to the Qfly fungal microbiome.

1.5 Methods for investigating the microbiome of insects

Traditionally, researchers used culture-dependent techniques to profile microbial communities. Unfortunately, the vast majority of microorganisms cannot be cultured or dependency on the host or on other microbial symbionts (co-metabolism etc), greatly limiting the sampling power of these techniques. Additionally, these techniques were biased towards fast-growing microbes that could be grown in isolation, on the media used. For example, only those microbes that are fast-growing under a particular pH might be found in a single assay (Andongma et al., 2015). In addition to pH, microbial growth is also affected by carbon and nitrogen source, media type (liquid/ solid), oxygen concentration and many other factors. Over the past several decades, methodological and technical advancements have revolutionised how microbial communities are profiled. These techniques target "barcoding" genes that allow taxonomic resolution within a specific taxonomical group. For example, in bacteria, the V4 region and other regions of the 16S ribosomal RNA gene is commonly used, while for fungi, the internal transcribed spacer region (ITS) of the 18S ribosomal RNA gene is used (Toju et al., 2012). Typically, these gene regions are targeted and amplified by Polymerase Chain Reaction (PCR) in order to increase their detection using subsequent molecular techniques. These techniques range from enzymatic molecular fingerprinting techniques (e.g., RFLP), hybridisation to oligonucleotides (e.g., Phylochip), and the direct sequencing of PCR products. Each of these approaches mentioned above has its own limitations associated with techniques to identify microbial communities in Qfly are well-documented (Morrow et al., 2015). Next-generation sequencing (NGS) technologies introduce a fast, high-throughput analysis procedure, capable of sequencing millions of DNA molecules in a single assay and allowing comprehensive sampling of complex communities. This approach has been applied to a range of fields (see Andongma et al., 2015; Chen et al., 2016; Ziganshina et al., 2016; Ziganshina et al., 2018). In the context of entomology, NGS has been used for identification of bacterial and fungal microbiomes in various insects including termites, ants, firebugs, beetles and bees (Toju and Fukatsu, 2011; Engel et al., 2012; Hulcr et al., 2012; Köhler et al., 2012; Poulsen and Sapountzis, 2012; Sudakaran et al., 2012; Boucias et al., 2013). In my study, I investigated the bacterial and fungal communities

in Qfly (wild and domesticated samples), along with host fruits, using NGS sequencing of 16s rRNA gene and ITS amplicons.

1.6 Thesis Objectives

It is well established that the gut microbiome plays an essential role in the fitness of tephritid fruit flies. Despite this, a vast knowledge gap remains in understanding the physiological and ecological diversity of the Qfly gut microbial community (Morrow et al., 2015; Deutscher et al., 2018; Woruba, 2018). Key research questions include: how do Qfly larvae acquire their microbial community? What role do fruit hosts play in the structuring of Qfly microbial communities in nature? Furthermore, to date, no complete culture-independent studies have been performed to characterize the bacterial and fungal communities associated with Qfly across all developmental stages of the wild-type Qfly. In addition, it is important to understand the impact of artificial larval diets on gut microbiome along with behavioural and fitness traits of Qfly, in order to reveal effects of domestication and diet on the Qfly quality control parameters before proceeding to industrial-scale mass rearing. Therefore, my PhD research aims are:

I. Comprehensively investigate the microbiome (both bacteria and fungi) of wild Qfly larvae from a range of fruits that have been infested in nature.
II. Explore the effect of fruit host on structure of the Qfly microbial communities.
III. Investigate the role of microbial transmission (vertical and horizontal) structuring these microbial communities.
IV. Identify the dominant gut bacteria and fungi at each of the developmental stages (metamorphosis) of the Qfly from a wild-type representative colony (G0).
V. Compare the microbial communities of domesticated Qfly reared on two different artificial larval diets to understand how larval diet affects gut microbiota diversity and community structure during the domestication process.
VI. Investigate the impact of artificial larval diets on behavioural and fitness traits of the domesticated Qfly colonies.

1.7 Thesis Structure

This thesis is formatted following a 'thesis by publication' structure and includes six chapters. The complete structure of the thesis is briefly outlined below:

Chapter 1 "Introduction" reviews the general identification and economic importance of Qfly in Australian horticulture, recent initiatives on pest control management, Sterile Insect Technique (SIT) (implementation and drawbacks), essential factors such as domestication, diet and knowledge of gut microbiome that can improve the Qfly mass rearing following SIT

Chapter 2 "Next-Generation Sequencing reveals relationship between the larval microbiome and food substrate in the polyphagous Queensland fruit fly" and has been published in *Scientific Reports,* (2019) 9:14292; https://doi.org/10.1038/s41598-019-50602-5

Chapter 3 "Fruit host-dependent fungal communities in the microbiome of wild Queensland fruit fly larvae" and has been formatted and submitted to *Frontiers in Microbiology (Fungi and their interaction)* (currently under review)

Chapter 4 "Gut microbiome of the Queensland fruit fly during metamorphosis" and has been formatted in accordance with style of *Frontiers in Microbiology*

Chapter 5 "Artificial larval diet modulates the microbiome of the Queensland Fruit fly during domestication process" and has been formatted in accordance with style of *Frontiers in Microbiology*

Chapter 6 "Discussion" integrates the research of the preceding chapters and highlights possible future research that would extend the findings of this thesis both for increased knowledge and for potential application.

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Chapter Two

This chapter has been published in Scientific Reports

SCIENTIFIC REPORTS
nature research

OPEN

Next-Generation Sequencing reveals relationship between the larval microbiome and food substrate in the polyphagous Queensland fruit fly

Received: 27 May 2019
Accepted: 16 September 2019
Published online: 01 October 2019

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Insects typically host substantial microbial communities (the 'microbiome') that can serve as a vital source of nutrients and also acts as a modulator of immune function. While recent studies have shown that diet is an important influence on the gut microbiome, very little is known about the dynamics underpinning microbial acquisition from natural food sources. Here, we addressed this gap by comparing the microbiome of larvae of the polyphagous fruit fly *Bactrocera tryoni* ('Queensland fruit fly') that were collected from five different fruit types (sapodilla [from two different localities], hog plum, pomegranate, green apple, and quince) from North-east to South-east Australia. Using Next-Generation Sequencing on the Illumina MiSeq platform, we addressed two questions: (1) what bacterial communities are available to *B. tryoni* larvae from different host fruit; and (2) how does the microbiome vary between *B. tryoni* larvae and its host fruit? The abundant bacterial taxa were similar for *B. tryoni* larvae from different fruit despite significant differences in the overall microbial community compositions. Our study suggests that the bacterial community structure of *B. tryoni* larvae is related less to the host fruit (diet) microbiome and more to vertical transfer of the microbiome during egg laying. Our findings also suggest that geographic location may play a quite limited role in structuring of larval microbiomes. This is the first study to use Next-Generation Sequencing to analyze the microbiome of *B. tryoni* larvae together with the host fruit, an approach that has enabled greatly increased resolution of relationships between the insect's microbiome and that of the surrounding host tissues.

Insects commonly have close relationships with a diverse microbiome that has substantial influence on their ecology and evolution through immunity development, pathogen resistance, gut physiology and fitness at every stage of the life cycle[1–5]. These relationships may be beneficial or harmful to the host health and fitness, depending on the composition of the microbiome[6–9]. Symbiotic and endosymbiotic bacteria can serve as an important source of essential nutrients to their host insects[10–12] and enhance resistance against pathogens, plant defences or pesticides[13–17]. Insect microbial communities often have a positive influence on egg maturation and production, physiological development and survival[2,18,19].

The existence of a symbiotic relationship between tephritid fruit flies and their microbiome has been known for almost 100 years[20,21]. As a prominent example, bacterial symbionts of *Bactrocera oleae* (olive fruit fly) play a vital role in the digestion of green olive, specifically protein hydrolysis[22]. *Candidatus Erwinia dacicola* in the larval microbiome of *B. oleae* provides essential amino acids and enables the larvae to develop in unripe olive that contain oleuropein, which inhibits development of other insects[23]. *Candidatus Erwinia dacicola* also increases reproduction in *B. oleae*[24]. The community of nitrogen fixing bacteria (e.g. Enterobacteriaceae) improves development and reproduction in *Ceratitis capitata* (Mediterranean fruit fly, or 'medfly')[10]. Numerous studies have

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OTU ID	%Fruit	%Larvae	Phylum	Class	Oder	Family	Genus	Species
OTU_1	15.2%	53.1%	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	*Swaminathania/Asaia*	
OTU_3	35.2%	7.0%	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	*Gluconobacter*	
OTU_2	28.5%	9.2%	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	*Gluconacetobacter*	*intermedius*
OTU_6	4.9%	2.0%	Firmicutes	Bacilli	Lactobacillales	Leuconostocaceae	*Leuconostoc*	
OTU_5	1.4%	5.3%	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	*Tatumella*	
OTU_368	3.6%	2.6%	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	*Acetobacter*	
OTU_7	0.0%	5.9%	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae		
OTU_70	0.1%	5.1%	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	*Klebsiella*	*oxytoca*
OTU_92	2.4%	1.9%	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae		
OTU_8	2.6%	1.0%	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	*Acetobacter*	
OTU_53	2.7%	0.4%	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae		
OTU_11	0.0%	2.8%	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	*Providencia*	
OTU_10	0.0%	1.4%	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae		
OTU_9	0.5%	0.9%	Proteobacteria	Gammaproteobacteria	Oceanospirillales	Halomonadaceae		
OTU_174	0.6%	0.1%	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae		
OTU_13	0.5%	0.2%	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	*Frateuria*	*aurantia*

Table 1. Taxonomic identification of the of the 16 most abundant bacterial OTUs in the larvae and fruit.

demonstrated that gut bacteria are associated with digestion, detoxification, immune response, metabolism, sexual behaviour, reproduction and survival in tephritid flies[18,23,25–29].

Bactrocera tryoni (Queensland fruit fly, or 'Q-fly') is a highly polyphagous tephritid fly that is widespread along the east coast of Australia where it is a significant pest of horticulture[30–32]. The most common gut bacterial families identified in *B. tryoni* include Enterobacteriaceae, Acetobacteraceae, Streptococcaceae, and Enterococcaceae[33–36]. These bacteria families are also common in other polyphagous fruit flies, including *B. neohumeralis*, *B. carambolae, B. jarvisi*, and *C. capitata*[9,34,36–38]. Several studies have investigated the bacterial communities of *B. tryoni* larvae and adults, providing partial identification of gut microbes[34,38,39]. A recent study of *B. tryoni* larvae used near full-length 16S analysis as a proof-of-concept study investigating the bacterial populations in the midgut from one type of fruit from different two locations[39]. In addition, pyrosequencing[34,40] and culture dependent methods have been applied to evaluate *B. tryoni* gut bacterial identifications[39]. Experimental techniques and conditions may influence the results of culture-dependent methods[41] and the biases and sampling limitations of techniques used to date to identify microbial communities in *B. tryoni* are well-documented[34]. With the advent of next-generation sequencing techniques we are now able to overcome these technical issues for a more comprehensive investigation of the *B. tryoni* microbial communities[39].

Despite clear evidence that the microbiome is a major mediator of fitness in tephritid flies[36,40,42–44], substantial knowledge gaps remain in the physiological and ecological diversity of the *B. tryoni* gut microbial community[34,38,39]. These knowledge gaps include how *B. tryoni* larvae acquire their microbial community and the ecological interaction between fruit hosts and *B. tryoni* larvae in nature. In the present study, we (i) comprehensively investigate the microbiome of wild *B. tryoni* larvae from a range of fruits that have been infested in nature, (ii) explore the effect of fruit host on structure of *B. tryoni* microbial communities, and (iii) assess the role of vertical transfer structuring these microbial communities. We profiled larval microbial communities by sequencing the 16S ribosomal RNA (rRNA) gene from whole insects using Next-Generation Sequencing (NGS). This technique is ideal for identifying the majority of cultivable and uncultivable microbes and, along with our sampling of multiple host fruit, enables the most comprehensive survey of *B. tryoni* microbial communities to date.

Results

Identification of wild larvae as *B. tryoni*. Sanger sequencing of the COI gene confirmed that all 36 wild larvae, collected from 5 different fruit type/origins were *B. tryoni*. Additionally, ~600 adult flies obtained from the collected fruits and were identified as *B. tryoni* by morphology. No other fly species was identified from the experimental samples.

Profile of *B. tryoni* larval microbiome. A total of 167 bacterial OTUs were detected in *B. tryoni* larvae. These represented 8 phyla, 18 classes, 27 orders, 53 families and 78 genera (Supplementary Data S1, S2). Despite this broad taxonomic range, the majority of these taxa were rare in abundance; only 16 OTUs (~5%) were classed as abundant, i.e. representing $\geq$1% of the microbiome in one or more larvae (Table 1). Further, an average of 97% of the larval microbiome was made up of proteobacterial taxa.

The majority of detected proteobacterial taxa belonged to just two families. The alphaproteobacterial *Acetobacteraceae* represented an average of 75% of the larval microbiome, and the Gammaproteobacterial Enterobacteriaceae represented an additional 21% (Supplementary Fig. 1). The next most abundant family was the Leuconostocaceae, from the phylum Firmicutes, which had an average relative abundance of 2% (Supplementary Fig. 1). Leuconostocaceae taxa represented $\geq$1% of microbiome in only 5 of the 36 larvae, and thus were only sporadically abundant. This contrasts with the alphaproteobacterial Acetobacteraceae and Gammaproteobacterial Enterobacteriaceae taxa, which represented $\geq$1% of the larval microbiome in 35 and 14 of 36 larvae, respectively. At a finer taxonomic resolution, the genus *Swaminathania/Asaia* constituted more than 50% of the larval

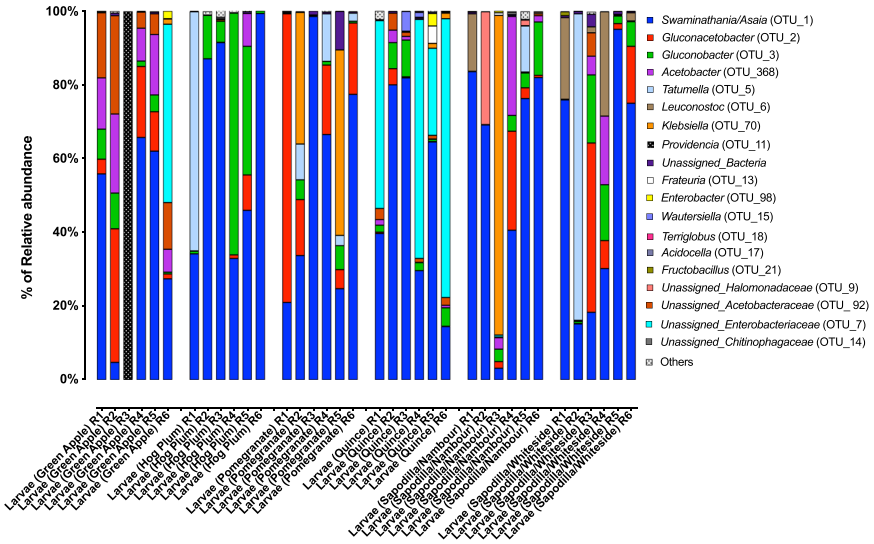


Figure 1. Relative abundance of gut bacterial taxa of *B. tryoni* wild larvae (genus level). The percentage of relative abundance of four or less than are included in "Others". Six wild larvae from five different types of fruits are plotted and R1 to R6 refers to the replicate number of each fruits.

microbiome (53%) and was abundant in 35 of 36 larvae samples (Fig. 1). Other genera representing an average abundance of ≥1% of the larval microbiome included *Gluconacetobacter* (9.1%), *Gluconobacter* (7%), *Tatumella* (5.2%), *Klebsiella* (4.9%), *Acetobacter* (3.8%), *Providencia* (2.8%) and *Leuconostoc* (2%) (Fig. 1, Fig. 2a). These genera, however, were abundant in <50% of the sampled larvae (Fig. 2a).

Larvae from different fruit types have different microbiomes. The microbiome of *B. tryoni* larvae varied among different types of fruit (Fig. 3a). While alphaproteobacterial Acetobacteraceae and Gammaproteobacterial Enterobacteriaceae were the most dominant bacterial families in *B. tryoni* larvae, overall (Supplementary Fig. 1a), the relative abundance of Unassigned Acetobacteraceae and Unassigned Enterobacteriaceae were found to differ significantly (FDR corrected, $P < 0.01$ and $P < 0.05$, respectively) between different types of fruit (Supplementary Data S3). *Acetobacter* ($P < 0.05$) was associated with the larval microbiome from all the types of fruit source except pomegranate. On the other hand, *Providencia* was observed only in one green apple larval sample (replicate 3) (Fig. 1). In addition, we only detected Bacilli (2%) in larvae from sapodilla fruit collected from Nambour, QLD and Whiteside, QLD. Further, only larvae from quince contained *Flavobacteria* (1%) (Fig. 1). Leuconostocaceae and Halomonadaceae were abundant in several larvae from sapodilla. Genus level relative abundance of *Leuconostoc* ($P < 0.001$), *Staphylococcus* ($P < 0.001$) and *Terriglobus* ($P < 0.01$) were significantly different in larvae from the five different fruits host. In addition, principal coordinate analysis (PCoA) of the microbial community structure of *B. tryoni* larvae from different fruits showed that larval microbial composition from green apple and quince were closer in the ordination plot than the other fruits (Fig. 3a).

Microbial communities in fruit samples. In parallel with larval microbiome analysis, samples of the fruit tissues (fruit flesh) from which these larvae were collected, were analyzed for their bacterial microbiome communities. A total of 66 bacterial taxa were detected in fruit samples. 32 fruit samples had sufficient sequencing depth to be included in the current study (>10,000 reads). Of the four samples with fewer reads which were excluded from the study, three were of hog plum and one was of sapodilla from Nambour, QLD. Bacterial taxa detected in fruits represented seven phyla, twenty-seven families and thirty-six genera (Supplementary Data S1, S2). As in the larval microbiome communities, phyla Proteobacteria and Firmicutes were the most abundant, comprising 99.8% of fruit flesh microbial communities. Proteobacteria represented ≥95% of the communities in all fruit samples tested. We detected 4 families with high average relative abundances in fruit samples; Acetobacteraceae (92%), Leuconostocaceae (5%), Enterobacteriaceae (1.8%) and Halomonadaceae (0.5%) (Supplementary Fig. 1b). At the genus level, only seven taxa had an average relative abundance of >1%. These were *Gluconacetobacter* (35.5%), *Gluconobacter* (28.5%), *Swaminathania/Asaia* (15.2%), *Acetobacter* (3.8%), Unassigned Acetobacteraceae (6.3%), *Leuconostoc* (4.9%), and *Tatumella* (1.4%) (Fig. 2b, Supplementary Fig. 2).

The relative abundance of *Gluconobacter* was the highest at 89.1% in hog Plum (Supplementary Fig. 2). This relative abundance was significantly greater than that found in the other fruits (FDR corrected $P < 0.0001$). Similarly, no significant difference of the relative abundance of *Gluconacetobacter* and *Gluconobacter* were observed among green apple, quince, and sapodilla (two locations). Unassigned Acetobacteraceae (FDR corrected $P < 0.0001$), Unassigned Enterobacteriaceae (FDR corrected $P < 0.05$), *Acetobacter* (FDR corrected $P < 0.05$), *Frateuria* (FDR corrected $P < 0.05$) and *Swaminathania/Asaia* (FDR corrected $P < 0.05$) were all significantly difference among fruit types (Supplementary Data S3). A close observation of the microbial community structure of the different fruit types evaluated by the principal coordinate analysis (PCoA) found significant different in microbial composition between fruit types. However, the ordination plot showed that the microbial composition of the few samples from green apple and quince were close and overlapped (Fig. 3c)

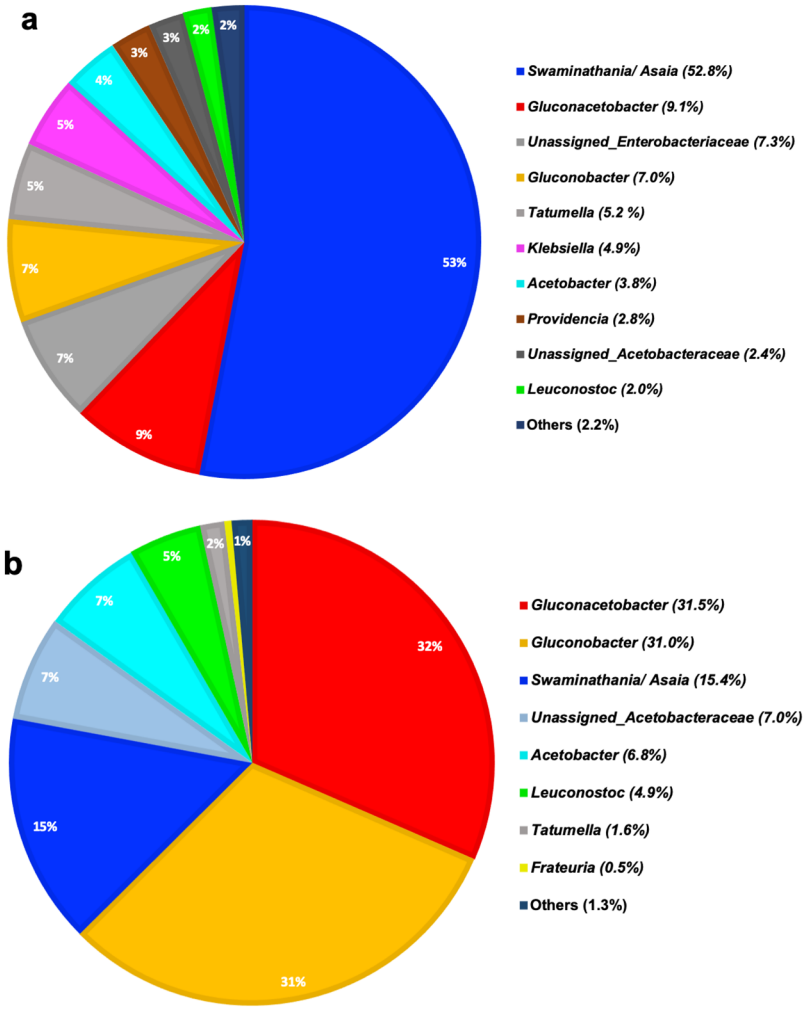


Figure 2. Percentage of mean relative abundance of the bacteria at the genus levels in (**a**) the *B. tryoni* wild larval samples and (**b**) fruit samples.

Larval microbiomes differ from those found in fruits. Biodiversity metrics, including total species, species richness, Pielou's evenness, Shannon's and Simpson's biodiversity indices, did not differ significantly between larval microbiomes and fruit (Supplementary Table 1). However, the Venn diagram showed that the average percentage of unique bacteria present only in larvae (61.7%) was much higher than the average percentage of unique bacteria present only in fruits (10.7%) (Supplementary Fig. 3). The percentage of bacteria present both in larvae and fruits was significantly higher than the bacteria found only in the fruits except in Quince (Fig. 4). The composition of the larval microbiome was significantly different from respective fruit flesh sample communities (PERMANOVA < 0.05, Table 2). This was reflected in the separation of the larval microbiome and bacterial community in the fruit flesh samples in the ordination plot (Fig. 3d). A number of differences in the relative abundance of abundant taxa were observed when comparing larval microbiomes with fruit flesh microbial communities. *Swaminathania/Asaia* was significantly more abundant in larvae compared to the fruits (FDR corrected $P < 0.0001$), while the opposite was observed for *Gluconacetobacter* (FDR corrected $P < 0.05$) and *Gluconobacter* (FDR corrected $P < 0.001$) (Fig. 2a,b, Supplementary Data S3). Surprisingly, two abundant bacterial genera, *Tatumella* and *Klebsiella*, were commonly observed in larvae but were rare in fruit (Fig. 2a,b). Unassigned bacteria (FDR corrected $P < 0.001$) and Unassigned proteobacteria (FDR corrected $P < 0.0001$) were found to be significantly different in relative abundance among fruits and larvae.

Geographic location did not influence larval microbiome. Principal coordinate analysis and PERMANOVA tests both indicated that bacteria in sapodilla fruits and larvae did not differ between geographic locations (PERMANOVA test, fruit $P = 0.151$, larvae $P = 0.094$; Fig. 3b). Otherwise, the microbial community differed significantly among fruit types (Table 2, Fig. 3c). In contrast, PERMANOVA analysis indicated that microbiome of *B. tryoni* larvae from different fruit sources had similar bacterial composition except for those from pomegranate (Table 2). However, significant variation in microbiome was observed between larvae from the same fruits (Table 2, Fig. 3a).

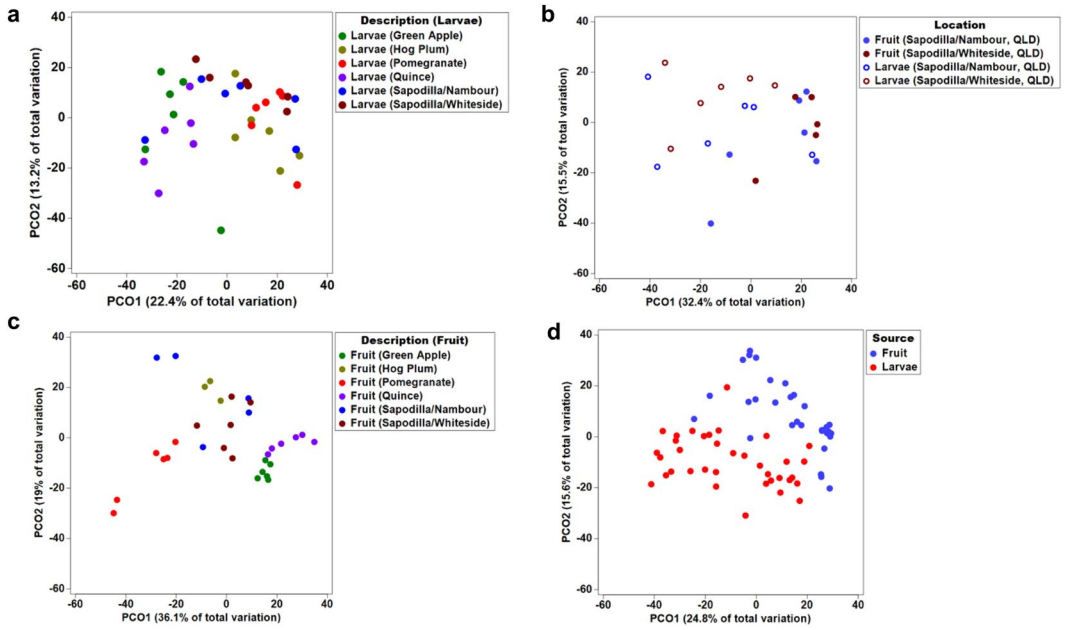
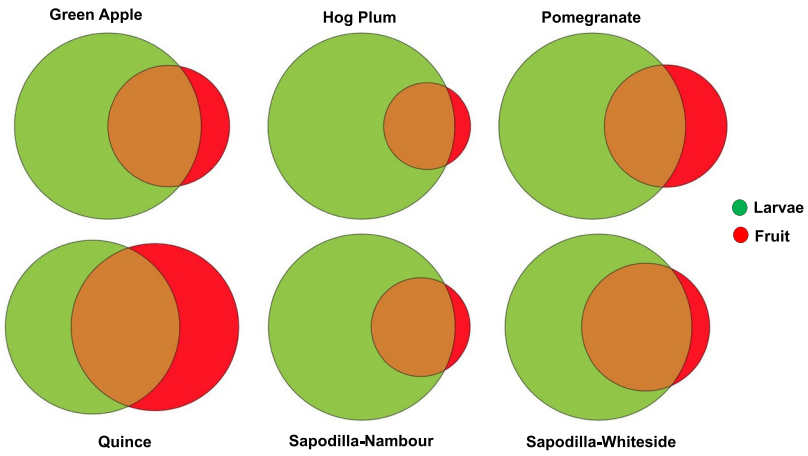


Figure 3. Principal co-ordinate analysis (**a**) the larval gut bacteria of *B. tryoni* from five type of fruit sources; (**b**) Bacterial community composition of the *B. tryoni* larvae collected from 2 different location (Sapodilla); (**c**) Bacterial community composition in the five different fruit; (**d**) bacterial population between larvae and fruit. Different color point indicates different fruit type and the larvae respectively.



Source name	Larvae (only)	Fruit (only)	Both
Green Apple	61.4%	9.1%	29.5%
Hog Plum	79.0%	3.2%	17.7%
Pomegranate	62.7%	13.7%	23.5%
Quince	33.9%	28.6%	37.5%
Sapodilla (Nambour)	72.9%	3.4%	23.7%
Sapodilla (Whiteside)	55.6%	5.6%	38.9%

Figure 4. Venn diagram of the percentage of the bacteria present in the larvae only, fruits only and common in both collected from five different types of fruit in the wild.

Discussion

This study presents comprehensive data on the microbiome of *B. tryoni* larvae collected from fruits that were infested in nature. By sampling from five different fruit types, we are able to explore meaningful ecological questions regarding the effect of host fruit on microbiome communities, while the inclusion of fruit flesh samples allowed us to explore the role of horizontal transfer in the microbial colonization. This is the first microbial survey to assess the microbiome of *B. tryoni* larvae from different types of fruits together with parallel assessment of the host fruit microbial community. We found that the larval microbiome composition differed substantially from the

	Fruit (Green Apple)	Fruit (Hog Plum)	Fruit (Pomegranate)	Fruit (Quince)	Fruit (Sapodilla/ Nambour)	Fruit (Sapodilla/ Whiteside)	Larvae (Green Apple)	Larvae (Hog Plum)	Larvae (Pomegranate)	Larvae (Quince)	Larvae (Sapodilla/ Nambour)	Larvae (Sapodilla/ Whiteside)
Fruit (Green Apple)												
Fruit (Hog Plum)	0.012											
Fruit (Pomegranate)	0.004	0.012										
Fruit (Quince)	0.001	0.012	0.002									
Fruit (Sapodilla/ Nambour)	0.003	0.111	0.003	0.002								
Fruit (Sapodilla/ Whiteside)	0.003	0.04	0.003	0.004	0.174							
Larvae (Green Apple)	0.003	0.017	0.001	0.003	0.011	0.006						
Larvae (Hog Plum)	0.002	0.015	0.002	0.005	0.005	0.006	0.003					
Larvae (Pomegranate)	0.003	0.015	0.002	0.007	0.003	0.002	0.003	0.007				
Larvae (Quince)	0.001	0.007	0.002	0.009	0.006	0.006	0.059	0.002	0.004			
Larvae (Sapodilla/ Nambour)	0.005	0.028	0.002	0.002	0.086	0.004	0.028	0.044	0.007	0.02		
Larvae (Sapodilla/ Whiteside)	0.002	0.013	0.002	0.002	0.004	0.004	0.004	0.004	0.006	0.001	0.483	

Table 2. PERMANOVA test (P values) from Pair-wise tests to compare the variation of the bacterial community between five different fru it and their larvae (*B. tryoni*).

microbial community of the fruit that larvae were obtained from. Our findings suggest that the larval gut acts as a strong environmental filter, and while there was overlap in microbial community of the fruit and larvae, taxa that were abundant in fruit were not necessarily abundant in the larvae. Despite substantial variation in the microbial community of individual *B. tryoni* larvae, the most abundant taxa in the larvae were consistent across the different fruit sources. Thus, the differences detected in PERMANOVA were driven by low abundance taxa within the larval microbiome. Our study suggests that the microbial communities inside the fruits strongly influence the structure of bacterial communities present in the *B. tryoni* larvae.

Analysis of the microbiome of *B. tryoni* larvae revealed that the bacterial community in the larvae was dominated by one phylum, Proteobacteria, with 97% of the total sequences assigned to these taxa. Phyla Proteobacteria and Firmicutes have previously been reported as common in the midgut of *B. tryoni* larvae collected from peach fruits in the field and in domesticated colonies[39], as well as in other fruit flies[40,45] and in other insects, including butterflies[46] and mosquitoes[47].

Seventy five percent of average bacterial relative abundance was from the family of alphaproteobacterial Acetobacteraceae. Fruits are an abundant source of sugar, and insects emerging from fruits commonly host acetic acid bacteria[48]. We observed a high abundance of five bacterial genera - *Swaminathania/Asaia, Gluconacetobacter, Gluconobacter, Acidocella*, and *Acetobacter* - among others in the larval microbiome. Other studies have also observed Acetobacteraceae in *B. oleae*[49], *Apis mellifera mellifera* (Hymenoptera: Apidae) (Honeybee)[50,51], *Saccharococcus sacchari* (Cockerell) (Homoptera: Pseudococcidae) (Pink sugar cane mealybug)[52], and *Drosophila*[53]. The alphaproteobacterial Acetobacteraceae helps to break down and digest complex glucose structure and lipid content present in larval diet[54]. *Swaminathania/Asaia* and *Acetobacter* are the two key bacteria commonly found in the gut of insects[26,48]. Previous studies have found that *Acetobacter pomorum* and *Swaminathania/Asaia* provide nutrients that improve larval development of *Drosophila* and *Anopheles gambiae* (Mosquito)[55,56]. Shin *et al.*[56] demonstrated that *A. pomorum* in *Drosophila melanogaster* stimulates insulin growth factor signaling to maintain metabolic homeostasis and physiological development by pyrroloquinoline quinone-dependent alcohol dehydrogenase (PQQ-ADH) activation. The addition of acetic acid as a metabolic product of PQQ-ADH in the diet may improve the metabolic homeostasis of flies. Previous studies report that *Swaminathania/Asaia* sp. produces acetic acid which is involved in nitrogen-fixing and improves metabolic homeostasis of *B. tryoni*[39,57]. Surprisingly, low populations of *Swaminathania/Asaia* were observed in wild *B. tryoni* adults[34], as well as in *B. oleae*[58]. In our study, the high dominance of *Swaminathania/Asaia* of 52.9% average relative abundance was observed in the presence of *Gluconacetobacter* and *Gluconobacter* in all 36 larvae samples. This suggests that Acetobacteraceae plays an important role in *B. tryoni* larvae.

The family of Gammaproteobacterial Enterobacteriaceae had an average relative abundance of 20.5% in *B. tryoni* larvae. *Enterobacter, Klebsiella*, and *Taumella*, are all major bacterial genera (average relative abundance > 0.1). From mammals to insects, Gammaproteobacterial Enterobacteriaceae commonly have mutualistic relationships in the host gut[26,59]. *Enterobacter* and *Klebsiella* have been reported in four *Bactrocera* species - *B. tryoni, B. neohumeralis, B. jarvisi*, and *B. cacuminata* - by using both pyrosequencing[34] and culture dependent methods[60]. Deutscher *et al.*[39] suggested that Gammaproteobacterial Enterobacteriaceae are crucial for survival of the larvae, transmitted vertically in *B. tryoni* and other tephritid. Further, these bacteria enhance metabolic activities in *C. capitata* and *B. oleae* larvae to support nitrogen fixation and pectinolysis[24,61]. Previous study also found Gammaproteobacterial Enterobacteriaceae enable the host *B. oleae* larvae to survive inside unripe olive fruits[24]. In *B. oleae* and *C. capitata* mass rearing programs using artificial larval diets, strains of Gammaproteobacterial Enterobacteriaceae have been added to the diet to improve pupal weight and mating performance, and decrease developmental time[62–66]. In contrast to beneficial bacteria, pathogens have also been

reported including *Providencia* which is able to cause infection in *C. capitata*[67] and *D. melanogaster*[68]. *Providencia*, a gram-negative opportunistic, non-spore forming pathogen[69], has been observed and isolated from many other insects including *Lucilia sericata* (Diptera: Calliphoridae) (Blow fly)[70], *Stomoxys calcitrans* L. (Diptera: Muscidae) (Stable fly)[71], *Anastrepha ludens* (Diptera: Tephritidae) (Mexican fruit fly)[72] and *B. oleae*[49]. In *D. melanogaster*, a strain of *Providencia*, *P. sneebia*, caused host mortality because of its ability to avoid detection by the insect host's immune system[68]. In the present study, *Providencia* was found only in one larva from a single green apple (Fig. 1).

The family Leuconostocaceae was observed in *B. tryoni* larvae along with the genera *Leuconostoc* and *Fructobacillus*. Although not many studies have reported lactic acid bacteria in insects, especially in tephritid, *Leuconostoc* has also been reported in adults of other tephritid including *C. capitata, B. neohumeralis, B. tryoni* and *B. cacuminata*[34,38,42]. Furthermore, in *Drosophila, Leuconostoc* has been identified in both wild and domesticated populations[73,74]. In our analysis, *Leuconostoc* was observed in all of the larval samples collected from sapodilla in Nambour, QLD and in Whiteside, QLD. In contrast, *Fructobacillus* was only observed in the larvae collected from sapodilla in Whiteside, QLD. This finding suggested that the *Leuconostoc* may be transmitted horizontally from the sapodilla fruit to the larval gut. We also found Weeksellaceae, Xanthomonadaceae, Halomonadaceae, Acidobacteriaceae, and Chitinophagaceae in some larvae samples. Previous studies detected Xanthomonadaceae in soil and plant samples[75,76]. Weeksellaceae has been found to be a dominant element of the microbiome in *B. carambolae* larvae and pupae[36] and *Colaphellus bowringi* (Coleoptera: Chrysomelidae) (Cabbage beetle)[28].

The bacterial community in green apple, hog plum, pomegranate, quince, sapodilla (Nambour, QLD) and sapodilla (Whiteside, QLD) was not different in total bacterial load or species richness, but differed in composition, which is not unexpected given the fruit composition. The genera *Gluconacetobacter* (35.5%), *Gluconobacter* (28.5%), *Swaminathania/Asaia* (15.2%), *Leuconostoc* (4.9%), *Acetobacter* (3.8%) and *Tatumella* (1.4%) were observed in all of the fruit samples. The microbial communities in host fruits depends on a wide diversity of influences including fruit physiology, larval density, and environment conditions[53,77]. Previous studies reported that female tephritid fruit flies (e.g. *B. oleae* & *C. capitata*) inoculate fruit with bacteria from the family of Gammaproteobacterial Enterobacteriaceae during oviposition[42,58,78]. In our investigation, only a few bacterial families dominated including Acetobacteraceae (91.9%), Leuconostocaceae (5%), Enterobacteriaceae (1.8%) and Halomonadaceae (0.5%). The infestation by *B. tryoni* and the over-ripe status of the fruit might be why we observed 91.9% of the average relative abundance of the Acetobacteraceae and its associated bacterial genera of *Gluconacetobacter* (35.5%), *Gluconobacter* (28.5%) and *Swaminathania/Asaia* (15.2%). The variation of bacterial population among types of fruits could arise from the level of decomposition that occurred during transportation of samples to the laboratory and holding of infested fruits until the larvae reached 3rd instar. Although we performed PERMANOVA analysis to observe difference in bacterial community structure among fruit types, no difference was found in sapodilla between the two sampled sites. While numerous comparisons of fruit types across multiple regions would be required for a detailed analysis, the available data suggest that fruit type may be a greater influence than geographic region in determining fruit microbial communities. In our study, we observed the presence of *Gluconacetobacter* and *Gluconobacter* bacteria both in green apple and quince without any significant difference in number. In contrast, overall bacterial relative abundance was significantly different between these two fruits.

We expected to find a correlation between microbial communities of larvae and their host fruit but, found very little evidence of such relationship. The bacterial community structure in larvae was significantly different not only from the same type of fruit, but to the other types of fruits as well. The only exception was found in sapodilla from Nambour, QLD. Yun *et al.*[41] found remarkable variation in the bacterial community of insects (e.g. Proteobacteria and Firmicutes) depending on the host environment. Gut bacterial relative abundances may vary with the natural surroundings and the associated oxygen level of the insect (e.g., wood feeding termites[79]). These observations are relevant to our findings. We found that there was no significant variation in the microbiome of *B. tryoni* larvae sampled across different types of fruit. The PERMANOVA pair-wise test did not detect significant variation in the basic bacterial community structure of larvae from different host fruit. Although there was a significant difference in bacterial relative abundance between green apple and quince fruits, no significant variation was present in the larval microbiome from these fruits. This could be related to both fruits being members of the Rosaceae. Previous studies of *B. oleae* and *C. capitata* found very low gut bacterial diversity in larvae collected from the field[24,42]. However, in *C. capitata* bacterial diversity was much higher in pupae and adults than in larvae[42]. Also, larvae of *B. dorsalis*[40] and *B. carambolae*[36] have also been reported to have substantially greater bacterial diversity than *B. oleae* and *C. capitata*. Gut bacterial diversity in larvae is generally less than the adults in insects. We found that the microbiome is very simply structured in wild *B. tryoni* larvae. It might be that the bacterial diversity is lower in *B. tryoni* larvae compared to the adults; further study of changes in bacterial diversity through *B. tryoni* metamorphosis will be required to assess this possibility.

Larvae may acquire bacteria from the fruits to develop the gut community. Diet has a significant effect on the gut microbiome composition in other insects[80], including *Helicoverpa armigera* (Lepidoptera: Noctuidae) (Cotton bollworm)[81], *Lymantria dispar* L. (Lepidoptera: Lymantriidae) (Gypsy Moth)[1] and *Heliconius erato* (Butterfly)[82]. We asked whether the bacterial microbiome of *B. tryoni* larvae comes from the host fruit and tested the difference in the bacterial communities of larvae and their host fruits. Based on principal coordination analysis and PERMANOVA tests, we found that the larval bacterial community (mostly from the gut) was significantly different from that of the host fruit. We further observed that the larval microbial community contains common bacteria of *Asaia, Gluconacetobacter, Gluconobacter*, and *Acetobacter* which were present in all larvae and fruit samples. However, the percentage of independent bacteria was significantly higher in larvae compared with each type of fruit (Supplementary Fig. 3). The vast majority of microbial taxa detected in larvae are not found in the fruit. Microbial communities associated with larvae were significantly more diverse than those of fruit. Previous studies have demonstrated that female tephritid fruit flies transmit gut bacteria during oviposition in the areas

Geographic location of collection	Fruit source and number of fruits collected	Collection date
Maroochy Research station, Nambour, QLD GPS: Lat 26°38′34.92", Long 152°56′22.99"	Hog Plum 26 pieces	1/02/17
Daboro Road, Whiteside, QLD, 4503. GPS: Lat 27°14′29.31", Long 152°55′8.49"	Sapodilla 52 pieces	1/02/17
Maroochy Research station, Nambour, QLD GPS: Lat 26°38′34.92", Long 152°56′22.99"	Sapodilla 68 pieces	1/02/17
Coomealla, NSW GPS: Lat 34° 5′50.97", Long 142° 3′7.21"	Pomegranate 37 pieces	5/05/17
St. Germains, Between Tatura and Echuca in Victoria GPS: Lat 36°10′48.86", Long 145° 8′50.74"	Green Apple 41 pieces	05/05/17
Downer road between Tatura and Toolamba in Victoria GPS: Lat 26°38′34.92", Long 152°56′22.99"	Quince 52 pieces	05/05/17

Table 3. Fruit types and origin for wild *Bactrocera tryoni* larvae collection. A total of six replicate larvae, and fruit flesh samples were collected from each fruit origin.

where the fly previously regurgitated[83]. This suggests that, as has been suggested previously[10,20,39,63], bacteria are transmitted vertically from the mother to the egg, and then larvae, during oviposition, and remain quite isolated from the surrounding host tissues.

Conclusion

The present study is the first detailed investigation of relationships between the bacterial ecology of *B. tryoni* larvae and their host fruit in nature. The abundant bacterial taxa in larvae were highly consistent across fruit types and geographic regions despite significant variation in overall bacterial microbiome composition.

Methods

Collection of larvae. *Bactrocera tryoni* larvae were collected from infested fruits at various geographic locations in New South Wales (NSW), Victoria (VIC) and Queensland (QLD), Australia (Table 3). All infested fruits were collected from under trees, and most were over-ripe. The fruit types collected included hog plum, sapodilla (from two different locations), pomegranate, green apple and quince (Table 3). The infested fruits were stored on racks in 60 L plastic bins (Award, Australia) that contained 250 g of fine vermiculate (Grade 1, Sage Horticultural, VIC, Australia) in a controlled environment laboratory (25 ± 0.2 °C, 65 ± 3% RH and 11 h: 1 h: 11 h: 1 h light: dusk: dark: dawn photoperiod). Samples of different fruit types and origins were kept separate to prevent cross-contamination. A total of 36 3rd instar *B. tryoni* larvae were collected from each of six replicate fruits from each of the five fruit types (see Table 3). Furthermore, six replicate samples of fruit tissues (fruit flesh) (1~2 mg mass) were collected from the same fruit from which larvae were collected.

Sample preparation. Upon collection, *B. tryoni* larvae were surface sterilized using 0.5% Tween 80 (Sigma-aldrich, Cat. No. 9005656), 0.5% Bleach (Sodium hypochlorite) (Sigma-Aldrich, Cat. No.7681529) and 80% Ethanol (Sigma-Aldrich, Cat. No. 65175) for 30 s, and rinsed 3 times in 1 M sterile phosphate-buffered saline (1x PBS) again for 30 s, following[39]. The PBS from the 2nd and 3rd washes were kept and 100 µL was spread-plated onto five types of microbial growth medium (de Man, Rogosa and Sharpe Agar, Tryptone Soya Agar, Macconkey Agar, Potato Dextrose Agar and Yeast-dextrose Agar medium) to check the performance of the sterilization method. All plates were incubated at 35 °C for 24 to 48 hr. Post sterilization, whole larvae were crushed using sterile pestles (Fisher Scientific, USA) and stored with Brain Heart Infusion (BHI) broth with 20% Glycerol solution and split into two separate cryovial tubes for COI gene identification and next generation sequencing analysis. All the samples preserved at −80 °C. Flesh from individual fruits was also preserved and stored under the same conditions. All procedures were completed in a sterile environment (Biological Air Clean Bench, safe 2020 1.2, Thermo Scientific, Germany).

Identification of larvae using mitochondrial Cytochrome Oxidase I (COI) gene. Identification of larvae was confirmed by sequencing the mitochondrial cytochrome oxidase I (COI) gene of all larval samples. DNA was extracted from crushed whole larvae using the Isolate II genomic DNA kit from Bioline (Cat. no. BIO-52066) following the manufacturer's protocol. DNA extract concentrations were then determined using the Invitrogen™ Qubit® dsDNA High Sensitivity (HS) Assay Kit (Life Technologies, USA). Standard LCO1490 /HCO2198 primers were used to amplify a 700 bp segment of the CO1 gene[84]. All PCR amplifications were performed in an Eppendorf thermocycler (Mastercycler, epgradient S, Eppendorf, Germany). Each 15 µL reaction was conducted in triplicate and contained 7.5 µL of MyTaq HS PCR master mix (Bioline, USA. Cat No. BIO-25045), 0.60 µL of forward (LCO1490F) and reverse primer (HCO2198R), and 1.5 µL of DNA extract of larval sample. The PCR profile included an initial denaturing step at 95 °C for 2 min, followed by 35 cycles of 94 °C for 30 s, 50 °C for 30 s and 72 °C for 90 s, and a final extension step of 72 °C for 5 min. Amplicons were visualised using electrophoresis on a 1% agarose gel (110 v, 45 min). Amplicons were then sent to the Australian Genomic Research Facility (AGRF) for Sanger sequencing. Sequence data were analysed by Geneious R10.2.3 to confirm that all larvae were *B. tryoni*. In addition to this molecular confirmation, microscopic examination of larval morphological features was carried out prior to DNA extraction[85]. Additional confirmation was gained through

stereomicroscopic (Leica MZ6 stereo-microscope, Germany) assessment of adult flies that developed from the larvae remaining in the infested fruits that larval and fruit flesh samples were obtained from[86].

Microbiome profiling. DNA extraction of the larvae samples for NGS analysis was completed using DNeasy Power Lyzer Power Soil Kit-100 (Qiagen, Germany) (Cat. no. 12855-100) following the manufacturer's protocol. DNA extracts were then quantified by Invitrogen™ Qubit® dsDNA High Sensitivity (HS) Assay Kit (Life Technologies, USA). PCR amplification and sequencing were performed by the Australian Genome Research Facility. Briefly, the V1-V3 16S rRNA region was amplified using primers 27 F (5′AGAGTTTGATCMTGGCTCAG-3′) and 519 R (3′ GWATTACCGCGGCKGCTG-5′). Amplification conditions were as in Fouts *et al.*[87] with slight modifications. Briefly, reactions contained 1X AmpliTaq Gold 360 mastermix (Life Technologies, USA), 0.20 µM of forward and 0.20 µM reverse primers and the total of 25 µL with DNA extract. PCR cycling conditions consisted of denaturation at 95 °C for 7 minutes, 35 cycles of denaturation at 94 °C for 45 s, annealing at 50 °C for 60 s and extension at 72 °C for 60 s, and a final extension of 72 °C for 7 minutes. A secondary PCR was used to adhere sequencing adaptors and indexes to the amplicons. Primerstar max DNA Polymerase used for secondary PCR amplicon generation from Takara Bio inc., Japan (Cat. No. #R045Q). The resulting amplicons were measured by florometry (Invitrogen Picogreen, Thermo Fisher Scientific, Australia) and normalized. The eqimolar amounts of each sample were pooled and quantified qPCR prior to sequencing (Kapa qPCR Library Quantification kit, Roche, Switzerland). The resulting amplicon library was sequenced on the Illumina MiSeq platform (San Diego, CA, USA) with 2 × 300 base pairs paired-end chemistry[88].

Sequence data processing. Paired-end reads were assembled by aligning the forward and reverse reads using PEAR (version 0.9.5)[89]. Primers were identified and trimmed. Quality filtering, clustering and taxonomic assignments were achieved using the 'usearch' tools[90,91] and rdp gold database as a reference (Ribosomal database project, https:// rdp.cme.msu.edu). The OTUs with taxonomic assignments to eukaryotic organelles (e.g., choloroplast) were removed from the dataset. We performed rarefaction to 10,000 reads per sample, repeating this 50 times and averaging the counts to obtain a representative rarefaction. This was achieved using an in-house python script. Those samples that had <10,000 reads were deleted. The data were then normalised as the percentage of relative abundance and is referred to as the OTU table (Supplementary Data S1). All the figures of the bacterial relative abundance in *B. tryoni* larval and fruit samples at different taxonomic levels were plotted in Microsoft excel version 16.18.

Statistical analysis. An OTU table, which contained the number of read counts for each OTU detected for each sample was imported into Primer-E v7[92,93] for analysis. In brief, all statistical testing was performed on fixed factors associated with fruit host (hog plum, sapodilla (from two different localities), pomegranate, green apple and quince) from which 6 replicates were collected. The DIVERSE function was used to generate univariate biodiversity metrics, including total species, species richness, Pielou's evenness and Shannon's and Simpson's biodiversity indices. Statistical differences between these metrics were assessed by one-way analysis of variance (ANOVA) and Tukey-Kramer post hoc analysis (see Supplementary Table 1) using JMP Statistical Software Version 10.0.0 (SAS Institute, Cary, NC, USA). To observe the taxonomic compositional differences amongst 16 s rRNA communities, the OTU table was first log transformed using Primer-E V7. A Bray-Curtis similarity matrix was derived from this transformed data and a permutational analysis of variance (PERMANOVA) pair wise comparison was conducted to compare all community samples. A p value of <0.05 was considered statistically significant. Ordination plots of these communities were visualised using principal coordinates analysis (PCoA) in Primer-E.

To explore which taxa were driving compositional differences between microbial communities from different groups, genera were investigated for statistically significant differences in their relative abundances. Relative abundance values were first arcsine square root transformed[94]. Subsequent statistical analyses were carried out using an in-house Python script, with the SciPy[95] and statsmodels[96] packages. Briefly, a t-test was used to compare relative abundances of genera between total larval microbiome communities and total fruit flesh microbial communities. A Benjamini–Hochberg adjustment to the resultant p value was made to adjust for false-discovery rate errors (FDR). FDR adjusted p-values of <0.05 were considered significant. ANOVA was used to compare genera relative abundances in larval microbiome communities from different fruit type/origin[97]. The resultant p value was again adjusted for FDR, and a posthoc Tukey's Honest Significant Difference test (Tukey's HSD) was used to test for significant pair-wise comparisons. This same approach was undertaken to compare genera in fruit flesh microbial communities from different fruit types/origins (Supplementary Data S3). Venn diagram plots for each fruit type were created using R 3.2.2 (R Development Core Team 2017). Percentage of the bacteria present in larvae and fruit samples in Venn diagrams were analysed using in the R package eulerr[98].

Data Availability

All sequences are publically available in NCBI under Bio-project PRJNA528521.

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Acknowledgements

This research was conducted as part of the SITplus collaborative fruit fly program. Project Raising Q-fly Sterile Insect Technique to World Standard (HG14033) is funded by the Hort Frontiers Fruit Fly Fund, part of the Hort Frontiers strategic partnership initiative developed by Hort Innovation, with co-investment from Macquarie University and contributions from the Australian Government. Rajib Majumder is supported by an International Macquarie University Research Excellence Scholarship (iMQRES) from Macquarie University (NSW, Australia). Special thanks to Dr. Ania Deutscher for her support on fruit samples collection and larval samples preparation. Dr. Bernie Dominiak reviewed and improved an earlier version of this manuscript.

Author Contributions

R.M. and T.C. designed the experiment. R.M. collected the data. R.M. and B.S. analysed the data. T.C. and P.W.T. supervised the project. All authors analysed the data, provided inputs into the writing of the manuscript, and approved the submitted version.

Additional Information

Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-019-50602-5.

Competing Interests: The authors declare no competing interests.

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SUPPLENTARY FIGURES AND TABLES

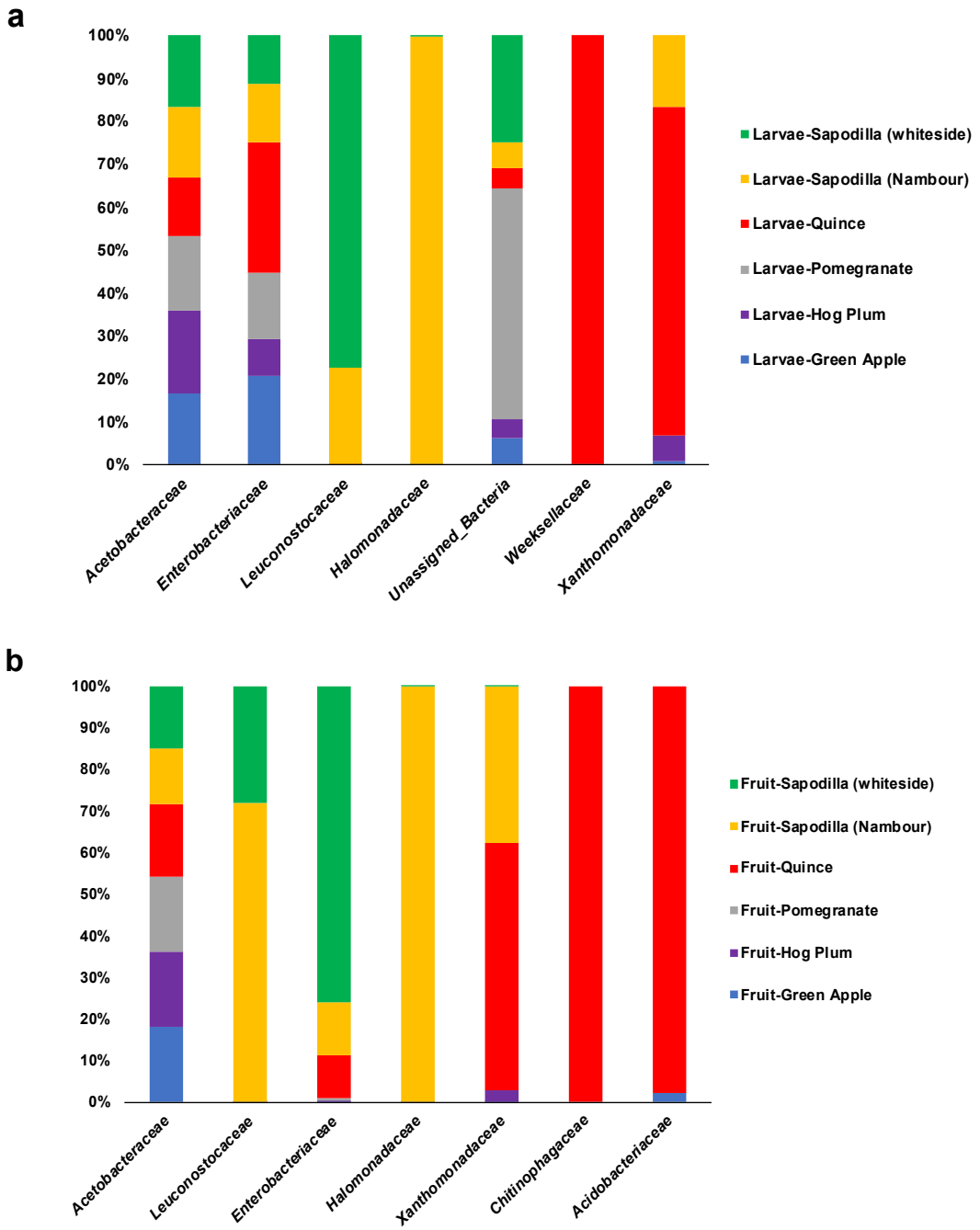

Figure S1. Percentage of mean Relative abundance of the bacteria at the family levels in samples of *B. tryoni* wild larvae and fruit samples; (a) relative abundance of bacteria at the family levels in samples of *B. tryoni* wild larvae obtained from five different types of fruit in the wild. (b) relative abundance of bacteria at the family levels in samples of five different types of the fruit

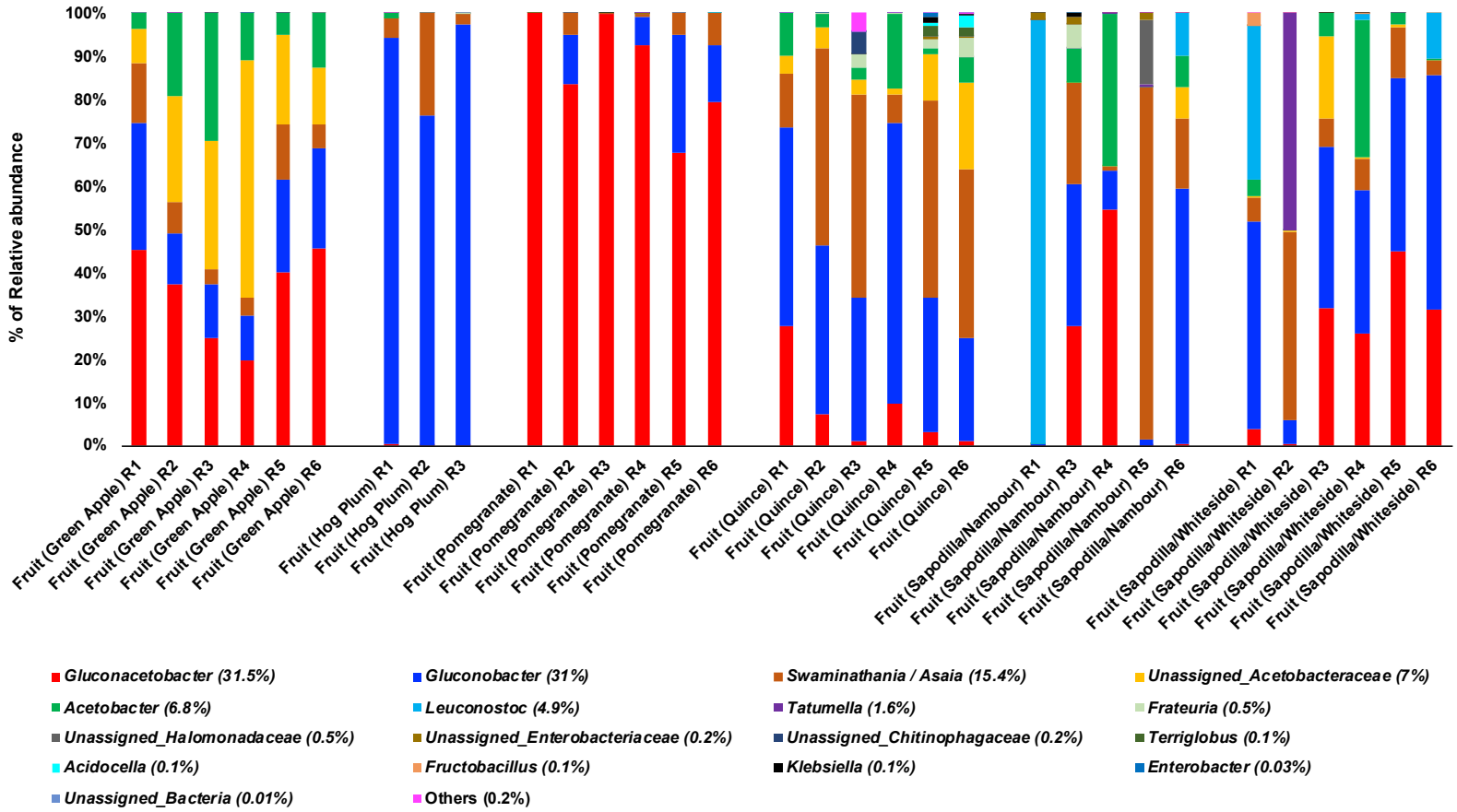

Figure S2. Relative abundance of bacterial taxa of 32 fruit samples. The percentage of relative abundance of four or less than are included in "Others". Each type of fruit sample has 6 identical replicates except Hog plum (3 replicates) and Sapodilla/Nambour (5 replicates). R1 to R6 refers to the replicate number of each fruit.

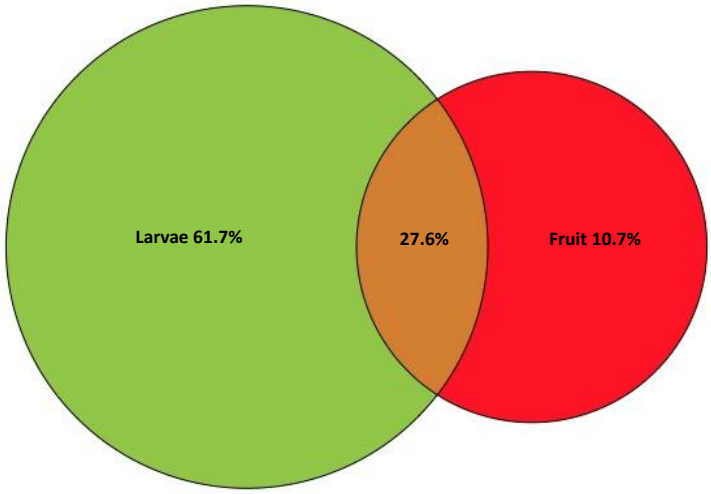

Figure S3. Venn diagram of the average percentage of the bacteria present independently in the larvae, fruit and common in both.

Table S1. Diversity indices of bacterial community composition in the five different fruit and the larval gut of *B. tryoni.* Different letters indicate significant Tukey's post hoc comparisons ($P < 0.05$)

Sample name	Total Species	Species Richness	Pielou's evenness	Shannon	Simpson
Fruit (Green Apple)	18.5 ± 0.43ab	1.9 ± 0.04ab	0.55 ± 0.01a	0.11 ± 0.04a	0.74 ± 0.17a
Fruit (Hog Plum)	16 ± 2.52ab	1.62 ± 0.27ab	0.13 ± 0.05c	0.34 ± 0.12cd	0.18 ± 0.09cd
Fruit (Pomegranate)	11.66 ± 2.03b	1.15 ± 0.22b	0.15 ± 0.05c	0.39 ± 0.13d	0.21 ± 0.07d
Fruit (Quince)	27.5 ± 3.28ab	2.88 ± 0.35ab	0.46 ± 0.02ab	1.49 ± 0.11ab	0.66 ± 0.02ab
Fruit (Sapodilla/Nambour)	18 ± 1.52ab	1.85 ± 0.16ab	0.32 ± 0.08abc	0.94 ± 0.26abcd	0.47 ± 0.12abcd
Fruit (Sapodilla/Whiteside)	18.33 ± 1.47ab	1.88 ± 0.16ab	0.42 ± 0.02ab	1.24 ± 0.08abc	0.65 ± 0.02abc
Larvae (Green Apple)	26.66 ± 4.27ab	2.78 ± 0.46ab	0.34 ± 0.07abc	1.17 ± 0.24abcd	0.54 ± 0.11abcd
Larvae (Hog Plum)	36 ± 4.83a	3.8 ± 0.52a	0.16 ± 0.04c	0.6 ± 0.16cd	0.33 ± 0.09bcd
Larvae (Pomegranate)	32.33 ± 3.05a	3.41 ± 0.33a	0.28 ± 0.02bc	0.93 ± 0.06abcd	0.45 ± 0.04abcd
Larvae (Quince)	33 ± 3.99a	3.51 ± 0.43a	0.25 ± 0.06bc	0.86 ± 0.21abcd	0.44 ± 0.1abcd
Larvae (Sapodilla/Nambour)	36.83 ± 9.83a	3.89 ± 1.06a	0.22 ± 0.03bc	0.79 ± 0.12bcd	0.39 ± 0.06abcd
Larvae (Sapodilla/Whiteside)	34.5 ± 4.23a	3.64 ± 0.45a	0.26 ± 0.06bc	0.94 ± 0.23abcd	0.45 ± 0.1abcd

Chapter 3

This chapter has been formatted and submitted to Frontiers in Microbiology (Fungi and their interaction) **(Currently under review)**

Fruit Host-dependent Fungal Communities in the Microbiome of Wild Queensland Fruit Fly Larvae

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Running head: Fungal microbiome analysis of Queensland fruit fly larvae

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ABSTRACT

Bactrocera tryoni (Froggatt), the Queensland fruit fly or 'Qfly', is a highly polyphagous tephritid fly that is widespread in Eastern Australia. Fungal microbial communities are abundant in Qfly, but few studies have characterised their fungal microbiome and none have used culture-independent high-throughput molecular sequencing techniques. We comprehensively identified and characterized the fungal community of Qfly larvae from five host fruits (sapodilla [from two different localities], hog plum, pomegranate, green apple, and quince) along the east coast of Australia using Next-Generation Sequencing on the Illumina MiSeq platform. To address an overarching question of the extent to which larval microbiomes are isolated from or adopted from host fruit, we focused on two core questions: (1) What fungi, yeast-like fungi, and yeast are present in Qfly larvae?; (2) Are the same fungi, yeast-like or yeast found in the host fruit? The most abundant fungal sequences were assigned to the family Saccharomycetaceae (88%), which are Ascomycota, followed by Metschnikowiaceae (9%) and Sporidiobolaceae (1%). Fungal amplicon data revealed substantial differences in the fungal microbiome of different host fruit types. The fungal communities in Qfly larvae varied amongst fruit in accord with the fungal community of that fruit. Overall, our results indicate that the fungal communities of Qfly larvae are mostly adopted from the fungal microbiome present in the host fruit. This is the first use of Next-Generation Sequencing to analyze both the fungal microbiome of fruit fly larvae from fruit infested in the field together with the host fruits. Through this culture independent approach, the present study makes a substantial contribution toward understanding the fungal ecology of fruit flies, and particularly the Qfly.

Key words: *B. tryoni*, Yeast, Yeast-like, Next generation Sequencing, Fungal ecology

INTRODUCTION

The gut microbiome plays a vital role in metabolic regulation, food digestion and the immune system of animals (Flint et al., 2012; Nicholson et al., 2012). In insects, the gut microbiome contributes to the extraction of nutrients from consumed food (Zhang et al., 2018), and can help to detoxify harmful compounds and protect from pathogens (Paine et al., 1997; Engel and Moran, 2013; Yun et al., 2014). The microbes associated with insects span all three domains of life: Bacteria, Archaea and Eukaryota. The eukaryotic microbes tend to be fungal, predominately yeasts. Yeast and yeast-like fungi play an important role in insect development and fitness by providing nitrogen compounds and degrading high molecular weight molecules (Malacrinò et al., 2015; Zhang et al., 2018). Additionally, fungi may produce pheromones inside insects, which can affect communication and mating performance (DeLeon-Rodriguez and Casadevall, 2016). In *Drosophila suzukii* and *D. melanogaster*, yeasts affect sexual maturation, oviposition rates and larval development (Stamps et al., 2012; Mori et al., 2017). Several species of yeast and yeast-like fungi enhance survival rates and shorten the developmental period in *D. melanogaster* (Anagnostou et al., 2010; Rohlfs and Kürschner, 2010). Fungal spores and yeasts supply nutrients to adult tephritid fruit flies (Boyce, 1934). Yeast supplements are routinely provided as a source of macronutrients (amino acids and carbohydrates) and micronutrients (vitamins, minerals and cholesterol) in the larval and adult diet used for the rearing of tephritid fruit flies (Nestel et al., 2004; Nestel and Nemny-Lavy, 2008; Fanson and Taylor, 2012; Nash and Chapman, 2014; Moadeli et al., 2018). On the other hand, fungi are also responsible for insect disease; more than 700 species of fungi have been identified as entomopathogenic (Hajek and St. Leger, 1994). For example, *Metarhizium anisopliae* fungus is pathogenic to *Drosophila* (Lu et al., 2015), and *Metarhizium anisopliae* (Metschnikoff) Sorokin and *Beauveria bassiana* (Balsamo) Vuillemin (Deuteromycotina, Hyphomycetes) are pathogenic to adults and pupae of the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann) (Lacey et al., 2001; Quesada-Moraga et al., 2006; Almeida et al., 2007; Ortu et al., 2009).

Some fungi have symbiotic relationships with insects (Gonzalez, 2014). Some ants cultivate fungus (a form of vegetative mycelium) which they use as a primary food source (Currie et al., 1999). Brazilian stingless bees, *Scaptotrigona depilis* and leaf cutter ants (two Genera, *Acromyrmex* and *Atta*) cultivate fungi inside the brood cell and a garden, respectively (Menezes et al., 2015; Yun et al., 2018). Fungi have been found to be abundant in nectar and

bee bread (a mixture of the honey and bee secretions which provides a rich source of protein) (Sammataro and Avitabile, 1998), which affects honey bee fitness (Gilliam and Prest, 1972; Gilliam, 1979; Sandhu and Waraich, 1985). Various polyphagous insect taxa have close relationships with gut fungal communities, including Lepidoptera, Chrysomelidae (Coleoptera), Curculionidae (Coleoptera), plant-galling Cecidomyiidae (Diptera) and all plant-feeding hemipteran families (Bissett, 1988; Six, 2003; Janson et al., 2008). Early studies found that fungal communities were not as dominant as bacterial communities in the gut microbiome of phytophagous insects (Fermaud and Menn, 1989; Six, 2003). Although the yeast and yeast-like fungi are known to have active roles in the ecology of tephritid fruit flies, much remains to be learned about the identities of these components of the insect microbiome, and their functional roles (Mori et al., 2017; Piper et al., 2017).

Queensland fruit fly, *Bactrocera tryoni* (Froggatt) ('Qfly') is the most economically damaging fruit fly species in Australia (Clarke et al., 2011; Dominiak and Daniels, 2012). Qfly is highly polyphagous and causes substantial economic damage to production and trade of commercial fruit and vegetables (Sutherst et al., 2000; Dominiak et al., 2003; Clarke et al., 2011; Dominiak and Daniels, 2012). Detailed knowledge of the microbiome is a key gap in understanding of Qfly biology. Several studies have recently investigated the Qfly microbiome and focused on the identification of the gut bacteria and their interactions with the host (Drew et al., 1983; Lloyd et al., 1986; Murphy et al., 1994; Thaochan et al., 2010; Morrow et al., 2015; Deutscher et al., 2018; Woruba, 2018, Majumder et al., 2019). To date only two studies have attempted to identify the fungal microbiome of Qfly, using traditional culture-dependent methods to isolate some fungal strains (Deutscher et al., 2016; Piper et al., 2017). These studies confirm the presence of yeast and yeast-like fungi but do not provide a detailed picture of the fungal community. Malacrinò et al. (2015) observed an abundance of fungi in adult males and females of olive fruit fly, *B. oleae* (Gmelin), using culture independent molecular techniques. Published cultivation-based studies of fruit fly fungal microbiome previously used selective media following traditional culture-dependent or independent methods. While these studies report on the particularly targeted species or group of fungi, they provide only a very limited account of the overall gut microbiome (Vaughan et al., 2000; Arias-Cordero et al., 2012; Malacrinò et al., 2015). To date, no culture-independent studies have been performed to characterize the fungal communities associated with Q-fly, and almost nothing is known of the relationship between the Qfly larval microbiome and that of its fruit hosts.

For analysis of fungal communities, the internal transcribed spacer (ITS) regions of the RNA gene is the most commonly used universal bar code marker (Toju et al., 2012). The ITS amplicon is helpful to identify the fungal community structure in the target sample using next-generation sequencing technologies (NGS analysis). NGS has already been used for identification of fungal biomes in various insects, including termites, ants, firebugs, beetles and bees, but not for Qfly (Toju and Fukatsu, 2011; Engel et al., 2012; Hulcr et al., 2012; Köhler et al., 2012; Poulsen and Sapountzis, 2012; Sudakaran et al., 2012; Boucias et al., 2013). In the present study, we investigated the fungal community of Qfly larvae and their host fruits using NGS analysis of ITS amplicon on the Illumina MiSeq platform. We particularly considered the similarity of insect and fruit fungal communities, and the likelihood of horizontal transfer.

MATERIALS AND METHODS

Collection of Qfly larvae

Qfly larvae were collected from infested fruits collected from various geographic locations of New South Wales (NSW), Victoria (VIC) and Queensland (QLD) in Australia (**Table 1**). Most infested fruits were over-ripe and were collected from under trees. The fruit types included: Hog Plum (*Spondias mombin*), Sapodilla (*Manilkara zapota*) (from two different locations, Nambour and Whiteside, QLD), Pomegranate (*Punica granatum*), Green Apple (*Malus pumila*) and Quince (*Cydonia oblonga*) (**Table 1**). The infested fruits were stored on wire racks in 60L plastic bins (45x24x66 cm, Award, Australia) that contained 250 g of vermiculite (Grade 1, Sage Horticultural, Victoria, Australia) in a controlled environment laboratory (25±0.20°C, 65±3% RH and 11: 1: 11: 1 light: dusk: dark: dawn photoperiod) at Macquarie University, Australia. Samples of different fruit types and origins were kept separate to prevent cross-contamination. A total of 36 Qfly 3rd instar larvae were collected in replicates of six from each of the five fruit types. Additionally, six replicate samples of fruit tissues (fruit flesh) (1~2 mg mass) were collected from the same fruit used to collect larvae.

Sample preparation

Larvae were surface sterilized with a solution of 0.5% (v/v) Tween 80 (Sigma-Aldrich, Cat. No. 9005656), 0.5% (v/v) Bleach (sodium hypochlorite) (Sigma-Aldrich, Cat. No.7681529) and 80% (v/v) Ethanol (Sigma-Aldrich, Cat. No. 65175) for 30s, and were rinsed 3 times in 1 M sterile phosphate-buffered saline (1x PBS) for 30s after collection (Majumder et al., 2019). Five types of microbial growth medium (de Man, Rogosa and Sharpe Agar, Tryptone Soya Agar, Macconkey Agar, Potato Dextrose Agar and yeast dextrose Agar medium) were used to check the performance of the sterilization method. The PBS collected from the 2nd and 3rd washes were kept and 100 µL was spread-plated onto the five growth medium plates and incubated in a 35°C incubator for 24 to 48 hr. After sterilization, sterile pestles (Fisher scientific, USA) were used to crush the whole larvae, which were then stored in Brain Heart Infusion (BHI) broth with 20% Glycerol solution at -80°C. The samples were split into two separate cryovial tubes (Simport Scientific, Canada) for Cytochrome Oxidase I **(**COI) gene identification and next gene sequencing analysis. Fruit flesh from individual fruit was also preserved and stored under the same conditions. All procedures were completed in a sterile environment (Biological Air Clean Bench, safe 2020 1.2, Thermo Scientific, Germany).

Larval identification using mitochondrial cytochrome oxidase I (COI) gene

Microscopic examination of larval morphology was carried out prior to DNA extraction (White and Elson-Harris, 1992). Additional confirmation was gained through examination of adult morphology after the emergence of adult flies (approximately 600) from the larvae remaining in the infested fruits that tested samples were obtained from (Leica MZ6 stereo-microscope, Leica®, Germany) (Plant Health Australia, 2011, Majumder et al., 2019).

Larval identification was confirmed by sequencing the mitochondrial cytochrome oxidase I (COI) gene of all samples. DNA was extracted from crushed whole larval samples using Isolate II genomic DNA kit from Bioline (Cat. no. BIO-52066) following the manufacturer's protocol. The concentration of DNA extract was determined using the Invitrogen™ Qubit® dsDNA High Sensitivity (HS) Assay Kit (Life Technologies). Standard LCO1490/HCO2198 primers were used to amplify a 700 bp segment of the CO1 gene (Folmer et al., 1994, Majumder et al., 2019). All PCR amplifications in the present study

were performed using an Eppendorf thermocycler (Mastercycler, epgradient S, Eppendorf, Germany) under the following conditions: each 15 µL reaction was conducted in triplicate and contained 7.5 µL of MyTaq HS PCR master mix (Bioline, USA. Cat No. BIO-25045), 0.60 µL of forward (LCO1490F) and reverse primer (HCO2198R), and 1.5 µL of DNA extract of larval sample. The PCR profile included an initial denaturing step at 95 °C for 2 min, followed by 35 cycles of 94°C for 30 s, 50°C for 30 s and 72 °C for 90s, and a final extension step of 72 °C for 5 min. Amplicons were visualised using electrophoresis on a 1% agarose gel (110v-45 min) (Majumder et al., 2019). Amplicons were then sent to the Australian Genomic Research Facility (AGRF) for Sanger sequencing. Sequence data were analysed by Geneious R10.2.3 to confirm Qfly larvae identification.

Fungal microbiome profiling

DNeasy Power Lyzer Power Soil Kit-100 (Qiagen, Germany) (Cat. no. 12855-100) was used to complete the DNA extraction process of the crushed whole larvae samples for NGS analysis following the manufacturer's protocol. Invitrogen™ Qubit® dsDNA High Sensitivity (HS) Assay Kit (Life Technologies, USA) was used to quantify the DNA extracts (Majumder et al., 2019). The Australian Genome Research Facility performed all the PCR amplification and sequencing procedures. In brief, the ITS region of RNA gene was amplified using the fungal-specific forward primer ITS1F (CTTGGTCATTTAGAGGAAGTAA) and the reverse primer ITS2 (GCTGCGTTCTTCATCGATGC). Reactions contained 1X AmpliTaq Gold 360 mastermix (Life Technologies, USA), 0.20 µM of forward and reverse primers and 25 µL of DNA. PCR cycling conditions included denaturation at 95ºC for 7 minutes, followed by 35 cycles of denaturation at 94ºC for 45 s, annealing at 50ºC for 60s and extension at 72ºC for 60s, with a final extension at 72ºC for 7 minutes. A secondary PCR was used to adhere sequencing adaptors and indexes to the amplicons. Primerstar max DNA Polymerase used for secondary PCR amplicon from Takara Bio inc. Japan (Cat. No. #R045Q). The resulting amplicons were measured by florometry (Invitrogen Picogreen) and normalized (Fouts et al., 2012, Majumder et al., 2019). The eqimolar amounts of each sample were pooled and quantified qPCR prior to sequencing (Kapa qPCR Library Quantification kit, Roche, Switzerland). The resulting amplicon library was sequenced on the Illumina MiSeq platform (San Diego, CA, USA) with 2 x 300 base pairs paired-end chemistry (Caporaso et al., 2010). The Illumina sequences were deposited in the NCBI GenBank under Bio-project PRJNA532489.

Sequence data processing

The Greenfield Hybrid Amplicon Pipeline (GHAP) was used to process amplicon sequences (Sutcliffe et al., 2018). GHAP is an in-house amplicon clustering and classification pipeline built around tools from USEARCH (Engel and Moran, 2013) and RDP (Cole et al., 2013). It was combined with locally-written tools for demultiplexing and generating OTU (operational taxonomic units) tables. This hybrid pipeline took files of reads and produced tables of classified OTUs and their associated read counts across all samples. The amplicon reads were demultiplexed, the read pairs merged and de-replicated. The merged reads were trimmed and clustered at 97% similarity to generate OTUs (Sutcliffe et al., 2018).

Representative sequences from each OTU were classified both by finding their closest match in the Warcup reference set of ITS sequences, and by using the RDP Naïve Bayesian Classifier and the Warcup training set (Deshpande et al., 2016). The use of two independent classification techniques can improve confidence in the taxonomic assignments. This process highlights those cases where a simple 'best match' might give a misleading result. Each OTU sequence was also classified with the RDP Classifier and compared with the UNITE training set to increase confidence in the classifications. The pipeline mapped the merged reads back onto the classified OTU sequences to obtain accurate read counts for each OTU/sample pairing and generated OTU tables complete with taxonomic classifications and species assignments. The OTU tables summarised overall taxonomic levels and combined the counts for identified taxa across all OTUs. The pipeline finally classified all the merged reads using the RDP Classifier, regardless of whether they were assigned to an OTU. This last step was carried out to provide confidence in the clustering and OTU formation steps by providing an independent view of the community structure. Each OTU was tested using the following criteria to ensure that OTU sequences were actually fungal. RDP taxonomic assignments using UNITE or Warcup included an assignment to a fungal order with >60% confidence. Alternatively, Blastn returned a similarity of >70% using the Geneious software (Geneious® 10.2.3, Biomatters Ltd.) to match with NCBI Insights fungal reference set.

Statistical analysis

The OTU table containing the number of read counts for each OTU detected for each sample was imported into Primer-E v7 for analysis (Clarke and Ainsworth, 1993; Sutcliffe et al.,

2018). In brief, all statistical testing was performed on fixed factors associated with fruit host [hog plum, sapodilla (from two different localities), pomegranate, green apple and quince] from which 6 replicates were collected. The DIVERSE function was used to generate univariate biodiversity metrics, including total species, species richness, Shannon's and Simpson's biodiversity indices. Statistical differences between these metrics were assessed in JMP Statistical Software Version 10.0.0 (SAS Institute, Cary, NC, USA) using one-way analysis of variance (ANOVA) and Tukey-Kramer's HSD post hoc analysis.

The OTU table was first log transformed using Primer-E V7 to observe the taxonomic compositional changes for fungal communities. A Bray-Curtis similarity matrix was derived from these transformed data and a permutational analysis of variance (PERMANOVA) pair wise comparison was conducted to compare all community samples. A p value of <0.05 was considered statistically significant. Further, ordination plots of these communities were visualised using principal coordinates analysis (PCoA) in Primer-E. Fungal Taxonomic plots for larvae and host fruit were modelled in Prism 8 (version 8.0.1(145), GraphPad software, Inc) (Majumder et al., 2019).

RESULTS

Identification of larvae

Analysis of the COI gene confirmed that all 36 larvae collected from the different fruit type/origins were Qfly. Furthermore, all of the approximately 600 adult flies developed from the collected fruits were identified as Qfly. No other species were identified from the collected samples. Additionally, the surface sterilization process of the larvae was found to be effective as there was no microbial growth detected in different growth medias after 24 to 48 hours incubation.

Fungal taxonomic identification in Qfly larvae

We sequenced the fungal microbiome of the 36 Qfly larvae, of which 29 were retained after quality control and rarefaction at 4,500 reads per sample. Using a cluster threshold of 97% sequence similarity, 62 fungal OTUs were obtained after rarefaction (**Supplementary file 1 & 2**). Among them, only 12 OTUs (~10%) were classed as abundant, i.e. representing ≥1%

of the microbiome in one or more larvae (**Table 2**). The fungal taxa detected in the larvae represented 2 phyla, 10 classes, 14 orders, 23 families and 30 genera. The most abundant Phylum observed in the larval microbiome was Ascomycota (98%), followed by Basidiomycota (2%). The class Saccharomycetes represented an average of 98% of the fungal microbial communities. Microbotryomycetes and Tremellomycetes contained only 1% of relative abundance respectively associated with the majority of Ascomycota taxa. At order level, the fungal taxa Saccharomycetales was found with 98% of relative abundance. The dominant fungal family found in larvae was Saccharomycetaceae with 88% relative abundance. Other observed families included Metschnikowiaceae (9%) and Sporidiobolaceae (1%). The most abundant fungi were associated with yeasts that contained the closest match to the genera of *Pichia*, *Trigonopsis, Clavispora, Candida, Kodamaea*, and *Cyberlindnera* (**Table 2 & Table S1**). Among the most abundant 12 fungal OTUs, the most abundant OTU was OTU 1 and was most closely related to *Pichia terricola* with 42% average relative abundance. The second most abundant was OTU 3 (21%) which was close to *P. fermentans*. The third most abundant was OTU 2 (13%) followed by OTU 5 (9%), which matched with *P. manshurica* and *Clavispora lusitaniae* respectively. The remaining OTUs all comprised less than 5% relative abundance (**Table 2**).

The fungal community present in Qfly larvae differed among fruit sources (**Figure 1**). *Pichia terricola* (OTU 1) was highly abundant in larvae from hog plum, followed by sapodilla. However, *P. terricola* was not detected in the larvae from green apples or quinces. In contrast, *P. fermentans* (OTU 3), *Trigonopsis vinaria* (OTU 4) and *P. kluyveri* (OTU 48) were found to be abundant (>1% relative abundance) only in larvae from green apple and quince. Further, *C. lusitaniae* (OTU 5) was abundant only in the larvae from pomegranate. Other taxa that were abundant in the larval microbiomes were only sporadically detected in fruit samples. For example, *Candida tropicalis* (OTU 15) was only found at a relative abundance of >1% in two larvae from sapodilla, while *Rhodotorula araucariae* (OTU 25) was identified in just 1 larva (from pomegranate) (**Figure 1**).

Microbial communities in host fruit

A total of 28 samples of fruit tissues (fruit flesh) had sufficient sequencing depth to be included in the present study (minimum of 4500 reads). After rarefaction, 92 fungal OTUs were obtained with a cluster threshold of 97% sequence similarity. The fungal taxa detected

in the fruit flesh represented 2 phyla, 12 classes, 14 orders, 26 families and 38 genera. The phylum Ascomycota was found the most abundant which represented ≥97% of the communities in all samples tested. The most abundant class observed in the fruit microbiome was Saccharomycetes (88%), followed by Dothideomycetes (7%). At order level, two fungal taxa of Saccharomycetales and Botryosphaeriales were observed with average of 88% and 7% relative abundance respectively. We observed 6 families associated with the most abundant OTUs: Saccharomycetaceae (78%), Metschnikowiaceae (8%), Botryosphaeriaceae (7%), Pestalotiopsidaceae (2%), Dipodascaceae (2%) and Sclerotiniaceae (1%).

The seven most abundant OTUs accounted for 92% of the total sequences and their relative abundances were different in the larvae from different fruit sources. OTU 1 (*P. terricola*) accounted for the highest average of 33% relative abundance and was most abundant in hog plum (88.12%), sapodilla (Whiteside) (26.26%), and sapodilla (Nambour) (68.62%) (**Table 2**). However, this OTU was less abundant in pomegranate, green apple and quince. OTU 3 (*P. fermentans)* and OTU 4 (*T. vinaria)* were highly abundant in green apple and quince compared with other fruits. OTU 5 (*C. lusitaniae)* was more prevalent and was highly abundant only in pomegranate. OTU 2 (*P. manshurica)* was associated with all fruit except green apple and quince (**Figure 2**). Overall, no OTU was commonly found in all types of fruit tested.

Diversity of the fungal microbiome in Qfly larvae and its host fruit

Total species, Species richness, Shannon and Simpson diversity indices represent the alpha diversity, which revealed that the fungal diversity in both the larvae and the fruit flesh of green apple and quinces was significantly higher than other fruit (**Figure 3**). The PERMANOVA analysis (pair-wise test with 999 permutations) based on Bray-Curtis similarities compared fungal microbial communities of larvae from five types of fruit hosts (**Table 3**). Principal coordinates analysis (PCoA) plots was created from the Bray-Curtis similarity matrix to visualize these differences (**Figure 4A-B**). The first PCoA axis explained 47.5% of the variation seen in larval microbial communities (**Figure 4A**). On this axis, the larvae from green apple and quince were clearly separated from all other fruit sources to form a tight and distinct cluster. On the second axis, which explained 19.7% of the variation, hog plum and pomegranate were separated, with a spread of sapodilla-sourced samples scattered between these sample clusters. The PERMANOVA results largely support these PCoA

clusters (**Table 3**). For example, green apple and quince larval microbiomes were significantly different from all other fruit sources, but not to one another. In contrast, sapodilla samples were highly disparate and overlapped in ordinations with both pomegranate and hog plum (**Figure 4A**). In addition, the microbiome communities of larvae from pomegranate were found to differ significantly from most other samples. However, these samples were not significantly different from the quince larval communities, despite their clear separation on the PCoA plot; this finding may reflect a lack of statistical power when using a sample number of just three, as occurred for the larvae from quince and pomegranate.

Microbiome variation with host taxon and geographic location

To further explore the trends observed for different fruits, we clustered samples based on (i) the taxonomic relationship between fruit (plant families) and (ii) geographic location. Principal coordinates analysis and PERMANOVA test both indicate that the fungal community, both in fruit and larvae, differed significantly with geographic location and host fruit (PERMANOVA test with 999 permutations, location p=0.001, and plant family p=0.001).

The fungal microbiome was not significantly different (p>0.05) between apple and quince, which are both from the plant family Rosaceae (**Supplementary Figure 1A-C**). Additionally, no significant variation (p>0.05) was found in the fungal communities of larvae from apple and quince. The PCoA ordination plot (PCO1 and PCO2 explained 45.9% and 17.5% of data variation, respectively) revealed a clear separation of the fungal communities in different plant families (represent a linked variable) (**Supplementary Figure 1A**). Among four plant families, alpha diversity was greatest in Rosaceae (**Supplementary Figure 1C**). Fungal OTUs had variable abundance in different plant families. OTU 1 (*P. terricola*) was highly abundant in the families Anacardiacea and Sapotaceae. In contrast, OTU 5 (*C. lusitaniae*) and OTU 3 (*P. fermentans*) were most abundant in Lythraceae and Rosacea, respectively. OTU 4 (*T. vinaria*) was abundant in Rosacea and OTU 2 (*P. manshurica*) was observed both in Sapotaceae and Lythraceae (**Supplementary Figure 1B**).

Additionally, the PCoA ordination plot (PCO1 and PCO2, explained, 45.9% and 17.5% of data variation), indicated that the fungal microbial community was significantly different between the regions from which the fruit was collected (**Supplementary Figure**

2A-C). Fungal populations of the samples from the same region (including the same type of fruits or different) had close clustering or over-lap in the ordination plot (**Supplementary Figure 2A**). The fungal microbiome in larvae and fruit flesh collected from VIC was more diverse and more evenly distributed compared to that of NSW and QLD (**Supplementary Figure 2C**). The fungal OTUs were differently abundant in different regions. Among 99 OTUs, the three most abundant OTUs, OTU 5 *C. lusitaniae,* OTU 1 *P. terricola* and OTU 3 *P. fermentans* were highly abundant in QLD, NSW and VIC respectively (**Supplementary Figure 2B**). Given that different fruit hosts were sampled from different geographic regions, we cannot at this time ascribe the variation specifically to host taxon or region.

DISCUSSION

The present study is the first comprehensive analysis of the fungal microbiome of Qfly larvae from taxonomically and geographically diverse host fruits. Qfly fungal communities have remained largely unknown, especially for fungal taxa that are difficult to isolate or culture for identification. Our data provide greatly increased insight to the yeast and yeast-like fungi present in Qfly larvae. Additionally, we explored the fungal microbiome of the host fruit flesh by sampling from five different fruit types collected from various geographic locations. This analysis has enabled us to understand the likely role of the host fruit microbiome in defining the Qfly larval microbiome. Together, these studies demonstrate a strong influence of the host fruit in defining the Qfly microbiome as larval fungal communities were very similar to those found in the host fruits. Our findings suggest that the fungal microbiome of Qfly larvae reflects horizontal transfer, most likely in part as a food source. Overall, we found the fungal communities of our larval samples to be more diverse than previous culture-based studies suggested (Deutscher et al., 2016). We identified abundant fungal taxa that were previously unknown from Qfly larvae (e.g., OTU 1 *P. terricola,* OTU 2 *P. manshurica*).

Based on alpha and beta diversity analysis, diversity was observed in fungal communities present in the most of the Qfly larvae sample which was not highly indexed. However, At the phylum level, only Ascomycota had a high relative abundance in the larvae. Ascomycota are commonly associated with plants and insects (Ravenscraft et al., 2019). We found that the major fungal sequences associated with the family Saccharomycetaceae belonged to the order Saccharomycetales. These fungi are single cell fungi known as the 'budding yeasts' or the 'true yeast' and are most common in sugar rich environments such as

ripe fruits (Ravenscraft et al., 2019). Our study detected numerous ascomycetes (e.g. OTUs 1, 2, 3, 4, 5) and basidiomycetes (e.g. OTU 25), confirming their ubiquity in Qfly larvae. The 12 most abundant fungal organisms found in the larvae were identified as yeasts. However, the form of colonization (infringement or biofilm) of the identified yeast inside the larvae is not known. It is likely that these yeasts have different abundance based on their host fruits. The most commonly identified yeast species were from the genus *Pichia*, with average of ~75% of the relative abundance of fungal OTUs in Qfly larvae. *Pichia* are also common in *Drosophila*, beetles and lepidopteran larvae (Marchesi, 2010; Kurtzman et al., 2011; Hamby et al., 2012; Ravenscraft et al., 2019). Previously, culture-based approaches based on isolation of yeast and yeast-like fungi from Qfly larvae also found yeast isolates mostly associated with the yeast genus *Pichia* (Deutscher et al., 2016). The genus *Pichia* is globally widespread and is found in diverse environments, including both pathogenic and commensal species. *Pichia terricola* (OTU 1) was abundant in the larvae from hog plum and was also highly abundant in sapodilla from Nambour, QLD. Conversely, *P. manshurica* (OTU 2) was abundant in sapodilla (from Whiteside, QLD). Biofilm forming yeast *P. fermentans* (OTU 3) was similarly abundant in both larvae from green apple and quince. *Pichia fermentans* can inhibit the phytopathogenic *Monilinia fructicola* to control brown rot disease in apple, yet acts as pathogen in peach (Giobbe et al., 2007). Additionally, *P. kluyveri* (OTU 48) and *P. sporocuriosa* (OTU 8) were abundant in the larvae from green apple. *Pichia kluyveri* and *P. terricola* have been reported in spotted-wing *Drosophila* (*D. suzukii*) isolated from cherries and raspberries (Hamby et al., 2012). *Pichia kluyveri, P. manshurica* and *P. terricola (Issatchenkia terricola*), are common fungi in mature fruits, vineyards and wineries (Bokulich et al., 2014). In our study, most of the remaining OTUs with confident taxonomic assignment were likely plant associated or yeast/yeast-like fungi mostly pathogenic to plant and insect. The yeast and yeast-like fungi usually play a vital role in insect development and nutrition, or use the insect as a vehicle for dispersal (Starmer and Lachance, 2011; Hamby et al., 2012; Quan and Eisen, 2018). *Clavispora lusitaniae* (OTU 5), an abundant entomopathogenic yeast from the family Metschnikowiaceae, was only observed in the larvae from pomegranate (Stefanini, 2018). Yeast of the family Metschnikowiaceae have been detected in *D. suzukii*, and also isolated from mature fruit and fermentative spoilage (Hamby et al., 2012).

Variation was found in identified yeast and yeast-like fungi amongst the different host fruits, and the fungal microbiome of Qfly larvae was generally similar to that of the host fruit.

One explanation for this is that the larvae might have a distinct dietary preference for particular fungi (Stefanini, 2018). This has been observed in other insects, including olive fruit fly *B. oleae* (Gmelin) (Malacrinò et al., 2015), and ground dwelling beetles (Coleoptera) (Kudo et al., 2019). Alternatively, this trend may indicate that some fungi are recruited into the mycobiome, and differentially survive in the larvae, while others are digested or expelled. Further studies are needed to resolve which of these environmental filters, if not both, underlie this trend. In Qfly larvae, all abundant fungal OTUs were associated with Saccharomycetales. However, within the fruit microbiome, with the exception of Saccharomycetales, some abundant fungal OTUs were very different from known fungal species sequences and so identification was not straightforward. Among them, OTU 10 and OTU 9 were identified as members of the plant pathogenic Leotiomycetes and Sordariomycetes, respectively (Alexopoulos et al., 1996; Ravenscraft et al., 2019).

We expected there would be a strong correlation between the fungal microbiome of the larvae and its hosts. However, we did not anticipate any major variation across the different types of fruit. There was no universal fungal community structure that was independent of host fruit. The yeast or yeast-like fungal community might vary with diverse factors such as environment, temperature, host nutrient composition, geographic locations and type of the host plants. There was no significant difference in the fungal community and its relative abundance in larvae and fruits of apple and quince, presumably because both plants belong to the same family (Rosaceae). Previous studies have investigated the diversity of microbes associated with fruits in New Zealand (Gayevskiy and Goddard, 2012), Australia (Prakitchaiwattana et al., 2004) and Europe (Čadež et al., 2010). Together, these studies found that yeast diversity varied significantly with fruit type, which is consistent with the present study. Gayevskiy et al. (2012) reported that fungal communities associated with grapes differed significantly by region. Our findings also support geographical variation as different fungal communities were found in both larvae and fruit collected from NSW, VIC and QLD. It may be that fungal communities vary within type of host fruits across geographic location. However, testing of this hypothesis would require significant further investigation that includes much greater assessment of the same suite of hosts from different locations. Further, A possible follow-up study for this work, would be to investigate the fruit properties associated with these different fruit types e.g. nutrient content, and correlate this with mycobiome changes. This may assist in resolving the influence of diet vs geography

We collected infested fruits mostly from the ground beneath trees, and most fruit were over ripe. Some fruit-based yeasts prefer acidic conditions, including *Hanseniaspora, Candida* and *Kazachstania* (Rosa and Peter, 2006; Komagata et al., 2014). Additionally, fungi play an essential role in saprotrophic functions by producing hydrolases that help to decompose fruit (Sutcliffe et al., 2018). The acid tolerant yeasts in fruit might readily colonize the acidic conditions of the Qfly larvae gut. Also, some soil-based fungi may be transferred from the soil to the fruits after the fall from the tree. Further emphasizing the likely dominance of horizontal transmission, Vijaysegaran et al. (1997) hypothesized that vertical transmission of yeast in Qfly was unlikely because of the absence of yeast in the alimentary canal of the adult Qfly. However, many authors note that insects, especially flies, carry fungi to new hosts (both internally and externally) and increase fungal diversity (Reuter et al., 2007; Goddard et al., 2010; Chandler et al., 2012; Malacrinò et al., 2015; Stefanini, 2018).

Diet is an essential factor influencing the gut microbial community of insects; e.g. cotton bollworm (*Helicoverpa armigera*) (Xiang et al., 2006), ground dwelling beetle (Coleoptera) (Kudo et al., 2019), gypsy moth (*Lymantria dispar* L.) (Broderick et al., 2004) and *Drosophila* (Colman et al., 2012). Fungal communities have potential ability to accumulate carbon, nitrogen and phosphorous which helps to process nutrition from the environment. (Gessner et al., 2010; Sutcliffe et al., 2018). Additionally, yeast and yeast-like fungi may be metabolically active and help to break down hydrocarbons and lipids (Coelho et al., 2010). Yeasts commonly produce extracellular enzymes which isolate nutritional components from the fruit substrate (Molnárová et al., 2014). Furthermore, yeast and yeast-like fungi can increase the probiotic bacterial communities by providing micronutrients and inhibiting deleterious gut bacteria (Rima et al., 2012). Ascomycetous yeast, *Yarrowia*, is found in the gut of larval and adult beetles and is important for sterol biosynthesis and fatty acid metabolism (Vogel et al., 2017). It might be that consumption of yeast or yeast-like fungi present in the fruits provide nutrients that support Qfly larval development.

Fruit fly larvae are known to feed on yeasts in fruit (Anagnostou et al., 2010). It is possible that fruit fly larvae disperse yeasts as they move through the fruit, increasing the availability of yeast as food. In the present study, the same fungi were abundant in the host fruit and the larvae, and it is highly likely that the fungal microbiome was transferred horizontally from the host fruit to the Qfly larvae. The host fruit fungal community

composition appears to largely dictate the larval fungal community composition. Deutscher et al. (2016) also isolated some yeasts using traditional culture dependent methods from the Qfly larva midgut, and mostly found representatives of Saccharomycetaceae. Deutscher et al. (2016) surmised that all of the yeast species in the larval gut were from the fruit. In our study, fungal microbiome analysis of both larvae and host fruits support the supposition of Deutscher et al. (2016). Qfly larvae likely use yeast or yeast-like fungi as a main food source, ingested with fruit flesh. In the laboratory, adult and larval fruit flies are provided Saccharomycetaceae yeast as a main food source (Loukas et al., 1985; Chang et al., 2001; Fanson and Taylor, 2012; Moadeli et al., 2017). For artificial rearing of the Qfly and other fruit flies, most popular larval diets include Torula yeast (*Candida utilis/Cyberlindnera jadinii*) or Brewer's yeast (*Saccharomyces cerevisiae*), both of which are Ascomycetous yeasts (Steiner, 1966; Nestel et al., 2004; Nestel and Nemny-Lavy, 2008; Chang and Cohen, 2009; Morrow et al., 2015; Moadeli et al., 2018; Mainali et al., 2019).

CONCLUSIONS

Our research aimed to ascertain the extent to which Qfly larvae and hosts share a common microbiome, and the extent to which the microbiome of larvae and hosts varies across host taxa. The findings indicate that the larvae harbour a diverse range of yeasts, most of which are previously undescribed and are also found in the host fruit. This relationship persisted despite massive variation in the fungal microbiome of different host fruit types and associated larvae. Overall, the Qfly larval fungal microbiome closely reflects that of the host fruit indicating horizontal transfer as a dominant influence. Our findings provide valuable insights for understanding the ecology of Qfly, in particular this species' ability to infest a vast diversity of fruit types, as well as for laboratory and factory-scale rearing.

SUPPLEMENTARY FILES

Supplementary file 1: OTU table

Supplementary file 2: Mapping file

AUTHORS CONTRIBUTIONS

RM and TC designed the experiment. RM collected the data. RM and BS analysed the data. TC and PWT supervised the project. All authors analysed the data, provided inputs into the writing of the manuscript, and approved the submitted version.

ACKNOWLEDGEMENTS

We thank to Dr Ania Deutscher for her support on fruit samples collection and larval sample preparation. Dr Bernie Dominiak reviewed and improved an earlier version of this manuscript.

CONFLICT OF INTERESTS

The authors have no conflict of interests to declare.

FUNDINGS

This research was conducted as part of the SITplus collaborative fruit fly program. Project *Raising Q-fly Sterile Insect Technique to World Standard* (HG14033) is funded by the Hort Frontiers Fruit Fly Fund, part of the Hort Frontiers strategic partnership initiative developed by Hort Innovation, with co-investment from Macquarie University and contributions from the Australian Government. Rajib Majumder is supported by an International Macquarie University Research Excellence Scholarship (IMQRES) from Macquarie University (NSW, Australia).

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FIGURES AND TABLES

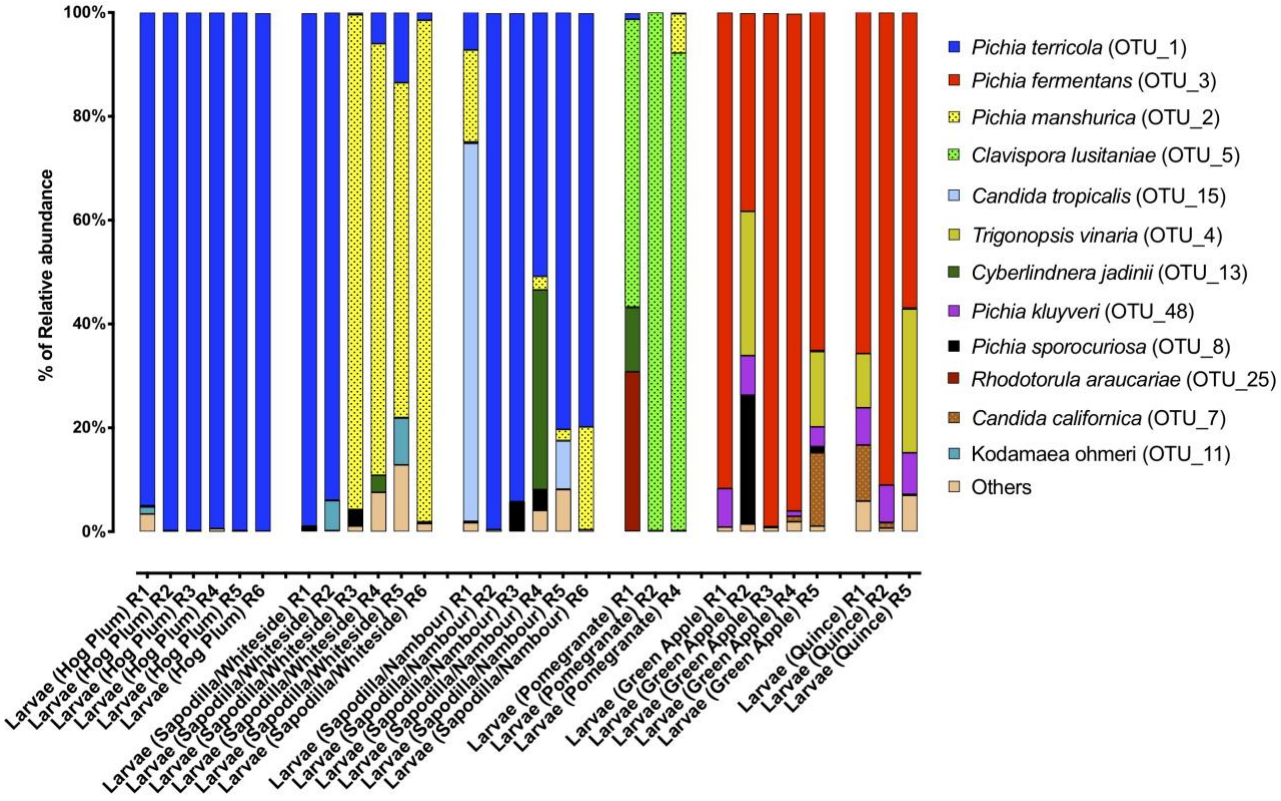

FIGURE 1 | Relative abundance of fungal taxa of Qfly larvae. The percentage of relative abundance of one or less than are included in "Others". Six larvae from each type of fruit are plotted and R1 to R6 refers to the replicate number of each larvae

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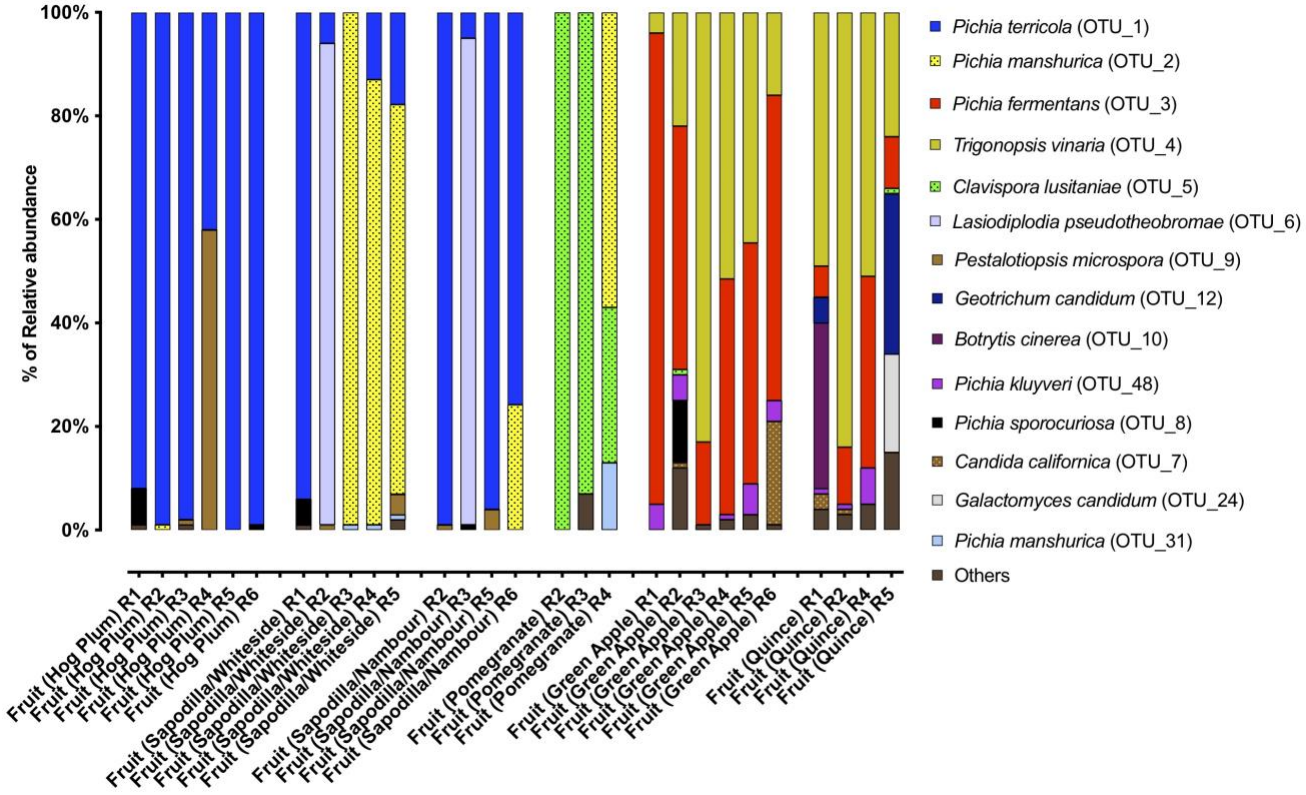

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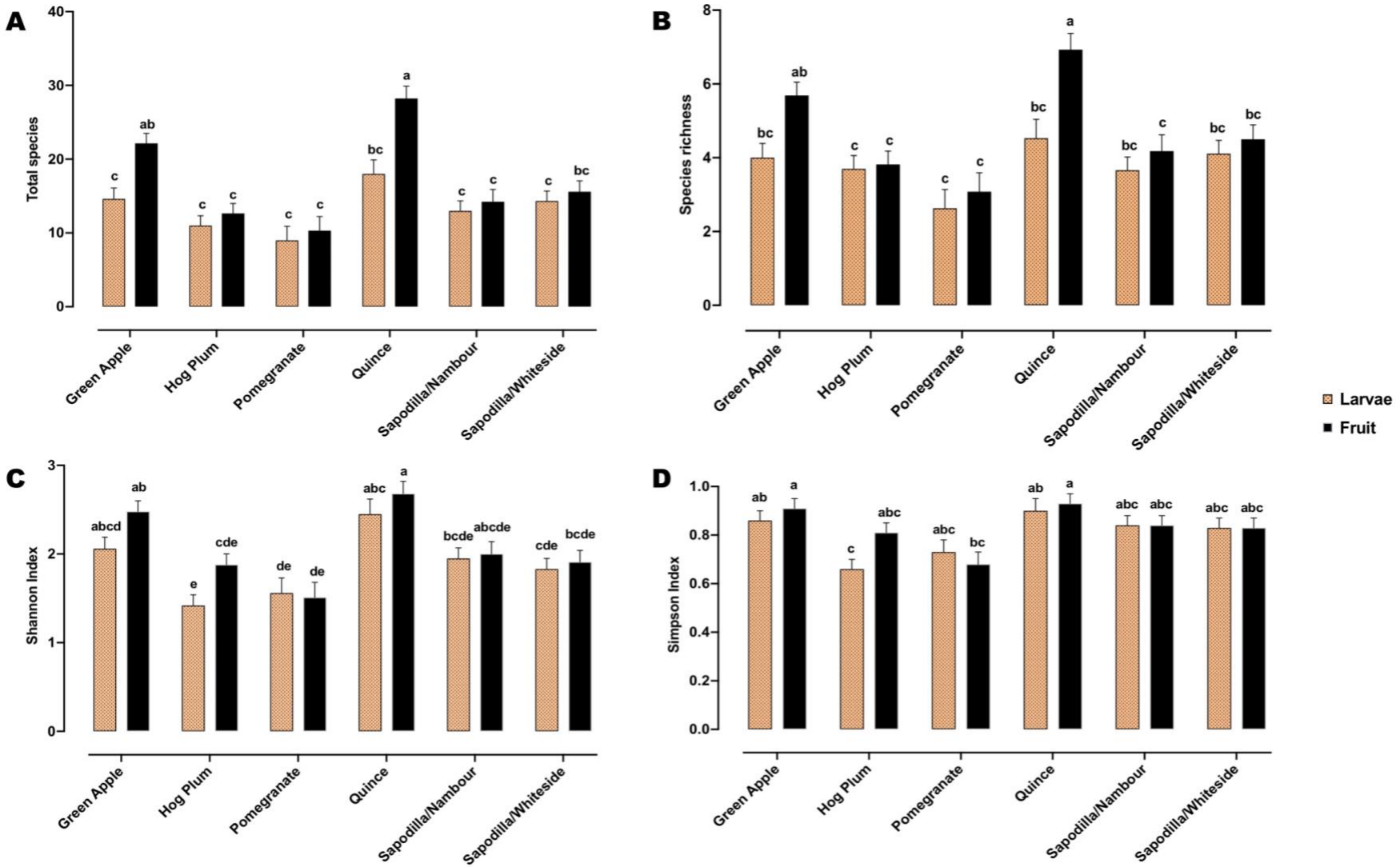
931 **FIGURE 2 |** Average Relative abundance of fungal taxa of different types of fruit samples.

932 The percentage of relative abundance of one or less than are included in "Others". Five

933 different types of fruit are plotted and R1 to R6 refers to the replicate number of each fruit



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935 **FIGURE 3 |** Alpha diversity of the fungal microbiome of the Qfly larvae and host fruit includes (A) Total species; (B) Species richness; (C)

936 Shannon; (D) Simpson indices. Different letters indicate significant Tukey's post hoc comparisons (p<0.05)

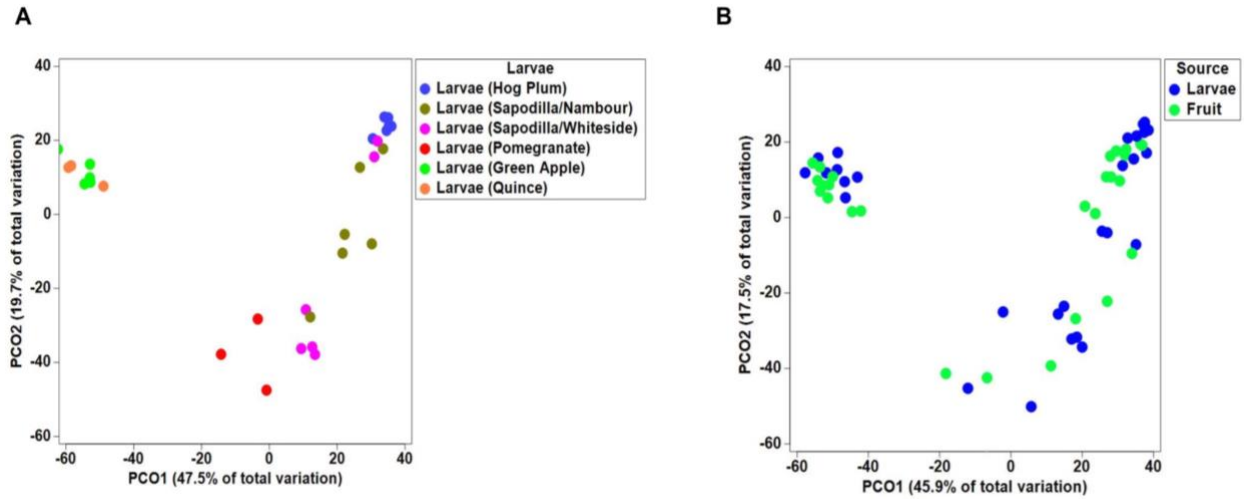

FIGURE 4 | Principal coordinates analysis (A) the fungi communities of Qfly larvae from five type of fruit sources; (B) fungal population between larvae and fruit. ITS next generation sequencing was performed for fungal identification. Different colour point indicates the larvae from different fruit respectively

TABLE 1 | Fruit types and origin for Qfly larvae collection. A total of six replicate larvae, and fruit flesh samples were collected from each fruit origin.

Geographic location of collection	Fruit source and number of fruits collected	Collection date
Maroochy Research station, Nambour, QLD GPS: Lat 26°38'34.92", Long 152°56'22.99"	Hog Plum 26 pieces	1/02/17
Daboro Road, Whiteside, QLD, 4503. GPS: Lat 27°14'29.31", Long 152°55'8.49"	Sapodilla 52 pieces	1/02/17
Maroochy Research station, Nambour, QLD GPS: Lat 26°38'34.92", Long 152°56'22.99"	Sapodilla 68 pieces	1/02/17
Commealla, NSW GPS: Lat 34° 5'50.97", Long 142° 3'7.21"	Pomegranate 37 pieces	5/05/17
St. Germains, Between Tatura and Echuca in Victoria GPS: Lat 36°10'48.86", Long 145° 8'50.74"	Green Apple 41 pieces	05/05/17
Downer road between Tatura and Toolamba in Victoria GPS: Lat 26°38'34.92", Long 152°56'22.99"	Quince 52 pieces	05/05/17

TABLE 2 | Top 18 most abundant fungal OTUs observed in the Qfly larvae and fruit samples

	Putative taxonomy	Closest blast match				Average Relative abundance (%)	
		Name	Accession ID	Similarity (%)	Overlap (%)	Larvae	Fruit
OTU_1	*Pichia terricola*	*Issatchenkia terricola* strain PMM08-3356-AL	KP132531	98	100	42	33
OTU_2	*Pichia manshurica*	*Pichia manshurica* strain CBS 11625	KP250849	98.5	100	13	12
OTU_3	*Pichia fermentans*	*Pichia fermentans* strain E224	KF468218	97	100	21	13
OTU_4	*Trigonopsis vinaria*	*Trigonopsis vinaria* culture CBS:4077	KY105765	100	100	3	15
OTU_5	*Clavispora lusitaniae*	*Clavispora lusitaniae* culture CBS:5094	KY102567	85.8	100	9	8
OTU_6	*Lasiodiplodia pseudotheobromae*	*Lasiodiplodia pseudotheobromae* strain CMW40982	KP872339	100	100	0	7
OTU_7	*Candida californica*	*Candida californica* strain P25B003	JX188103	99.4	81.91	1	1
OTU_48	*Pichia kluyveri*	*Pichia kluyveri* strain PMM10-1742L	KP132503	97.1	100	1	1
OTU_8	*Pichia sporocuriosa*	*Pichia sporocuriosa* culture CBS:9199	KY104646	98.5	100	1	1
OTU_9	*Pestalotiopsis microspora*	*Pestalotiopsis microspora* strain AZ21	MH712224	100	100	0	2
OTU_10	*Botrytis cinerea*	*Botrytis cinerea* isolate BOT 01	MF405181	99.3	100	0	1
OTU_11	*Kodamaea ohmeri*	*Kodamaea ohmeri* strain PMM10-1288L	KP132356	88.4	100	1	0
OTU_15	*Candida tropicalis*	*Candida tropicalis* isolate LMICRO118	KJ451642	99.2	100	3	0
OTU_13	*Cyberlindnera jadinii*	*Cyberlindnera jadinii* strain ICMP 21923	MH393499	99.2	100	2	0
OTU_31	*Pichia manshurica*	*Pichia manshurica* strain ICMP 21927	MH393500	98.6	100	0	1
OTU_12	*Geotrichum candidum*	*Geotrichum candidum* strain 42415bDRJ	MF782775	99.5	100	0	1
OTU_24	*Galactomyces candidum*	*Galactomyces candidum* culture CBS:11616	KY103454	96.2	100	0	1
OTU_25	*Rhodotorula araucariae*	*Rhodotorula araucariae* culture CBS:6031	AF444510	94	100	1	0

983 **TABLE 3 |** PERMANOVA test (*p* values) from Pair-wise tests to compare the variation of the fungal community between five different host
984 fruit and their larvae
985

	Larvae (Hog Plum)	Larvae (Sapodilla/ Whiteside)	Larvae (Sapodilla/ Nambour)	Larvae (Pomegranate)	Larvae (Green Apple)	Larvae (Quince)	Fruit (Hog Plum)	Fruit (Sapodilla/ Whiteside)	Fruit (Sapodilla/ Nambour)	Fruit (Pomegranate)	Fruit (Green Apple)	Fruit (Quince)
Larvae (Hog Plum)												
Larvae (Sapodilla/Whiteside)	0.005											
Larvae (Sapodilla/Nambour)	0.007	0.21										
Larvae (Pomegranate)	0.013	0.012	0.014									
Larvae (Green Apple)	0.002	0.004	0.002	0.016								
Larvae (Quince)	0.017	0.012	0.012	0.091	0.586							
Fruit (Hog Plum)	0.01	0.046	0.083	0.012	0.002	0.012						
Fruit (Sapodilla/Whiteside)	0.004	0.83	0.098	0.014	0.008	0.022	0.009					
Fruit (Sapodilla/Nambour)	0.001	0.109	0.517	0.043	0.009	0.038	0.476	0.277				
Fruit (Pomegranate)	0.012	0.008	0.013	0.591	0.021	0.095	0.009	0.02	0.033			
Fruit (Green Apple)	0.006	0.002	0.005	0.022	0.065	0.186	0.006	0.004	0.003	0.011		
Fruit (Quince)	0.006	0.004	0.003	0.025	0.011	0.154	0.005	0.008	0.032	0.026	0.029	

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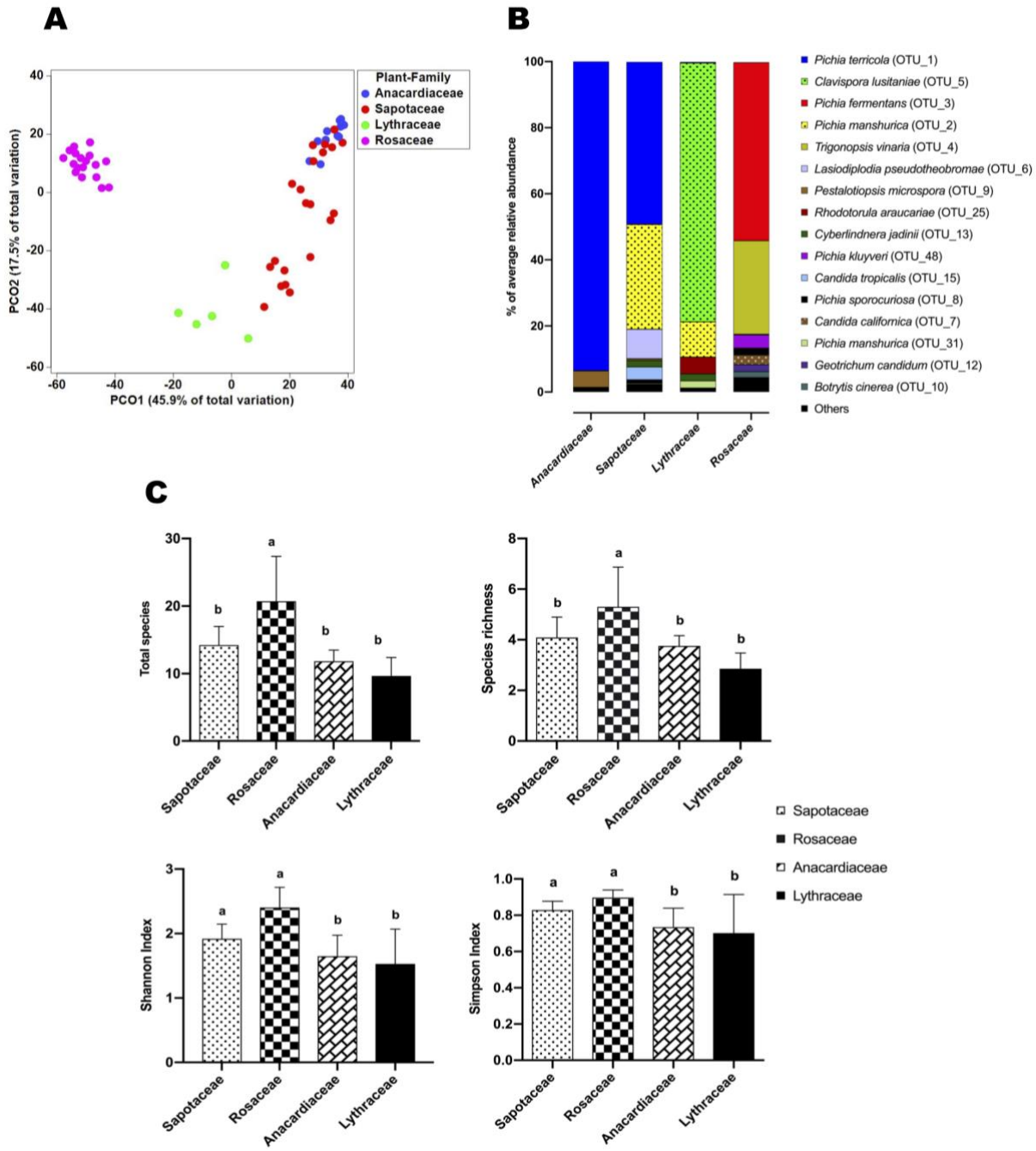
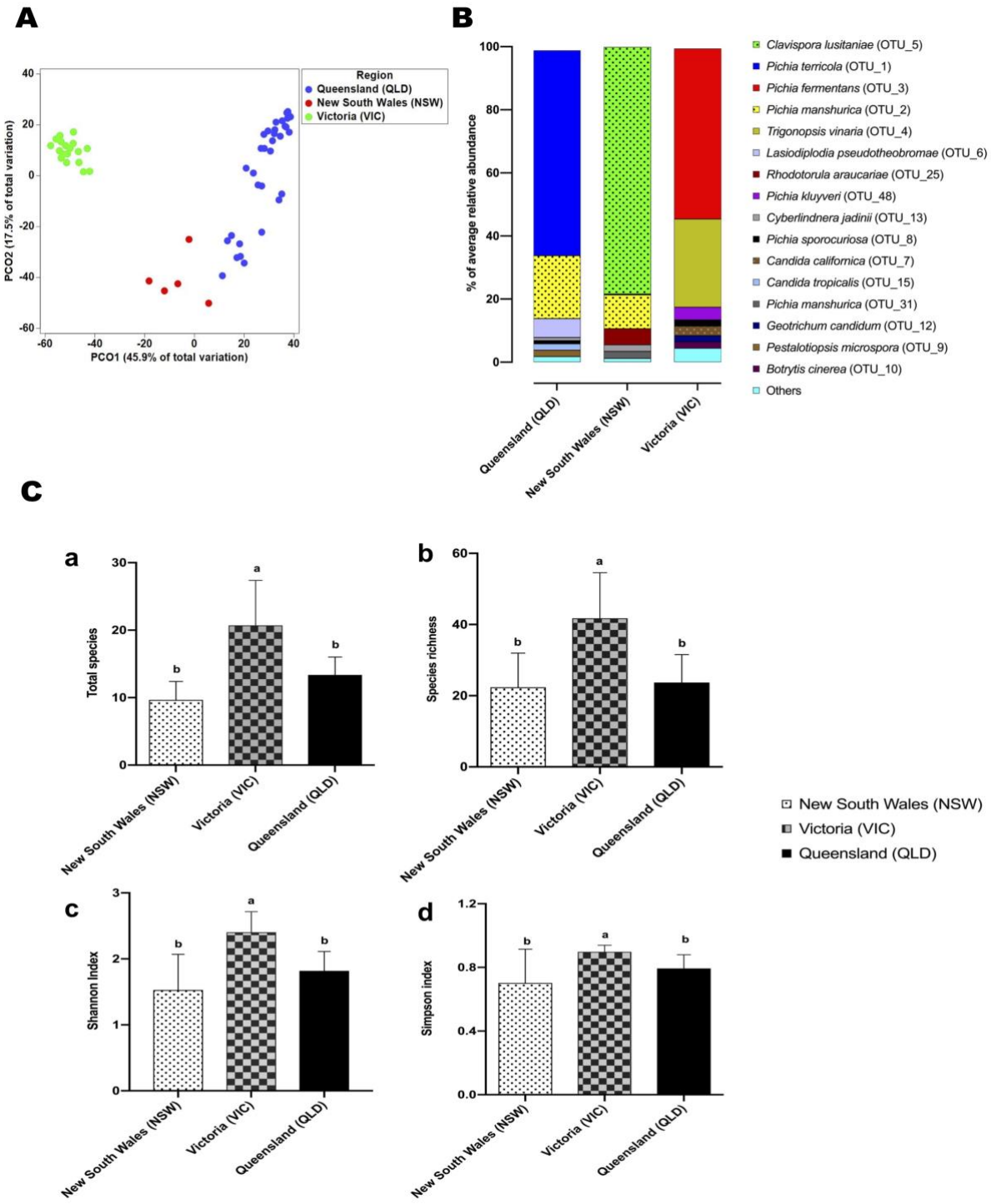
SUPPLEMENTARY FIGURES AND TABLES



SUPPLEMENTARY FIGURE S1 | Fungal microbiome of the Qfly larvae from host fruit from 4 different plant families. (A) Principal coordinates analysis; (B) fungal taxa of the Qfly larvae collected from 4 different plant families; (C) Alpha diversity of the fungal microbiome of the Qfly larvae and host fruits of different plant families. Different letters indicate significant Tukey's post hoc comparisons (p<0.05)



SUPPLEMENTARY FIGURE S2 | Fungal microbiome of the wild Qfly larvae from five type of fruit sources from 3 different region in Australia. (A) Principal coordinates analysis; (B) fungal taxa of the larvae of the Qfly collected from 3 different region in Australia; (C) alpha diversity of the fungal microbiome of the Qfly larvae and host fruit in different region. Different letters indicate significant Tukey's post hoc comparisons (p<0.05)

SUPPLEMENTARY TABLE S1 | Top 18 fungal OTUs with taxonomic classification observed in the Qfly larvae and fruit

OTU ID	Domain	Phylum	Class	Oder	Family	Genus	Species
OTU_1	Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	*Pichia*	*terricola*
OTU_2	Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	*Pichia*	*manshurica*
OTU_3	Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	*Pichia*	*fermentans*
OTU_4	Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	*Trigonopsis*	*vinaria*
OTU_5	Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Metschnikowiaceae	*Clavispora*	*lusitaniae*
OTU_6	Fungi	Ascomycota	Dothideomycetes,	Botryosphaeriales	Botryosphaeriaceae	*Lasiodiplodia*	*pseudotheobromae*
OTU_7	Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	*Candida*	*californica*
OTU_48	Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	*Pichia*	*kluyveri*
OTU_8	Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	*Pichia*	*sporocuriosa*
OTU_9	Fungi	Ascomycota	Sordariomycetes	Amphisphaeriales	Pestalotiopsidaceae	*Pestalotiopsis*	*microspora*
OTU_10	Fungi	Ascomycota	Leotiomycetes	Helotiales	Sclerotiniaceae	*Botrytis*	*cinerea*
OTU_11	Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	*Kodamaea*	*ohmeri*
OTU_15	Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	*Candida*	*tropicalis*
OTU_13	Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	*Cyberlindnera*	*jadinii*
OTU_31	Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	*Pichia*	*manshurica*
OTU_12	Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Dipodascaceae	*Geotrichum*	*candidum*
OTU_24	Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Dipodascaceae	*Galactomyces*	*candidum*
OTU_25	Fungi	Basidiomycota	Microbotryomycetes	Sporidiobolales	Sporidiobolaceae	*Rhodotorula*	*araucariae*

Chapter 4

This chapter has been formatted in accordance with style of Frontiers in Microbiology

Gut microbiome of the Queensland fruit fly during metamorphosis

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Running head: Gut microbial profiling of the *B. tryoni* during metamorphosis

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ABSTRACT

Bactrocera tryoni (Queensland fruit fly, or 'Qfly') is a highly polyphagous tephritid fly that is a serious economic pest in Australia. Qfly biology is intimately connected to the gut microbiome (both bacteria and fungi) although changes in the microbial community across developmental stages from the larvae to adults are still unknown. To address this knowledge gap, we used high throughput Next Generation Sequencing (NGS) to analyse both 16s rRNA and ITS amplicon on the Illumina MiSeq to comprehensively characterize the gut bacteria and fungi of the Qfly at each developmental stage. We observed that the bacterial family of Enterobacteriaceae is highly dominant only in the adults. The majority of fungal taxa present in the various developmental stages of the Qfly are yeasts or yeast like fungi. Comparative analysis between larvae, pupae and the adults gut microbiome revealed diverse microbial communities in larvae and adults. We found that microbial communities are similar in Qfly larvae and pupae, and between adult males and females. However, the larval microbiome was markedly distinct from that of adults. Specific bacterial and fungal taxa are present in the larvae and adult gut which is likely related to differences in their nutritional biology. This study implemented high throughput NGS to analyze both the bacterial and fungal microbiome of Qfly through development from larvae, to pupae, to adult.

Keywords: Gut bacteria and fungi, Yeast and yeast like, *B. tryoni*, Next generation sequencing

INTRODUCTION

From humans to insects, the gut microbiome plays an important role in host health and metabolism (Ottman et al., 2012; Oh et al., 2014). The symbiotic relationship between insects and their gut microflora is very complex, but is essential to insect health (Janson et al., 2008). In a study on *Drosophila,* Shin et al. (2011) identified that the gut microbiome influenced host gene expression, affecting host fitness through body development, nutritional metabolism, and stem cell activity. The interactions between hosts and gut microbial communities have been investigated in diverse insects, including beetles (Kudo et al., 2019), mosquitoes (Coon et al., 2014), butterflies (Ravenscraft et al., 2019), silk worms (Chen et al., 2018), house flies (Zhao et al., 2017) and red palm weevil (Tagliavia et al., 2014). Together, this body of research shows that a vast array of insect metabolic and behavioural functions are linked to the functioning of the microbiome. (Dillon and Dillon, 2004; Douglas, 2009; Calderón-Cortés et al., 2012; Hammer and Bowers, 2015; Ravenscraft et al., 2019. A comprehensive understanding of the target insect's gut microbiome is an essential step to understand the insect's biology but may also be a first step towards developing novel pest management strategies (Dillon and Dillon, 2004).

Although insect microbiomes are often analysed fractionally (either bacteria or fungi), it is important to recognise that they are comprised of both bacteria and fungi, and so have substantial taxonomic breadth. Additionally, different microbial taxa have different relationships with host insects. For example, the bacteria *Providencia*, a gram-negative opportunistic, non-spore forming pathogen (Galac and Lazzaro, 2011) and the fungi *Metarhizium anisopliae* are known insect pathogens (Lu et al., 2015). On the other hand, bacteria from the family Acetobacteraceae (includes Acetic acid bacteria) have a mutualistic relationship with honey bee *Apis mellifera* (Hymenoptera: Apidae) (Mohr and Tebbe, 2006; Babendreier et al., 2007) and pink sugar cane mealybug *Saccharococcus sacchari* (Cockerell) (Homoptera: Pseudococcidae) (Ashbolt and Inkerman, 1990), both of which have a sugar-based diet. In order to more comprehensively understand the different roles of these taxa in insect host health, an understanding of the diversity of gut microbiota is essential. Additionally, gut associated fungi are an essential source of amino acids, vitamins and enzymes, playing a role in insect detoxification, metabolism and pheromone production pathways (Dowd, 1989; Dowd, 1991; Vega and Blackwell, 2005; Nguyen et al., 2007). Our

knowledge of insect gut microbial communities, and their functional roles, is still in its infancy.

In addition to this complexity in microbial taxonomic and functional diversity, temporal changes also occur in the microbiome of insects. Perhaps the most dramatic example is the change in microbial communities through metamorphosis. Metamorphosis is a conspicuous and abrupt transformation process in which an insect undergoes a complex remodelling of its external and internal morphology (Truman and Riddiford, 1999; Grimaldi et al., 2005; Janson et al., 2008). All holometabolous insects undergo a metamorphosis process starting with a larval stage followed by a pupal stage and finally an adult stage. Numerous holometabolous insects are economically important, both as a source of production (e.g., honey bee) and as agricultural pests (e.g., many tephritid flies) (Yong et al., 2017b; c; Ventura et al., 2018), as well as vectors of infectious diseases (e.g., sandfly, mosquito) and as important experimental models (e.g., *Drosophila melanogaster*) (Truman and Riddiford, 1999; Grimaldi et al., 2005). The gut microbiota changes during metamorphosis [e.g., butterflies (Ravenscraft et al., 2019); silkworms (Chen et al., 2018); ground dwelling beetles (Kudo et al., 2019); long horn beetles (Mohammed et al., 2018)], most likely due to the anatomical transformation undertaken during this period (Johnston and Rolff, 2015). In tephritid fruit flies, changes in the gut microbiota through development have been investigated in *Bactrocera carambolae* (Yong et al., 2017a), *B. dorsalis* (Andongma et al., 2015) and *B. latifrons* (Yong et al., 2017c), but to date there is only fragmentary knowledge of how the gut microbiome (both bacteria and fungi) changes through development in Queensland fruit fly, *B. tryoni* ('Qfly').

Qfly is highly polyphagous and is the most economically damaging insect pest of Australian horticulture (Hancock et al., 2000; Clarke et al., 2011; Stringer et al., 2017). Due to its economic importance, numerous studies have been conducted on Qfly biology and physiology, including their ecology (Fletcher, 1974; Clarke et al., 2011; Nguyen et al., 2019), domestication (Meats et al., 2004; Gilchrist et al., 2012; Pérez et al., 2018), production quality traits (Dominiak et al., 2007; Collins and Taylor, 2011), behavior (e.g. mating performance) (Tychsen, 1977; Vijaysegaran et al., 1997; Pérez-Staples et al., 2007; Radhakrishnan et al., 2009), larval and adult nutritional requirement (Fanson et al., 2009; Fanson and Taylor, 2012) and microbiome (Thaochan et al., 2010; Morrow et al., 2015; Deutscher et al., 2018; Woruba, 2018; Majumder et al., 2019). Bacteria associated with wild

and domesticated larvae (Deutscher et al., 2018; Majumder et al., 2019), pupae (Fitt and O'Brien, 1985) and adult Qfly (Thaochan et al., 2010; Morrow et al., 2015; Woruba, 2018) have been described. Also, yeasts associated with domesticated larvae (Deutscher et al., 2016) and adults (Piper et al., 2017) have been described. Some of these studies have used culture-dependent approaches to profile the microbiota (Fitt and O'Brien, 1985; Thaochan et al., 2010; Deutscher et al., 2018), while others have used high-throughput sequencing technologies to circumvent the well-documented biases of culture-based methods (Morrow et al., 2015). However, there has been no comprehensive study of changes in the wild Qfly gut microbiome through metamorphosis from larvae, to pupae, to adults. Such a detailed study is important to overcome the highly constrained interpretation of comparisons across studies of different life stages that have been undertaken in different laboratories and applying different techniques.

In the present study we applied high-throughput sequencing technology to profile the bacterial 16S ribosomal RNA gene (rRNA) and fungal internal transcribed spacer region (ITS) obtained from larvae, pupae and the gut of adult Qfly. We identify the dominant gut bacteria and fungi at each of these stages, comparing these communities to understand how gut microbiota diversity and community structure changes through metamorphosis. We predicted that there would be some common dominant bacterial and fungal taxa that represented the microbiome of Qfly across the three developmental stages, while other taxa would be specific to a developmental stage.

MATERIALS AND METHODS

Qfly sample collection

Infested pomegranates, green apples and quinces were collected from different geographic locations in New South Wales (NSW) and Victoria (VIC), Australia (**Table 1**). The infested fruits were collected from under trees, and most were over-ripe. After collection, all fruits were stored in 60L plastic bins (Award, Australia) that contained a layer of vermiculite (1.0cm depth) (Grade 1, Sage Horticultural, VIC, Australia) in a controlled environment laboratory (25±0.20°C, 65±3% RH and 11h: 1h: 11h: 1h light: dusk: dark: dawn photoperiod). The emerged adult flies were supplied with hydrolysed yeast (MP Biomedicals,

Cat. no 02103304) and commercial sucrose (CSR® White Sugar), and were placed in mesh cages (Megaview Bugdorm 44545, 47.5 x 47.5 x 47.5 cm, Taiwan). This Qfly colony of Generation 0 (G0) was considered as wild-type, where larvae and pupae were collected from the natural host fruits although adult flies fed on hydrolysed yeast with sugar (2:1) and water for 15 days. Each developmental stage of the Qfly from G0, 3rd instar larvae (N=6), 8 days old pupae (N=6) and 15 days old sexually mature adults both male (N=6) and female flies (N=6) were collected for next generation sequencing.

Sample preparation

For sample processing, Qfly larvae, pupae and adult flies (male and female separately) were surface sterilized with 0.5% Tween 80 (Sigma-Aldrich, Cat. No. 9005656, USA), 0.5% Bleach (sodium hypochlorite) (Sigma-Aldrich, Cat. No.7681529, USA) and 80% ethanol (Sigma-Aldrich, Cat. No. 65175, USA) for 30s, and rinsed 3 times in 1M sterile phosphate-buffered saline (1x PBS) for 30s (Majumder et al., 2019). The PBS from the 2nd and 3rd washes were kept and 100 µL spread-plated on to five types of microbial growth medium (de Man, Rogosa and Sharpe Agar, Tryptone Soya Agar, Macconkey Agar, Potato Dextrose Agar and Yeast-dextrose Agar medium) (Sigma-Aldrich, USA) to confirm surface sterilization of the insects. All plates were incubated at 35°C for 24 to 48 hr. Post sterilization, the guts of adult flies were dissected under a stereomicroscope (Leica MZ6, Leica®, Germany). Using sterile pestles, larvae, pupae, and dissected guts from the adults were homogenised separately in a sterile solution of Brain Heart Infusion (BHI) broth (Oxoid Ltd, UK, Lot # 1656503) and 20% glycerol (Sigma Aldrich®, Lot # SHBG2711V, USA) and each sample split into two separate cryovial tubes (Simport Scientific, Canada) for both COI gene identification and next generation sequencing analysis. All samples were preserved at -80°C for future use. All procedures were completed in a sterile environment (Biological Air Clean Bench, safe 2020 1.2, Thermo Scientific, Germany). Furthermore, a complete experimental design was explained in a flowchart (**supplementary figure 3)**

Qfly identification using mitochondrial Cytochrome Oxidase I (COI) gene

The mitochondrial cytochrome oxidase I (COI) gene sequencing of all samples for Qfly identification were performed. DNA was extracted from Qfly samples using Isolate II

genomic DNA kit from Bioline, USA (Cat. no. BIO-52065) following the manufacturer's protocol. DNA extract concentrations were then determined using the Invitrogen™ Qubit® dsDNA High Sensitivity (HS) Assay Kit (Life Technologies, USA). Standard LCO1490/ HCO2198 primers were used to amplify a 700 bp segment of the CO1 gene (Folmer et al., 1994). All PCR amplifications in the present study were performed in an Eppendorf thermocycler (Eppendorf, Germany) using the following conditions: each 15 µL reaction was conducted in triplicate and contained 7.5 µL of MyTaq HS PCR master mix (Bioline, USA. Cat No. BIO-25045), 0.60 µL of forward (LCO1490F) and reverse primer (HCO2198R), and 1.5 µL of DNA. The PCR profile included an initial denaturing step at 95°C for 2 min, followed by 35 cycles of 94°C for 30s, 50°C for 30 s and 72 °C for 90s, with a final extension step of 72 °C for 5 min. Amplicons were visualised using electrophoresis on a 1% agarose gel (110v-45min). Amplicons were then sent to the Australian Genome Research Facility (AGRF) for Sanger sequencing. Sequence data were analysed by Geneious R10.2.3 to confirm Qfly identification. In addition to this molecular confirmation, microscopic examination of larval morphological features was carried out prior to DNA extraction (White and Elson-Harris, 1992). Additional confirmation was gained through morphological observation of emerged adult flies under a stereomicroscope (Leica MZ6, Germany) (Plant Health Australia, 2011).

Qfly microbiome profiling

DNeasy Power Lyzer Power Soil Kit-100 (Qiagen, Germany) (Cat. no. 12888-100) was used for each sample following the manufacturer's procedure. DNA extracts were then quantified by Invitrogen™ Qubit® dsDNA High Sensitivity (HS) Assay Kit (Life Technologies, USA). PCR amplification and sequencing were performed by the Australian Genome Research Facility. For the bacterial identification, the V1-V3 16S rRNA region was amplified using primers 27F (5′AGAGTTTGATCMTGGCTCAG-3′) and 519R (3′ GWATTACCGCGGCKGCTG-5′) and for the fungi, the ITS region of RNA gene was amplified using the fungal-specific forward primer ITS1f (CTTGGTCATTTAGAGGAAGTAA) and the ITS2 reverse primer (GCTGCGTTCTTCATCGATGC). Reactions contained 1X AmpliTaq Gold 360 mastermix (Life Technologies, Australia), 0.20 µM of each forward and reverse primer with 25 µL of DNA. PCR cycling conditions consisted of denaturation at 95ºC for 7 min, 35 cycles of 94ºC

for 45s, 50ºC for 60 s and 72ºC for 60s, and a final extension of 72ºC for 7 minutes. A secondary PCR was used to adhere sequencing adaptors and indexes to the amplicons. Primerstar max DNA Polymerase used for secondary PCR amplicon generation from Takara Bio Inc. Japan (Cat. No. #R045Q). The resulting amplicons were measured by fluorimeter (Invitrogen Picogreen, Thermo Fisher Scientific, Australia) and normalised (Fouts et al., 2012). The eqimolar amounts of each sample were pooled and quantified qPCR prior to sequencing (Kapa qPCR Library Quantification kit, Roche, Switzerland). The resulting amplicon library was then sequenced on the Illumina MiSeq platform (San Diego, CA, USA) with 2 x 300 base pairs paired-end chemistry (Caporaso et al., 2010). The Illumina sequences were deposited in the NCBI GenBank under Bio-project PRJNA556787.

Sequence data processing

Both bacterial 16s rRNA and fungal ITS amplicons were processed by first aligning forward and reverse reads using PEAR (version 0.9.5) (Zhang et al., 2013). For the 16S rRNA data, quality filtering, clustering and taxonomic assignments were achieved using the 'usearch' tools (Edgar, 2010; Edgar et al., 2011) and rdp_gold database as a reference (Cole et al., 2013). Any 16S rRNA OTUs with taxonomic assignments to eukaryotic organelles (e.g., chloroplast) were removed from the dataset. The ITS sequence data processing was completed, and sequences were quality filtered, using Usearch tools. Full length duplicate sequences were removed and sorted by abundance. Singletons or unique reads in the data set were discarded. Sequences were clustered followed by chimera filtering using the "Unite" database as reference. To obtain the number of reads in each OTU, reads were mapped back to OTUs with a minimum identity of 97%. Qiime taxonomy was assigned using Unite database (Kõljalg et al., 2005) (Unite Version7.1 Dated: 22.08.2016). An in-house python script was applied for rarefaction. To maintain equal sequence depth among all samples, we then rarefied to 14,000 reads per sample for bacteria and 1000 reads for fungi, repeating these 50 times and averaging the counts to obtain a representative rarefaction. Samples with <14,000 reads and <1000 reads for bacteria and fungi, respectively, were deleted. The data were then normalised as the percentage of relative abundance and is henceforth referred to as the OTU table (**Supplementary file 1**). All the figures of bacterial average relative abundance in different taxonomic levels were plotted in Prism 8 (version 8.0.1(145), GraphPad software, Inc).

Statistical analysis

The bacterial and fungal OTU tables were imported into Primer-E v7 for analysis (Clarke and Ainsworth, 1993; Sutcliffe et al., 2017). The average relative abundance of the bacterial community members in the larval and pupal microbiome, as well as that in adult males and females was analyzed. The DIVERSE function was used to generate univariate biodiversity metrics, species richness, Pielou's evenness and Shannon's and Simpson's biodiversity indices. Statistical differences between these metrics were assessed in JMP Statistical Software Version 10.0.0 (SAS Institute, Cary, NC, USA) using one-way analysis of variance (ANOVA) and Tukey-Kramer's post hoc analysis. To observe the taxonomic compositional changes in the bacterial and fungal communities, the OTU table was first log transformed using Primer-E V7. A Bray-Curtis similarity matrix was derived from this transformed data and a permutation analysis of variance (PERMANOVA) pairwise comparison was conducted to compare all community samples. A p value of <0.05 was considered statistically significant. Further, ordination plots of these communities were visualised using principal coordinates analysis (PCoA) in Primer-E.

RESULTS

Identification of the fruit fly as Qfly

We confirmed that all larval, pupal and adult fly samples included in this study were Qfly by both morphological and molecular testing. The genetic testing was done by analyzing the mitochondrial cytochrome oxidase I (COI) gene using Sanger sequencing and confirmed that all 24 Qfly samples were Qfly. Additionally, approximately 600 adult Qfly developed in the colony were identified as Qfly. No other fly species were identified from the experimental samples. Furthermore, the surface sterilization process of the larvae was found to be effective as there was no microbial growth detected in different growth media after 24 to 48 hours incubation. In our study, we used 15 days old mature flies. Previous studies from our group have observed that bacterial and fungal numbers are too low for 16S and ITS analysis when flies are newly emerged and so we have allowed 15 days for the fly microbiome to be sufficiently established before sampling (result not shown).

Gut bacterial alpha and beta diversity of Qfly during metamorphosis

After rarefaction to 14,000 reads per sample and quality control, 74 bacterial OTUs were detected (**Supplementary file 1**). These taxa spanned 6 phyla, 14 classes, 38 families and 49 genera, however, despite this broad taxonomic range, the majority were rare in abundance. Only 11 genera were classed as abundant (**Table 2**).

Bacterial alpha biodiversity metrics were compared between the developmental stages (**Figure 4A-D**). Shannon indices were significantly different between larvae and adults (both male and female flies) ($p<0.05$) (**Figure 4A**). None of the bacterial alpha diversity metrics showed significant differences between adult males and females. Species richness of the bacteria was highest in larvae and lowest in the adult male gut microbiome (**Figure 4C**). Beta diversity of the bacterial communities at each Qfly stage was assessed by PERMANOVA analysis (pair-wise test with 999 permutation) based on Bray-Curtis similarities (**Supplementary file 3**). Additionally, a principal coordinate analysis (PCoA) was plotted with Bray-Curtis similarity matrix to visualize variation among host microbial communities (**Figure 3A**). PCoA and PERMANOVA both found no evidence of differences between the bacterial communities of the larval and pupal microbiome (PERMANOVA test, $p=0.578$) and the same was observed among adult male and female gut microbiome (PERMANOVA test, adult male and female $p=0.472$, **Figure 3A**). However, a clear structural difference was observed in bacterial communities of Qfly larvae and adults (PERMANOVA < 0.05, **Figure 3A**, **Supplementary file 3**).

Gut bacterial communities associated with the Qfly metamorphosis

At the phylum level, the most abundant taxa in the larval microbiome was Proteobacteria (98.20%), followed by Bacteroidetes (1.70%) and Actinobacteria (0.01%). In adult flies, Proteobacteria represented an even larger percentage of the microbiome, 99.95% for females and 100% for males. Adult female flies were the only samples to host Firmicutes (0.04%). Actinobacteria and Unassigned bacterial phylum were only found in the larvae and pupal microbiome.

At the family level, the most prevalent bacterial taxa were the gammaproteobacterial Enterobacteriaceae, which represented an average of 76.1% of the relative abundance at all developmental stages. The alphaproteobacterial Acetobacteraceae was observed to be highly abundant both in larvae (48.58%) and pupae (42.98%), but were substantially less abundant in adult males (0.18%) and females (1.09%). The gammaproteobacterial Xanthomonadaceae was only observed in the larvae (0.03%) and pupae (0.33%). Conversely, gammaproteobacterial Enterobacteriaceae were more abundant in adults (males 99.78% and females 98.80%) compared with the larvae (49.59%) and pupae (56.22%).

At the genus level, bacterial taxa with average relative abundance were listed as *Swaminathania/Asaia* (17%), *Erwinia* (10.5%), *Providencia* (5.6%), *Acetobacter* (2.9%), *Gluconobacter* (2.2%) and unassigned Acetobacteraceae (1.1%) (**Figure 1, Table 2, supplementary figure 1**). Other observed bacteria were classified as *Wautersiella,* unassigned Comamonadaceae, unassigned Xanthomonadaceae and unassigned bacteria with very low average relative abundance (<1%). Unassigned Enterobacteriaceae revealed the highest average relative abundance (60.1%). Few highly abundant sequences belonging to Enterobacteriaceae were blasted again with Geneious R10.2.3 and NCBI to reconfirm the genus level and the bacterial genus *Enterobacter* was then observed. *Swaminathania/Asaia* was highly abundant both in larvae (29.2%) and pupae (37.6%) but had relatively low abundance in adult guts (male 0.2% and female 1.1%). In contrast, the average relative abundance of the *Enterobacter* was particularly high in adults (males 80.4% and females 94.8% respectively) compared with larvae and pupae. *Erwinia* was detected with 22.6% and 18.1% relative abundance in larvae and pupae respectively (**Table 2**). However, average relative abundance of only 1.1% was observed in adult females and *Erwinia* was not detected in adult males.

Gut fungal alpha and beta diversity during Qfly metamorphosis

The fungal microbiome of 24 Qfly samples were sequenced, of which 22 were retained after quality control and rarefaction at 1000 reads per sample. A total of 96 fungal ITS OTU sequences were identified with taxa spanning 4 phyla, 11 classes, 35 families and 40 fungal genera (**Supplementary file 1**). Among them, only 14 fungal genera (~10%) were listed as abundant (**Table 3**).

The species richness, Pielou's evenness, Shannon's and Simpson's biodiversity indices were illustrated in the alpha diversity of the fungal communities (**Figure 5A-D**). Generally, the fungal communities associated with the Qfly were less diverse than the bacterial communities. The fungal communities were only significantly different in species richness across the developmental stages ($P<0.05$) (**Figure 5C**). The larval and pupal fungal microbiome had higher species richness compared to adults (**Figure 5C**). PERMANOVA analysis (pair-wise test with 999 permutation) and a principal coordinate analysis (PCoA) were examined to address the beta diversity of the fungal communities (**Supplementary file 3, Figure 3B**). PCoA axes PCO1 and PCO2 accounted for 27.6% and 12.8% of total variation seen in fungal communities. PERMANOVA results suggest that fungal species groups at each developmental stage were significantly distinct ($P<0.05$). Additionally, PCoA clusters reflected the variation of the fungal communities at the different developmental stages. There was no significant difference between the larval and pupal microbiome, but the fungal community was significantly different ($P<0.05$) between larvae and adults.

Overall, both bacterial and fungal diversity followed a trend of decreasing from the larval stage to the adult stage. Additionally, The PCoA plots also showed similar results with the bacterial and fungal communities of the larvae and pupae clustering together and separating from the adults and adult male and female gut microbial communities clustering closely together.

Gut fungal communities associated with the Qfly metamorphosis

The most abundant fungal phylum was Ascomycota (94.28%), followed by Basidiomycota (4.94%). In a complex community of fungi, Unassigned fungi was abundant in larvae (0.15%) and pupae (2.90%) but not in adults. The phylum Basidiomycota was observed in the adult male gut but was rarely observed in other developmental stages.

Trichocomaceae and unassigned fungi were the two dominant fungal families found with average relative abundance of 21.40% and 20.05% respectively. Other observed families included Incertae sedis (23.16%), Pichiaceae (9.71%), Nectriaceae (4.92%), Saccharomycetaceae (4.86%), unassigned Tremellomycetes (4.86%), Cladosporiaceae (4.79%), Trichomonascaceae (2.83%), Debaryomycetaceae (1.57%) and Didymosphaeriaceae (1.03%). Trichocomaceae was highly abundant in adult males (47.77%)

compared to other developmental stages. Additionally, unassigned fungi were abundant. The fungal family Pichiaceae was highly abundant in larvae and pupae, but not in adults. Cladosporiaceae and Debaryomycetaceae were both only abundant in adult females and pupae. In contrast, Trichomonascaceae was not abundant in the fungal microbiome of the larvae but was present in other life stages.

The most abundant fungi were associated with the form of yeasts that contained the closet match of the genera (relative abundance >1%) of *Penicillium*, unassigned fungi, *Candida, Pichia, Cyberlindnera, Gibberella,* unassigned Tremellomycetes*, Cladosporium, Zygosaccharomyces, Zygoascus, Meyerozyma* and *Pseudopithomyces* (**Figure 2, Table 3, Supplementary figure 2**). Compared to the adult female (10.8%) and larvae (0.4%), adult males (47.8%) harbored a much higher proportion of the fungal genus *Penicillium.* Unassigned Tremellomycetes (19.4%), *Gibberella* (19.6%) and *Pseudopithomyces* (4.1%) were abundant only in the adult male gut microbiome (**Table 3**). Unassigned fungi comprised a quite substantial proportion of relative abundance (20.1%) overall. *Candida* and *Pichia* were abundant prominently in the larval and pupal microbiome but not in adults. This result indicated that certain fungi present in the larval and pupal stages did not transmit to the adults. *Cyberlindnera* (32.3%) was abundant only in the female gut and was completely absent in males and other developmental stages. Conversely, Qfly larvae contained *Zygosaccharomyces* at relative abundance of 16.1% but these were not found in other life stages.

DISCUSSION

The present study identifies and characterizes the microbial communities present in the different developmental stages of wild-type Qfly at the point of entry into laboratory rearing. The use of high-throughput sequencing methods to profile both bacterial and fungal elements of the microbiome circumvents the well-known difficulties in isolating microbes through traditional, culture-dependent methods. Indeed, a number of the taxa identified in both bacterial and fungal datasets were highly novel, with no closely related cultured representatives. This approach enabled us to assess biodiversity independent of culturing, and thus interrogate how the Qfly gut microbiota changes through development and between the adult sexes. Our research suggests that both bacterial and fungal communities of larvae and pupae are significantly different from the adults of the Qfly.

Bacterial communities and diversity in Qfly during metamorphosis

The PCoA plot revealed that bacterial communities in larvae and pupae cluster closely and clearly separated from the adults (both male and female). The pupal stage, however, appeared as transitional, exhibiting no significant differences when compared with both larvae and adults (**Figure 3A, Supplementary file 3**). During the pupal period, the gut microbiome undergoes minimum metabolic activity. Like other holometabolous insects (e.g., Bark beetle *Dendroctonus rhizophagus*), morphological changes during Qfly metamorphosis might impact on the bacterial community structure (Morales-Jiménez et al., 2012). Previous research on butterfly gut bacteria during metamorphosis are consistent with our findings (Hammer et al., 2014). PERMANOVA analysis found no significant difference between the adult male and female gut bacterial communities. We expected to find a correlation among larval and adult microbial communities but observed significant ($P<0.05$) differences in the Shannon indices. Similarly, Moll et al., (2001) found that the gut community structure of the mosquitooes (Diptera: Culicidae) *Anopheles punctipennis* (Say), *Culex pipiens* (L.), and *Aedes aegypti* (L.) changes rapidly during metamorphosis. The environment, diet and developmental time can all be key factors affecting gut microbial diversity in insects (Yun et al., 2014; Chen et al., 2016; Martinson et al., 2017), and might each contribute to the results of the present study. Our results are also consistent with previous findings in gut microbial analysis across developmental stages of *B. dorsalis* (Zhao et al., 2018; Stathopoulou et al., 2019) and *B. carambola* (Yong et al., 2017a).

This study revealed that the Proteobacteria phylum was the most dominant bacterial taxa found across the three developmental stages of the larvae, pupae and adults (male and female both) of the Qfly. This trend has also been observed in other *Bactrocera* species, including in studies of *B. carambola* metamorphosis (Yong et al., 2017a). Further, these findings are consistent with the high abundance of Proteobacteria reported in adults of other tephritid species, including *B. cacuminata, B. dorsalis, B. jarvisi, B. minax, B. neohumeralis,* and *Ceratitis capitata* (Wang et al., 2014; Andongma et al., 2015; Yong et al., 2017b) as well as in other studies of Qfly (Morrow et al., 2015; Woruba, 2018). Within the Proteobacteria phylum, different families were associated with different life stages. For example, the Enterobacteriaceae was found to be the most dominant family in adult Qfly, but in both the

larvae and pupae the Acetobacteraceae was the most abundant family. Woruba (2018) also found Enterobacteriaceae associated with the phylum of Proteobacteria as a dominant family present in the gut microbiome of wild Qfly adults. Enterobacteriaceae are known to be transmitted to Qfly larvae via vertical transmission during oviposition (Deutscher et al., 2018; Majumder et al., 2019). These bacteria present in *B. oleae* larvae, fix nitrogen and perform pectinolysis in the gut (Behar et al., 2008; Ben-Yosef et al., 2014). Additionally, previous studies of *C. capitata* larvae showed that supplementation of *Enterobacter* spp. in the diet decreases developmental time, and increases pupal weight and mating performance during mass rearing (Ben Ami et al., 2010; Gavriel et al., 2011; Hamden et al., 2013; Augustinos et al., 2015). Within the Enterobacteriaceae family, a number of interesting trends were observed at the genus level. For example, we identified the pathogenic bacterium *Erwinia* in the larvae, pupae, and adult female Qfly microbiome. *Erwinia* works on nitrogen fixation and helps insect to sustain in an environment with limited oxygen (Martínez-Falcón et al., 2011). It is likely that these functions are required at all life stages in the Qfly, potentially explaining the consistency in their relative abundances across the dataset. In comparison, the pathogenic bacterium *Providencia* was only found in adult male and female gut microbiota. Surprisingly, *Providencia, Enterobacter* and *Swaminathania/Asaia* were observed in both male and female Qfly gut microbiome collected from the wild (Woruba, 2018).

On the other hand, we observed dominance of all three are major bacterial genera *Swaminathania/Asaia, Acetobacter* and *Gluconobacter* associated with alphaproteobacterial Acetobacteraceae. These acetic acid bacteria work on carbohydrate metabolism and were observed highly abundant only in immature stages (larvae and pupae) of the Qfly, having very low abundance in adults. A similar observation was reported for *B. dorsalis* (Andongma et al., 2015). Previously, *Swaminathania/Asaia* was also detected very poorly in the adult phase of the both Qfly (Morrow et al., 2015; Woruba, 2018) and *B. oleae* (Sacchetti et al., 2008). Although the role of the *Swaminathania/Asaia* is still unknown in tephritid (Deutscher et al., 2018; Woruba, 2018), these bacterial taxa are also observed in the adult *D. melanogaster* and *B. oleae*, mosquitoes of the genera *Anopheles* and *Aedes*, and the honeybee *Apis mellifera* (Crotti et al., 2010). In addition, Chouaia et al. (2010) observed that, lack of the *Swaminathania/Asaia* spp. delayed larval development in *Anopheles stephensi.* *Acetobacter pomorum* and *Swaminathania/Asaia* supply essential nutrients that improve larval development in *Drosophila* and *Anopheles gambiae* mosquitoes (Shin et al., 2011; Mitraka et al., 2013). Therefore, we hypothesize the reason behind life stage-specific

variation in abundance is that *Swaminathania/Asaia* might be essential to be a symbiont during the larval stages to maintain proper larval development but much less important in adult stages. Further, it might be that the larval gut bacterial communities (mostly *Swaminathania, Acetobacter* and *Gluconobacter*) are favored by the plant-based diet rich with carbohydrate whereas adults consume more protein (Stathopoulou et al., 2019). Considering this, the bacterial communities in adults (mostly from the bacterial family Enterobacteriaceae) may be more focused on protein metabolism than the larval microbiome. Additionally, among all the identified bacteria in the Qfly, a large proportion of the unidentified bacteria from the Enterobacteriaceae and Acetobacteraceae were also detected.

Fungal communities and diversity through Qfly metamorphosis

The mycobiome of the Qfly during development includes various types of fungi and yeast. In the present study, many unidentified fungi and yeast, which are generally difficult to isolate or culture following traditional methods, were identified. Our previous study on the fungal microbiome of Qfly larvae using NGS found yeast and yeast-like fungi to be abundant in Qfly (data not shown). Furthermore, while using the fungal ITS amplicon to identify fungal species is mostly accepted in scientific communities, it is very difficult to distinguish very similar fungi (Bellemain et al., 2010; Kiss, 2012; Schoch et al., 2012; Malacrinò et al., 2015). To our knowledge, this study on the fungal microbiome of Qfly across life stages using NGS is the first not only in Qfly but also in any other *Bactrocera* species.

In our study, alpha diversity analysis and PERMANOVA analysis indicated that fungal diversity has general trends that are similar to those for bacterial diversity during development. Species evenness, Shannon and Simpson indices all indicated a simple structure in the fungal community across stages. The fungal species richness was significantly different between the larvae and adult male gut microbiome. Among all developmental stages of the Qfly, species richness was highest in the larvae followed by pupae, while adult males contained the lowest number of fungal species.

Yeast and yeast like fungi produce proteins, carbohydrates, and lipids that can provide important nutrition to host insects (Martin, 1987; Nardon and Grenier, 1989; Vega and Blackwell, 2005). Yeast and yeast like fungi present in the insect gut also contribute to amino acid and fatty acid metabolic pathways and, consequently, development through

538 metamorphosis can be compromised if yeasts are not present (Vega and Blackwell, 2005;
539 Carvalho et al., 2010). Although Qfly do not feed during the pupal stage, they maintain a
540 microbiome that might support metabolic activities during the pupal stage. Based on PCoA
541 ordination plots, the fungal microbial communities varied across the Qfly developmental
542 stages with larvae being clearly separated from the adults but similarity between larval and
543 pupal stages. It might be that the morphological transformation of the Qfly from the larval
544 stage to pupal stage causes reduction of metabolic activities which impact on the fungal
545 communities that transmit to the adult stage (Morales-Jiménez et al., 2012; Zhang et al.,
546 2018). The structure of the gut microbial communities of insects can be modified through
547 enzyme production according to host morphology during metamorphosis and diet (Franzini et
548 al., 2016). Our findings are somewhat similar to those of Hu et al. (2015) for fungal
549 community structure of the Chinese white pine beetle (*Dendroctonus armandi*) across
550 different developmental stages.
551
552 We identified that at the phylum level, Ascomycota was present at the highest
553 abundance in every developmental stage of the Qfly gut microbiome. Our study found the
554 maximum number of ascomycetes associated genera of *Penicillium, Candida, Pichia,*
555 *Cyberlindnera, Gibberella, Cladosporium, Zygosaccharomyces. Zygoascus, Meyerozyma,*
556 *Aspergillus* and *Saccharomyces* (**Figure 2**). These fungi are mostly identified as single cell
557 fungi commonly known as the budding yeasts (Ravenscraft et al., 2019). In our study,
558 different types of yeast were found at different development stages of Qfly. Therefore, it
559 might be that the Qfly ingest yeast as a food source during larval and adult stages and few
560 strains are able to transmit across all developmental stages. The fungal taxa *Candida* and
561 *Pichia* were highly abundant in the larval and pupal stage but were comparatively rare in
562 adults. Similarly, a comprehensive fungal analysis of adult wild *B. oleae* did not identify any
563 fungi associated with *Candida* and *Pichia* genera (Malacrinò et al., 2015). In contrast, these
564 fungi have been reported in other insects including spotted wing *Drosophila*, *D. suzukii*
565 (Matsumura) (Diptera: Drosophilidae) (Hamby et al., 2012) and *Agrilus mali* (Coleoptera:
566 Buprestidae) (Zhang et al., 2018). Similarly, the yeast genus *Saccharomyces* was only found
567 in adult female gut of the Qfly. *Saccharomyces* have also been detected in the adult stage of
568 *B. oleae* (Diptera: Tephritidae) (El Haidani et al., 2008). Vega and Blackwell, (2005)
569 demonstrated that *Saccharomyces* and *Candida* produce digestive enzyme including *β*-
570 glucosidases, xylases, and cellulases to help the host insect in digestion. These yeasts can
571 play a vital role on detoxification of the toxic compounds from plants (Yun et al., 2014).

572

573 The genus *Penicillium* was dominant mostly in the adult gut. However, the average
574 relative abundance of *Penicillium* was significantly lower in larvae and gradually increased
575 from the pupal microbiome to adults. Previously, *Penicillium* has been detected in other fruit
576 flies including *C. capitata* (Diptera: Tephritidae) (Konstantopoulou and Mazomenos, 2005)
577 and *B. oleae (*Diptera: Tephritidae) pupae and adults (both male and female gut) (Malacrinò
578 et al., 2015). Deutscher et al., (2016) isolated *Penicillium* from the midgut of Qfly larvae
579 using culture-based methods. Various toxigenic species are included under the fungal genus
580 *Penicillium* and mostly produce mycotoxin. The *Penicillium* species *P. chrysogenum* and *P.*
581 *notatum* are used to produce the commercial antibiotic Penicillin (Sweeney and Dobson,
582 1998; Demain and Fang, 2000). However, Konstantopoulou and Mazomenos, (2005)
583 demonstrated the *Penicillium* toxin was not toxic to insects. Furthermore, *Cladosporium,*
584 *Zygoascus* and *Meyerozyma* were also found to be abundant in adults mostly in female gut
585 fungal communities. The genus *Cladosporium* has also been found to be abundant in the gut
586 of both male and female *B. oleae* (Malacrinò et al., 2015). *Cladosporium* associated with
587 sooty mould communities are mainly abundant in plant phylloplane and carpoplane (Frisullo
588 and Carlucci, 2011; Flessa et al., 2012). Additionally, Bensch et al. (2012) demonstrated that
589 the fungal genus *Cladosporium* can cause plant diseases. *Cladosporium* was poorly abundant
590 in the pupal stage of Qfly but highly abundant in adult females. It might be that
591 *Cladosporium* starts colonization during the pupal stage and then becomes abundant in the
592 adults. The opposite was found in genus *Zygoascus*. This yeast was highly abundant in the
593 pupal stage but was much less abundant in the Qfly adult gut microbiome. The yeast genus
594 *Zygoascus* has also been reported in beetles (Suh et al., 2005). The fungal genera
595 *Meyerozyma* and *Aspergillus* were abundant in pupae. *Meyerozyma* has also been found in
596 burying beetles (*Nicrophorus vespilloides)* (Vogel et al., 2017) and in the hindgut of carrion
597 beetles (Coleoptera, Silphidae) (Kaltenpoth and Steiger, 2014). *Aspergillus* has also been
598 detected in *B. oleae* (Malacrinò et al., 2015). In our study, some plant pathogens were found
599 to be associated with some Qfly developmental stages. We predicted that Qfly might act as a
600 host carrier of these fungal pathogens and ingest them with food or from the environment.
601 For example, fungal species of *Colletotrichum* causes olive anthracnose that greatly effects
602 on the quality of both fruits and oil (Cacciola et al., 2012; Schena et al., 2014).
603 *Colletotrichum* are sourced by *B. oleae* from olive fruits and are present for at least a part of
604 their life cycle with this pest fruit fly as well as infesting the fruit, *B. oleae* might be an
605 important disease carrier that spreads *Colletotrichum* among olive fruits. (Moral et al., 2009;

Malacrinò et al., 2015). Unidentified fungi were found in every developmental stage of Qfly but were highly abundant in the larvae. These yeasts are likely transmitted horizontally to the larvae from infested fruits during ingestion of fruit flesh.

CONCLUSIONS

The present study demonstrates that gut microbial communities both of bacteria and fungi differ between the larvae and adults of the Qfly. Our findings contribute to increased understanding of microbiome (both bacteria and fungi) present in the Qfly through development. This knowledge may enable us to manipulate the gut bacteria and fungi to improve artificial diet both for the larvae and adults, thereby improving the quality of artificially reared Qfly, and may also provide useful starting points for the development of pest management solutions.

SUPPLEMENTARY FILES

Supplementary file 1: OTU table (both bacteria and fungi)
Supplementary file 2: Mapping file (both bacteria and fungi)
Supplementary file 3: PREMANOVA data (both bacteria and fungi)

AUTHORS CONTRIBUTIONS

RM and TC designed the experiment. RM collected the data. RM and BS analysed the data. TC and PWT supervised the project. RM wrote the manuscript. All authors provided inputs into the writing of the manuscript, and approved the submitted version.

ACKNOWLEDGEMENTS

We thank Dr Ania Deutscher for her support on fruit samples collection and larval sample preparation.

CONFLICT OF INTERESTS

The authors have no conflict of interests to declare.

FUNDINGS

This research was conducted as part of the SITplus collaborative fruit fly program. Project *Raising Q-fly Sterile Insect Technique to World Standard* (HG14033) is funded by the Hort Frontiers Fruit Fly Fund, part of the Hort Frontiers strategic partnership initiative developed by Hort Innovation, with co-investment from Macquarie University and contributions from the Australian Government. RM is supported by an International Macquarie University Research Excellence Scholarship (IMQRES) from Macquarie University (NSW, Australia).

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FIGURES AND TABLES

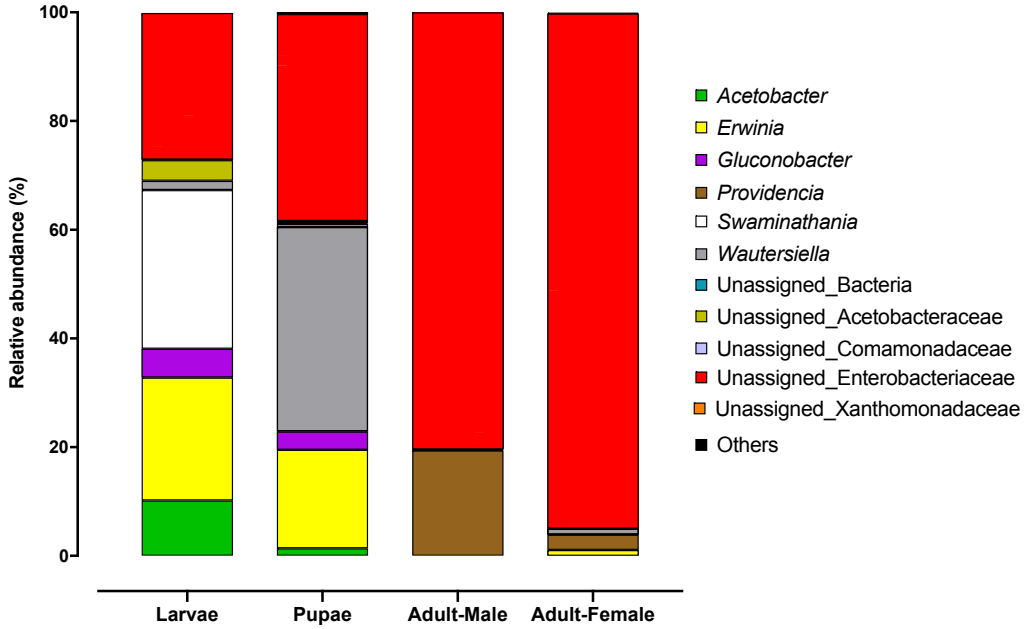

FIGURE 1 | Percentage of average relative abundance of the bacterial genera present in the different developmental stages of the Qfly

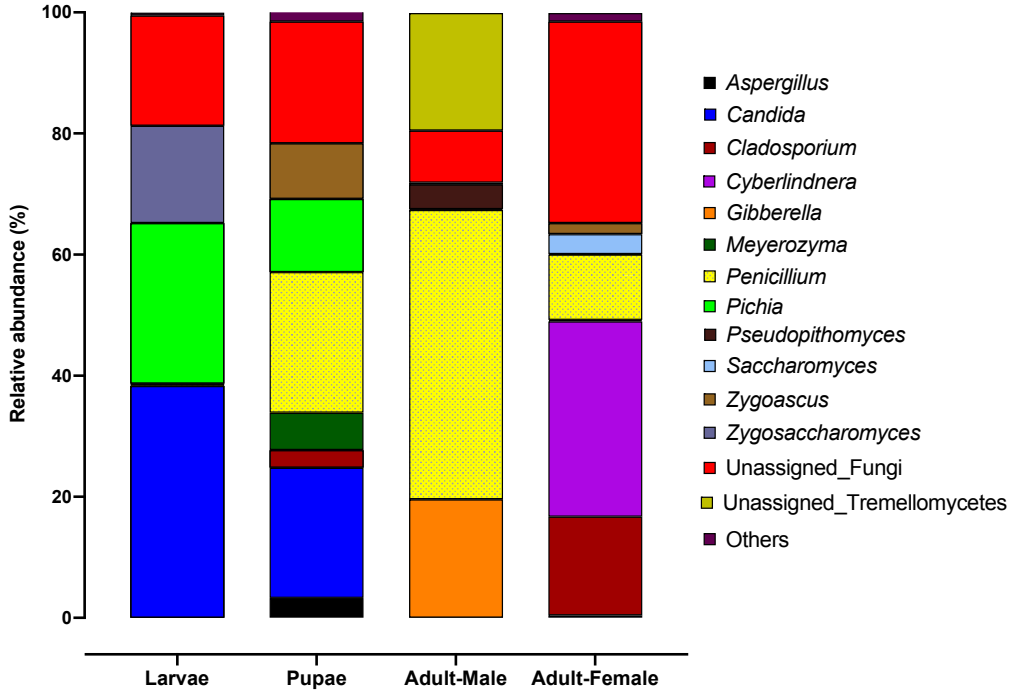

FIGURE 2 | Percentage of average relative abundance of the fungal genera present in the different developmental stages of the Qfly

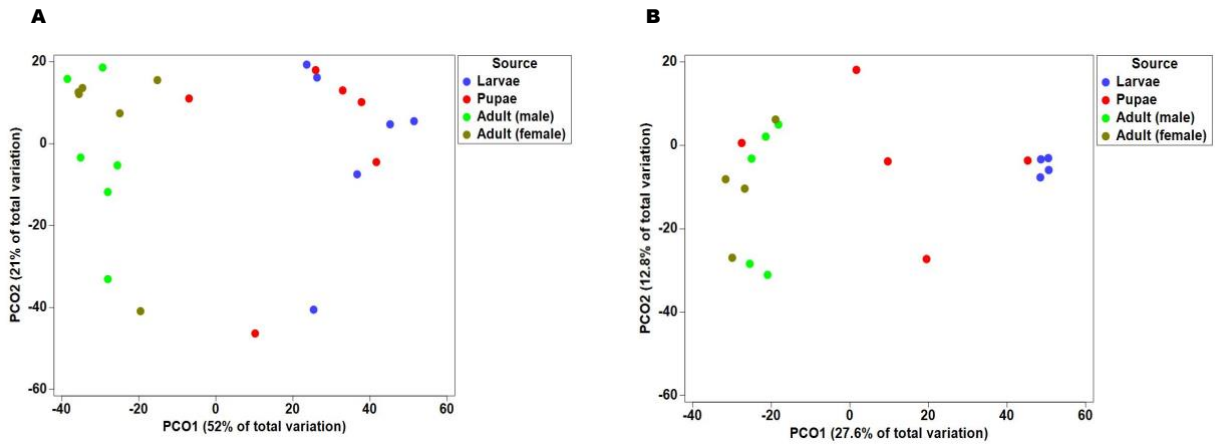

FIGURE 3 | Principal coordinates analysis of the the Qfly in the different developmental stages. (A) Bacterial communities; (B) fungal communities. Different color indicates the microbial communities in the different life stages of the Qfly

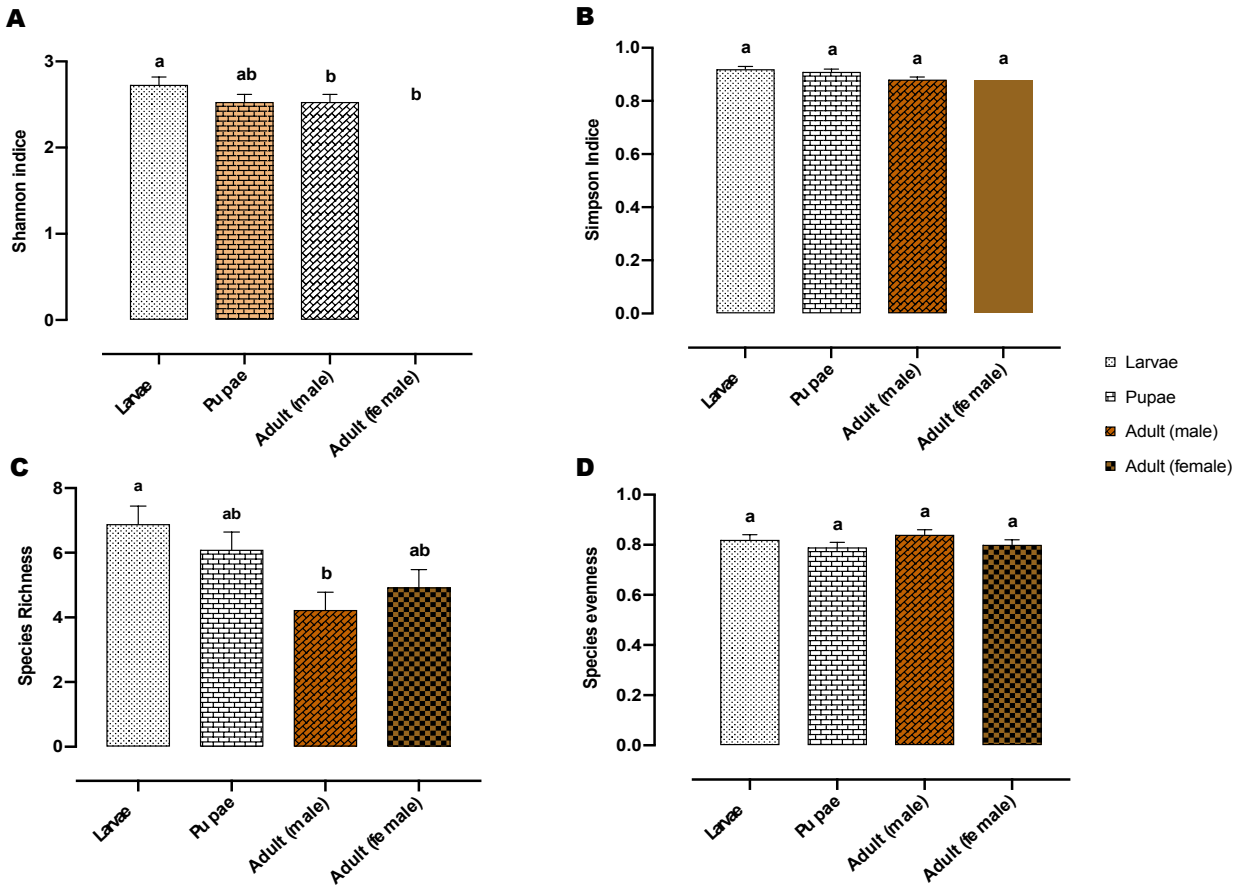

FIGURE 4 | Alpha diversity of the Bacterial microbiome of the Qfly developmental stages A) Shannon index; B) Simpson index; C) Species richness and D) Species evenness. Different letters indicate significant Tukey's post hoc comparisons ($p<0.05$)

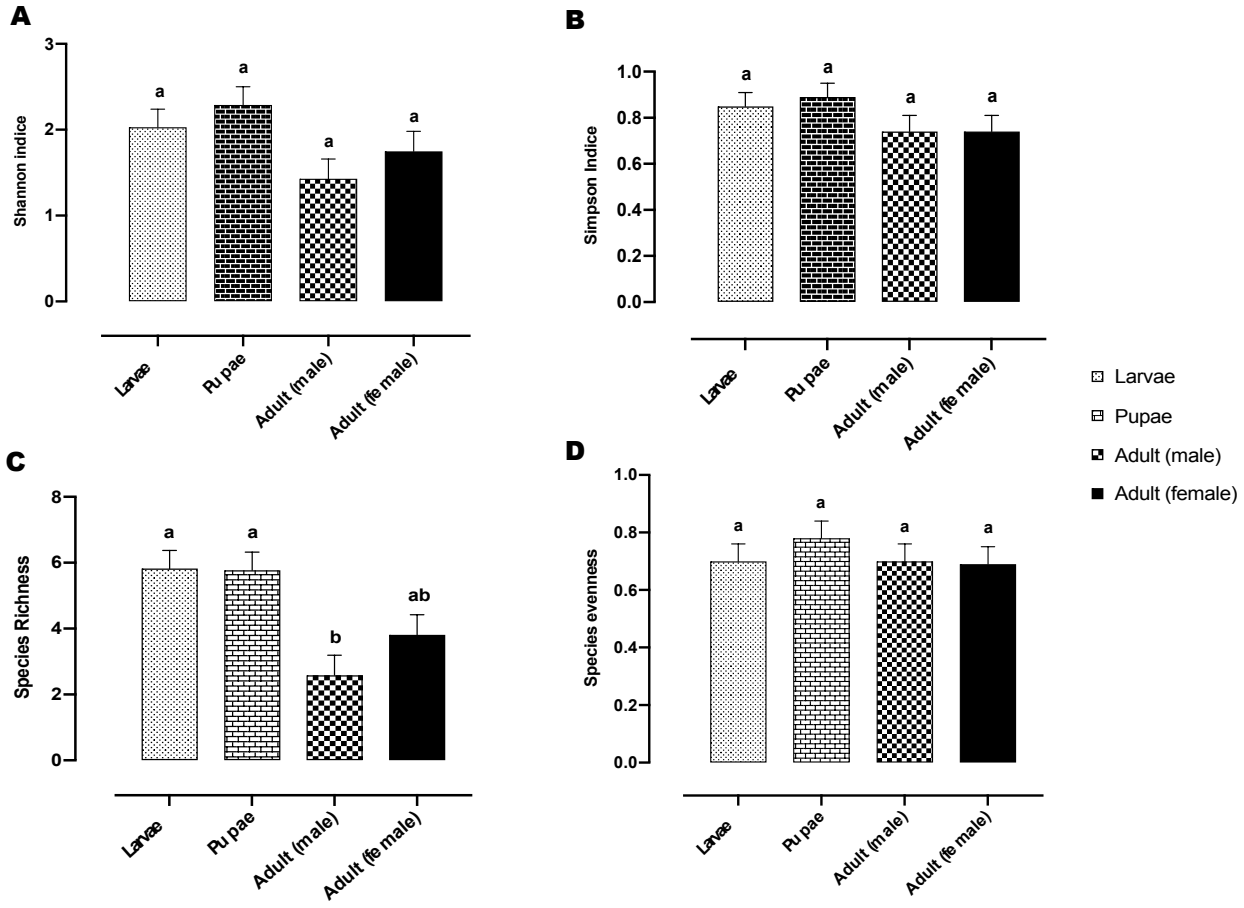

FIGURE 5 | Alpha diversity of the fungal microbiome of the Qfly developmental stages includes A) Shannon indices; B) Simpson indices; C) Species richness & D) Species evenness. Different letters indicate significant Tukey's post hoc comparisons (p<0.05)

TABLE 1 Fruit types and origin for wild *Bactrocera tryoni* larvae collection. A total of six replicate larvae, and fruit flesh samples were collected from each fruit origin.

Geographic location of collection	Fruit source and number of fruits collected	Collection date
Coomealla, NSW GPS: Lat 34° 5'50.97", Long 142° 3'7.21"	Pomegranate 37 pieces	5/05/17
St. Germains, Between Tatura and Echuca in Victoria GPS: Lat 36°10'48.86", Long 145° 8'50.74"	Green Apple 41 pieces	05/05/17
Downer road between Tatura and Toolamba in Victoria GPS: Lat 26°38'34.92", Long 152°56'22.99"	Quince 52 pieces	05/05/17

TABLE 2 | Taxonomic identification of the of the 11 most abundant bacterial OTUs in the Qfly across all developmental stages

Domain	Phylum	Class	Oder	Family	Genus	Larvae	Pupae	Adult male	Adult female
Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae		27.0%	38.1%	80.4%	94.8%
Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	*Swaminathania*	29.2%	37.6%	0.2%	1.1%
Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	*Erwinia*	22.6%	18.1%	0.0%	1.1%
Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	*Providencia*	0.00%	0.00%	19.4%	2.8%
Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	*Acetobacter*	10.2%	1.4%	0.0%	0.0%
Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	*Gluconobacter*	5.3%	3.4%	0.0%	0.0%
Bacteria	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae		3.8%	0.6%	0.0%	0.0%
Bacteria	Bacteroidetes	Flavobacteriia	Flavobacteriales	Weeksellaceae	*Wautersiella*	1.7%	0.0%	0.0%	0.0%
Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Comamonadaceae		0.0%	0.4%	0.0%	0.0%
Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae		0.0%	0.3%	0.0%	0.0%
Bacteria						0.1%	0.1%	0.0%	0.0%

TABLE 3 | Taxonomic identification of the 14 most abundant fungal OTUs in the Qfly across all developmental stages

Domain	Phylum	Class	Oder	Family	Genus	Larvae	Pupae	Adult male	Adult female
Fungi	Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	*Penicillium*	0.4%	23.2%	47.8%	10.8%
Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Incertae-sedis	*Candida*	38.3%	21.5%	0.0%	0.4%
Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Pichiaceae	*Pichia*	26.5%	12.1%	0.1%	0.1%
Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Incertae-sedis	*Cyberlindnera*	0.0%	0.0%	0.0%	32.3%
Fungi	Ascomycota	Sordariomycetes	Hypocreales	Nectriaceae	*Gibberella*	0.0%	0.0%	19.6%	0.1%
Fungi	Basidiomycota	Tremellomycetes				0.0%	0.0%	19.4%	0.0%
Fungi	Ascomycota	Dothideomycetes	Capnodiales	Cladosporiaceae	*Cladosporium*	0.0%	2.9%	0.0%	16.3%
Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	*Zygosaccharomyces*	16.1%	0.0%	0.0%	0.0%
Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Trichomonascaceae	*Zygoascus*	0.0%	9.2%	0.3%	1.8%
Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Debaryomycetaceae	*Meyerozyma*	0.0%	6.2%	0.0%	0.1%
Fungi	Ascomycota	Dothideomycetes	Pleosporales	Didymosphaeriaceae	*Pseudopithomyces*	0.0%	0.0%	4.1%	0.0%
Fungi	Ascomycota	Eurotiomycetes	Eurotiales	Trichocomaceae	*Aspergillus*	0.0%	3.3%	0.0%	0.0%
Fungi	Ascomycota	Saccharomycetes	Saccharomycetales	Saccharomycetaceae	*Saccharomyces*	0.0%	0.0%	0.0%	3.3%
Fungi						18.2%	20.1%	8.6%	33.3%

SUPPLEMENTARY FIGURES AND TABLES

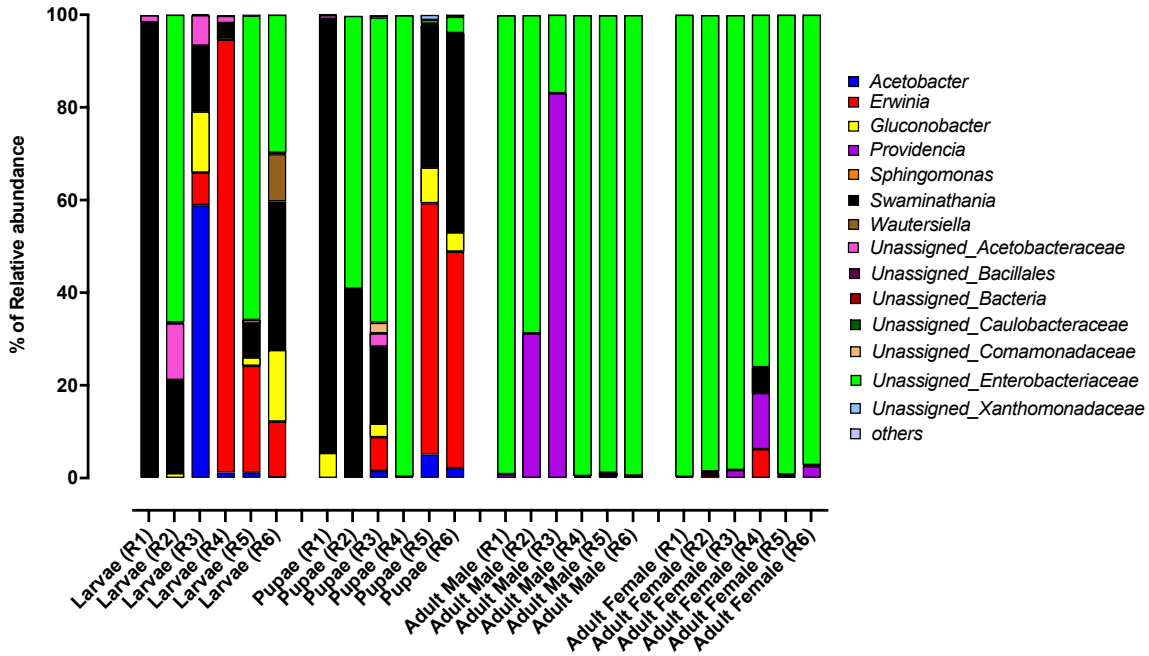

Figure S1 | Relative abundance of the bacterial genera of the Qfly across all developmental stages in G0. The percentage of relative abundance of 0.1 or less are included in "Others". Six replicates (R1-R6) of the developmental stages of fruits are plotted and R1 to R6 refers to the replicate number the of each types of developmental stages

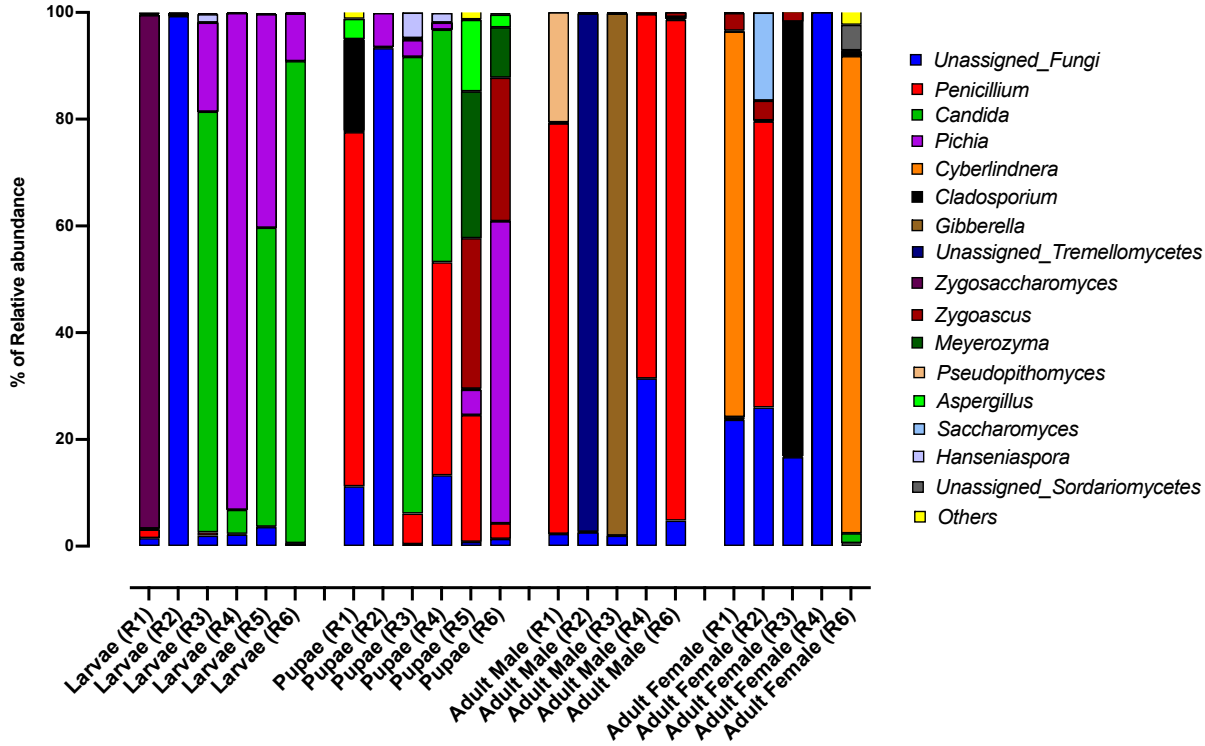

Figure S2 | Relative abundance of the fungal genera of the Qfly across all developmental stages in G0. The percentage of relative abundance of 0.1 or less are included in "Others". Six replicates (R1-R6) of the developmental stages of fruits are plotted and R1 to R6 refer to the replicate number of each type of developmental stages

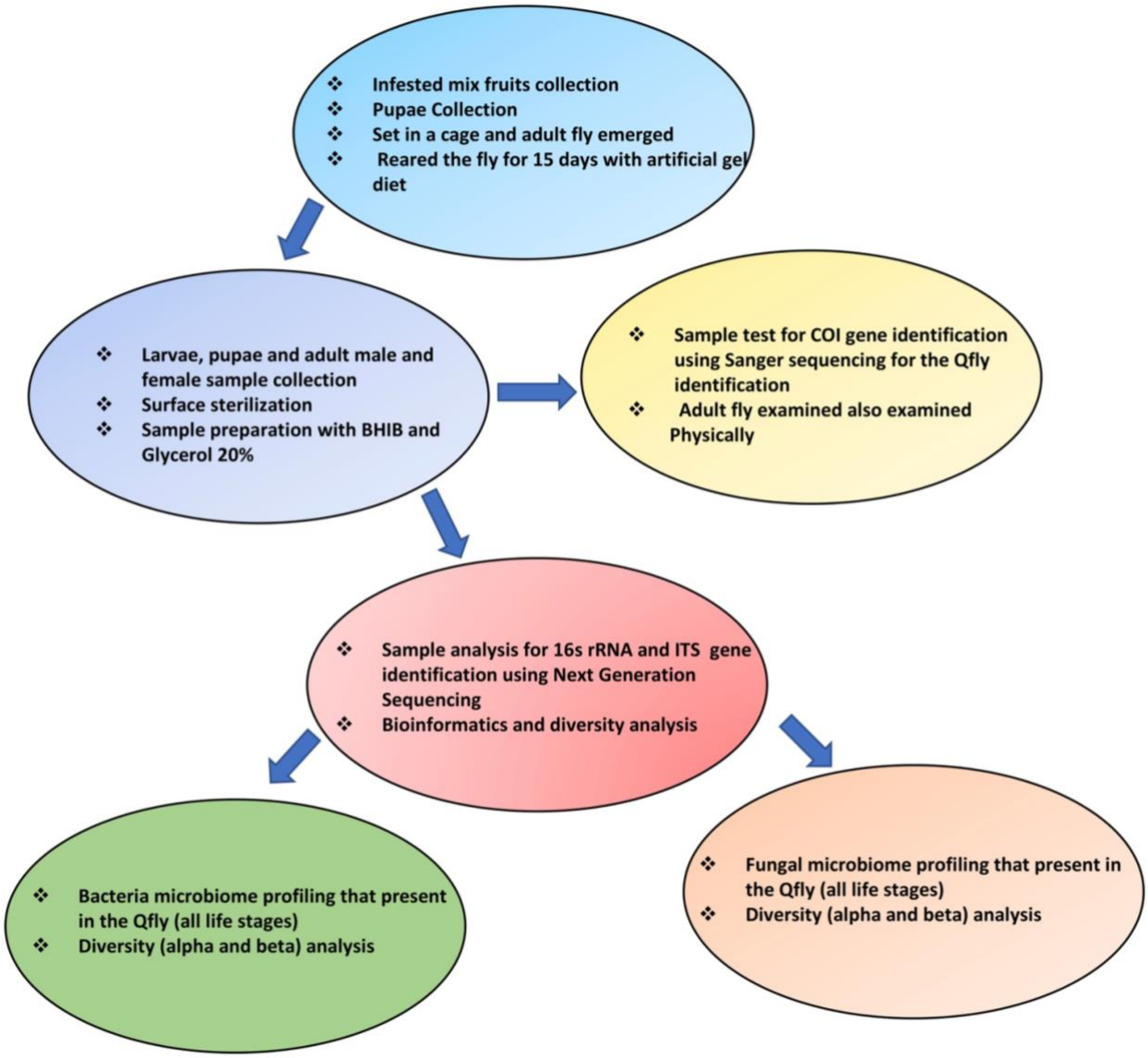

Figure S3 | Experimental Flowchart

Chapter 5

This chapter has been formatted in accordance with style of Frontiers in Microbiology

Artificial larval diet modulates the microbiome of Queensland fruit fly during the domestication process

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Running head: Gut microbiome of the domesticated Queensland fruit fly across developmental stages

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ABSTRACT

Larval diet during artificial rearing has a significant effect on fruit fly biology. The Queensland fruit fly (aka 'Qfly'), *Bactrocera tryoni* (Froggatt) (Diptera: Tephritidae) is one of the greatest challenges for horticultural growers in Australia, and sterile insect technique (SIT) is being developed as a strategy to manage outbreaks and to reduce populations in endemic areas. Laboratory domestication and artificial rearing of Qfly colonies is essential for SIT, however, artificial larval diets are known to affect the microbiome of Qfly, which may then affect fly physiology and behaviour. In this study, the Qfly microbiome was assessed in colonies reared, for five generations, on carrot and gel-based artificial diets. All developmental stages were assessed (larvae, pupae, adult males and females) from generation five (G5), along with fly quality and key behavioural traits (mating probability, stress tolerance). This study aimed to (1) describe differences in bacterial communities at each Qfly developmental stage in colonies from each diet and (2) compare these differences with quality and behavioral traits from the same Qfly colonies. At the finer taxonomic resolutions (OTU, genus) the bacterial communities of the Qfly were significantly different between the two larval diets. However, communities converged at higher taxonomic levels (e.g. phylum to family level). Interestingly, OTUs assigned to putatively pathogenic genera (e.g. *Morganella, Citrobacter, Providencia, Burkholderia*) were observed as highly abundant in all developmental stages of Qfly reared on the gel diet, when compared to the carrot diet. In contrast, there was a greater percentage of egg hatching, heavier pupal weight and a higher percentage of fliers from the Qfly reared on gel diet, compared to carrot diet. Mating performance and survival under stress was similar between Qfly colonies. Overall, our findings reveal that the artificial larval diet strongly influences the microbiome, performance and behaviour of Qfly. This is the first study to use Next-Generation Sequencing to analyze the microbiome of each stage of Qfly when larvae are reared on different artificial diets through the early stages of domestication.

Keywords: Gut bacteria, Next-Generation Sequencing, Developmental stages, Domestication, Mating probability, Stress tolerance

INTRODUCTION

Insects brought into the laboratory from nature and reared over multiple generations are confronted by a new environment that is very different from the native habitat, and are exposed to significant selection pressures that lead to adaptation to the laboratory environment ('domestication') (Chambers, 1977; Hoffmann et al., 2001). In tephritid fruit flies, adaptation to artificial rearing conditions has been reported to have significant influence on numerous life history traits, including stress tolerance and reproductive behaviour (Cayol et al., 2000; Pérez et al., 2018). Mass reared fruit flies tend to mature at a much younger age than wild type flies, and may have reduced sexual competitiveness or compatibility with wild populations (Moreno et al., 1991; Mangan, 1997; Miyatake, 1998; Meats et al., 2004). These changes resulting from domestication, are anticipated to have important implications for the success of the sterile insect technique (SIT) program.

SIT is an environmentally friendly pest management technique in which millions of sterile insects are released to mate with wild populations, inducing reproductive failure in females of pest populations (Knipling, 1955; Hendrichs et al., 1995; Vreysen et al., 2006). Tephritid fruit flies are amongst the world's most damaging insect pests (Norrbom et al., 1999). SIT has been an effective means to manage some of the most economically damaging fruit flies including Mediterranean fruit fly (Medfly) *Ceratitis capitata* (Wiedemann) (Reyes et al., 2007), melon fly *Zeugodacus cucurbitae* (Coquillett) (Kakinohana, 1994; Yosiaki et al., 2003), Oriental fruit fly *Bactrocera dorsalis* (Hendel) (Orankanok et al., 2007), and Mexican fruit fly *Anastrepha ludens* (Loew) (Orozco-Dávila et al., 2015). SIT has been implemented to supress or eradicate Queensland fruit fly (Qfly) *Bactrocera tryoni* (Knipling, 1955; Fanson et al., 2014; Stringer et al., 2017) in Australia. With increased restrictions on the use of insecticides due to concerns about environmental and human health (Dominiak and Ekman, 2013), there has been substantial interest and investment in development of SIT as a sustainable and environmentally benign solution to protect 'fruit fly free' regions and to suppress pest abundance in endemic areas.

Insects commonly host a large variety of microbes that can influence insect health (Jurkevitch, 2011). Microbial communities are often abundant in insect digestive systems (Dillon and Dillon, 2004), especially bacteria (Broderick et al., 2004; Robinson et al., 2010). In many cases, symbiotic bacteria have been found to provide nutrition that contributes to

insect host fitness (Dillon and Dillon, 2004; Baumann, 2005). Microorganisms may provide amino acids (Nogge, 1981), essential vitamins (Douglas, 1998), as well as nitrogen and carbon compounds (Benemann, 1973; Dillon and Dillon, 2004) to insect hosts. The gut microbiota may have the ability to alternate between mutualism/commensalism and parasitism in response to changes in their host's diet (De Vries et al., 2004). Further, elimination of bacteria can sharply reduce fly fitness (Ben-Yosef et al., 2008). Thus, the tephritid-microbe symbiotic relationship is of significant ecological and evolutionary importance. The insect microbiome can vary across life stages, as well as with diet and the local environment (Yun et al., 2014). Different microflora may be abundant in the host and so, the host diet is a major exogenous factor that might directly influence the composition of the insect gut microbial community and its metabolic capabilities (Chandler et al., 2011; Broderick and Lemaitre, 2012; Mason et al., 2014; Majumder et al., 2019). Additionally, variation in the diet composition (protein, carbohydrate and lipids) can strongly influence both the gut microbiome biodiversity and community structure (Broderick et al., 2004; Woruba, 2018; Ravenscraft et al., 2019). Increasing our knowledge of these relationships may identify ways to enhance the quality of artificial diets, with the goal of improving performance in laboratory or mass-reared insects. To date, only a handful of studies have analysed the microbiome of tephritid fruit flies reared on artificial diet (Behar et al., 2008b; BenAmi 2010, Andongma et al., 2015; Morrow et al., 2015; Yong et al., 2017a; Deutscher et al., 2018; Woruba, 2018). To our knowledge, however, there are no studies assessing the effect of the different artificial larval diets on the gut bacterial community of tephritid fruit flies across the developmental stages of larva, pupa and adult.

Larvae of the highly polyphagous Qfly develop in diverse host fruits (Hancock et al., 2000; May and Drew, 2003). In Qfly, the bacterial microbiome is largely transmitted vertically from the mother to the offspring when eggs are laid (Deutscher et al., 2018). Descriptions are available characterising the bacteria associated with wild and domesticated Qfly larvae (Deutscher et al., 2018; Majumder et al., 2019), pupae (Fitt and O'Brien, 1985) and adult flies (Thaochan et al., 2010; Morrow et al., 2015; Woruba, 2018). However, the effects of larval diet on changes in the Qfly microbiome through the early stages of domestication process are not known. Different type of traditional solid diets has been used for rearing Qfly that includes a biological bulking agent like wheat meal, dehydrated carrot or lucerne chaff (Finney, 1956; Jessup, 1999; Dominiak et al., 2002; Dominiak et al., 2008). Carrot-based diet mostly uses in moderate scale rearing and lucerne chaff-based diet has used

in factory-scale rearing (Jessup, 1999; Fanson et al., 2014) but now uses a gel larval diet (Moadeli et al., 2017; Moadeli et al., 2018a; Moadeli et al., 2018b; c). Mainali et al. (2019) found that Qfly reared on the gel diet produce better quality flies compared to solid diet containing carrot or lucerne chaff. Previous studies have assessed the bacterial populations inside Qfly larvae and Qfly adults reared on carrot diet and lucerne chaff diet (Morrow et al., 2015; Deutscher et al., 2018; Woruba, 2018b). However, there has been no studies investigating the microbial communities in all developmental stages of the Qfly reared on a gel diet compared to other larval diets.

In the present study, next generation sequencing was used to investigate bacterial diversity and abundance in the microbiome of Qfly larvae, pupae and adults from colonies that have been established from wild material and maintained through five generations of laboratory rearing on either the carrot or gel larval diets. This study greatly improves our understanding of how choice of artificial diet affects the microbiome of laboratory reared flies, and has significant implications for factory-scale rearing, such as is required for SIT programs.

MATERIALS AND METHODS

Qfly sample collection

Infested pomegranate, green apple and quince were collected from different geographic locations in the states of New South Wales (NSW) and Victoria (VIC) in Australia (**Table 2**). The infested fruits were collected from under trees, and most were over-ripe. After collection, all fruits were stored in buckets (60L, 447 x 236 x 663 mm, Award, Australia) containing fine vermiculite (1.0 cm depth) (Grade 1, Sage Horticultural, VIC, Australia) in a controlled environment laboratory (25±0.20°C, 65±3% RH and 11h: 1h: 11h: 1h light: dusk: dark: dawn photoperiod). Approximately 600 adult Qfly were obtained from these fruits. The emerged adult flies were supplied with hydrolysed yeast (MP Biomedicals, Cat. no 02103304) and commercial sucrose (CSR® White Sugar), and water through a moist sponge. Two replicate populations, each of ca. 300 flies were placed in mesh cages (Megaview Bugdorm 44545, 47.5 x 47.5 x 47.5 cm, Taiwan) in a controlled environment room and reared for five generations (G1 to G5).

Artificial diet preparation and domestication

The two Qfly colonies were reared on two artificial larval diets, carrot and gel (Moadeli et al., 2017; Mainali et al., 2019) [see Table S1 and S2 for the recipe of the carrot and gel diets respectively, Fig S1]. The carrot diet was prepared by mixing all ingredients using a food mixer for 15 min (5 min slow and 10 mins fast) and kept at room temperature for 12-24h before use. The gel diet was prepared as in Moadeli et al. (2017), by mixing all the dry ingredients using a blender (Kenwood, Australia) for 5 mins. Water was mixed with agar and the solution boiled. Boiled agar and the dry mixture were then mixed together. We transferred 150 g of carrot diet and 150 mL of gel diet into larvae rearing containers (17.5 cm long, 12 cm wide and 4 cm deep).

At each generation, eggs were collected using an artificial oviposition device comprising a 300 mL semi-transparent white soft plastic bottle (low density polyethylene). The oviposition device had numerous 1 mm holes through which females could oviposit, and contained 20 mL of water to maintain humidity and a few drops of natural apple juice to attract the female flies and encourage egg laying (Collins et al., 2008). Eggs were collected from 12-16 days old mature flies between 9 am and 3 pm on a single day. The oviposition device was rinsed with distilled water to wash out the eggs. The eggs were then collected using a 15 mL falcon tube and 250 ml of eggs in suspension were transferred to larval diet using a 1000 µL pipette (ca. 3500 eggs, ca. 23 eggs per gram of diet) (Moadeli et al., 2017). Third instar larvae (N=12), 8 days old pupae (N=12) and 15 days old sexually mature male (N=12) and female adult flies (N=12) were collected from generation 5 (G5) for sequencing.

Sample preparation

For sample processing, Qfly larvae, pupae and adult flies (male and female separately) from the G5 colonies were surface sterilized using 0.5% Tween 80 (Sigma-Aldrich, Cat. No. 9005656, USA), 0.5% Bleach (Sodium hypochlorite) (Sigma-Aldrich, Cat. No.7681529, USA) and 80% Ethanol (Sigma-Aldrich, Cat. No. 65175, USA) for 30s, and rinsed 3 times in 1M sterile phosphate-buffered saline (1x PBS) again for 30s (Deutscher et al., 2018). The PBS from the 2nd and 3rd washes were kept and 100 µL spread-plated on to five types of microbial growth medium (de Man, Rogosa and Sharpe Agar, Tryptone Soya Agar, Macconkey Agar, Potato Dextrose Agar and Yeast-dextrose Agar medium) (Sigma-aldrich,

USA) to check the performance of the sterilization method. All plates were incubated at 35°C for 24 to 48 hr. Post sterilization, the guts of adult flies were dissected using a stereomicroscope (Leica MZ6 stereo-microscope, Leica®, Germany). Using sterile pestles, larvae, pupae, and dissected guts from the adults were homogenised separately in a solution of Brain Heart Infusion (BHI) broth (Oxoid Ltd, UK, Lot # 1656503) and 20% Glycerol (Sigma Aldrich®, Lot # SHBG2711V, USA) and each sample was stored in a separate cryovial tube (Simport Scientific, Canada). All the samples are preserved at -80°C. All procedures were completed in a sterile environment (Biological air clean bench, safe 2020 1.2, Thermo Scientific, Germany).

Qfly microbiome profiling

DNA was extracted using the DNeasy Power Lyzer Power Soil Kit-100 (Qiagen, Germany) (Cat. no. 12855-100) following the manufacturer's protocol. DNA extracts were then quantified in the Invitrogen™ Qubit® dsDNA High Sensitivity (HS) Assay Kit (Life Technologies, USA). PCR amplification and sequencing were performed by the Australian Genome Research Facility. For the bacterial identification, the V1-V3 16S rRNA region was amplified using primers 27F (5′AGAGTTTGATCMTGGCTCAG-3′) and 519R (3′ GWATTACCGCGGCKGCTG-5′). Reactions contained 1X AmpliTaq Gold 360 mastermix (Life Technologies, Australia), 0.20 µM of each forward and reverse primer and 25 µL DNA. PCR cycling conditions consisted of denaturation at 95ºC for 7 minutes, 35 cycles of 94ºC for 45 s, 50ºC for 60 s and 72ºC for 60 s, and a final extension of 72ºC for 7 minutes. A second PCR was used to adhere sequencing adaptors and indexes to the amplicons. Primerstar max DNA Polymerase was used to generate a second PCR amplicon (Takara Bio inc. Japan; Cat. No. #R045Q). The resulting amplicons were measured using a fluorimeter (Invitrogen Picogreen, Thermo Fisher Scientific, Australia) and normalized (Fouts et al., 2012). The eqimolar amounts of each sample were pooled and quantified qPCR prior to sequencing (Kapa qPCR Library Quantification kit, Roche, Switzerland). The resulting amplicon library was then sequenced on the Illumina MiSeq platform (San Diego, CA, USA) with 2 x 300 base pairs paired-end chemistry (Caporaso et al., 2010).

Sequence data processing

The Greenfield Hybrid Amplicon Pipeline (GHAP) was used to process bacterial 16s rRNA amplicon sequences (Sutcliffe et al., 2018). GHAP is a publicly available (https://doi.org/10.4225/08/59f98560eba25) amplicon clustering and classification pipeline built around tools from USEARCH (Engel and Moran, 2013) and the Ribosomal Database Project (RDP; Cole et al., 2013). GHAP was used to generate biome tables of classified OTUs and their associated read counts across all samples. A 97% similarity threshold was used for OTU clustering and classifications were achieved using the RDP Naïve Bayesian Classifier against the RDP 16S training set.

All OTUs which were assigned to "Mitochondria" at the Order level were removed from the dataset before downstream processing. To maintain equal sequence depth among all samples, the above biome table was rarefied to 10,000 reads per sample, repeating this 50 times and averaging the counts to obtain a representative rarefaction. This was achieved using an in-house python script. Those samples with <10,000 reads were excluded. The data were then normalised as the percentage of relative abundance, and are henceforth referred to as the OTU table (**Supplementary file 1**). All the figures of bacterial relative abundance at different developmental stages and between generations in colonies reared on different diets were plotted in Prism 8 (version 8.0.1(145), GraphPad software, Inc).

Experimental design for Qfly quality control measures and behaviour

The experimental design of quality control measures and behaviour of the Qfly were based on standard procedures (Collins et al., 2008; FAO/IAEA/USDA, 2014; Moadeli et al., 2017; Adnan et al., 2018; Mainali et al., 2019). Briefly, we performed the following experiments.

Egg hatching

A 100 µL pipette was used to collect the eggs which were counted under a stereomicroscope (Leica MZ6 stereo-microscope, Leica, Germany) using a soft paintbrush. Five sets of 50 eggs from G5 of each Qfly colony were spread on a 1 cm x 3.5 cm strip of soaked black filter paper. We poured 25 mL of gel diet and 25 g of carrot diet separately into a 90 mm Petri dish (see **Supplementary Figure S1**) with five replicates for each diet. Petri dishes were covered

and were kept in the controlled environmental room. Four days later, the number of the unhatched eggs was counted. The percentage of egg hatch was calculated as [(N of egg hatched/ (N unhatched + N hatched)) x100].

Pupal recovery

We poured 25 ml of gel diet and 25 g of carrot diet separately into 90 mm Petri dish with five replications for each diet. Eggs were collected as described previously. We counted 50 eggs under the stereo microscope and immediately transferred them to the diet surface. All plates were placed in the laboratory environment with lids on. The lid was removed when larvae were ready to pupate. Plates were then placed into separate plastic containers (12 L) containing fine vermiculite (Grade 1, Sage Horticultural, Victoria, Australia) and with a mesh lid. The pupae were collected every two days. Pupal recovery was calculated as the total number of pupae divided by the number of hatched eggs multiplied by 100 (Mainali et al., 2019)

Larval, pupal, and adult weight

Third instar larvae were collected to assess larval weight as the mean weight of four sets of 30 larvae from each colony. Eight days old pupae were weighed as for the larvae. To assess adult fly weight (male and female), approximately 300 pupae from each colony were placed in a mesh cage (Megaview Bugdorm 44545, 47.5 x 47.5 x 47.5 cm, Taiwan). No food or water were supplied in the cages. After 2 hours of emergence, adult males and females were collected separately and placed in a -20°C freezer. We used a microbalance (Sartorius ME5, Germany) to measure the weight of adult flies as the mean weight of two sets of 30 adult males and females.

Sex ratio

We collected 800 Qfly pupae (8 days old) from the carrot and the gel diet reared colonies to measure the sex ratio of each colony. We counted 100 pupae and placed these pupae in a 32.5 x 32.5 x 32.5 cm size mesh cage (Megaview Bugdorm- 43030F) on uncovered 55 mm Petri dishes two days before emergence. We collected and froze all the flies after emergence and

304 measured sex ratio under a stereomicroscope (Leica MZ6 stereo-microscope, Leica,
305 Germany) by dividing the number of males by the total number of adults.
306
307 **Flight ability test**
308
309 Adult fly emergence, percentage of fliers and the rate of fliers was assessed. Two days prior
310 to fly emergence, we placed 100 pupae (8 days old) in five replicates from each diet (total
311 1000 pupae) separately in 55 mm Petri dishes without lids. The dishes containing the pupae
312 were placed in the centre of 90 mm Petri dish lids with black filter paper on the base. We
313 used a black 100 mm tall acrylic flight ability tube (94 mm inner diameter, 3 mm thickness)
314 coated with unscented talcum powder inside and placed over the 90 mm Petri dish lid. Fine
315 talcum powder on the tube interior prevented the flies from walking out during the
316 experiment. The whole setup with pupae was placed in a mesh cage (dimensions 32.5 x 32.5
317 x 32.5 cm size, Megaview Bugdorm- 43030F) in the laboratory. We placed all the cages on
318 shelves close to 20-watt fluorescent lights (ca. 1250 lx at the top and ca. 900 lx at the base of
319 the flightability tubes). A control tube was placed 6 cm away from the flightability tube
320 containing pupae. During fly emergence, we removed the flies that flew out from the tube
321 every day in the morning and afternoon to minimize fly back. All collected flies were stored
322 in a -20°C freezer for later assessment. We collected and counted the flies until all emergence
323 was complete (4-5 days). Data was collected on five categories; (1) not emerged (un opened
324 pupal case), (2) partially emerged (part of adult body stuck in pupal case), (3) deformed (fly
325 fully emerged but with damaged or deformed wings), (4) non-fliers (morphologically normal,
326 but unable to escape from the flightability tube), and (5) fliers (normal fly found outside of
327 the flightability tube inside the cage and fly back) (Collins et al., 2008). We followed the
328 standard (FAO/IAEA/USDA, 2014) protocol to calculate the percentage of adult fly
329 emergence, percentage of the flier and the rate of the flier.
330
331 *Percentage of adult emergence:* ((no. of pupae - (no. of not emerged + no. of part
332 emerged)/no. of pupae) x 100)
333 *Percentage of fliers*: calculated as (no. of pupae – (no. of not emerged + no. of part
334 emerged)/no. of pupae) x 100)*;*
335 *Rate of fliers:* (percentage fliers/percentage emergence) x 100
336

Mating performance

For mating trials, approximately 400 pupae from each colony were placed in a mesh cage (Megaview Bugdorm 44545, 47.5 x 47.5 x 47.5 cm) for emergence. After emergence, cages were supplied with water-soaked cotton wool in a 70 ml sample container. Food was provided as dry granular sucrose (CSR® White Sugar) and yeast hydrolysate (MP Biomedicals, Cat. no 02103304) (3:1) on a 90 mm Petri dish *ad libitum*. To obtain mature flies (12-17 days old) to pair with experimental flies, ca. 400 pupae from a separate laboratory colony (reared on gel larval diet for >25 generations) were placed in separate mesh cages (Megaview Bugdorm 44545, 47.5 x 47.5 x 47.5 cm) for adult emergence. Similar to experimental treatment, selected flies were supplied with water-soaked cotton wool in a 70 ml sample container, dry granular sucrose and yeast hydrolysate as food on a 90 mm Petri dish *ad libitum*. Both experimental and mature adult flies were sorted according to sex within 3 days after emerging by collecting and transferring individual flies in glass tubes to clear plastic 12 L cages that had a mesh-covered ca. 80 cm^2 window for ventilation. Approximately 160 flies were sorted into each 12 L cage, with this relatively low density to avoid effects of crowding. No calling, courting, or mating was observed in cages prior to separating the sexes.

Mating trials were conducted when flies were sexually mature (12-16 days old). On the mating day, four hours before the onset of dusk, 40 males and 40 females from each experimental group were placed individually in clear plastic 1.25 L containers with a mesh-covered window (ca. 28 cm^2) for ventilation. Each fly was individually paired with a sexually mature (12-16 days old) fly of the opposite sex. Virgin flies of this age fed a diet of sugar and yeast hydrolysate show a high level of sexual receptivity (Pérez-Staples et al., 2007; Prabhu et al., 2008). Periodic observations were carried out after pairs were set up, and continuous observations began 90 minutes prior to the onset of dusk. The time of onset of copulation for each mating pair was recorded to assess mating latency (time from the start of dusk until the onset of mating, in minutes) and observations continued until the last pair had separated to assess mating duration for each mating pair.

Stress tolerance

Immediately after emergence (0-2hrs), 40 females and 40 males were placed in individual, 5-mL (75 mm×10 mm) round bottom plastic labelled vials (Lab Australia Pty Ltd, Australia). Flies were given no access to food or water after being placed in the vials until death. The number of dead flies was recorded by visually inspecting the vials every 3 hr. Flies were considered dead when they were incapable of holding onto the inner surface of the plastic vial, and when no movement of their legs or mouthparts was observed after the vials were gently flicked with a finger. Dead flies were removed at each assessment.

Statistical analysis

Microbiome data analysis

The bacterial OTU table was imported into Primer-E v7 (Clarke and Ainsworth, 1993; Sutcliffe et al., 2017) for analysis. In brief, all statistical testing was performed on fixed factors associated with various developmental stages (larvae, pupae, adult male and female) from which 12 replicates were collected. The DIVERSE function was used to generate univariate biodiversity metrics, species richness, Pielou's evenness and Shannon's and Simpson's biodiversity indices. Statistical differences between these metrics were assessed in JMP Statistical Software Version 10.0.0 (SAS Institute, Cary, NC, USA) using one-way analysis of variance (ANOVA) and Tukey-kramer post hoc analysis. To observe the taxonomic compositional changes for the bacterial and fungal communities, the OTU table was first log transformed using Primer-E V7. A Bray-Curtis similarity matrix was derived from this transformed data and a permutation analysis of variance (PERMANOVA) pairwise comparison was conducted to compare all community samples. A p value of <0.05 was considered statistically significant. Further, ordination plots of these communities were visualised using principal coordinates analysis (PCoA) in Primer-E.

To determine whether significant differences occurred in the relative abundance of bacterial communities at all developmental stages of the Qfly, from carrot and gel diet reared colonies, ANOVA and post-hoc Tukey-Kramer tests were performed. Benjamini–Hochberg was used to correct for multiple testing false-discovery rate (FDR) and an alpha threshold of 0.05 on FDR corrected ANOVA P-values was used to determine significance.

405 **Fitness and behavioural data analysis**

406

407 We performed all analysis regarding fitness traits and sexual performance of Qfly in
408 JMP statistical software (Version 10.0.0, SAS Institute, Cary, NC, USA). Qfly quality control
409 measures were analysed with ANOVA and pair-wise Student's *t*-tests. Prior to the analysis,
410 distribution patterns were observed for all quality control data using JMP statistical software.
411 Figures of quality control measures were plotted using Prism 8 software (1995-2018
412 GraphPad software, Inc., USA).

413

414 Mating probability (binary outcome) was assessed using nominal logistic regression
415 with significance tested using likelihood ratio tests (*G*-test). Main effects included in the
416 model were diet (nominal) and sex (nominal). Model parameter estimates were inspected to
417 identify effects. Mating latency and mating duration (continuous outcomes) were analyzed
418 for each treatment using least squares regression including diet (nominal) and sex (nominal).
419 The survival data for the individual vial stress tolerance experiments were subjected to least
420 square regression analysis including diet (nominal) and sex (nominal). Post-hoc pairwise
421 survival comparisons were then conducted to assess differences within factors.

422

423 **RESULT**

424

425 **16s rRNA sequence reads and OTUs**

426

427 We sequenced the bacterial microbiome of 96 Qfly samples from G5 reared on the carrot and
428 gel diet. This included larvae (n=12), pupae (n=12), adult males (n=12) and adult females
429 (n=12) from each diet. Among them, 79 were retained after quality control and rarefaction at
430 10,000 reads per sample (9 and 8 samples were removed from carrot and gel diet,
431 respectively). After rarefaction and quality control a total of 472 bacterial OTUs were
432 detected across the 79 samples (**Supplementary data file 1; 2**). Among them, only 14 OTUs
433 (~2%) were classed as abundant, i.e. representing ≥1% of the microbiome (**Table 2**).

434

435 **Gut bacterial diversity of Qfly**

436

437 Bacterial alpha diversity metrics, species richness and Shannon biodiversity indices, were
438 calculated for each Qfly developmental stage from each of the two larval diets (**Figure 1A–**

B). Both biodiversity metrics were insensitive to larval diet, with no significant differences for any of the developmental stages (**Figure 1**). In contrast, the beta diversity shows significant differences between diets at all developmental stages (PERMANOVA; $P<0.05$) (**Supplementary file 3**). To visualize this variation of Qfly bacterial communities, across different life stages, and between the carrot and the gel diet, principal coordinates analysis (PCoA) of Bray-Curtis similarity matrix was plotted (**Figure 2**). The PCoA ordination plot suggested that each developmental stage of the Qfly, both from carrot and gel diet, had a distinct microbiota population. In the PCoA scatter plot, PCO1 captured 36.1% of the total variance in the dataset and corresponded with the separation of larval and pupae samples from those of the adult (**Figure 2**). The second axis, PCO2 captured a further 15.6% of variance in the data, and corresponded with the separation of communities associated with Qfly reared on different diets (**Figure 2**).

Gut bacterial communities associated with two artificial diets

The bacterial taxa detected in the Qfly reared on the carrot and gel diet represented a total of 5 phyla, 12 classes, 61 families and 139 genera. We analyzed the relative abundance of the bacterial community members present in the Qfly microbiome across larval, pupal and adult (both male and female) stages, reared on the carrot versus gel larval diets (**Supplementary data file 1**). At the phylum level, very little difference was seen between the two diets, or developmental stages. For example, Proteobacteria represented ~90% of all microbial communities. One exception to this was the decrease of Actinobacteria in larvae, with an average relative abundance of 3.8% for those reared on the carrot diet, compared to 0.05% on the gel diet.

At the family and genus level, strong taxonomic trends could be observed when comparing the two diets across developmental stages. In the larval stage, the relative abundance of Enterobacteriaceae was ~99% for those reared on the gel diet. In contrast, the relative abundance of this family was <0.1% for those reared on the carrot diet (**Supplementary file 1**). Instead, the microbiomes of the carrot diet fed larvae were dominated by Acetobacteraceae, which represented almost 100% of the population, but was almost undetectable in the gel fed larvae (0.01%). These trends were mirrored at the genus level, with *Morganella* and *Providencia* (Enterobacteriaceae) significantly more abundant in the larvae reared on the gel diet compared with the carrot diet (**Figure 3A-B; 4**), and

Swaminathania/Asaia (Acetobacteraceae) accounting for ~99.9% of the larval microbiome for colonies fed on the carrot diet, but was only a minor component (<1%) of microbiomes from gel fed larvae (**Figure 4**).

Due to the pathogenesis of species within the *Morganella* genus, those OTUs assigned to this genus (n=16) were blasted against the NCBI database in order to assess their similarity with pathogenic species/strains. These OTUs overwhelmingly matched *Morganella morganii* strain JCM1672 with a similarity of >98.0%. Interestingly, *Morganella, Providencia* and *Swaminathania/Asaia* were also detected in the pupae and adults, and showed similar trends to those in the larvae. Specifically, *Swaminathania/Asaia* had a significantly higher relative abundance in pupae/ adults reared on the carrot diet (42%) compared with those reared on gel (<0.01%; FDR corrected P<0.0001), while *Morganella* and *Providencia* were more abundant in the Qfly colony reared on the gel diet. Despite being statistically significant in the larvae and adults, these differences in the Enterobacteriaceae genera were not significant for the pupae.

Another notable trend within the pupal stage (**Figure 3A-B**) is the higher relative abundance of the Micrococcaceae in the gel fed pupae (14%), compared with the carrot diet (<1%). As with the Acetobacteraceae, this trend was driven by a single genus, *Arthrobacter* (**Figure 4**). Despite the fact that this genus was more abundant in the pupal developmental stage compared with the larvae, it was also significantly more abundant in the larvae fed on a gel diet (0.5% vs 0%; **Figure 4**). In addition, although *Burkholderia* was found highly abundant in the pupal stage, significant abundance was detected both in the male and female reared on the carrot diet compared to the gel diet (**Figure 4**).

In the adult stage, a number of diet-associated differences were detected that were unique to this developmental stage and/or the sex of adults (**Figure 3A-B**). For example, in both male and female adults, *Orbus* was significantly more abundant in colonies reared on the carrot diet (**Figure 4**). It is notable, however, that this genus was of far greater relative abundance in the males compared with females. There were a number of additional diets associated changes that only occurred in one adult gender. For example, *Kluyvera, Aeromonas,* and *Erwinia* were all significantly more abundant in the adult females reared on gels, compared with carrot. Conversely, *Citrobacter* was only significantly more abundant in adult males reared on the gel diet (**Figure 4**).

Quality control and behaviour

Percentage of egg hatch and pupal recovery

The percentage of egg hatching was not significantly different ($F_{1,8} = 2.82$, $p = 0.132$) between the carrot and gel diet reared Qfly (gel diet 71.20 ± 1.86%; carrot diet 66.80 ± 1.86%). No significant difference was observed in the pupal recovery rate between the Qfly reared on the two different larval diets ($F_{1,8} = 1.73$, $p = 0.225$; gel diet 63.60 ± 3.02%; carrot diet 58 ± 3.02%).

Larval, pupal and adults (male and female) fly weight (mg)

Larval weight was significantly higher ($F_{1,58} = 73.51$, $p < 0.001$) in the Qfly reared on the carrot diet compared to the gel diet (**Figure 5A**). Also, pupal weight was significantly greater ($F_{1,58} = 62.16$, $p < 0.001$) in the Qfly reared on the gel diet (**Figure 5B**). No significant difference ($F_{1,58} = 0.07$, $p > 0.792$) was found in the adult male body weight of the Qfly reared on the carrot and the gel diet. In contrast, adult female body weight was significantly greater ($F_{1,58} = 13.89$, $p < 0.001$) in the Qfly reared on carrot diet (9.09 ± 0.14) compared to Qfly reared on the gel diet (8.36 ± 0.14).

Flight ability

There was no significant difference between the carrot and the gel diet reared Qfly in the percentage of adult emergence ($F_{1,8} = 2.86$, $p > 0.129$) or rate of the fliers ($F_{1,8} = 0.978$, $p > 0.352$). In contrast, the percentage of fliers was significantly different ($F_{1,8} = 5.389$, $p=0.0488$) between the Qfly reared on the carrot diet and the gel diet (**Figure 6A-B**). In all flight ability measures, the mean value of the Qfly fed on the gel diet was greater than the carrot diet (**Figure 6A-B**)

Sex ratio

The percentage of male Qfly was significantly higher on the gel diet compared to the carrot diet ($F_{1,8} = 6.64$, $p = 0.033$) (**Figure 7**).

Mating performance

Mating probability did not vary significantly between carrot and gel diet reared flies (df=1, χ_2= 0.02, p =0.87). Similar to mating probability, there was no significant difference between the two diets in mating latency ($F_{1,51}$= 0.66, p =0.42) and mating duration ($F_{1,51}$= 2.2, p=0.14). Sex of the flies did not influence mating propensity ($F_{1,51}$= 0.02, p =0.87), mating latency ($F_{1,51}$= 2.97, p =0.09) and also mating duration ($F_{1,51}$= 1.63, p =0.20) of the fly.

Stress tolerance

Survival of the flies that were subjected to starvation did not significantly differ between flies reared on the carrot and the gel diet ($F_{1,156}$= 0.05, p =0.82). In addition, survival under stress did not vary with the sex of flies ($F_{1,156}$= 0.25, p =0.62).

DISCUSSION

This study represents a comprehensive investigation into how two artificial laboratory diets effect the Qfly microbiome across all developmental stages, along with adult fitness and behaviour. By analyzing the microbiome of each life stage (i.e., larvae, pupae and adults - both male and female), we are able to explore meaningful questions regarding the effect of these diets at all developmental stages of the Qfly. Our results demonstrate that artificial larval diet strongly modulates the microbial community structure (beta diversity) across all developmental stages, but did not affect total biodiversity (as assessed by alpha diversity metrics: species richness and Shannon's diversity index). The bacterial phyla Proteobacteria and Firmicutes have previously been reported as common in the midgut of Qfly larvae and the adults in domesticated colonies reared on carrot diet (Deutscher et al., 2018; Woruba, 2018; Majumder et al., 2019), as well as in other fruit flies including *B. neohumeralis* , *B. jarvisi*, *B. cacuminata* and *C. capitata* (Morrow et al., 2015). Our results are consistent with these previous findings; however, it is noteworthy that variations in important metabolic (diet) and physiological (developmental stage) states had a limited impact on microbial community composition at the phylum level. Analyses at the family and/or genus level are far more informative of such variations in Qfly populations.

Previous studies have shown that metamorphosis has a strong effect on the fruit fly microbiome (Andongma et al., 2015; Yong et al., 2017a; b). This is consistent with the magnitude of metabolic and physiological change that occurs during metamorphosis, and indeed, in this study we observed a similar effect on each developmental stage (PCoA, **Figure 2A-B**). Despite the strength of this effect, however, a number of bacterial families and genera showed consistent trends at all developmental stages when comparing artificial diets. Specifically, the gel diet was associated with an increased relative abundance of Enterobacteriaceae genera *Morganella* and *Providencia*, while these taxa were almost non-existent in colonies reared on the carrot diet (**Figure 3A-B**). Conversely, the carrot diet resulted in an increased abundance in the Acetobacteraceae genus *Swaminathania/Asaia*, which was negligible in the colonies reared on the gel diet. Previous studies of wild Qfly larvae (Majumder et al., 2019; Woruba 2018) and adults (Deutscher et al., 2018; Majumder et al., 2019) suggest that Enterobacteriaceae and Acetobacteraceae are naturally abundant in the microbiome. In contrast to these globally effected taxa, there were a number of taxa whose response to diet was limited to a specific developmental stage. This included *Orbus* and *Enterobacter* two bacterial genera that were significantly abundant in adult male and female respectively fed on the carrot diet. Conversely, *Kluyvera, Aeromonas,* and *Erwinia* were highly abundant in gel diet reread males. Similarly, Woruba (2018) found that male and female Qfly from the wild had significant differences not only in bacterial diversity but also in bacterial composition. Our research also revealed a significant ($P<0.05$) difference in the microbial beta diversity between male and female Qfly reared on the carrot diet, while the opposite was observed in the gel diet reared adults based on PERMANOVA (**Supplementary file 3**). A possible explanation is that in the natural environment and the carrot diet reared domesticated colonies, the main ingredient of the available diet was plant based.

Diet is an essential factor influencing the gut microbiome in fruit flies (Deutscher et al., 2018; Majumder et al., 2019) as well as other insects, for example: cotton bollworm (*Helicoverpa armigera*) (Xiang et al., 2006), the ground dwelling beetle (Kudo et al., 2019), gypsy moth (*Lymantria dispar* L.) (Broderick et al., 2004) and *Drosophila* (Colman et al., 2012). In our study the starting material, rearing environments, adult diet and the generations were the same, the only differing factor was the larval diet. This single difference resulted in substantial variation in the bacterial composition across all developmental stages. In both the carrot diet and the gel diet, although the antimicrobial agents of sodium benzoate and citric

608 acid were common (Mainali et al., 2019), the antifungal Nipagen (methylparaben) was only
609 used in gel diet (Moadeli et al., 2017). Moreover, the yeast concentration used in the gel diet
610 was almost double than that of the carrot diet (**Table S1; S2**). Yeast and yeast like fungi are
611 considered to be key for providing amino acids to the larvae (Martin, 1987; Nardon and
612 Grenier, 1989; Vega and Blackwell, 2005; Moadeli et al., 2018a). Previous studies found that
613 the bacteria Enterobacteriaceae help improve metabolic activities in *C. capitata* and *B. oleae*
614 larvae in supporting nitrogen fixation and pectinolysis (Behar et al., 2008b; Ben-Yosef et al.,
615 2015). In addition, it might be that larvae reared on the gel diet consume more yeast and the
616 high abundance of Enterobacteriaceae are needed for protein hydrolysis (Pavlidi et al., 2017).
617 Additionally, while the gel diet has added cane sugar, in the carrot diet sugar is only as
618 naturally present in the carrot, and this could underlie the presence of *Swaminathania/Asaia*.
619 Previous studies found that Acetobacteraceae helps to break down and digest complex
620 glucose structure and lipid content of the larval diet (Huang and Douglas, 2015). At the
621 initiation of the domestication process, the wild type larvae fed on the host fruits.
622 *Swaminathania/Asaia* has been transferred and become abundant in the larval microbiome of
623 the domesticated colony reared on the carrot diet which contains a suitable source of plant-
624 based carbohydrate. Deutscher et al. (2018) and Majumder at al. (2019) also observed the
625 same findings. This suggests that bacteria from the alphaproteobacterial Acetobacteraceae are
626 needed for the digestion of complex natural plant-based carbohydrate and are not needed with
627 an artificial gel diet, hence their absence. Therefore, bacterial taxonomic composition in the
628 carrot diet reared colony was similar to the wild counterparts. In contrast, bacterial taxonomic
629 composition was highly altered across all developmental stages of the Qfly reared on the gel
630 larval diet.
631
632 *Providencia*, a pathogenic bacterial genus, was abundant in all developmental stages
633 of the Qfly reared on the gel diet but was absent in the carrot diet reared Qfly. *Providencia* is
634 a gram-negative opportunistic, non-spore forming pathogen (O'Hara et al., 2000), and has
635 been also observed and isolated from many other fruit fly species including *A. ludens*
636 (Diptera: Tephritidae) (Kuzina et al., 2001) and *B. oleae* (Diptera: Tephritidae) (Kounatidis et
637 al., 2009). *Providencia* was also identified in the domesticated colony of *C. capitata*
638 (Guerfali et al., 2018) and reported to cause infection. However, to date, there is no evidence
639 of any pathogenic effect of *Providencia* on the Qfly. Similarly, *Morganella*, a pathogenic
640 bacterium, was also detected mostly abundant in the larval stages (~99%) and less then 2% in
641 other life stages of the Qfly fed on the gel diet but was absent in carrot diet fed Qfly (**Figure**

642 **3A-B**). *Morganella* was first identified by Fulton, (1943) and Brenner et al., (1978), and
643 included within the family Enterobacteriaceae. Salas et al. (2017) also observed *Morganella*
644 *morganii* as a lethal pathogenic bacterium in domesticated *A. ludens* larvae. Surprisingly, this
645 pathogenic bacterial species was also detected in the wild Qfly (adult) (Woruba 2018). After
646 a careful blast of these bacterial sequences in NCBI, we found the same bacterial strain
647 present in the larval microbiome from the Qfly reared on the gel diet. Our study revealed that
648 although the gel diet contained more antimicrobial agents, pathogenic bacteria genera
649 including *Morganella, Enterobacter, Citrobacter, Providencia* and *Burkholderia* were
650 detected at higher abundance in the Qfly fed on the gel diet compared to the carrot diet
651 (**Figure 3A-B**). Surprisingly, most of these bacteria had also been identified (e.g.,
652 *Morganella, Citrobacter, Providencia*) in wild Qfly adults (Woruba 2018). It might be that,
653 due to domestication and continuous rearing on the same larval diet, these bacteria build a
654 mutualistic relationship with the host and improve the quality of the Qfly across all
655 developmental stages. For example, *Burkholderia* strains present in the stinkbug *Riptortus*
656 *pedestris* that helps to protect its host from Fenitrothion (insecticide) (Kikuchi et al., 2012).
657 *Citrobacter* present in *B. dorsalis* was worked on Trichlorphon degradation (Cheng et al.,
658 2017). We hypothesize that these pathogenic strains could be controlled in part by the host
659 genetics along with nutritional components present in the gel based larval diet (Woruba
660 2018). Other putative pathogenic bacteria found across all developmental stages of the Qfly
661 fed on gel diet might follow the same trend. However, it is not known what type of genetic
662 factors control these bacterial pathogeneses. We also hypothesize that if these bacteria are
663 mostly found in the wild Qfly (Woruba 2018), then maybe they are not pathogenic and are
664 normally abundant as gut residents.
665
666 The relationship between the insect and its symbionts may be beneficial or harmful to
667 the host health and fitness, and this depends on the composition of the insect's microbiome
668 (Kaufman et al., 2000; Marchini et al., 2002; Feldhaar, 2011; Hammer et al., 2017).
669 Symbiotic and endosymbiotic bacteria are important sources of essential nutrients to their
670 host insects (Behar et al., 2005; Behar et al., 2008a). Further, the nutritional components
671 present in the larval diet has a significant impact on the developmental parameters of fruit
672 flies (Krainacker et al., 1987; Vargas et al., 1994). In fruit fly, larval, pupal and adult body
673 weight are commonly used measures of quality (Sharp et al., 1983; Churchill-Stanland et al.,
674 1986; Fanson et al., 2014). We found that pupal weight was higher in Qfly from the gel diet.
675 Pupal weight is considered as a key factor as a quality parameter in mass rearing of the Qfly

(Dominiak et al., 2010). Greater pupal weight is generally expected to correspond to larger, healthier, adults (Mohamed et al., 2016). Mainali et al. (2019) also stated that the gel diet might be better than carrot diet. In our study, the gel diet produced flies with better flight ability performance. Previous studies of Moadeli et al. (2017) and Moadeli et al. (2018a) also reported higher percentage of fly emergence and percentage of fliers in Qfly reared on the gel diet. Mainali et al. (2019) also observed similar results that support our findings.

The nutritional components present in the artificial diet including proportion of yeasts and sugar, fatty acids and minerals can have a strong influence on fruit fly development (Krainacker et al., 1987; Vargas et al., 1994; Aluja et al., 2001; Plácido-Silva et al., 2006; Moadeli et al., 2018a; Moadeli et al., 2018b; c). Moadeli et al. (2018a) reported that brewer's yeast present in the gel diet had a better effect on larval development rather than Torula yeast. On the other hand, in tephritid flies, numerous studies have demonstrated that gut bacteria are associated with digestion, detoxification, immune response, metabolism, sexual behaviour, reproduction and survival in tephritid flies (Dillon and Dillon, 2004; Hosokawa et al., 2007; Engel and Moran, 2013; Ben-Yosef et al., 2015). Various strains of Enterobacteriaceae have been added to artificial larval diets to improve pupal weight and mating performance, and decrease developmental time in *B. oleae* and *C. capitata* in mass rearing programs (Sacchetti et al., 2008; Ben Ami et al., 2010; Gavriel et al., 2011; Hamden et al., 2013; Augustinos et al., 2015). It could be that Enterobacteriaceae improve larval, pupae and adult quality. In the present study, the same bacterial family of Enterobacteriaceae was observed in Qfly microbiome in both artificial diets at all developmental stages but they mostly contained different bacterial genera. It might be that the different bacterial genera of the same family in Qfly fed the carrot or gel diet reflect their varying ability to adopt the changes in the gut environment due to different diet during domestication from the wild. This may result in functional redundancy of some microbiome components, where loss of one type of bacterium could be replaced by different kind of bacteria (pathogenic/non-pathogenic) with similar functions (e.g., improve metabolic activity or physiological development) (Moya and Ferrer, 2016).

CONCLUSION

The present study aimed to explore the influence of two artificial larval diets on the Qfly microbiome across all developmental stages. Overall our findings suggest that the artificial

larval diet strongly effects the microbial community structure of the Qfly across the developmental stages as well as fly fitness and behaviour. This knowledge may help to guide manipulation of bacteria to improve the artificial diet quality prior to release of sterile flies or during production phases to boost the quality of the domesticated Qfly for SIT.

SUPPLEMENTARY FILES

Supplementary file 1: OTU table
Supplementary file 2: Mapping file
Supplementary file 3: PREMANOVA data

AUTHORS CONTRIBUTIONS

RM, and TC designed the experiment. RM collected the data. RM, BS and SMA analysed the data. TC and PWT supervised the project. All authors analysed the data, provided inputs into the writing of the manuscript, and approved the submitted version.

ACKNOWLEDGEMENTS

We thank Dr Bishwo Mainali for his valuable suggestions on quality control experiments and analysis.

CONFLICT OF INTERESTS

The authors have no conflict of interests to declare.

FUNDINGS

This research was conducted as part of the SITplus collaborative fruit fly program.
Project *Raising Q-fly Sterile Insect Technique to World Standard* (HG14033) is funded by the Hort Frontiers Fruit Fly Fund, part of the Hort Frontiers strategic partnership initiative developed by Hort Innovation, with co-investment from Macquarie University and contributions from the Australian Government. Rajib Majumder is supported by an

International Macquarie University Research Excellence Scholarship (IMQRES) from Macquarie University (NSW, Australia).

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FIGURES AND TABLES

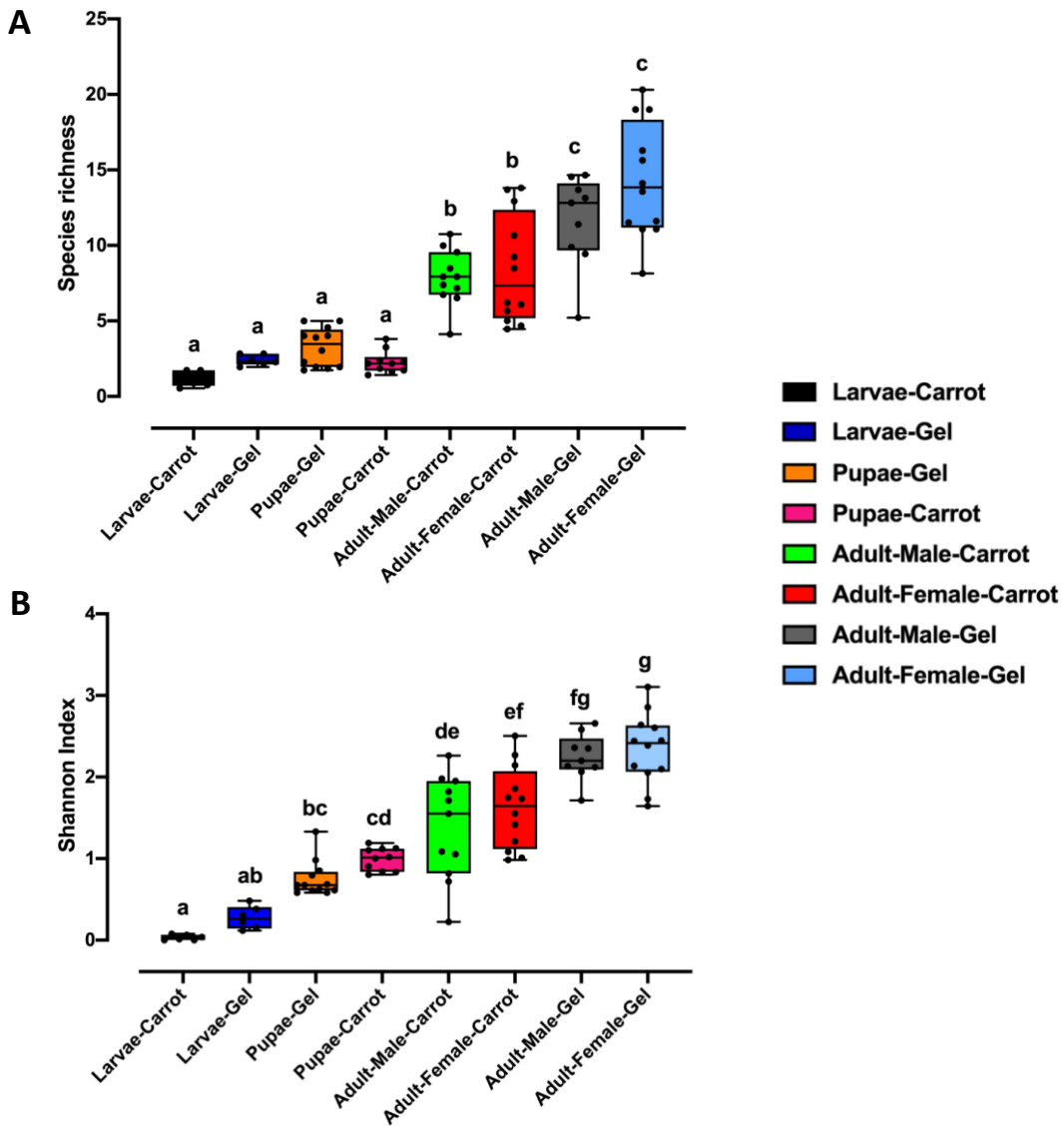


FIGURE 1 | Alpha diversity of the bacterial microbiome of the *B. tryoni* developmental stages at G5 reared on two different artificial diets, A) Species richness; B) Shannon Index. Different letters indicate significant Tukey's post hoc comparisons ($P<0.05$)

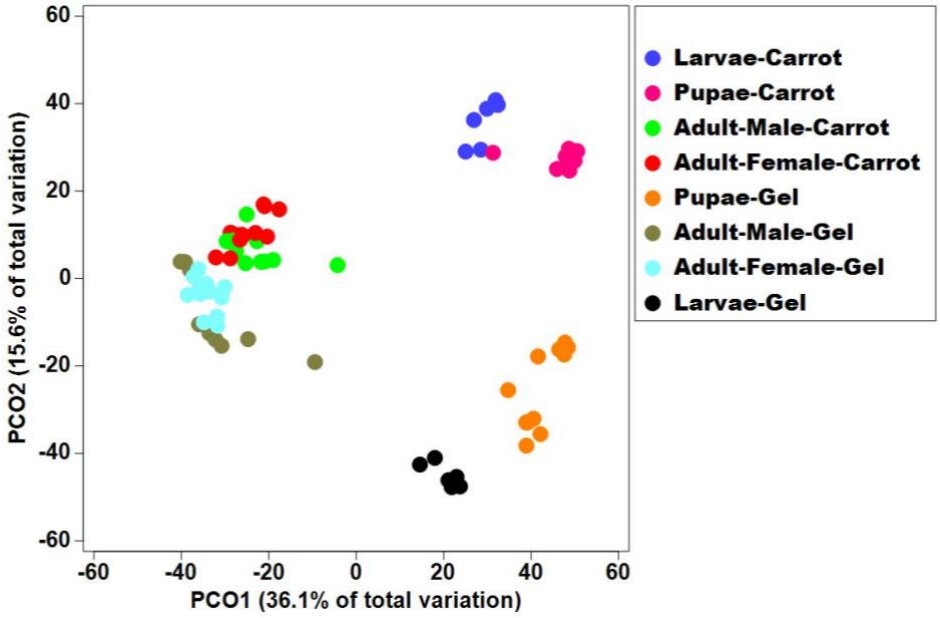


FIGURE 2 | Principal coordinates analysis of the bacterial communities in the Qfly developmental stages at G5 reared on carrot diet and gel diet. Different color indicates the microbial communities in different life stages of the Qfly

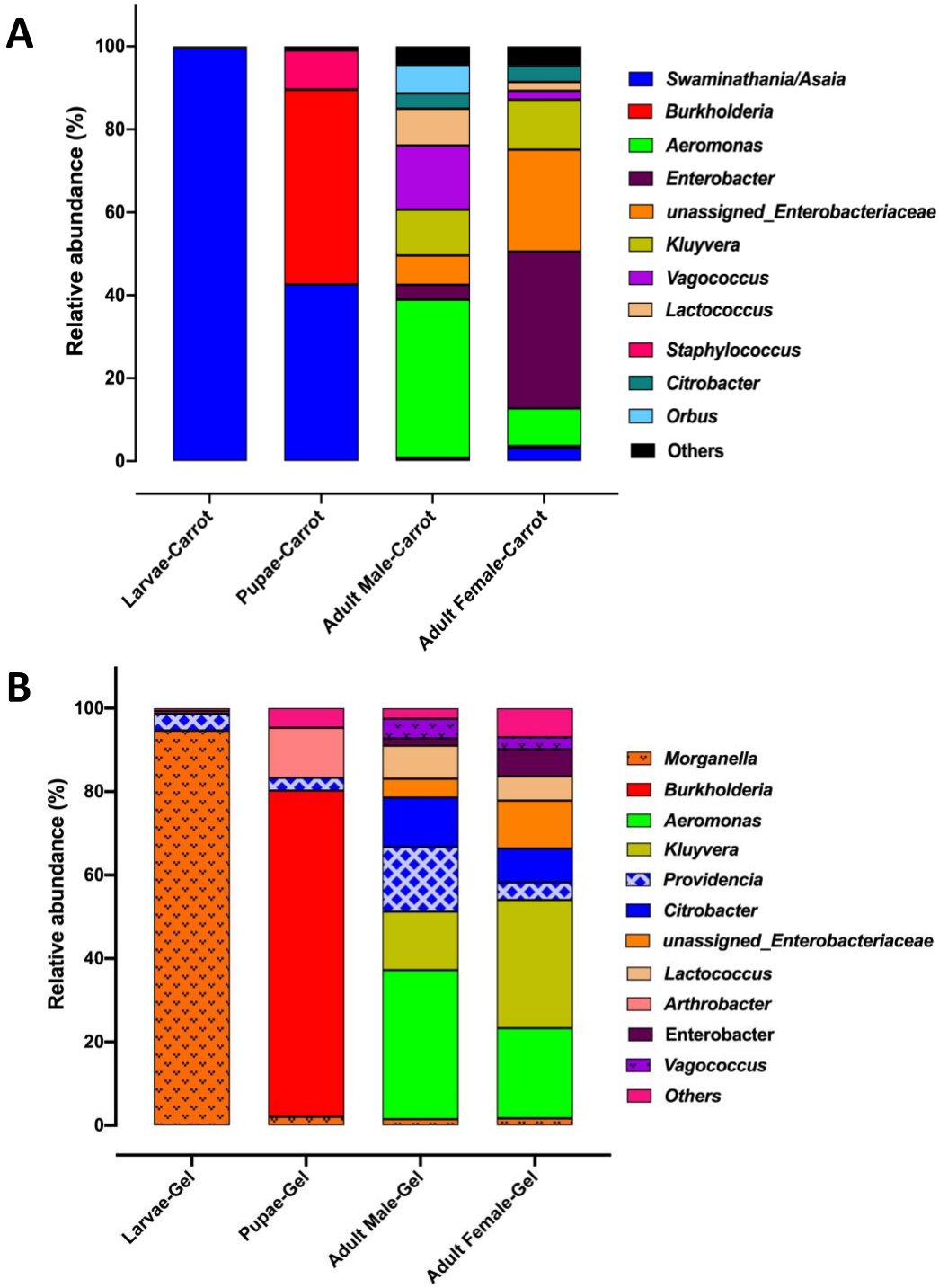


FIGURE 3 | The relative abundance of the bacterial microbiota in Qfly at different developmental stages from G5 reared on A) Carrot based larval diet; B) Gel based larval diet.

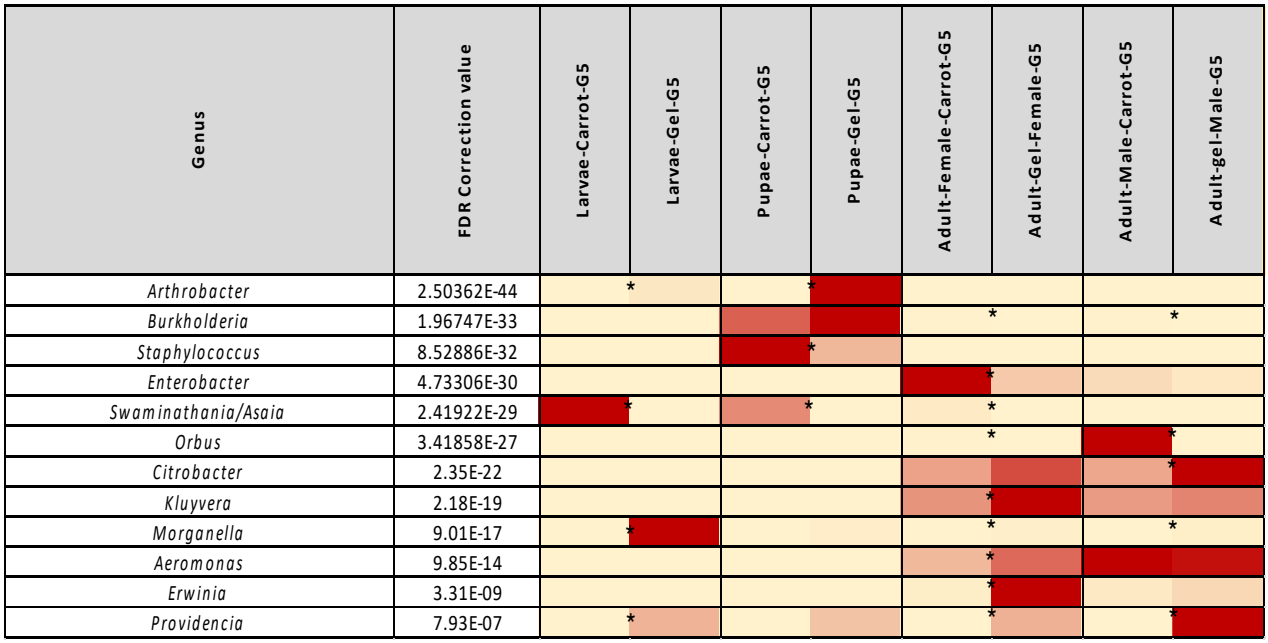


FIGURE 4 | Heatmap representing the average relative abundance of the 12 most abundant (≥1%) bacterial genera of Qfly from the carrot and gel-based diet groups on 16S rRNA gene amplicon data. Relative abundances for each genus are represented by a colour on a spectrum from yellow to deep red. Lowest values are highlighted yellow, highest are represented as a deep red. Asterisks (*) indicate relative abundances of each developmental stage is significantly different between the carrot and gel diet.

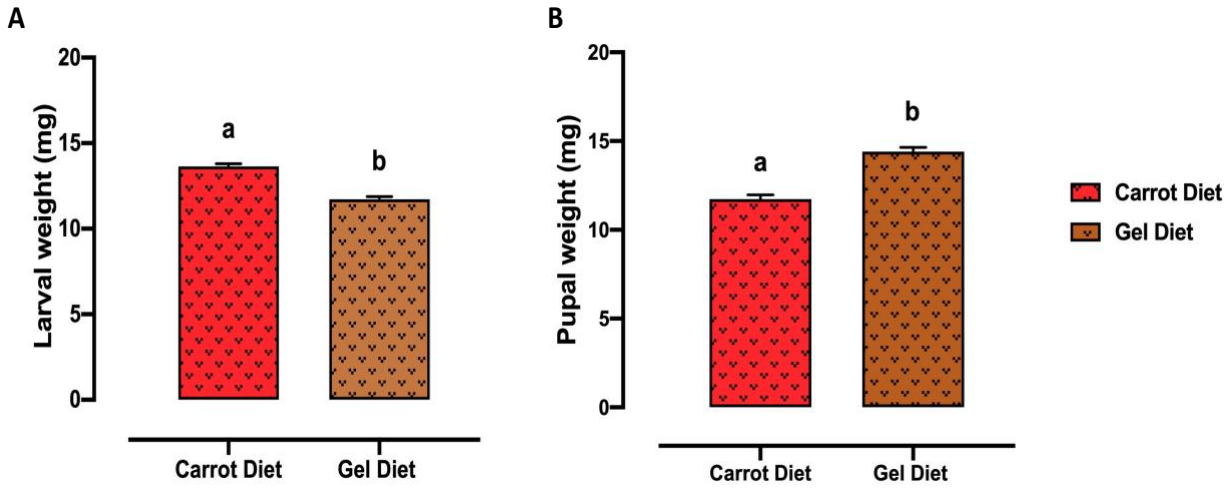


FIGURE 5 | A) Larval weight and B) Pupal weight of Qfly from G5 reared on carrot diet and gel diet. Different letters indicate significant Student's *t*-test comparisons ($p < 0.05$)

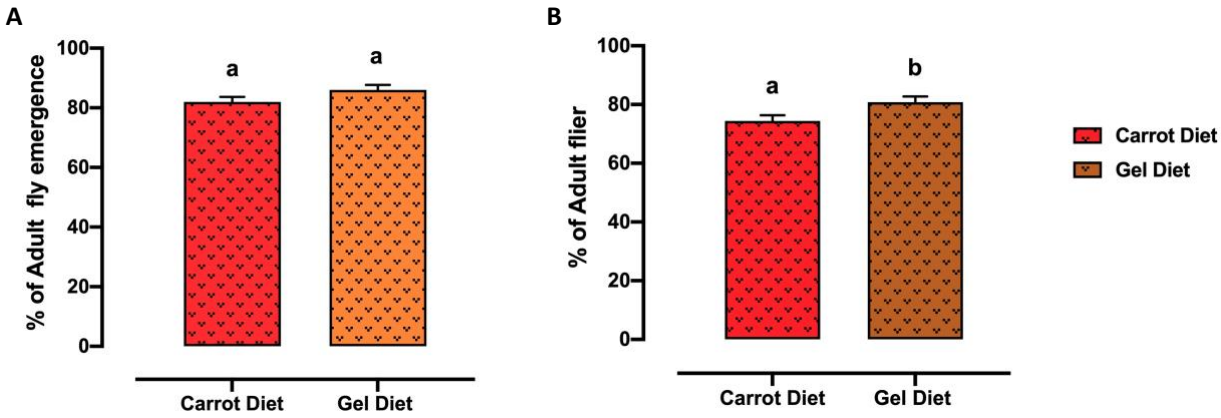
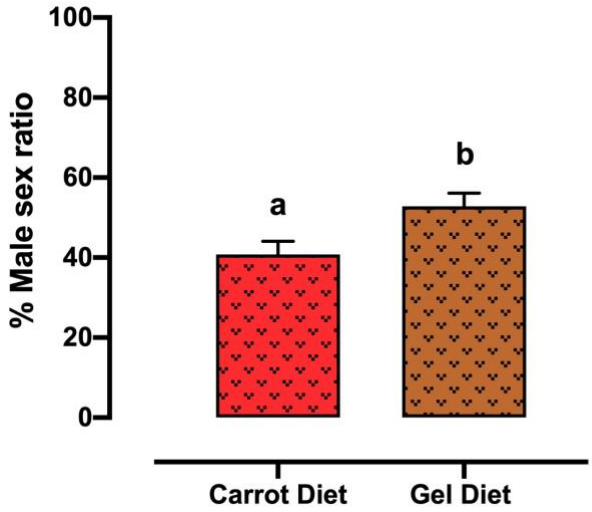


FIGURE 6 | Flight ability test: A) Percentage of emergence and B) Percentage of fliers of the Qfly (G5) reared on the carrot and gel diet. Different letters indicate significant Student's *t*-test comparisons ($p < 0.05$)



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1178 **FIGURE 7 |** Percentage of the male sex ratio of the Qfly from G5 reared on the carrot and

1179 gel diet. Different letters indicate significant Student's *t*-test comparisons ($p < 0.05$)

TABLE 1 | Taxonomic identification of the 14 most abundant bacterial OTUs in the Qfly from carrot and gel diet. Percentage of the carrot and gel diet represents the percentage of the average relative abundance of each genus present in the Qfly sample from the two different diets.

OTUs	Phylum	Class	Oder	Family	Genus	% carrot Diet	% Gel Diet
OTU_19	Actinobacteria	Actinobacteria	Actinomycetales	Micrococcaceae	*Arthrobacter*	0.00	3.14
OTU_3	Proteobacteria	Betaproteobacteria	Burkholderiales	Burkholderiaceae	*Burkholderia*	12.02	19.55
OTU_6	Firmicutes	Bacilli	Bacillales	Staphylococcaceae	*Staphylococcus*	2.35	0.57
OTU_7	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	*Enterobacter*	10.36	2.06
OTU_1	Proteobacteria	Alphaproteobacteria	Rhodospirillales	Acetobacteraceae	*Swaminathania/Asaia*	36.40	0.00
OTU_30	Proteobacteria	Gammaproteobacteria	Orbales	Orbaceae	*Orbus*	1.73	0.00
OTU_59	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	unassigned_Enterobacteriaceae	7.93	4.00
OTU_100	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	*Citrobacter*	1.91	4.99
OTU_5	Firmicutes	Bacilli	Lactobacillales	Enterococcaceae	*Vagococcus*	4.40	1.89
OTU_36	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	*Kluyvera*	5.77	11.19
OTU_9	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	*Morganella*	0.00	24.98
OTU_15	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	*Lactococcus*	2.75	3.44
OTU_8	Proteobacteria	Gammaproteobacteria	Aeromonadales	Aeromonadaceae	*Aeromonas*	11.79	14.32
OTU_14	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	*Providencia*	0.00	6.70

TABLE 2 | Fruit types and origin for wild *Bactrocera tryoni* larvae collection. A total of six replicate larvae, and fruit flesh samples were collected from each fruit origin.

Geographic location of collection	Fruit source and number of fruits collected	Collection date
Coomealla, NSW GPS: Lat 34° 5'50.97", Long 142° 3'7.21"	Pomegranate 37 pieces	5/05/17
St. Germains, Between Tatura and Echuca in Victoria GPS: Lat 36°10'48.86", Long 145° 8'50.74"	Green Apple 41 pieces	05/05/17
Downer road between Tatura and Toolamba in Victoria GPS: Lat 26°38'34.92", Long 152°56'22.99"	Quince 52 pieces	05/05/17

SUPPLEMENTARY FIGURES AND TABLES

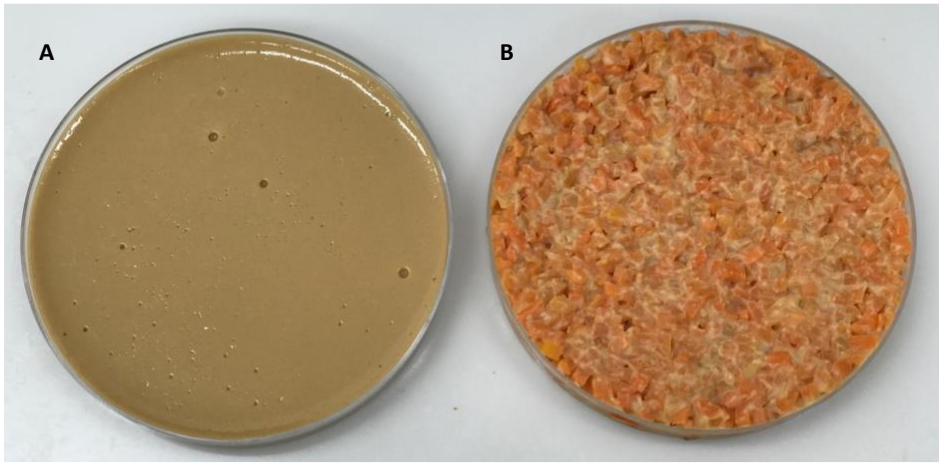

FIGURE S1 | Artificial larval diet A) Gel based diet; B) Carrot based diet

TABLE S1 | Gel based larval diet recipe

Ingredients	**1kg diet preparation**	**Company name and catalogue number**
Brewer's Yeast (g)	204	SF Health foods, Australia
Sugar (g)	121.8	MP Biomedicals LLC, France, Cat. no02902978
Agar (g)	10	Sigma Aldrich®
Citric Acid (g)	23.1	Sigma Aldrich®
Nipagen (g)	2	Southern Biological (Cat no MC11.2)
Sodium benzoate (g)	2	Sigma Aldrich®
Wheat Germ Oli (mL)	2	Melrose laboratories Pty Ltd, Australia
Mili -Q-water (mL)	1000	Milli-Q water

1262 **TABLE S2 |** Carrot based larval diet recipe

1263

Ingredients	**1kg diet preparation**	**Company name and catalogue number**
Carrot (dehydrated diced) (g)	280	H.J. Langdon, Australia
Torula yeast (g)	89.55	H.J. Langdon, Australia, product code 45014
Citric acid (g)	13.43	Sigma Aldrich®
Sodium Benzoate (g)	3.72	Sigma Aldrich®
water (mL)	1000	Milli-Q water

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Chapter 6

General Discussion

6.1 Summary of thesis objectives

The research described in this thesis focused on the comprehensive characterization of the bacterial and fungal microbiome of the tephritid pest Queensland fruit fly (Qfly; *Bactrocera tryoni*), both in the wild, and during the domestication process. To achieve this, I addressed the following research objectives:

I. Characterize the diversity of the bacterial community in the microbiome of wild Qfly larvae.
II. Characterize the diversity of the fungal community in the microbiome of wild Qfly larvae.
III. Assess the influence of the fruit host microbiome on gut colonisation of wild Qfly larvae by bacterial and fungal symbionts.
IV. Describe and compare the gut microbiota of Qfly across developmental stages.
V. Describe and compare the effects of two artificial larval diets on gut microbiota of domesticated Qfly across developmental stages.

6.2 The microbiome of wild Qfly larvae

Understanding the abundance and diversity of bacteria and fungi associated with wild Qfly larvae, and its various host fruits, is important foundation information for understanding Qfly physiology and ecology. In chapters 2 and 3, I comprehensively identify the bacteria (Chapter 2) and fungi (Chapter 3) associated with Qfly larvae collected from multiple host fruit. Through the use of culture-independent next generation sequencing, this provided new insights into the natural diversity of microbiota associated with Qfly larvae in the wild, the transmission processes involved in structuring these communities (i.e. vertical vs horizontal transfer), and the role of fruit hosts in these processes.

6.2.1 The diversity of bacterial and fungal microbiota in wild Qfly larvae

In Chapter 2, a total of 167 bacterial OTUs were detected in Qfly larvae. This is extremely high when compared to previous culture-based surveys of Qfly larvae, which typically detected between 10 to 15 different species (Deutscher et al., 2018). This disparity demonstrates the power of culture-independent surveys for microbiome analyses, and supports the approach undertaken in this thesis. In comparison to their bacterial counterparts,

a total of 62 fungal OTUs obtained in fungal microbiota communities were varied and for the first time identified in Qfly larvae in the wild.

At the OTU level, Shannon's biodiversity index for bacterial microbiota in wild Qfly larvae were an average of 0.837 ± 0.08. This contrasted the fungal microbiota, in which Shannon's biodiversity indices were significantly greater (1.846 ± 0.08; $p<0.05$, Figure 1A). This difference appears to be driven by an increase in community evenness for fungal microbiota, which was found to differ significantly ($p<0.05$, Figure 1B), and not species richness ($p>0.05$).

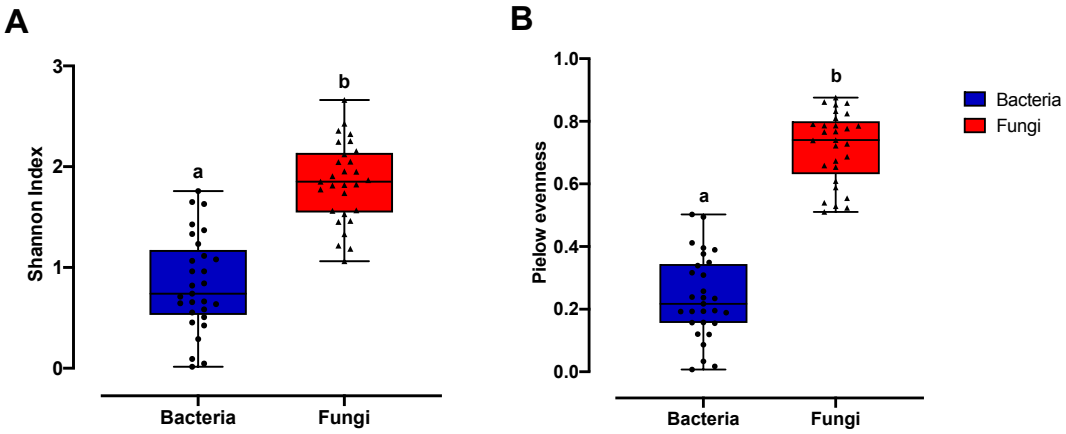

Figure 1: A) Alpha biodiversity of the bacterial and fungal microbiota of wild Qfly larvae (as determined by Shannon's biodiversity index); B) Community evenness of the bacterial and fungal microbiota of wild Qfly larvae (as determined by Pielous' evenness index). Different letters indicate significant Student's *t*-test comparisons ($p< 0.05$)

The majority of bacterial OTUs detected in this study belonged to the Proteobacteria. In particular, OTUs assigned to the *Swaminathania/Asaia, Gluconacetobacter, Gluconobacter*, and *Acetobacter* genera were identified across all larval samples, at relatively high abundances. This suggested that they are common bacteria within this environmental niche. The bacterial family of alphaproteobacterial Acetobacteraceae and gammaproteobacterial Enterobacteriaceae represented at an average of 75% and 21% in the larval microbiome respectively. In addition, Leuconostocaceae with an average relative abundance of 2% were also detected. At the genus level, *Swaminathania/Asaia* constituted more than 50% of the larval microbiome (53%), with other abundant genera including

Gluconacetobacter (9.1%), *Gluconobacter* (7%), *Tatumella* (5.2%), *Klebsiella* (4.9%), *Acetobacter* (3.8%), *Providencia* (2.8%) and *Leuconostoc* (2%). Although the biodiversity was found higher in the fungi, however, vast majority of the fungal OTUs mostly associated with the fungal family Saccharomycetaceae. For fungal microbiota communities, the dominant fungal families with the greatest relative abundances in larvae were Saccharomycetaceae (88%), Metschnikowiaceae (9%) and Sporidiobolaceae (1%). The most abundant fungi were found as the closest match to the genera of *Pichia*, *Trigonopsis, Clavispora, Candida, Kodamaea*, and *Cyberlindnera.* Most of these fungi were associated with the family Saccharomycetaceae belonging to the order Saccharomycetales which are single cell fungi known as the 'budding yeasts' or the 'true yeast'. This study has enabled us to understand the taxonomic identification and diversity of the Qfly larval microbiome.

6.2.2 The transmission process of the microbiome to the Qfly larvae

Previous studies have demonstrated that the structure and composition of the insect microbiome is influenced by diet (Broderick et al., 2004; Xiang et al., 2006; Colman et al., 2012; Hammer et al., 2014). Chapters 2 and 3, however, represent the first study to investigate this relationship in wild Qfly larvae. This research suggests that the microbial communities of fruits strongly influence the structure of bacterial communities present in the Qfly larvae. Many of the abundant microbial taxa within the larval microbiome (see section 6.2.1), were also identified in the host fruits, albeit at different relative abundances. For example, *Swaminathania/Asaia* OTU 1 was abundant in both larvae and fruit, but made up an average of ~55% of the larval microbiome, and just 15% of the fruit bacterial community (Chapter 2). Similarly, in the fungal communities, fungal OTU 1 was most closely related to *Pichia terricola* with 42% and 33% average relative abundance observed in larvae and host fruit, respectively (Chapter 3).

For the bacterial microbiome communities, the percentage of bacterial OTUs unique to the larval microbiome was significantly greater than those unique to the fruit flesh. This not only resulted in greater biodiversity in the larvae compared with host fruits, but also suggests that some of the bacteria in the larval microbiome are transmitted vertically. That is, females transmit gut bacteria during oviposition, possibly by regurgitating in the same area of the host fruit as has been observed by Courtice (1984). Thus, my research suggests that

bacteria are transmitted vertically from the mother to the egg during oviposition, which is subsequently transferred to the larvae.

In contrast, the fungal microbiota of wild Qfly larvae did not differ significantly from that of the host fruit. Fruit fly larvae are known to feed on yeasts in fruit, using the yeast as a protein source (Deutscher et al., 2016). Further, the larvae may play a role in distributing yeasts through the host fruit (Malacrinò et al., 2015). Therefore, there may be feedback between the fungal communities detected in larvae and fruit flesh, with very different underlying ecology to that identified in the bacterial communities. This finding leads to the conclusion that the fungal microbiome of the Qfly larvae mostly reflects horizontal transfer, most likely in part as a food source.

6.2.3 The role of host fruit in shaping wild Qfly microbiota

By sampling larvae from five different fruit types, this research explored meaningful ecological questions regarding the effect of host fruit on the Qfly microbiome. In fungal host fruit communities, a substantial amount of variation was observed and, as the fungal microbiome of Qfly larvae was generally similar to that of the host fruit, this was reflected in the wild Qfly larvae fungal microbiome. Similarly, there was significant variation found in the bacterial microbiome of Qfly larvae sampled across different types of fruit. Despite substantial variation in the bacterial community of individual Qfly larvae, the most abundant taxa in the larvae were consistent across the different fruit sources. Thus, the differences detected in PERMANOVA were driven by low abundance taxa within the larval microbiome. In addition, the findings of this thesis support that the host plant family and geographical variation influenced the fungal communities found in both larvae and fruit collected from NSW, VIC and QLD in Australia. However, testing of this hypothesis would require significant further investigation that includes a large study cohort of samples of hosts from different locations and plant families. Overall, this research contributed comprehensive knowledge of the Qfly larval microbiome and the interaction with its host fruits.

6.3 Gut microbiome of Qfly during metamorphosis

Metamorphosis is a conspicuous and abrupt transformation process where the insect undergoes a complex remodelling of both of its external and internal morphology and is

based on different developmental stages like larvae, pupae and adults (Figure 2) (Truman and Riddiford, 1999; Grimaldi et al., 2005; Johnston and Rolff, 2015). In Chapter 4, I undertook the first comprehensive investigation into the gut microbiome at different developmental stages of Qfly. Previous studies of the insect gut microbiome during metamorphosis have been reported on domesticated insects, including butterfly (Ravenscraft et al., 2019), beetle (Kudo et al., 2019) and several fruit fly species including *B. carambola*e (Yong et al., 2017a), *B. dorsalis* (Andongma et al., 2015) and *B. latifrons* (Yong et al., 2017c). These studies have all focused on the bacteria only. However, this thesis comprehensively revealed both the bacterial and fungal profile of the Qfly through development. In Chapter 5, I also investigated the compositional changes of the bacterial microbiome across developmental stages (larvae, pupae, adults) of Qfly but compared the impact of on two artificial larval diets during domestication at generation 5 (G5).

Chapter 4 showed that during metamorphosis of the Qfly in the wild-type colony (G0), the bacterial microbiome was significantly different between the larval stage and adult ($P<0.05$). This same trend was observed in domesticated colonies of G5 reared from the two different larval diets studied in Chapter 5. For example, in wild-type colony of Qfly, *Swaminathania/Asaia* (~30%) and *Acetobacter* (~11%) were detected in the larval stage but present at very low relative abundance (<1%) in the adult. Woruba (2018) also observed *Swaminathania/Asaia* in wild Qfly adult (both male and female) with very low abundance. This finding supports that although in wild-type colony G0, flies were kept for 15 days in the laboratory the microbiome inherited from the wild was observed as abundant. Similarly, in the domesticated colony reared on carrot diet, *Swaminathania/Asaia* was highly abundant in the larval and pupal stages but in very low abundance (~4%) in the adult stage. On the other hand, the opposite was found for bacteria associating to the Enterobacteriaceae, which were more abundant in the adults and less in larval stages both G0 and domesticated G5 colonies. These results also reflect the bacterial vertical transmission process from the adult mother to the larvae (Deutscher et al., 2018).

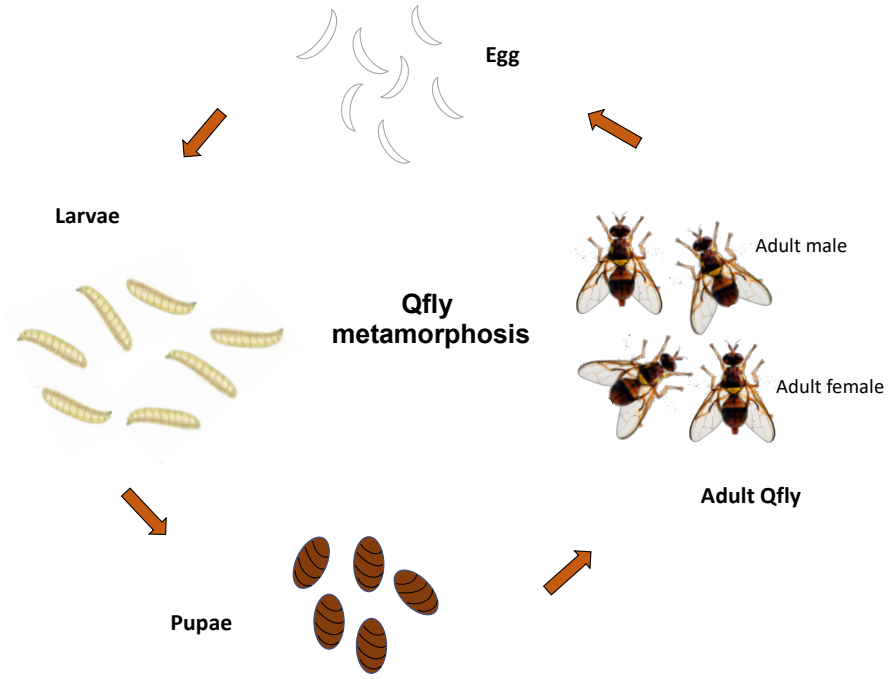

Figure 2: Qfly developmental stages (Metamorphosis). Flies of the opposite sex are figured for adult male and female. (Adult fly picture was collected from this link: https://area-wide-management.com.au/about/the-pest/)

Considering the fungal population, the fungal genera of *Candida* and *Pichia* were abundant prominently in the larval and pupal microbiome but not in adults. In contrast, the fungal genus *Penicillium* was found to be highly abundant in adult males (47.8%) but of relatively low abundance in larval stages (0.4%). Further, unassigned Tremellomycetes (19.4%), *Gibberella* (19.6%) and *Pseudopithomyces* (4.1%) were abundant (>1%) only in the adult male gut microbiome. Similarly, *Cyberlindnera* was relatively abundant (32.3%) in the female gut, but was not detected in males and other developmental stages. This study revealed that during metamorphosis certain fungi that were present in the larval and pupal stages did not transmit to the adults after eclosion. The differing diet preferences and morphology of these stages are possible explanations for such trends. Similar results were also observed in the bacterial microbiome of *B. dorsalis* (Zhao et al., 2018; Stathopoulou et al., 2019) and *B. carambola* (Yong et al., 2017b). This is the first comprehensive analysis of fungal symbionts of Qfly across all development stages.

6.4 Diet modulates the bacterial diversity in Qfly during the domestication process

Previous studies of other insects have reported differences between life stages in the microbiome composition and diversity, although the direction of changes in diversity and the bacterial taxa affected were species-specific (Dillon and Dillon, 2004; Wong et al., 2011; Engel and Moran, 2013; Yun et al., 2014). Findings of this study provided insights into the impact of diet on the symbionts at each life stage through the transition from nature to the laboratory environment. To observe the artificial larval diet effect through the domestication process (Chapter 5), the G0 wild-type colony of the Qfly (described in Chapter 4) was used and domesticated on two different artificial larval diets (carrot-based diet and gel-based diet) up to G5 in a controlled environment laboratory. For this discussion section, I have further analysed and compared the diversity and taxonomic classification of the bacterial microbiome of the Qfly between G0 and G5 colonies reared on two different artificial larval diets.

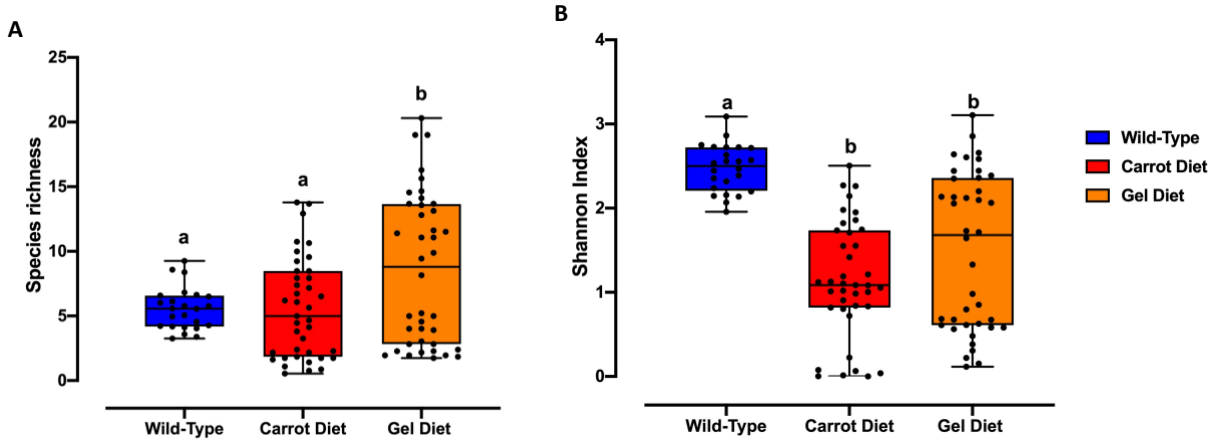

Figure 3. A) Alpha biodiversity of the bacterial and fungal microbiota of wild Qfly larvae; B) Community evenness of the bacterial and fungal microbiota of wild Qfly larvae. Different letters indicate significant Tukey's post hoc comparisons ($p < 0.05$)

This research found that both species richness (Figure 3A) and Shannon index (Figure 3B) were significantly different ($p < 0.05$) between the G0, G5 (carrot diet) and G5 (gel diet) (Figure 3A-B). In contrast, the Shannon indices showed no significant difference between the Qfly from the carrot diet and the gel diet reared G5 flies. These results shown that flies reared on both artificial diets had altered bacterial microbiome at G5 due to domestication process.

Interestingly, species richness was significantly ($P<0.05$) higher in the gel diet compared with the carrot diet reared G5 flies as well as G0. Bacterial species richness was similar in the Qfly microbiome of G0 and carrot diet reared G5. In this study, domesticated Qfly colonies were reared using artificial diet, where carrot diet fed colony received plant-based ingredients (e.g., dried carrot dice) compared to a synthetic gel-based diet. Additionally, G0 colonies were established with the wild larvae fed on natural fruits they were collected in. Therefore, it might be that the common bacterial species (e.g., *Swaminathania/Asaia*) transfer from G0 (wild-type) to carrot diet reared G5 colony during domestication. This study clearly demonstrated that the gel diet strongly influenced the microbiome of Qfly at all developmental stages during domestication from G1. Chapter 5 showed that the overall microbial community composition was different between the carrot diet and the gel diet reared Qfly at all developmental stages and changed across the developmental stages in both colonies at G5. Interestingly, the bacterial alpha and beta diversity observed in both domesticated Qfly colonies (G5) reared on the carrot and gel diet exhibited a similar diversity trend to wild-type Qfly microbiome described in Chapter 4, whereby microbial diversity in larvae was significantly different from adults. Consistently, in wild-type Qfly the larvae and pupae were not significantly different and this was similar in the carrot and gel diet domesticated colonies at G5**.** Overall, these results revealed that the artificial larval diet has substantial impact on compositional changes in the microbiome of the Qfly during domestication process.

6.5 Microbiome changes in Qfly through the domestication process

In Chapter 5, a strong effect of the artificial larval diet on the relative abundance of the bacterial taxa in the Qfly microbiome from G5 (both carrot and gel diet) was found. Here I compared the relative abundance of the bacterial taxa of the Qfly from G5 to the wild-type Qfly samples of G0 (Figure 4A-C). This research showed that in G0, larval and pupal microbiomes were dominated by the genus *Swaminathania/Asaia,* whereas, the adult microbiomes were dominated by *Enterobacter* from the family of the Enterobacteriaceae. Previous studies also detected Enterobacteriaceae as a bacterial family present in the wild Qfly (only adults) microbiome (Woruba, 2018). In the G5 fed on the carrot diet, larval and pupal microbiomes contained a high relative abundance of *Swaminathania/Asaia*, however, pupae also contained high relative abundance of *Staphylococcus* and *Burkholderia*, and there were no dominant patterns of these bacteria found in adults.

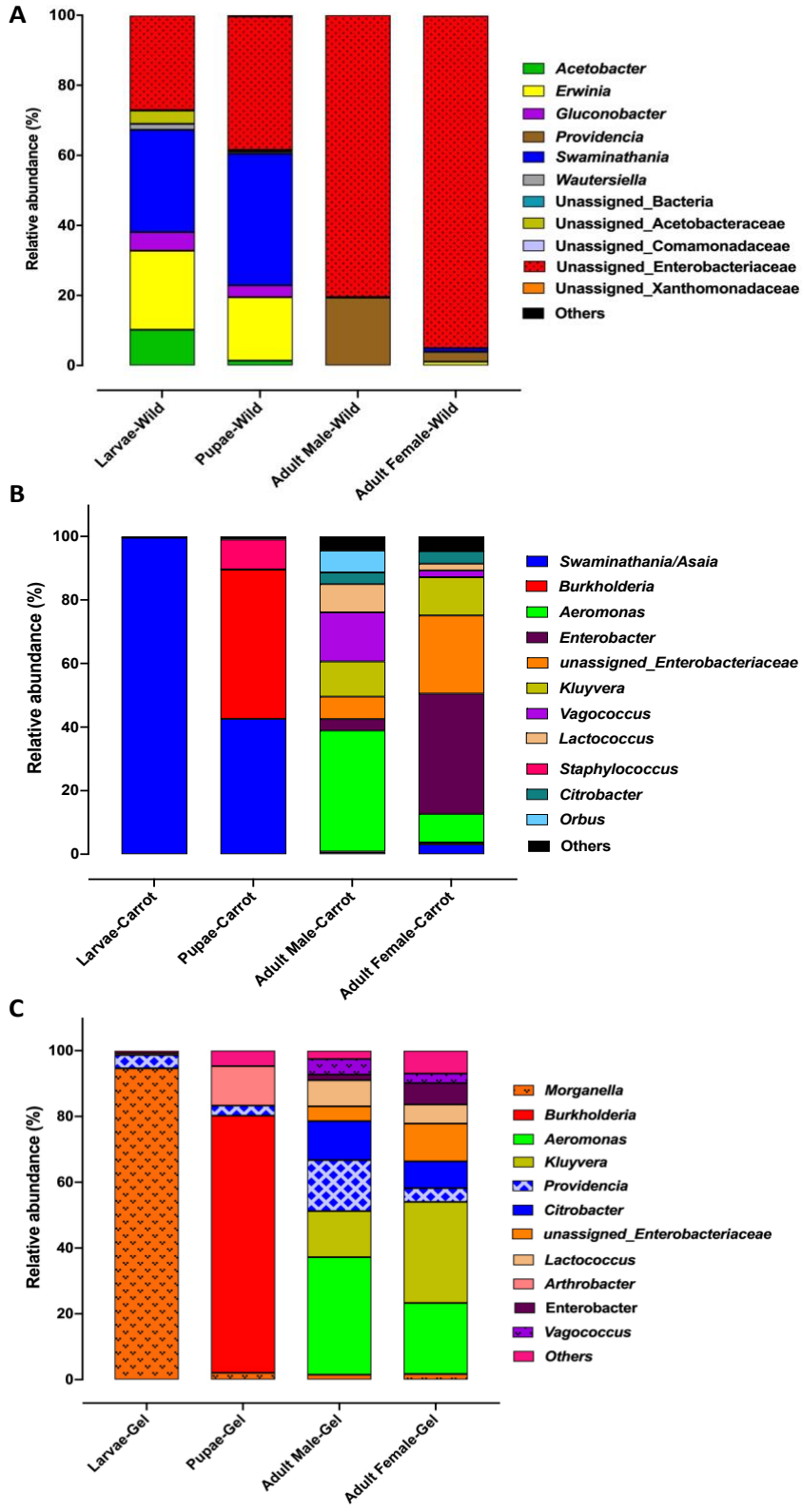

Figure 4: The relative abundance of bacterial microbiota in Qfly at different developmental stages in A) Wild-type (G0); B) Carrot diet (G5); and C) Gel diet (G5)

In contrast, *Swaminathania/Asaia* was not observed in any developmental stages of the Qfly from the gel diet reared colony of G5. *Swaminathania/Asaia* was observed as one of the dominant populations in wild larvae, with the presence of a number of other abundant bacterial genera groups, including *Erwinia, Acetobacter* and *Gluconobacter*. Surprisingly, none of these bacteria were found in the Qfly microbiome from both colonies at G5. The pathogenic bacterium, *Erwinia* was abundant in the pupal stages of the G0 but was not detected in the pupal stages of the G5 reared on both artificial larval diets. However, another pathogenic bacterium, *Burkholderia,* was abundant in the pupal stage of the Qfly reared on both carrot and gel diet. Further, the putative pathogenic bacterial genera *Pseudomonas* and *Klebsiella* were not detected in any colonies of G5 whereas they were abundant in wild-type populations of G0. Conversely, pathogenic bacteria including *Burkholderia, Aeromonas, Kluyera* and *Citrobacter* were found only in G5 samples, being absent in G0.

After careful comparison of the bacterial microbiome of the Qfly between the wild-type G0 colony with domesticated colonies of G5 from carrot and gel diet, this research suggests that the artificial diet has altered host-symbiont interactions at all developmental stages through the domestication process, a trend which continues within the domesticated generations. Chapters 2 and 3, demonstrated that the microbiome (mostly in the gut) of Qfly larvae is influenced by diet in the wild. Based on this, Chapter 5 also showed that during the domestication process, the artificial diet is a primary factor that modulate the overall microbiome in the Qfly during development. Artificial larval diet containing various antimicrobial and synthetic ingredients (Moadeli 2017, 2018a-c) have been used to rear colonies of the Qfly which might impact on the microbial communities and abundance.

In Figure 4, a clear demonstration of the taxonomic variation has been shown, where microbial population of the wild larvae was found to be more diverse than the domesticated colonies. The opposite was found in the adult microbiome, where various species of bacteria associated with Enterobacteriaceae were found but rapidly changed and were replaced by different species of bacteria from the same family. The continuous rearing of Qfly on an artificial larval diet might alter the microbial community structures over generations from the wild. In this study, larval diet was altered from G1, but the adult diet remained the same. Therefore, it revealed that during the domestication process, alteration of the Qfly microbiome started from the use of artificial larval diet at G1. Pathogenic bacteria mostly associated with the family Enterobacteriaceae were found highly abundant in domesticated

colonies reared on gel diet compared to the carrot diet. It might be possible that, these pathogenic bacteria altered their relationship with the Qfly, and under domestication contributed to Qfly development. Overall, these findings indicated that continuous domestication process, the transition from the wild to laboratory flies, impacts on the microbiome structure. Based on these research findings, future research is needed to identify whether similar trends are observed for the fungal population. These findings are fundamental to understanding how the domestication process along with diet can be manipulated to impact the microbial communities in Qfly used in SIT.

Previous research in tephritid fruit flies has demonstrated that adaptation of the artificial rearing conditions has a significant influence on microbiome (Chapter 5) and several life history traits, including stress tolerance and sexual indices (Cayol et al., 2000; Pérez et al., 2018; Mainali et al., 2019). Based on the significant effect of the artificial larval diet on microbiome changes in Qfly, I also determined whether the artificial larval diets had impact on behaviour and fitness traits of the domesticated Qfly at G5. Here this research reveals that the artificial larval diet significantly influenced quality control parameters during the Qfly domestication. There was a greater percentage of egg hatching rate, heavier pupal and adult weight (both for male and female), higher percentage of the fly emergence and fliers, and high male sex ratio in the gel diet fed G5 colony compared to carrot diet reared Qfly colony at G5. In addition, similar effects were observed on mating performance and survival under stress. As previously discussed in section 6.4.2, the number of putative bacteria (pathogenic and non-pathogenic) were rapidly altered in the Qfly microbiome from G0 to G5 colonies reared on both artificial diets. Thus, this finding is consistent with our observations in the microbiome of Qfly and may in fact be linked with fly development. Future studies to integrate behavioural/ trait-based assessments with the characterisation of microbiomes would help resolve this knowledge-gap. Further, inoculum of bacterial communities known to improve fitness may be a practical application of such research. So overall, this research indicated that artificial larval diets during domestication process influence not only the microbial communities but also strongly influence the physiological and behavioural changes in Qfly.

6.6 Implications for application

A comprehensive knowledge of the Qfly microbiome in the wild and during the domestication process across all developmental stages is the main outcome of my PhD thesis. This includes understanding of the diversity and the abundance of the gut microbiome present in the Qfly, which is strongly influenced by its host. The findings from this research contributed to a detailed understanding of how artificial larval diet effect on the gut microbiome of the Qfly reared from nature to artificial laboratory facilities. Possible manipulation of the selected beneficial bacteria and yeast with artificial diet, or direct application could improve the quality of mass reared Qfly as a part of SIT. Beyond the scope of Qfly SIT application, these findings are significant beyond their application in pest control and contribute to progress in the research of microbiome, host-insect interaction and ecology in fruit fly species, and more widely in other insects and animals.

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