Characterisation and isolation of gut bacterial communities from Queensland fruit fly (*Bactrocera tryoni*) across different environments to improve the sterile insect technique

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BSc, MPhil

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Dedication

In fond memory of an astute gentleman that was the late Moses G Woruba MBE. He was a dedicated and passionate agriculturalist who served selflessly and with distinction by the virtues of his faith in his God and his love for humanity, friendship and family. Thank you for being our father and friend.

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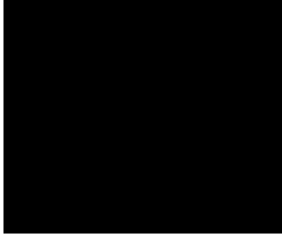
Finally, to Chief Kondom Agaundo, it is now tomorrow. And your son has come.

Salute!

Deane Nabre Woruba

Statement of Authentication

The work presented in this thesis is, to the best of my knowledge and belief, original except as acknowledged in the text. I hereby declare that I have not submitted this material, either in full or part, for a degree at this or any other institution.



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Preface

This thesis is an outcome of a project supported by the Australian Government's Cooperative Research Centre Programme through the Plant Biosecurity Cooperative Research Centre (PBCRC). The project was a collaboration between the NSW Department of Primary Industries (NSW DPI) and Western Sydney University. Apart from the field sampling, the laboratory research work was carried out at the Elizabeth Macarthur Research Institute (EMAI) of NSW DPI, and the Hawkesbury Institute for the Environment (HIE) of Western Sydney University.

The thesis is a series of chapters written in the format of independent manuscripts for submission to peer-reviewed journals. This means that some of the fundamental content, in particular in the introduction of every chapter, has some repetition so that each chapter can also be read as a stand-alone piece. Chapter 1 (Literature Review) and Chapter 2 have previously been submitted for publication to journals (Journal of Pest Science and BMC Microbiology, respectively) and have been returned with reviewer comments that have been included in the development of these two thesis chapters. Both chapters will be resubmitted to the journals for consideration. Chapters 3, 4 and 5 are also planned to be submitted to peer-reviewed journals after submission of this thesis. The end of Chapter 1 describes the overall scope and aims of the thesis. The research findings of the thesis have been synthesised in Chapter 6 together with recommendations for future research.

Thesis abstract

The digestive tracts of pest fruit flies (Tephritidae: Diptera) contain a diverse range of bacteria. Since the 1980's there has been increasing interest in the role of microbial symbionts in tephritid fruit fly performance, rising sharply in the past decade.

The sterile insect technique (SIT) is an environmentally safe insect pest management method that has been implemented against several tephritids and other pest insects of economic significance. The efficacy of SIT relies upon sterile males outcompeting field males to copulate with field females, which then fail to reproduce, resulting in suppression of pest populations. Mass production and sterilisation by irradiation can adversely affect several male fly traits. SIT has also been developed and deployed for the control of Queensland fruit fly, *Bactrocera tryoni* (Froggatt), Australia's most significant horticultural pest species.

Research has shown that bacteria play an important role in tephritid biology, and some bacterial isolates can improve performance traits, including mating competitiveness. However, little is known about the application of symbiotic bacteria in enhancing tephritid performance in SIT operational programs, and this is particularly the case for *B. tryoni*.

Symbiotic bacteria supplied to mass-reared fruit flies may help overcome some of these issues. However, the effects of tephritid ontogeny, sex, diet and irradiation on their microbiota are not well known.

The aim of this PhD was to establish the diversity and abundance of bacterial symbionts in the gut of *B. tryoni* collected from different laboratory and field environments. In the first experimental chapter (Chapter 2) I used next-generation sequencing to characterise the bacterial community composition and structure within *B. tryoni* by generating 16S rRNA gene amplicon libraries derived from the dissected guts of 58 individual teneral and mature, female and male, irradiated (sterile) and unirradiated (fertile) adult flies reared on artificial larval diets in a laboratory or mass-rearing environment, and fed either a full adult diet (i.e. sugar and yeast hydrolysate) or a sugar only adult diet. Gut bacteria in teneral flies were less abundant and less diverse than in mature adults and impacted by colony origin. In contrast, mature adult flies had increased abundances for some gut bacteria, indicative of either endogenous proliferation or acquisition of these bacteria from the adult diet and environment.

This suggested that bacterial populations in fruit flies experience significant bottlenecks during metamorphosis and are re-established in the adult development. Therefore, the time prior to sexual maturity may be ideal to target for probiotic manipulation of fly microbiota to increase adult fly performance in SIT programmes. Operational taxonomic units (OTUs), belonging to the families Enterobacteriaceae (8 OTUs) and Acetobacteraceae (1 OTU) were most prevalent. Enterobacteriaceae dominated laboratory-reared tenerals from a colony fed a carrot-based larval diet, while Acetobacteraceae dominated mass-reared tenerals from a production facility colony fed a lucerne chaff based larval diet. As adult flies matured, Enterobacteriaceae became dominant irrespective of larval origin. The inclusion of yeast in the adult diet strengthened this shift away from Acetobacteraceae towards Enterobacteriaceae. Interestingly, irradiation increased 16S rRNA gene sequence read volume. Therefore, irradiation augmented bacterial abundance in mature flies. This implies that either some gut bacteria were compensating for damage caused by irradiation, or irradiated flies had lost their ability to regulate their bacterial load.

In Chapter 3, I investigated the gut bacteria of field-collected *B. tryoni* of native and invasive populations from across tropical, sub-tropical and temperate Australia and New Caledonia using next-generation 16S rRNA gene amplicon sequencing. There were significant differences in bacterial communities between sexes with the bacterial community composition in males being more diverse. Gut bacterial communities in females more similar across habitats. Across all field populations, Enterobacteriaceae was the most dominant bacterial family. The endosymbiont *Wolbachia* was detected in male gut samples collected from tropical rainforest in the Atherton Tablelands.

In Chapter 4, I compared the gut bacterial communities of *B. tryoni* of field-collected individuals and individuals reared in controlled environments in Australia and New Caledonia. This was to identify core bacteria of field collected flies that either were lacking or variable in abundance in flies reared in controlled environments. These bacterial taxa could be potential bacterial candidates to use as probiotics in improving mass-reared irradiated flies for SIT. At the level of OTUs, the field flies were more diverse than flies from controlled environments. However, at the bacterial family level, the flies had very similar bacterial communities, indicative of substitution of some bacterial taxa for related taxa across sampling locations. Furthermore, we found that the laboratory flies contained the same bacterial genera as the field flies but at different abundance. It was concluded that the

challenge to improve the microbiome for increased performance of sterile *B. tryoni* in SIT will not necessarily require the supplementation of bacteria but the facilitation of existing microbiota so that released sterile flies have a similar bacterial abundance to that of field flies.

In Chapter 5, I have isolated and cultured gut bacteria from 20 *B. tryoni* individuals across three regions (Cairns, Brisbane and Sydney), and from one laboratory population. These bacterial isolates were then characterised using near full-length Sanger sequencing of the 16S rRNA gene. The flies from the field had many more different bacteria than flies reared in the controlled environment. The most common bacteria isolated was *Citrobacter* with *Enterobacter, Klebsiella*, *Providencia* and *Kluyvera* were also common.

Overall my research contributes to the wider research effort on the microbiota of tephritid pest fruit flies. Recent advances in sequencing technology have enabled more insights into the diversity and dynamics gut bacterial communities of insects and the roles they play in insect development. Gut bacteria have been demonstrated to improve the performance of tephritid fruit flies and thus are a promising target in improving the sterile insect technique used in tephritid fruit fly management.

Chapter 1: Literature Review - Gut bacteria of tephritid fruit fly pests of relevance to the sterile insect technique

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Abstract

The digestive tracts of pest fruit flies (Tephritidae: Diptera) contain a diverse range of bacteria. Since the 1980's there has been a steadily increasing interest in the role of microbial symbionts in tephritid fitness and performance, rising sharply in the past decade. The sterile insect technique (SIT) is an environmentally safe insect pest management method that has been implemented against several tephritids and other pest insects of economic significance. The efficacy of SIT relies upon sterile males outcompeting field males to successfully copulate with field females, which then fail to reproduce, resulting in suppression of pest populations. Mass production and sterilisation by gamma irradiation can adversely affect several male fly traits. Research has shown that bacteria play an important role in tephritid biology, and some bacterial isolates can improve performance traits, including mating competitiveness of sterile tephritids. However, little is known about the application of symbiotic bacteria in enhancing tephritid fitness in SIT operational programs. Here, we review the current knowledge about symbiotic gut bacteria of tephritids and their potential as probiotic supplements in SIT programs. Further, we discuss the different effects of the same bacterial species on fly performance. Understanding the diversity, biology and ecology of these bacteria is crucial in the identification and utilisation of candidates for use as probiotics to increase the effectiveness of SIT programs. The potential for use of the Queensland fruit fly, *Bactrocera tryoni* as a model for studying host/microbiota interactions of tephritids is also discussed.

1.1: Introduction

Fruit flies of the family Tephritidae (Diptera) contain approximately 5,000 species across 500 genera (Norrbom 2010), of which approximately 70 species are considered agricultural pests (White and Elson-Harris 1992). The major tephritid fruit fly pest genera include *Anastrepha, Bactrocera, Ceratitis, Dacus* and *Rhagoletis* (White and Elson-Harris 1992).

With the global push for environmentally-friendly pest control measures (World Trade Organization 2016), the current control measures are focused on environmentally soft, sustainable and targeted approaches (Dominiak and Ekman 2013). Several management tools are available for tephritid fruit fly pests including surveillance (trapping), protein bait spraying, male annihilation technique, biological control agents and the sterile insect technique (SIT) (Jessup et al. 2007; Vargas et al. 2008). SIT is recognised as a highly effective strategy against tephritid outbreaks in non-endemic areas (Meats et al. 2003; Raphael et al. 2014), and has been successful in managing tephritid pest populations when combined with other approaches in area-wide integrated pest management (AW-IPM) programs (Jang et al. 2008; Kakinohana et al. 1993; Kuba et al. 1993; Manrakhan et al. 2011).

SIT involves the release of large numbers of reproductively sterile individuals of a target insect pest species into a field population (Knipling 1955). The success of SIT relies upon the sterile males locating and successfully mating with the field females resulting in embryonic mortality and suppression of the pest population if sterile males outcompete field males in mating with field females.

Tephritid fitness and performance are therefore crucial to the efficacy of SIT, and these factors are demonstrably affected by both mass-rearing and gamma irradiation (Balock et al. 1963; Collins et al. 2008; Follett and Armstrong 2004). Sexual competitiveness is clearly weaker in mass-reared sterile flies (Lance et al. 2000), with an associated loss of genetic diversity occurring during domestication (Gilchrist et al. 2012) as well as a loss of microbial diversity (Morrow et al. 2015b). Irradiation is believed to perturb the microbiome of tephritids, resulting in a reduction in fly fitness (Ben Ami et al. 2010; Lauzon and Potter 2012). There is now a growing body of research on the role of microbial symbionts in tephritid performance (Augustinos et al. 2015; Behar et al. 2009; Ben-Yosef et al. 2010;

Drew and Lloyd 1987; Fitt and O'Brien 1985; Naaz et al. 2016), and the prospect of using beneficial bacteria to improve the attractiveness and mating success of mass-reared tephritids (Ben Ami et al. 2010; Gavriel et al. 2010; Hamden et al. 2013; Liu et al. 2016a; Niyazi et al. 2004; Sacchetti et al. 2014; Yuval et al. 2010). This review focuses on the role of bacterial gut symbionts and their potential value in the improving the mass-reared sterile male tephritid fruit flies used in SIT.

1.2: Microbial diversity in tephritid fruit flies

Insects are associated with a wide range of symbiotic bacteria (Dillon and Dillon 2004). However, the high incidence and prevalence of associations between insects and bacteria have been underestimated (Weinert et al. 2007). This is likely due to limitations in sampling efforts and the enormous diversity of insects. Yet, for some insect species, symbiotic bacteria are vital for survival. One example is the well-studied obligate symbiotic relationship between aphids and *Buchnera* bacteria that are housed inside special host tissues (called bacteriome) and cannot be cultured outside the aphid host. Aphids rely on the presence of *Buchnera*, and when treated with antibiotics, develop slowly and are unable to reproduce (Douglas 1992). Similarly, the symbiotic relationship between some groups of ant species and bacteria has been identified as one of the contributing factors to their evolution as herbivores, particularly through facilitation of host plant use and colonisation of new environments (Russell et al. 2009).

Tephritids were amongst the first insects studied for their symbiotic bacterial association, with microbial symbiont research carried out on the olive fruit fly, *Bactrocera oleae* (Rossi) in the early 20th century (Petri 1910). Despite notable research on bacteria isolated from 37 tephritid species by Stammer (1929), it was not until the 1970s when there was a resurgence in research into bacteria-tephritid associations (Boush et al. 1972; Dean and Chapman 1973; Hagen 1966). Since the 1980s, there has been an increased effort to investigate bacteria-tephritid interactions, mostly in economically-significant fruit-feeding tephritid pests including the Mexican fruit fly *Anastrepha ludens* (Loew), the Mediterranean fruit fly *Ceratitis capitata* (Wiedemann), the oriental fruit fly *Bactrocera dorsalis* (Hendel), the olive

fruit fly *B. oleae*, the peach fruit fly *Bactrocera zonata* (Saunders) and the Queensland fruit fly *Bactrocera tryoni* (Froggatt) (see Table 1.1).

Research has largely focused on bacteria in the digestive system of tephritids, and these bacteria predominantly belong to the class Gammaproteobacteria (Table 1.1). However, some tephritid species are also associated with other bacteria such as the intracellular, maternally inherited *Wolbachia* endosymbionts (Alphaproteobacteria), which inhabit the reproductive tissues and profoundly influence host reproductive biology (Riegler and Stauffer 2002; Werren 1997). Reproductive manipulation by *Wolbachia* is being investigated as a biological control agent in the incompatible insect technique (Riegler and Stauffer 2002; Zabalou et al. 2004) that could also be implemented as an auxiliary method to SIT, rendering irradiation unnecessary (Zabalou et al. 2004). In addition, *Wolbachia* and other endosymbiotic bacteria can impact host immune function in many ways such as resisting infection (Eleftherianos et al. 2013). Future research will need to investigate how maternally inherited endosymbionts, including *Wolbachia*, interact with or impact the gut microbiota of fruit flies.

Recent research has also demonstrated the importance of yeasts (Deutscher et al. 2016). However, the overall knowledge about the role and impact of fungi other than as a component in artificial larval and adult diets (Pérez-Staples et al. 2009; Reynolds et al. 2014; Taylor et al. 2013a; Weldon et al. 2008) is still very limited.

1.3: The tephritid fruit fly gut and bacterial occupancy

As in almost all insects, the tephritid gut system is divided into three primary regions, which are more distinct in adults: foregut, midgut (or ventriculus), and hindgut (Chapman et al. 2013; Drew et al. 1983). Inside the insect, most of the gut bacteria are found in the midgut lumen, between the gut epithelial tissues and the peritrophic membrane, a thin highly permeable membrane that contains the food particles and is bathed by the midgut juices containing the digestive enzymes and microbial symbionts (Mazzon et al. 2008). Acidity is an important factor impacting bacterial occupancy in insect guts (Dillon and Dillon 2004; Engel and Moran 2013), and compared to other insects, the tephritid midgut is relatively acidic with an average pH of 3.4 (Terra and Ferreira 1994). Therefore, tephritid gut bacteria can probably tolerate and function in low pH environments. Another digestive tract region of

interest is the oesophageal bulb in the foregut. Its content with bacteria has been investigated in the olive fruit fly *B. oleae* (Hagen 1966; Marchini et al. 2002; Savio et al. 2012) where these bacteria may contribute to the external digestion in flies (Sacchetti et al. 2014).

1.4: Functions of symbiotic bacteria in tephritid fruit fly pests throughout their development

The fruit-feeding tephritid pest species are either polyphagous, oligophagous, or monophagous (Fletcher 1987). The larval diet of fruit-feeding tephritids is bound to the carbohydrate-rich and protein-poor host fruit into which eggs are oviposited, and therefore it is expected that bacteria and yeasts in the digestive system of larvae, and introduced to fruits by adult flies compensate for this imbalance (Ben-Yosef et al. 2014; Deutscher et al. 2016). The adult tephritid diet also consists of carbohydrate-rich fruit juices, honeydew, nectar, fruit and plant exudates. Protein sources for adult flies include microorganisms found on host plant leaf surfaces (Drew and Yuval 2000) and protein from other sources such as bird faeces (Christenson and Foote 1960; Drew and Yuval 2000).

Due to the varied diets and requirements of larvae and adults of tephritid species, the bacterial composition of tephritids is likely to vary throughout their development. The transition through larval stages involves several moults and during pupation a complete remodelling of the gut; thus substantial turnover and possibly depletion of the gut bacteria occurs throughout development (Engel and Moran 2013). In *C. capitata*, gut bacteria are abundant in larvae, pupae and adults, but with a significantly higher bacterial load detected in 30-day old adults compared to the other developmental stages including one day old adults (Aharon et al. 2013). Similarly, mature *B. tryoni* adults have a much higher diversity and abundance of bacteria compared to recently eclosed (teneral) adults (Woruba et al, submitted 2018 – Chapter 2). Feeding behaviour may explain the abundance of bacteria in mature flies - tephritid fruit flies regurgitate during feeding, as observed on *B. tryoni* (Drew et al. 1983; Drew and Lloyd 1987) and *Anastrepha* species (Aluja et al. 1989). This behaviour of regurgitating is due to the feeding behaviour and the type of mouthparts which tephritid fruit flies possess; it favours the uptake of bacteria and fluids over larger particle food sources which may include pollen and fungal spores (Vijaysegaran et al. 1997). Therefore, the

microbial gut community in the adult tephritid gut may be distinct from the larval microbial gut community, also as the adult fly feeds on more varied diets.

Some bacteria are known to successfully persist throughout all life stages of tephritids. For example, gut bacterial species *Pantoea agglomerans* and *Klebsiella pneumoniae* were successfully cultured from all life stages of *C. capitata* with transmission through 21 successive generations (Lauzon et al. 2009). If a bacterial symbiont is present in all life stages of fly populations and across generations, then it can be expected that it may have adapted to its host. This might also imply that these bacterial symbionts play versatile roles during host development. However, the genetic diversity within these individual bacterial species has not yet been studied and it is therefore not understood how such bacterial strain diversity is shared within and across host populations geographically throughout development. Understanding the bacteria that are transmitted through the stages, and those missing in mass-reared tephritids could ultimately assist in identifying a single, or consortium of bacteria that could be fed to both the larvae and adult stages in a mass-rearing facility and is an area which warrants further work.

The most important role of symbiotic bacteria in tephritids is the digestion of complex compounds and provision of nutrients for the host (Bateman 1972). Gut bacteria provide their host insect with usable forms of nutrients, either by synthesising the nutrients or breaking-down the nutrients into more host-accessible forms (Ben-Yosef et al. 2010; Frago et al. 2012). Symbiotic bacteria are also reported to enable their fruit fly host to overcome plant defensive compounds (Ben-Yosef et al. 2015), confer insecticide resistance (Cheng et al. 2017), promote host fitness (Ben-Yosef et al. 2010), reduce larval development time (Augustinos et al. 2015), increased female fecundity (Sacchetti et al. 2014), enhance mating success (Ben Ami et al. 2010), and increase longevity (Behar et al. 2008b).

1.5: Protein metabolism

Proteins are a key source of nutrition for insects. Despite being a common element occupying an estimated 78% of the Earth's atmosphere, nitrogen as the key element in proteins, is paradoxically limited for herbivores (Dixon and Kahn 2004; Galloway et al. 2004). Amino acids, the constituents of proteins, are nitrogen-containing compounds that are required for

insect growth and reproduction (Fagan et al. 2002; Nardi et al. 2002). Proteins and amino acids are important in the physiology and behaviour of tephritid fruit flies (Fletcher 1987; Yuval et al. 2007). Fruit as the primary diet of fruit-feeding tephritids has a high carbon to nitrogen (C:N) ratio, due to a high carbohydrate and low protein content. Yet tephritids require protein for development and reproduction, including sexual maturation (Meats and Leighton 2004; Perez-Staples et al. 2007; Perez-Staples et al. 2008) and mating competitiveness (Blay and Yuval 1996; Kaspi and Yuval 2000; Yuval et al. 2007). Symbiotic bacteria found in the alimentary tract play an important role in providing the much needed protein or amino acids to overcome this imbalance in the primary diet (see review by Fletcher (1987)).

Gut bacteria may provide proteins to tephritids by fixing atmospheric nitrogen into forms usable by the host insect, or by converting or assisting the conversion of nitrogenous compounds in the alimentary tract, into forms that the host insect can then utilize. Termites, for example, harbour functional diazotrophic (nitrogen-fixing) gut microbiota (Benemann 1973). Likewise, diazotrophic bacteria of the family Enterobacteriaceae, particularly of the genera *Klebsiella* and *Enterobacter* which are localised in the midgut, promote nitrification in *B. tryoni* (Murphy et al. 1988; Murphy et al. 1994) and *C. capitata* (Behar et al. 2009).

In larval *Bactrocera jarvisi*, the gut bacterium *Serratia liquefaciens*, secretes proteases that contribute to protein metabolism in the gut (Fitt and O'Brien 1985). This bacterium has also been isolated from adult specimens of *B. tryoni*, *B. jarvisi*, *Bactrocera neohumeralis* (Hardy), *Bactrocera cacuminata* (Hering) (Fitt and O'Brien 1985; Lloyd et al. 1986) and *A. ludens* (Martinez et al. 1994), and it is possible that it plays a comparable role in these species. Similarly, the symbiotic bacterium *Pseudomonas melophthora* provides amino acids to its host, the apple maggot fly, *Rhagoletis pomonella* (Walsh) (Miyazaki et al. 1968).

Due to a limited amount of protein in fruits, the foraging habit of adult tephritids is geared towards protein-rich diets, and gut bacteria may allow the extraction of proteins from these sources (Ben-Yosef et al. 2014; Lauzon et al. 2009).

1.6: Carbohydrate metabolism

Adult tephritid fruit flies are considered to be opportunistic feeders and, besides protein sources, consume high carbohydrate sources including plant exudates, hemipteran honeydew, and nectar (Bateman 1972; Drew and Yuval 2000; Fletcher 1987). Some tephritids are known to have limitations converting some forms of carbohydrates. For example, *C. capitata* cannot readily process polysaccharides (Silva et al. 2006). Some bacteria are efficient converters of polysaccharides and may play a role in the larval development of apple maggot *R. pomonella* (Rossiter et al. 1982). In this species, the gut bacterial species *Klebsiella oxytoca* and *Enterobacter cloacae* were responsible for the degradation of polysaccharides, cellulose and pectin into forms which *R. pomonella* larvae can utilise (Rossiter et al. 1982). More recent work on *C. capitata* demonstrated the presence of pectinolytic Enterobacteriaceae, which are key agents in fruit rot (Behar et al. 2008a). These bacteria break down pectin in the host fruit and convert the compounds into forms that the larvae can utilise for development (Behar et al. 2008a). Many of these carbohydrate-metabolizing gut Enterobacteriaceae are also found in adult tephritids and may perform the same function as they do in the larvae (Behar et al. 2009).

1.7: Reproductive performance

The success of SIT depends upon the ability of released sterile males to compete with resident males for females in the field, and achieve a successful copulation. However, sterile male tephritids are known to be less competitive and successful than field males (Weldon et al. 2010). It is possible that gut bacteria may influence mating performance and reproductive development (Engel and Moran 2013), and therefore manipulation of the microbiome in sterile flies may overcome such negative effects.

Fecundity is a fitness indicator for female tephritids (Krainacker et al. 1989). Bacteria have been credited for improving egg production in *B. oleae* deprived of essential amino acids (Ben-Yosef et al. 2010; Sacchetti et al. 2014). However, laboratory reared female populations of *B. tryoni* provided with *K. oxytoca* and *K. pneumoniae* (obtained from non-tephritid sources) displayed no reproductive benefits (Meats et al. 2009). Such differences in observed effects is most likely due to the different bacterial species and strains tested in different tephritid hosts.

Bacteria are known to influence the oviposition choice of gravid female tephritids and therefore may play a role in host location (Díaz-Fleischer et al. 2000). Similarly, in other dipterans such as stable flies, *Stomoxys calcitrans* (Diptera: Muscidae), the host bacterial community greatly influences oviposition choice (Romero et al. 2006), as their larvae do not develop in the absence of bacteria (Lysyk et al. 1999; Schmidtmann and Martin 1992; Watson et al. 1993). It is likely that bacteria might have similar effects on tephritids, thus ensuring the emerging offspring have the best chance of survival (Díaz-Fleischer et al. 2000).

Mating behaviour of tephritids is also affected by bacteria, for example in *C. capitata*, laboratory-reared males provided with a diet that included *P. agglomerans* and *K. pneumoniae*, demonstrated a significant mating advantage over those without the bacteria (Niyazi et al. 2004). Sterile male *C. capitata* fed the bacterium *K. oxytoca* were also observed to initiate mating sooner than sterile males that were not exposed to this bacterium (Ben Ami et al. 2010). Male *C. capitata* fed *P. agglomerans* and *K. pneumoniae* together with the standard diet, have higher mating success than males fed the standard diet alone (Ben-Yosef et al. 2008; Niyazi et al. 2004). Gut bacteria may emit volatile cues that play a vital role in informing the female of the male's health and nutritional status and thus influence mate choice (Behar et al. 2009).

1.8: Attraction and communication

The attraction of tephritids to bacteria (and consequently also between fruit flies) can be classified under two broad, but intertwined categories; direct attraction to bacterial volatiles originating from catabolism of substrates, and interactions involving bacterial volatiles that affect adult behaviour. Foraging fruit flies have long been known for their attraction to volatiles originating from the break-down of protein substrates (Drew and Lloyd 1991; Lauzon 2003). The most commonly produced volatile that is attractive to tephritids is ammonia (Behar et al. 2009), a volatile known to be produced by *P. agglomerans* isolated from *Anastrepha suspensa* (Epsky et al. 1998). This association is thought to be an evolutionary adaptation for locating protein sources in the field (Lauzon 2003; Robacker et al. 1998).

Furthermore, volatiles of bacterial origin may represent more than just a guide to locate food sources for adult flies and serve as semiochemicals mediating more complex behaviours affecting fly fitness. Female *C. capitata*, are known for aggregated oviposition, a trait that is dependent on olfactory cues from bacterial origins (Díaz-Fleischer et al. 2000). Aggregated oviposition might benefit *C. capitata* larvae as their crowded development inhibits growth of pathogenic agents that can proliferate in rotting hosts (Rohlfs and Hoffmeister 2003). Conversely, overcrowding and unsynchronised egg hatching results in competition amongst larvae (Behar et al. 2009), which can later determine the size and fitness of the emerged fruit fly. Therefore, bacteria that are deposited with the egg may also produce volatile cues that provide arriving gravid females with information on the density and age of eggs already laid (Behar et al. 2009). Such information can assist the females to make optimal reproductive decisions.

1.9: Longevity

Bacteria have been studied for their role in providing protein to tephritids. Much of the work on bacteria and longevity was focused on *C. capitata* (Behar et al. 2009; Behar et al. 2008a; Ben-Yosef et al. 2008). When antibiotics were supplied to adult *C. capitata*, the flies had a longer life span (Ben-Yosef et al. 2008). More recently, *B. oleae* adults fed a diet enriched with *Pseudomonas putida*, considered a tephritid symbiont with beneficial effects, experienced shorter lifespans (Sacchetti et al. 2014). Some *Pseudomonas* spp. are known as insect pathogens (Ben Ami et al. 2010) and therefore, it is possible that *P. putida* could be pathogenic for *B. oleae*, thus impacting longevity. Although there is a growing body of work on the effects of protein supply on longevity (Fanson et al. 2009; Pérez-Staples et al. 2009; Perez-Staples et al. 2008; Prabhu et al. 2008; Yuval et al. 2007), the role of bacteria in this context require further research.

1.10: Bacterial strains and isolates

Bacterial species within a genus can differ in biological properties, such as serologic or biochemical reactions, phage or bacteriocin sensitivity, pathogenicity, or other characteristics

(Baron 1996). In addition, strains can have varying effects on host insects. For example, *P. agglomerans*, *K. oxytoca* and *Enterobacter cloacae*, as part of a bacterial consortium, were classified as "attractive" bacteria as they made host trees more attractive to *B. tryoni* and *B. neohumeralis* (Drew and Lloyd 1987). However, these bacteria were also observed to have different effects on other tephritids. Another isolate of *E. cloacae* was found to be attractive to *Bactrocera zonata* (Reddy et al. 2014). Yet another strain of *E. cloacae*, isolated from *Anastrepha fraterculus*, is known to be pathogenic to the citrus pest *Phyllocnistis citrella* Stainton (Lepidoptera: Gracillariidae) and has been evaluated as a biological control agent for the management of this pest (Campos et al. 2007). *Pantoea agglomerans* was observed to increase male mating efficiency (Niyazi et al. 2004) and *K. oxytoca* increased longevity in *C. capitata* (Behar et al. 2008b). It is because of these diverse and idiosyncratic effects in host species that the isolation and selection of bacterial strains should occur from the target fly species.

1.11: Case study: Beneficial bacteria in Queensland fruit fly

1.11.1: The Queensland fruit fly, *Bactrocera tryoni*

With the exception of being invasive in New Caledonia, French Polynesia, Pitcairn Islands and Cook Islands (Secretariat of the Pacific Community 2012), the distribution of the Queensland fruit fly, *B. tryoni* is confined to Australia where it is native. *Bactrocera tryoni* originated in the northern and eastern tropical and subtropical rainforests of eastern Australia, and now occurs throughout Australia including Queensland, New South Wales, Victoria and the Northern Territory (Clarke et al. 2011; Dominiak and Daniels 2012; Drew 1989; Vargas et al. 2015), with the exception of South Australia and Tasmania, which are recognised as fruit fly free, while Western Australia has *C. capitata* (Dominiak and Daniels 2012; Raphael et al. 2014). The successful spread of *B. tryoni* in Australia can be credited largely to its polyphagous nature, ability to adapt to different climatic conditions and the expansion of horticultural production areas during the 19th century (Meats 1981). It is thought that much of this movement has occurred through human assisted transport (Dominiak and Coombes 2009).

The fact that *B. tryoni* is distributed from tropical to temperate Australia (Dominiak and Daniels 2012) and has a large host range from native wild fruits to horticultural crops (Hancock et al. 2000) provide an ideal opportunity to sample across wide climatic and habitat gradients, including both native and invasive ranges, to understand the varying factors that affect the microbial gut communities of a significant pest tephritid. These make *B. tryoni* an ideal model system for investigating the role of bacterial gut symbionts in fruit fly biology, ecology and pest management.

1.11.2: Identification of *Bactrocera tryoni* gut bacteria

Early research into the gut bacteria of *B. tryoni* focussed on the isolation of culturable bacteria that could be utilised in *B. tryoni* management (Drew et al. 1983; Drew and Lloyd 1987; Lloyd et al. 1986). However, understanding the complete microbial community (including yet unculturable microbes) could provide an insight into the key bacteria necessary for the development and survival of *B. tryoni*.

Until recently, gut bacteria of *B. tryoni* were isolated using culture dependent methods and characterised using mostly morphological and physiological traits (Drew et al. 1983; Drew and Lloyd 1987; Lloyd et al. 1986) (Table 1.1). Furthermore, the detection of bacterial species was limited by the choice of isolation media. Almost all of the gut bacteria of *B. tryoni* have been identified as Enterobacteriaceae. These studies have largely used biochemical tests and the analytical profile index (API) 20E, which are designed for the identification of Enterobacteriaceae (Holmes et al. 1978).

Sequencing of the bacterial 16S rRNA gene PCR products was also performed on bacteria after they were isolated by culturing on bacteriological culture media, peptone yeast extract agar (PYEA) and tryptone soya agar (TSA) (Thaochan et al. 2009), limiting the detection of bacterial species that are capable of aerobic growth on these media. In more recent work by Morrow et al. (2015b), 16S rRNA gene amplicon NGS was used for the characterisation of the entire bacterial diversity and community composition of six Australian tephritid species. As the first study to describe the *B. tryoni* microbiota using a non-culture dependent isolation technique it contrasted the microbial communities of different tephritid species with diverse host plant use and specialisation, and across field and laboratory populations. Overall, it

appeared that specialist species, and lab-adapted lines had a smaller bacterial community than generalist and field- collected flies. However, Morrow et al. (2015b) used a small number of samples, which were then pooled for analysis in order to maximise representation of diversity in a small sample size while keeping sequencing costs low. Furthermore, amplicon NGS is generally limited in its taxonomic resolution power, typically only allowing assignment to the family level because of short amplicon sizes. Ultimately, more extensive profiling and characterisation of the complete microbial community consisting of both culturable and non-culturable will provide an improved understanding of the bacterial diversity and composition required for the development and survival of *B. tryoni*, and mating competitiveness in the field.

1.11.3: Overview of *Bactrocera tryoni* symbiotic bacteria

The host effects of bacteria that have been identified from *B. tryoni* eggs, larvae, pupae or adults (Table 1.1) may be neutral, beneficial or pathogenic. Broadly, beneficial bacteria include those that are a direct source of nutrition, convert otherwise unavailable nutrients, aid digestion, support host development and behaviour, but may also include bacteria that enhance sterile adult male performance.

From the perspective of identifying and developing beneficial bacteria of *B. tryoni* to improve the success of SIT, two points should be considered. Given the bacterial diversity of insect gut ecosystems, it could be anticipated that the addition of a single bacterium may not alone increase tephritid performance (Ben-Yosef et al. 2010). Furthermore, while the microbiological and molecular characterization has focused on the level of bacterial families and genera in *B. tryoni* populations, it may be important to further characterize these bacteria at strain levels. It is for these reasons that it is important to not only study both the diversity of the bacterial community in terms of families and genera present through 16S rRNA gene amplicon NGS, but also the diversity at the species level.

Bacteria isolated from *B. tryoni* that are known to influence tephritid fruit fly behaviour have only been classified based on morphological and biochemical characteristics (API 20E but not based on sequence information) as *E. cloacae*, *K. oxytoca*, *P. agglomerans*, *Pantoea fluorescens* and *Serratia marcescens* (Drew et al. 1983; Drew and Lloyd 1987; Howie 2007;

Meats et al. 2009) (for a full list and the reported effects of bacteria of *B. tryoni* see Appendix 1.1). Other isolated bacterial species that are attractive to *B. tryoni* which have not been tested for fitness and performance effects were identified (without molecular identification approaches) as *Stenotrophomonas maltophilia* and *Citrobacter freundii*. *Stentropomonas maltophilia* was found to be attractive to *B. zonata* (Reddy et al. 2014) and *C. freundii* was found to be a good attractant for *B. dorsalis* (Wang et al. 2014b). Due to the attraction of the two related *Bactrocera* species, it is possible that *S. maltophilia* and *C. freundii* might also be attractive to *B. tryoni*. Understanding the functional significance of these and other attractive bacteria will enable their use in Queensland fruit fly pest management system as a lure of *B. tryoni*.

Table 1.1: Bacteria of *Bactrocera tryoni* isolated by culture-dependent methods. With the exception of the identification performed by Thaochan et al. (2009) identification of bacterial isolates was based on morphological and biochemical characteristics only. The National Centre for Biotechnology Information taxonomy database was used for bacterial classification and synonyms (NCBI 2011)

Lineage	Species	Synonyms	Bactrocera tryoni host	Source of isolation	Literature
Bacteroidetes/Chlorobi group; Bacteroidetes; Flavobacteria; Flavobacteriales; Flavobacteriaceae	*Flavobacterium* sp.	NA	*Psidium guajava, Prunus persica, Pyrus communis*	Adult head and adult abdomen	Fitt and O'Brien (1985)
Proteobacteria; Gammaproteobacteria; Aeromonadales, Enterobacteriales, Enterobacteriaceae	*Enterobacter cloacae*	*Cloaca cloacae, Bacterium cloacae, Bacillus cloacae, Aerobacter cloacae*	*P. guajava*	Oesophageal bulb, crop and midgut	Drew and Lloyd (1987)
			P. guajava, P. persica, P. communis Lab	Adult head	Fitt and O'Brien (1985)
			P. guajava, P. persica	Crop, midgut, oesophageal bulb and faeces	Lloyd et al. (1986)
			Wild (unspecified)	Crop and midgut	Murphy et al. (1994)
			Annona reticulata, P. guajava, Eriobotrya japonica	Crop and midgut	Thaochan et al. (2009)
Proteobacteria; Gammaproteobacteria; Aeromonadales, Enterobacteriales, Enterobacteriaceae	*Enterobacter sakazakii*	yellow-pigmented *Enterobacter cloacae, Enterobacter sakazakii, Cronobacter sakazakii* subsp. *sakazakii*	*A. reticulata, P. guajava, E. japonica*	Crop and midgut	Thaochan et al. (2009)
			P. guajava, P. persica	Crop, midgut, oesophageal bulb and faeces	Lloyd et al. (1986)
Proteobacteria; Gammaproteobacteria;	*Kluyvera intermedia*	*Kluyvera cochleae, Enterobacter intrermedium,*	*A. reticulata, P. guajava,*	Midgut	Thaochan et al. (2009)

Aeromonadales, Enterobacteriales, Enterobacteriaceae		*Enterobacter intermedius, Enterobacter intermedium*	*E. japonica*		
Proteobacteria; Gammaproteobacteria; Aeromonadales, Enterobacteriales, Enterobacteriaceae	*Lelliottia amnigena*	*Enterobacter amnigenus*	*A. reticulata, P. guajava, E. japonica*	Crop and midgut	Thaochan et al. (2009)
Proteobacteria; Gammaproteobacteria; Aeromonadales, Enterobacteriales, Enterobacteriaceae	*Pantoea agglomerans*	*Pseudomonas herbicola, Pantoea herbicola, Erwinia milletiae, Erwinia herbicola, Enterobacter agglomerans, Bacterium herbicola*	*P. guajava, P. persica*	Crop, midgut, oesophageal bulb and faeces	Lloyd et al. (1986)
Proteobacteria; Gammaproteobacteria; Aeromonadales; Aeromonadaceae	*Aeromonas hydrophilia*	*Pseudomonas hydrophila, Proteus ichthyosmius, Proteus hydrophilus, Bacterium hydrophilum, Bacillus hydrophilus fuscus, Aeromonas liquefaciens, Aeromonas dourgesi*	*P. guajava, P. persica, P. communis*	Adult head	Fitt and O'Brien (1985)
Proteobacteria; Gammaproteobacteria; Aeromonadales' Enterobacteriales; Enterobacteriaceae;	*Citrobacter freundii*	The Bethesda group of bacteria, The Ballerup group of bacteria, *Salmonella hormaechei, Salmonella ballerup, Escherichia freundii, Citrobacter ballerupensis, Bacterium freundii*	*P. guajava, P. persica*	Crop, midgut, oesophageal bulb and faeces	Lloyd et al. (1986)
			A. reticulata, P. guajava, Eriobotrya japonica	Crop and midgut	Thaochan et al. (2009)

Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae	*Hafnia alvei*	*Hafnia alvei* sensu stricto genomosp. *Enterobacter hafniae, Enterobacter alvei, Enterobacter aerogenes* subsp. *hafniae*	*A. reticulata, P. guajava, E. japonica*	Crop and midgut	Thaochan et al. (2009)
Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae	*Klebsiella oxytoca*	*Bacillus oxytocus perniciosus*	*P. guajava*	Oesophageal bulb, crop and midgut	Drew and Lloyd (1987)
			P. guajava, P. persica	Crop, midgut, oesophageal bulb and faeces	Lloyd et al. (1986)
			Wild (unspecified)	Crop and midgut	Murphy et al. (1994)
			A. reticulata, P. guajava, E. japonica	Crop and midgut	Thaochan et al. (2009)
Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae	*Klebsiella pneumoniae* spp. *ozaenae*	*Klebsiella pneumoniae ozaenae, Klebsiella pneumoniae (*subsp. *ozaenae), Klebsiella ozaenae, Bacterium ozaenae, Bacillus ozaenae, Bacillus mucosus azaenae*	*P. guajava*	Oesophageal bulb, crop and midgut	Drew and Lloyd (1987)
			A. reticulata, P. guajava, E. japonica	Crop and midgut	Thaochan et al. (2009)
Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae	*Klebsiella pneumoniae*	*Hyalococcus pneumonia, Bacterium pneumoniae crouposae, Bacillus pneumoniae*	Lab	Adult head and abdomen	Fitt and O'Brien (1985)
Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae	*Proteus mirabilis*	NA	*P. guajava, P. persica*	Crop, midgut, oesophageal bulb and faeces	Lloyd et al. (1986)

Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae	*Proteus vulgaris*	NA	*P. guajava, P. persica, P. communis* Lab	Adult abdomen	Fitt and O'Brien (1985)
			P. guajava, P. persica	Crop, midgut, oesophageal bulb and faeces	Lloyd et al. (1986)
Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae	*Raoultella ornithinolytica*	*Klebsiella ornithinolytica*	*A. reticulata, P. guajava, E. japonica*	Midgut	Thaochan et al. (2009)
Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae	*Raoultella terrigena*	*Klebsiella terrigena*	*A. reticulate, P. guajava, E. japonica*	Crop and midgut	Thaochan et al. (2009)
Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae	*Serratia liquefaciens*	*Aerobacter liquefaciens*	*P. guajava, P. persica, P. communis* Lab	Adult head and Adult abdomen Egg and pupae	Fitt and O'Brien (1985)
Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae	*Serratia marcescens*	*Aerobacter liquefaciens*	Lab	Abdomen	Fitt and O'Brien (1985)
			A. reticulata, P. guajava, E. japonica	Crop and midgut	Thaochan et al. (2009)
Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae	*Serratia odorifera*	NA	*A. reticulata, P. guajava, E. japonica*	Midgut	Thaochan et al. (2009)

Proteobacteria; Gammaproteobacteria; Pseudomonadales; Moraxellaceae;	*Acinetobacter calcoaceticus*	*Neisseria winogradskyi, Moraxella calcoacetica, Micrococcus calcoaceticus, Acinetobacter genomospecies 1, Acinetobacter genomosp. 1*	*P. persica* Lab	Egg Adult head	Fitt and O'Brien (1985)
Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae	*Pseudomonas fluorescens*	*Liquidomonas fluorescens, Bacterium fluorescens, Bacillus fluorescens liquefaciens, Bacillus fluorescens*	*Morus nigra*	Crop, stomach and faeces	Drew et al. (1983)
			Lab *P. guajava,* *P. persica,* *P. communis*	Adult head Pupae	Fitt and O'Brien (1985)
Proteobacteria; Gammaproteobacteria; Xanthomonadales; Xanthomonadaceae	*Stenotrophomonas maltophilia*	*Xanthomonas maltophilia, Xanthomonas maltiphilia, Stenotrophomonas africana, Pseudomonas maltophilia, Pseudomonas maltiphilia, Pseudomonas betle, Pseudomonas beteli*	*P. guajava,* *P. persica,* *P. communis*	Adult head	Fitt and O'Brien (1985)
Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudomonadaceae	*Pseudomonas oryzihabitans*	group Ve-2, *Flavimonas oryzihabitans*	*A. reticulata,* *P. guajava,* *E. japonica*	Crop	Thaochan et al. (2009))
Proteobacteria; Gammaproteobacteria; Enterobacteriales; Enterobacteriaceae	*Providencia rettgeri*	*Shigella rettgeri, Proteus rettgeri, Bacterium rettgeri*	*P. guajava*	Oesophageal bulb, crop and midgut	Drew and Lloyd (1987)
			P. guajava, *P. persica*	Crop, midgut, oesophageal bulb and faeces	Lloyd et al. (1986)
			A. reticulata, *P. guajava,* *E. japonica*	Midgut	Thaochan et al. (2009)

1.12: Potential use of bacteria to improve fruit fly sterile insect technique

The use of beneficial bacteria may improve performance of sterile male tephritid flies that have suffered from the effects of domestication, mass-rearing and/or sterilization via irradiation. Recent studies have provided an increased understanding of the abundance and diversity of bacteria residing within the gut of several tephritid pest species, as well as their potential roles and effects on several host traits. The intimate association of bacteria with their tephritid hosts led to the proposed concept of manipulating bacteria to improve the performance of sterile tephritids in SIT. Beneficial bacteria additives to larval diets of mass-reared flies was known to improve adult size and other morphometric traits of *C. capitata* and thus gave mass-reared males an advantage in mating tests (Hamden et al. 2013). Beneficial bacteria may also be utilised in improving mass production of sterile flies. For example, female *B. oleae* used in mass production increased their egg production when exposed to beneficial bacterial isolates (Sacchetti et al. 2014). The provision of a pre-release probiotic, to enhance sterile male mating success, may decrease the required number of sterile male flies and over flooding ratio, leading to a decrease in the cost of SIT programs.

Identification of the differences in gut microbiota of field, mass-reared and irradiated tephritids, is key in selecting candidate isolates that may be suitable as beneficial bacteria in SIT programs. The natural distribution of *B. tryoni* across wide vegetation and climatic gradients in Australia (including its original natural habitat) makes this tephritid pest species a useful model to dissect host-microbiota interactions. Selecting a particular bacterial strain, or a consortium of bacterial strains, is a challenge in developing probiotics for SIT programs. This is because limited resolution in the microbiological and molecular characterisation have sometimes resulted in the misidentification of bacterial species at the genus and species level, while the characterisation of actual strain diversity is still lacking. Not all strains of a bacterial species have the same physiological attributes within and across species, and thus may impact flies differently. The effects of bacterial symbionts, on target organisms, are often strain-specific (Foligné et al. 2013; Fuller 1991). The identification will require utilizing genotypic and phenotypic characterization of bacterial strains beyond the 16S rRNA gene characterisation, because these gene fragments are too conserved to differentiate bacterial intraspecific diversity.

Bacterial strains for use in improving in SIT may have a better chance of success if isolated from the target host. However, identification of bacterial strains that perform a desirable function in other species should not be discounted. The ideal bacterial candidates for use in SIT will be those that are adaptable to delivery in the existing systems. This may include incorporation into SIT mass-rearing diet systems, and pre-release supplementation. Adult tephritids in SIT programs are often held for several days post-eclosion when they are fed a pre-release diet, as used in *Anastrepha obliqua* and *A. ludens* (Gómez et al. 2013) and *B. tryoni* (Reynolds et al. 2014). This post-teneral period offers opportunities for interventions, such as the introduction of a probiotic. Probiotic candidate bacteria must also be suitable to their incorporated task, whether that involves incorporation into a diet, or the process of mass production, delivery (in what form and how) and shelf life longevity. Importantly, bacterial candidates for use as probiotics must also be safe to humans and the environment that they will come in contact. At the time of this review, the studied bacterial probiotic candidates have not yet been evaluated for their adaptability to existing tephritid mass-rearing SIT systems. Such concepts are prerequisites in determining the suitability of potential candidates.

Bacteria have the potential to improve tephritid SIT programs. The recent advances in molecular, microbiological and biochemical characterisation tools will assist with accurate identification of probiotic candidates. These tools will inform and drive research into insect physiology, ecology and behaviour, and also bacteria handling and management under existing systems so bacteria can be efficiently incorporated in tephritid SIT programs.

1.13: Thesis scope

This thesis aims to establish fundamental knowledge and fill knowledge gaps about gut bacteria of Queensland fruit fly (*B. tryoni*) with the perspective that these findings will be useful for the improvement of SIT against this tephritid pest. Specifically, the thesis intends to assist in the isolation and identification of key gut bacteria, to obtain an understanding of the dynamics of gut bacterial communities in this major tephritid pest and how the factors of pupal origin, adult development stage, irradiation, adult diet, habitats within and across

climatic regions, and native and invasive populations, affect gut bacterial communities of *B. tryoni*.

The thesis sets out to address the following themes:

- **i)** Investigate the abundance and diversity of gut bacteria in tenerals and mature adult *B. tryoni* in captivity
- **ii)** Investigate the diversity of gut bacteria between irradiated and unirradiated teneral and mature adult *B. tryoni* in captivity
- **iii)** Investigate the impact of larval rearing environments (diets) and adult diets on the gut bacteria of mature adult *B. tryoni* in captivity
- **iv)** Investigate the gut bacterial communities of field-collected populations of *B. tryoni* from native and invasive populations within and across climatic regions and habitats
- **v)** Compare the gut bacterial communities of captive populations of *B. tryoni* to the gut bacterial communities of field-collected populations to identify missing or deficient bacteria
- **vi)** Culture and identify isolates of Enterobacteriaceae from field collected individuals across climatic regions and habitats as candidates for probiotic development to fill in gaps that might be missing in microbiomes of captive populations

The knowledge gained from this thesis will be vital in identifying the beneficial bacteria of *B. tryoni*. Identified beneficial bacterial isolates can be utilised as candidates in developing tephritid probiotics.

Chapter 2: Diet and irradiation effects on the bacterial community composition and structure in the gut of domesticated teneral and mature Queensland fruit fly, *Bactrocera tryoni* (Diptera: Tephritidae)

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Abstract

Background

Mass-rearing, domestication and gamma irradiation of tephritid fruit flies used in sterile insect technique (SIT) programmes can negatively impact fly quality and performance. Symbiotic bacteria supplied as probiotics to mass-reared fruit flies may help overcome some of these issues. However, the effects of tephritid ontogeny, sex, diet and irradiation on their microbiota are not well known.

Results

We have used next-generation sequencing to characterise the bacterial community composition and structure within Queensland fruit fly, *Bactrocera tryoni* (Froggatt), by generating 16S rRNA gene amplicon libraries derived from the guts of 58 individual teneral and mature, female and male, sterile and fertile adult flies reared on artificial larval diets in a laboratory or mass-rearing environment, and fed either a full adult diet (i.e. sugar and yeast hydrolysate) or a sugar only adult diet. Overall, the amplicon sequence read volume in tenerals was low and smaller than in mature adult flies. Operational taxonomic units (OTUs), belonging to the families Enterobacteriaceae (8 OTUs) and Acetobacteraceae (1 OTU) were most prevalent. Enterobacteriaceae dominated laboratory-reared tenerals from a colony fed a carrot-based larval diet, while Acetobacteraceae dominated mass-reared tenerals from a production facility colony fed a lucerne chaff based larval diet. As adult flies matured, Enterobacteriaceae became dominant irrespective of larval origin. The inclusion of yeast in the adult diet strengthened this shift away from Acetobacteraceae towards Enterobacteriaceae. Interestingly, irradiation increased 16S rRNA gene sequence read volume.

Conclusions

Our findings suggest that bacterial populations in fruit flies experience significant bottlenecks during metamorphosis. Gut bacteria in teneral flies were less abundant and less diverse, and impacted by colony origin. In contrast, mature adult flies had selectively increased abundances for some gut bacteria, or acquired these bacteria from the adult diet and environment. Furthermore, irradiation augmented bacterial abundance in mature flies. This implies that either some gut bacteria were compensating for damage caused by irradiation or irradiated flies had lost their ability to regulate bacterial load. Our findings suggest that the adult stage prior to sexual maturity may be ideal to target for probiotic manipulation of fly microbiota to increase adult fly performance in SIT programmes.

2.1: Background

In the quest to understand the association between bacteria and their insect hosts, one of the first associations studied was a tephritid fruit fly pest, the olive fly, *Bactrocera oleae* (Rossi), (Diptera: Tephritidae) and its gut microbiota (Petri 1910). Over the past decade, there has been increasing interest in symbiosis of bacteria with tephritids, particularly the potential manipulation of this association for pest management (Lauzon 2003; Yuval et al. 2010). One such prospect involves the use, or manipulation of microbial symbionts as part of the sterile insect technique (SIT) (Yuval et al. 2010).

SIT involves mass rearing (leading to domestication (Gilchrist et al. 2012)) and the release of irradiated (sterile) individuals of the target pest species into wild pest populations in the field (Knipling 1955). The success of SIT relies upon sterile males locating and successfully copulating with field females, resulting in embryonic mortality and a decline of the pest population. However, released sterile tephritid males are less competitive than their field male counterparts due to the processes of mass-rearing and exposure to irradiation (Collins et al. 2008).

Tephritids are holometabolous insects with different nutritional environments and requirements during their developmental stages (Taylor et al. 2013b). To attain sexual maturity and achieve good sexual performance certain nutrients are relevant, particularly at the adult stage (Yuval et al. 2002). For example, yeast, as a protein source, is known to affect adult male and female tephritid fitness and performance differently during the development (see review (Yuval et al. 2007)). Fruit flies harbour symbiotic bacteria in their gut and evidence suggests that they are involved in the fly's nutritional status. As environmental factors are known to shape the composition and structure of bacterial communities in tephritids (Morrow et al. 2015b), tephritid development may also impact their gut microbiome and therefore condition how resources are used. Furthermore, diets and exposure to irradiation are known to affect the performance of mass-reared adult tephritids (Niyazi et al. 2004). Although it is known that exposure to irradiation damages the tephritid gut (Lauzon and Potter 2012), little is known about how this affects the gut microbiome. In this sense, a supplementation of symbiotic bacteria to mass-reared irradiated tephritids is expected to improve their performance (Hamden et al. 2013; Sacchetti et al. 2014). Therefore, an

improved understanding of the gut bacterial communities, and how they are impacted by insect development and environmental factors (such as diets and irradiation) may lead to the identification of beneficial symbiotic gut bacteria and how these may be promoted in flies, e.g. through probiotic supplementation.

In Australia, SIT is used in an integrated approach to control the serious horticultural pest, Queensland fruit fly, *Bactrocera tryoni* (Froggatt) (Diptera: Tephritidae) (Dominiak et al. 2003; Jessup et al. 2007). The aim of the present study was to investigate the bacterial community composition and structure within the gut of domesticated populations of *B. tryoni* flies and determine the effects of colony origin, adult developmental stage, sex, adult diets, rearing environment, and exposure to gamma irradiation on gut microbiota. We hypothesised that diet and irradiation impact the gut microbiome. We used 16S rRNA gene amplicon next-generation sequencing (NGS) to characterise the gut bacterial communities of teneral (immature) and mature adult *B. tryoni*, irradiated and unirradiated, maintained on varying adult diets in order to understand the bacterial population dynamics across adult development and to identify an optimal time point for adult probiotic supplementation to enhance adult fruit fly performance for SIT.

2.2: Methods

2.2.1: Treatment of teneral and mature adults

The flies for the characterisation of the bacterial communities were sampled from two laboratory colonies of *B. tryoni* in late January 2015. These two colonies were originally sourced from two different field-collected lines and then independently maintained at two rearing facilities that used larval diets comprising different bulking agents. The first *B. tryoni* colony was from the Fruit Fly Production Facility (FFPF) of the Elizabeth Macarthur Agricultural Institute (EMAI), NSW Department of Primary Industries (NSW DPI) in Camden, New South Wales (NSW). This colony was sourced from a line derived from *B. tryoni* infested fruits collected in the NSW Central Coast region in 2013 and established at the NSW DPI's Central Coast Primary Industries Centre (CCPIC), in Ourimbah, NSW. At the FFPF, mass-reared individuals (>5 million/week; 5,000 larvae per litre larval diet

[personal communication S Balagawi]) of this two year old colony were reared on standard fruit fly larval growth medium using lucerne chaff as the bulking agent, torula yeast, white cane sugar, water, citric acid, sodium benzoate and methyl paraben (Fanson et al. 2014).

The second *B. tryoni* colony was from the Hawkesbury Institute for the Environment (HIE), Western Sydney University, Richmond, NSW. This colony originated from a CCPIC line established from infested fruits collected in the field in the NSW Central West region in 2009. At HIE, laboratory-reared individuals (<500/cohort; approximately 3,000 larvae per litre larval diet) of this six year old laboratory colony (Morrow et al. 2015b) were reared on a larval diet consisting of dehydrated ground carrot as the bulking agent, torula yeast, water, hydrochloric acid and methyl paraben (Meats et al. 2004). A key compositional difference between the two larval diets was the bulking agents (lucerne chaff versus ground carrot) that have minimal nutritional function, but rather provide a matrix to allow aeration and heat dissipation as the larvae feed and develop within the diet.

To cause sterility, half of the late-stage FFPF pupae were irradiated in a ^{60}Co in-ground gamma Technology Research Irradiator at the Australian Nuclear Science and Technology Organisation (ANSTO) in Lucas Heights, NSW, at the current recommended dose of 60-65 Gy and a dose rate of approximately 6 Gy min^{-1}, while a second group of FFPF pupae were not irradiated and remained fertile. All pupae of the HIE cohort were fertile, i.e. unirradiated.

Adult female and male flies were sampled from three replicates of nine experimental treatment groups based on larval rearing environment (FFPF and HIE populations reared on different larval diets), adult developmental stage (teneral or mature adults), irradiation status (irradiated or unirradiated) and adult diet (sugar only, or full diet, i.e. 3:1 ratio of white sugar and yeast hydrolysate) (Table 2.1). In preparation for this, approximately 100 pupae from each of the experimental populations were set up in Petri dishes in separate 30 cm x 30 cm x 30 cm mesh covered cages (Bugdorm, Taiwan) in a controlled glasshouse chamber at HIE at 25 ± 3°C, 65 ± 15% RH and a 10:14 h light: dark photoperiod. The cages were monitored three times daily and flies sampled as teneral and mature adults. Tenerals were not provided with water or food and were sampled between 6 and 12 hours post eclosion (tenerals less than 6 hours old were not used as their digestive systems were soft and disintegrated when dissected). Captive adult *B. tryoni* reach maturity by 10 days (Meats et al. 2004; Perez-Staples et al. 2007). Therefore, mature adults were sampled at 14 days, and were provided

with water and either a full adult diet (sugar and yeast hydrolysate [3:1]) or a sugar only adult diet from eclosion. All adult diets were provided as 2% agar in a Petri dish (Reynolds et al. 2010). The adult diets were replaced every second day.

Table 2.1: *Bactrocera tryoni* experimental treatment groups. Treatment group abbreviations represent treatments for individual samples with the first letter indicating the colony origin of either EMAI-FFPF (E) or HIE (H) from which the pupae were collected, the second letter indicating the larval diets of either carrot (C) or lucerne chaff (L), the third letter identifies the adult diet of either a full adult diet yeast consisting of yeast hydrolysate and sugar (3:1) (Y), sugar only (S) or nil (N) as in the case of tenerals who were not fed, the fourth letter indicates if the pupae were irradiated (I) or unirradiated (U), the fifth letter denotes the sex, either male (M) or female (F). Adult diets were provided in a 1% agar matrix).

Treatment group	Life stage	Colony origin	Larval diet bulking agent	Adult diet	Irradiation	Sex
ELNIF	Teneral	EMAI-FFPF	Lucerne chaff	Nil	Irradiated	Female
ELNIM	Teneral	EMAI-FFPF	Lucerne chaff	Nil	Irradiated	Male
ELNUF	Teneral	EMAI-FFPF	Lucerne chaff	Nil	Unirradiated	Female
ELNUM	Teneral	EMAI-FFPF	Lucerne chaff	Nil	Unirradiated	Male
HCNUF	Teneral	HIE	Carrot	Nil	Unirradiated	Female
HCNUM	Teneral	HIE	Carrot	Nil	Unirradiated	Male
ELSIF	Mature	EMAI-FFPF	Lucerne chaff	Sugar only	Irradiated	Female
ELYIF	Mature	EMAI-FFPF	Lucerne chaff	Full diet	Irradiated	Female
ELSIM	Mature	EMAI-FFPF	Lucerne chaff	Sugar only	Irradiated	Male
ELYIM	Mature	EMAI-FFPF	Lucerne chaff	Full diet	Irradiated	Male
ELSUF	Mature	EMAI-FFPF	Lucerne chaff	Sugar only	Unirradiated	Female
ELYUF	Mature	EMAI-FFPF	Lucerne chaff	Full diet	Unirradiated	Female
ELSUM	Mature	EMAI-FFPF	Lucerne chaff	Sugar only	Unirradiated	Male
ELYUM	Mature	EMAI-FFPF	Lucerne chaff	Full diet	Unirradiated	Male
HCSUF	Mature	HIE	Carrot	Sugar only	Unirradiated	Female
HCYUF	Mature	HIE	Carrot	Full diet	Unirradiated	Female
HCSUM	Mature	HIE	Carrot	Sugar only	Unirradiated	Male
HCYUM	Mature	HIE	Carrot	Full diet	Unirradiated	Male

2.2.2: Gut dissection

At least three females and three males of *B. tryoni* from each of the nine experimental treatment groups (Table 2.1) were selected for gut dissections. Insects were placed in 250mL specimen jars and, within 30 minutes of sampling, were anaesthetised with carbon dioxide for 1 min. The insects were then surface sterilised by sequentially immersing for 1 minute in each of 70% ethanol, sterile distilled water, 0.05% sodium hypochlorite and lastly sterile distilled water, before individuals were placed on a sterile concave glass slide that had been surface treated by wiping with 70% ethanol and 0.05% sodium hypochlorite. The glass slide was placed on top of ice in a plastic Petri dish, which was then viewed under a stereomicroscope. Two pipette drops of sterile phosphate-buffered saline (PBS) were placed on top of the insect before dissection with sterile forceps. The dissection involved firstly removing the wings, the legs and the exoskeleton after softening by immersion in PBS for 1 minute. The intact gut of the insects was then gently removed and placed in a clean 1.5mL microcentrifuge tube and immediately transferred to a freezer (-20°C) for a maximum of 1 hour. Afterwards, samples were stored at -80°C until required.

2.2.3: DNA extraction, library preparation and 16S rRNA gene amplicon sequencing

DNA from each of 58 individual gut samples stored at -80°C was extracted using the QIAmp DNA mini kit (Qiagen), including RNase treatment, and eluted in 50µL nuclease-free water. DNA integrity was examined by gel electrophoresis. The DNA solutions were reduced to a volume between 15 and 20µL using a vacuum concentrator. DNA concentration and purity were assessed using Qubit 2.0 Fluorometry and Nanodrop spectrophotometry. Each genomic DNA sample was also PCR amplified using the eubacterial 16S rRNA gene primers 63F and 1227R, and insect mitochondrial COI with primers Pat and Dick to ensure the DNA did not contain inhibitors that would interfere with amplification.

The DNA samples were then submitted for high-throughput sequencing at the HIE Next-Generation Sequencing Facility for 16S rRNA gene amplification of 7ng DNA using primers 341F – 5' CCTACGGGNGGCWGCAG 3' and 805R – 5' GACTACHVGGGTATCTAATCC 3', which span the variable V3 and V4 regions of the 16S rRNA gene producing a fragment

of approximately 464bp. Library preparation for 58 samples was performed with the Nextera XT kit, and sequencing of 2 x 300bp paired ends was performed on a 384-multiplexed Illumina MiSeq run.

2.2.4: Sequence analyses

The data was analysed using the open-source bioinformatics pipeline QIIME (Caporaso et al. 2010). The raw data of the 58 libraries received in fastq format were examined using FastQC v0.11.5 (Andrews 2010), which showed that trimming of at least 10bp from the 3' ends of R1 reads and 90bp from R2 reads would improve the quality of the merged sequences. Therefore, the reads were trimmed using the *trimfq* command of seqtk (Li 2016), removing the primer and the final 10bp (-b 17 –e 10) from the forward (R1) reads, as well as the primer and final 90bp from the reverse (R2) reads (-b 21 –e 90). FLASH v1.2.11 (Magoč and Salzberg 2011) was used to join the trimmed, paired reads into single sequences with a minimum overlap of 10bp.

The operational taxonomic units (OTUs) were assigned using the *pick_open_reference_otus.py* command which also removes singletons. Chimeric sequences were detected and removed using ChimeraSlayer (Haas et al. 2011).

After singleton and chimera removal, the number of sequence reads per library and alpha diversity indices were compared by pairwise ANOVA and plotted by using *base R* commands in R (R Development Core Team 2017). Due to the significant difference in sequence read numbers obtained per library, following quality control, the data were split into two groups defined as teneral adults and mature adults, and then the sequences for each group were normalised to the lowest number of sequences found in each group using the command *single_rarefaction.py*. The rarefaction curves to assess coverage were created by the *rarecurve* command of the Vegan package (Oksanen et al. 2017) in R.

Beta diversity across the samples was analysed by the phylogenetic distance-based measurement, UniFrac and the abundance distance-based measurement, Bray-Curtis. The distance matrix values for unweighted UniFrac (presence and absence of taxa), weighted UniFrac (presence, absence and abundance of taxa) and Bray-Curtis (compositional

dissimilarity based on counts) for the samples were calculated in QIIME. Then, the distance matrices were imported into R for statistical analysis of treatment effects and plotting of the principal component analysis (PCoA) and relative abundance. The ellipses in the PCoA plots were created using the *ordiellipse* command of the Vegan package and the heatmap plots were created using the *levelplot* command of the Lattice package (Sarkar 2008) in R.

2.3: Results

2.3.1: Sequence read analyses

A total of 58 libraries from 19 teneral and 39 mature adult *B. tryoni* were high-throughput amplicon sequenced for approximately 460bp of their bacterial 16S rRNA gene with the primers 341F and 805R. This generated 2,453,686 raw sequence reads (Appendix 2.4: Chapter 2 OTU table). After filtering and OTU picking, 1,088,483 (44.4%) sequences remained and this large reduction in sequence read numbers was likely due to the reads being of low quality at the 3' ends, which affects the number of read pairs that are merged into a complete sequence fragment, both by reducing the amount of overlap found in reads producing a larger merged sequence (i.e. ~426bp), or by having too much overlap in smaller sized sequences (i.e. ~403bp) and mismatches preventing the reads from being merged. Standardised trimming parameters were applied across all samples in order to minimise bias in merging the paired reads. Following chimera removal, sequences were reduced to 1,018,739 (41.5%) ranging from 11 to 19,606 in tenerals and 7,850 to 57,800 in mature adults. Clustering at 97% identity across all samples, produced 324 OTUs across the entire dataset, including 44 OTUs in tenerals and 309 OTUs in mature adults.

The comparative number of 16S rRNA gene sequence reads across libraries can be used as an indicator of the relative bacterial load across samples. The total sequence reads, or bacterial loads were higher in mature adults ($\bar{x}$ = 25,190.36 ± 1,674.84 SE) than in tenerals ($\bar{x}$ = 1911.32 ± 1076.351 SE) ($F_{1,57}$ = 85.15, p <0.001; Fig. 2.1 and Appendix 2.2). The colony origin affected sequence reads in tenerals ($F_{1,12}$ = 5.23, p <0.05) where FFPF tenerals ($\bar{x}$ = 1,167.00 ± 544.80 SE) had more reads than HIE tenerals ($\bar{x}$ = 22.14 ± 4.01 SE). The irradiation of pupae also resulted in a higher count of sequence reads in mature adults ($F_{1,25}$ =

4.89, $p = < 0.05$) with irradiated matures ($\bar{x} = 31{,}403.08 \pm 3{,}676.84$ SE) having more sequence reads than unirradiated matures ($\bar{x} = 22{,}367.69 \pm 1{,}780.03$ SE). The other parameters of sex and adult diet (for mature adults only), had no discernible impact on bacterial sequence read count (Appendix 2.2).

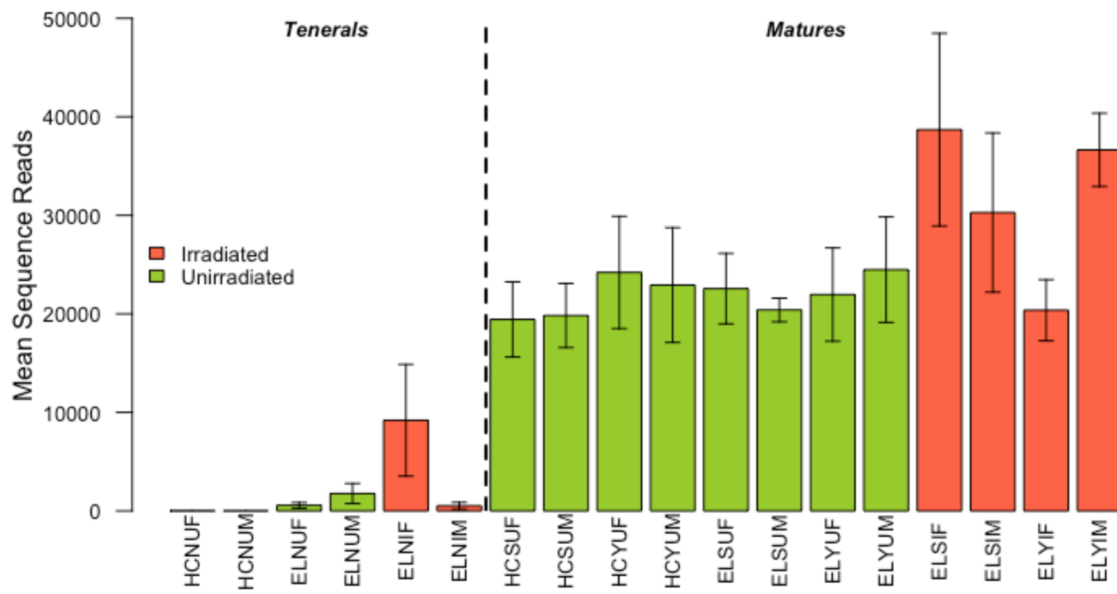


Figure 2.1: Mean of counts of 16S rRNA gene reads by treatment groups. Letter codes are as per Table 1.1.

2.3.2: Alpha diversity

Rarefaction curve (Fig. 2.2) and Good's coverage (Appendix 2.1) indicated that microbial communities of the mature adults were well captured by the sequencing coverage. The reads from the mature fly samples were rarefied to 5,500 and were represented by 309 observed OTUs. The most OTU diverse mature sample was one unirradiated female, kept on a full adult diet (containing yeast hydrolysate and sugar), originating from a FFPF pupa (ELYUF02) that contained 102 OTUs. The rest of the mature samples were much less diverse and contained between 12 and 44 OTUs. The sequences from the tenerals clustered into 44 OTUs following rarefaction to 10 sequences per sample (Fig. 2.3), but only nine out of 19 samples achieved adequate sequence coverage at this low value.

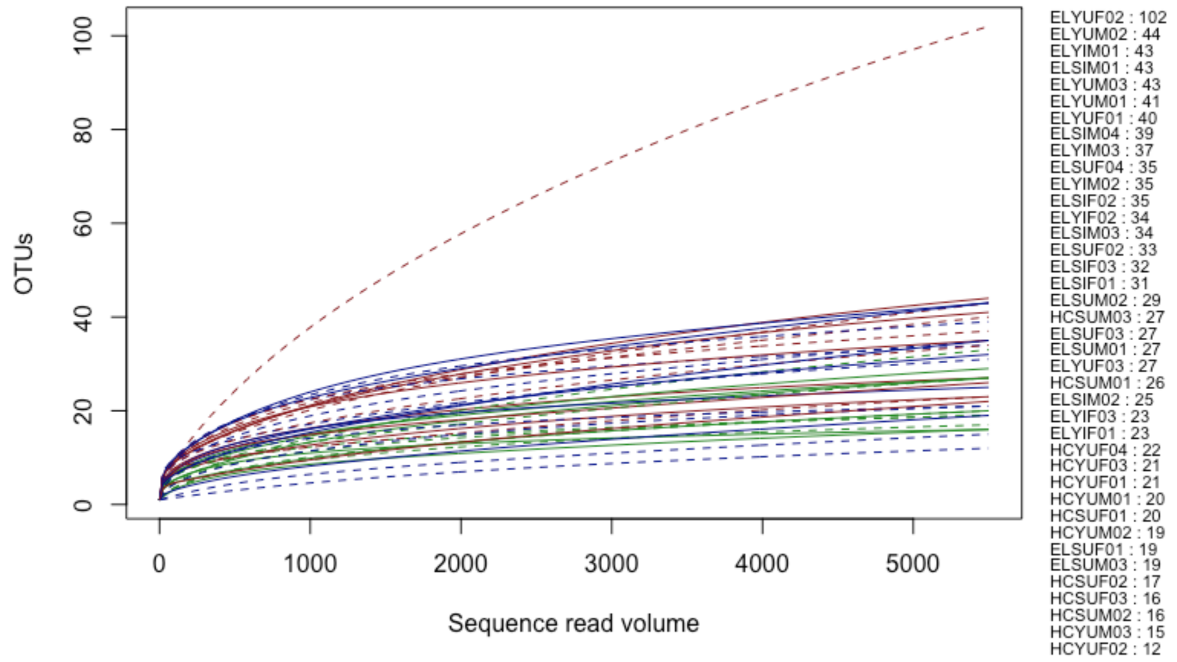


Figure 2.2: Rarefaction curves for mature *Bactrocera tryoni.* Figures to the right of the graph indicate the order of lines as sorted by number of OTUs. Sample name letter codes are as per Table 1.1.

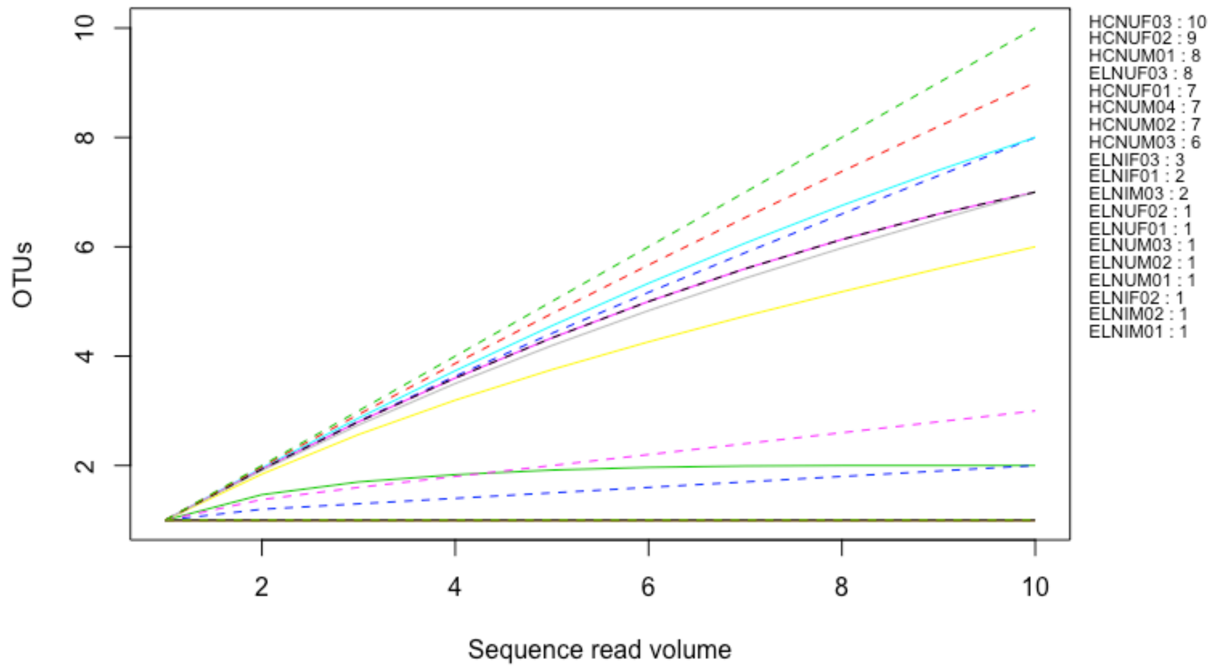


Figure 2.3: Rarefaction curves for teneral *Bactrocera tryoni*. Figures to the right of the graph indicate the order of lines as sorted by number of OTUs. Sample name letter codes are as per Table 1.1.

Colony origin significantly influenced the number of OTUs in both mature ($F_{1,25} = 9.055$, $p < 0.01$) and teneral flies ($F_{1,12} = 20.93$, $p < 0.001$) (Appendix 2.3). Mature adult flies derived from FFPF pupae had more OTUs ($\bar{x} = 37.39 \pm 5.86$ SE) than mature flies derived from HIE pupae ($\bar{x} = 19.39 \pm 1.18$ SE). Contrary to the mature adults, HIE tenerals ($\bar{x} = 7.71 \pm 0.52$ SE) were more OTU diverse than FFPF tenerals ($\bar{x} = 2.17 \pm 1.17$ SE). Sex, irradiation, and adult diets were observed to not affect the number of OTUs in adult flies.

2.3.3: Beta diversity

Beta diversity measurements were applied to sequences clustered at 97% similarity using the weighted and unweighted UniFrac and Bray-Curtis distances (Fig. 2.4). In the tenerals, the PCoA of all three measurements indicated an emerging pattern of separation between samples based on colony origin. This pattern was also visible in mature flies. Further to this, the Bray-Curtis distance PCoA within the irradiated mature flies showed a separation between flies fed a full adult diet, and those fed an adult diet of sugar only. No sex effect was observed in the PCoAs.

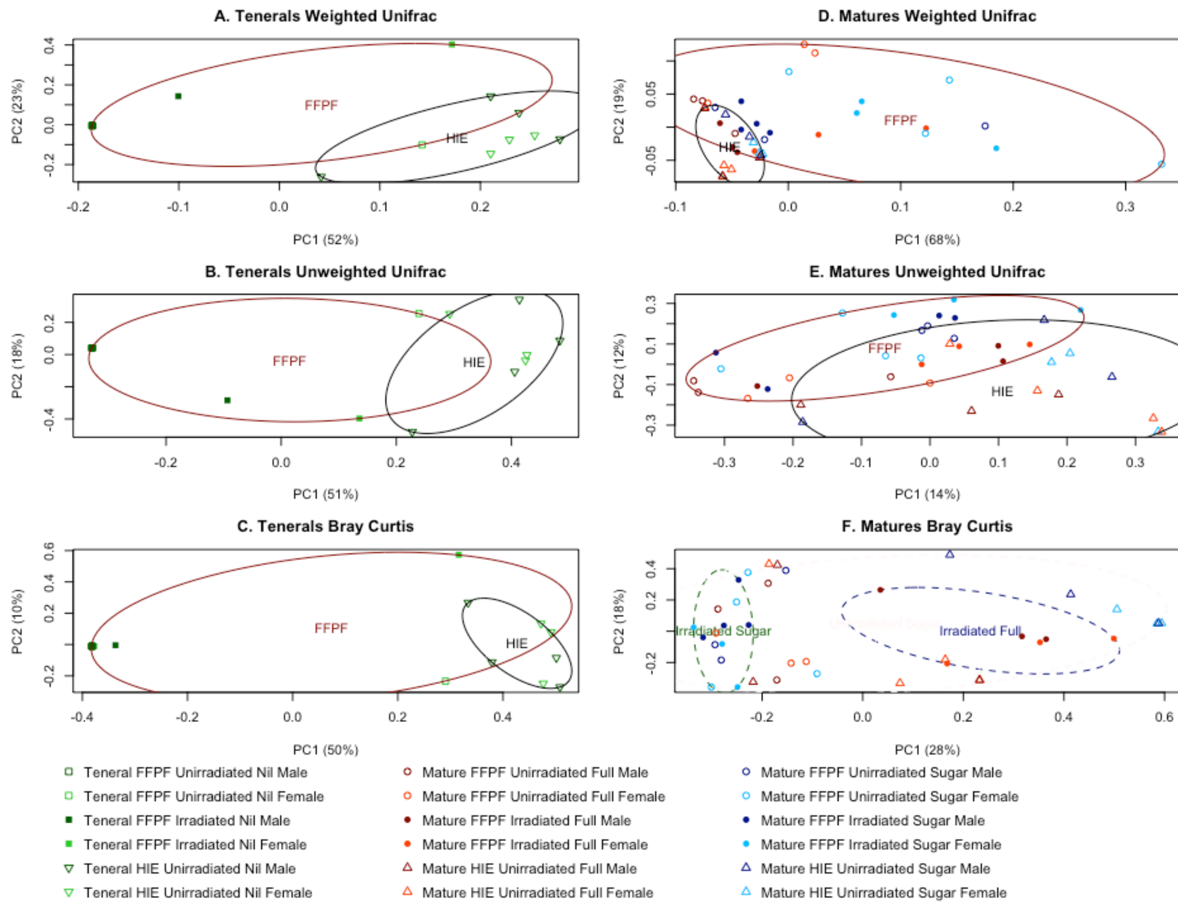


Figure 2.4: Principal coordinate analysis of A) weighted UniFrac distances of tenerals, B) unweighted UniFrac distances of tenerals, C) Bray-Curtis distances of tenerals, D) weighted UniFrac distances of matures, E) unweighted UniFrac distances of matures, and F) Bray-Curtis distances of matures.

2.3.4: Identity of dominant bacterial OTUs

For the entire dataset, the nine most abundant OTUs represented over 80% of the rarefied combined mature and teneral sequence reads (Table 2.2). Based on the BLAST search of the short 16S rRNA gene amplicons, these dominant OTUs likely belonged to the Enterobacteriaceae genera *Enterobacter* (1 OTU), *Pluralibacter/Klebsiella* (2 OTUs), *Proteus* (1 OTU), *Providencia* (2 OTUs) and *Serratia* (2 OTUs), and to the Acetobacteraceae genus *Asaia* (1 OTU).

In mature flies, the most abundant and OTU diverse bacterial family was Enterobacteriaceae, comprising 116 OTUs (Fig. 2.6 & 2.7). The high abundance of Enterobacteriaceae in matures was mostly due to 8 OTUs that accounted for over 86% of the total rarefied mature adult sequence reads (Table 2.2). The second most abundant bacterial family in the mature flies was Acetobacteraceae, where one of the 11 OTUs accounted for 11% of the total rarefied mature adult sequence reads. Based on a BLAST search, this dominant Acetobacteraceae OTU belonged to the genus *Asaia*.

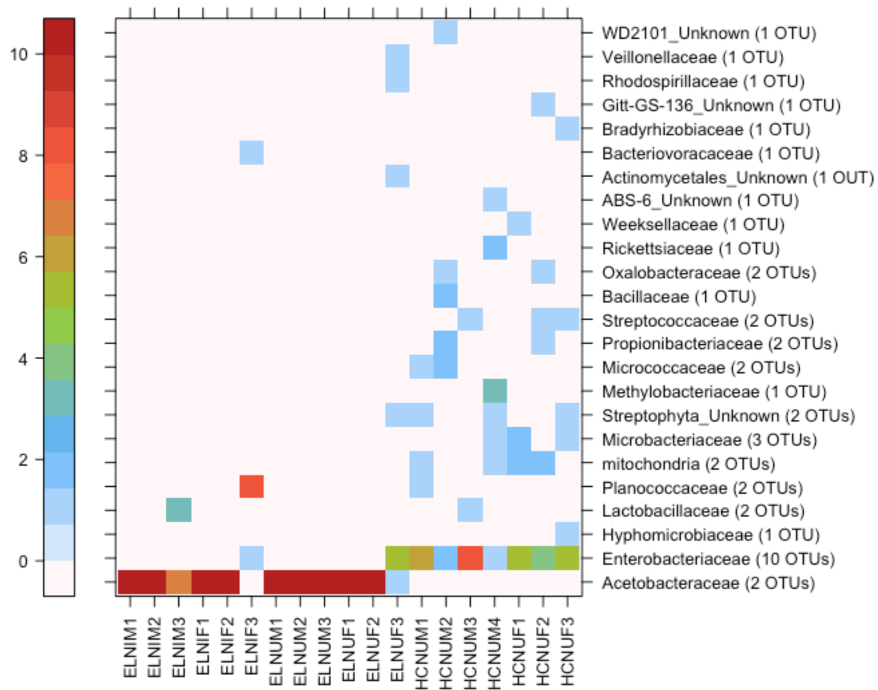


Figure 2.5: Relationship between individual teneral *Bactrocera tryoni* and bacterial families. Sample name letter codes are as per Table 2.1.

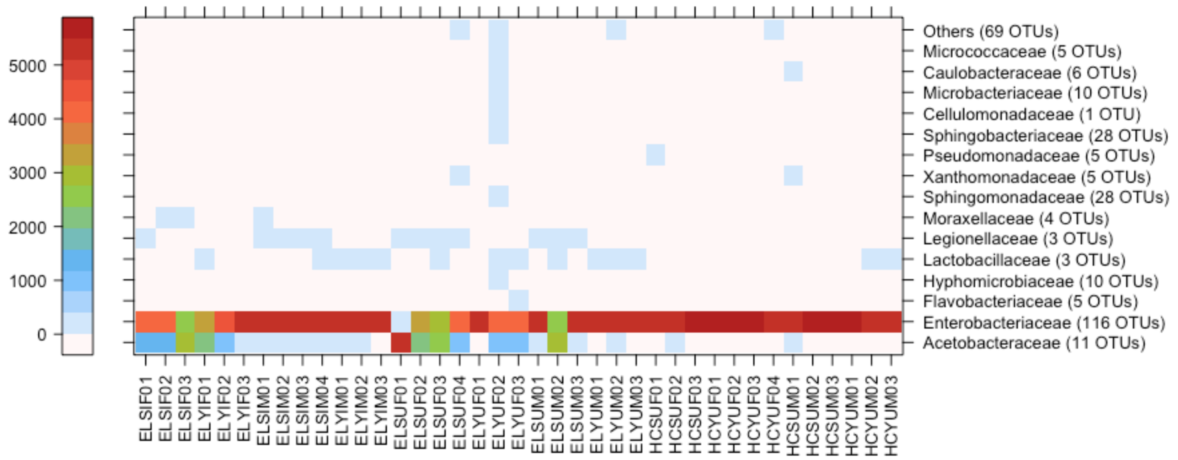


Figure 2.6: Relationship between individual mature *Bactrocera tryoni* and major bacterial families. Sample name letter codes are as per Table 2.1.

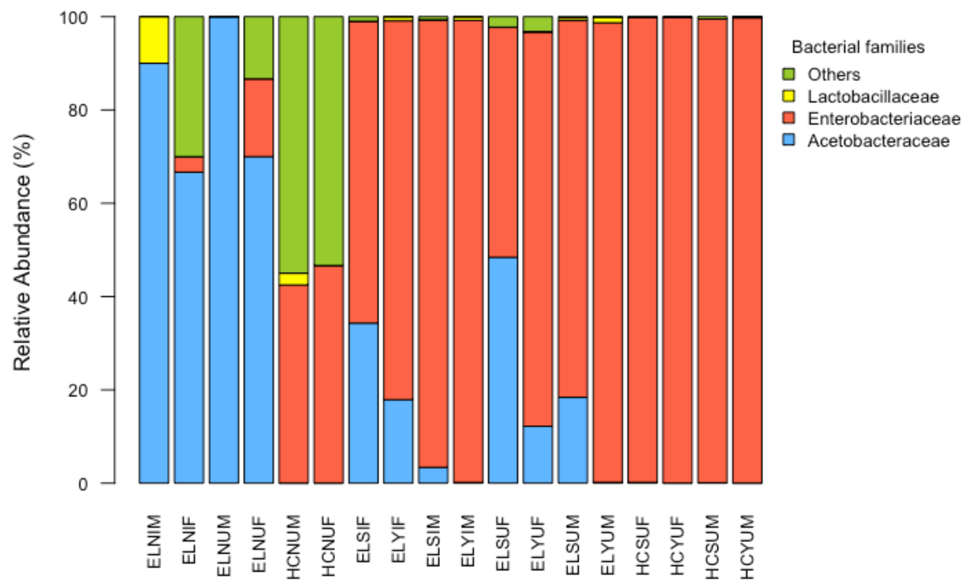


Figure 2.7: Relative abundance of bacterial families in teneral and mature *Bactrocera tryoni* treatment groups represented by 16S rRNA gene sequences after rarefaction of tenerals to 10 sequence reads and mature adults to 5,500 sequence reads. Letter codes are as per Table 2.1.

Table 2.2: Major OTUs (excluding OTUs less than 1%) in teneral and mature adult *Bactrocera tryoni* and their BLAST hits

OTU ID	Tenerals		Mature		Combine		Query cover (%)	E value	Identity score (%)	Closest NCBI BLAST hit
	Reads	Abundance	Reads	Abundance	Reads	Abundance				
4418165	5	2.63%	44809	20.89%	44814	20.87%	100%	0.0	99%	*Pluralibacter gergoviae* strain BYK-7 16S rRNA gene, complete sequence; *Pluralibacter gergoviae* strain FB2, complete genome; *Klebsiella oxytoca* strain CAV1015, complete genome; *Klebsiella oxytoca* strain CAV1099, complete genome
1122622	8	4.21%	35835	16.71%	35843	16.70%	100%	0.0	100%	*Providencia rettgeri* strain RB151, complete genome
3101394	6	3.16%	33454	15.60%	33460	15.59%	100%	0.0	100%	*Providencia rettgeri* strain RB151, complete genome
470879	7	3.68%	32797	15.29%	32804	15.28%	100%	0.0	100%	*Proteus* sp. strain JP20 16S rRNA gene, partial sequence
814266	97	51.05%	25055	11.68%	25152	11.72%	100%	0.0	100%	*Asaia bogorensis* NBRC 16594 DNA, complete genome
4477719	1	0.53%	10079	4.70%	10080	4.70%	100%	0.0	99%	*Pluralibacter gergoviae* strain BYK-7 16S rRNA gene, complete sequence; *Pluralibacter gergoviae* strain FB2, complete genome; *Klebsiella oxytoca* strain CAV1015, complete genome; *Klebsiella oxytoca* strain CAV1099, complete genome
1108706	2	1.05%	9058	4.22%	9060	4.22%	100%	0.0	100%	*Serratia marcescens* strain B3R3, complete genome
572750	0	0.00%	7304	3.41%	7304	3.40%	100%	0.0	100%	*Enterobacter* sp. Amlc14 16S rRNA gene, partial sequence
4343005	0	0.00%	6554	3.06%	6554	3.05%	100%	0.0	100%	*Serratia marcescens* strain B3R3, complete genome

The HIE tenerals harboured 10 OTUs classified to Enterobacteriaceae and were dominated by the same Enterobacteriaceae OTUs that were highly abundant in the mature flies (Table 2.2). The FFPF tenerals contained two Acetobacteraceae OTUs, but were dominated by one OTU that accounted for 51% of the total rarefied teneral sequence reads (Fig. 2.5 & 2.7). The dominant Acetobacteraceae OTU in tenerals, as in matures, was *Asaia*. Other notable OTUs in tenerals included Planococcaceae (according to the short 16S rRNA gene amplicon possibly a *Staphylococcus* spp.), and mitochondrial 16S rRNA gene from Poaceae (grasses), probably from the cane sugar used in the FFPF larval diet, and hits to a chloroplast 16S rRNA gene. *Asaia* or other Acetobacteraceae were not found in teneral and mature HIE flies.

The relative abundance plot (Fig. 2.7) suggested a pattern in mature FFPF flies (which were dominated by Acetobacteraceae in the teneral stage) that favoured, irrespective of irradiation, the proliferation of Enterobacteriaceae (and reduced relative presence of Acetobacteraceae) when fed the full adult diet over those fed the sugar only adult diet. Furthermore, for FFPF matures, it appeared that females had higher relative abundance of Acetobacteraceae than males when fed sugar however this was not observed when FFPF flies were fed a full adult diet.

2.4: Discussion

We used 16S rRNA gene amplicon sequencing to characterise the bacterial community composition and structure of individual adult *B. tryoni* and to evaluate the impact of colony origin, adult diets and irradiation on the bacterial community across two adult developmental stages. Tenerals consistently had reduced total bacterial titres when compared with mature adult flies. This may be due to the bottleneck that bacterial populations experience as a consequence of the emptying of gut content prior to pupation during holometabolous metamorphosis. An overall low bacterial count in larvae, pupae and teneral flies was also observed for Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann), (Diptera: Tephritidae) (Aharon et al. 2013). Another possible explanation for the differences found between teneral and mature flies may be that larvae are less mobile and restricted to one diet while pupae are a non-feeding, largely sessile stage and, therefore, have reduced exposure to diverse

microbial communities compared with adults. Due to their mobility, adult flies have the potential to forage from diverse food sources across environments with variable microbial communities (particularly in the field but also to some extent when in a captive colony).

Unexpectedly, irradiated mature *B. tryoni* had a higher bacterial sequence read volume than unirradiated individuals, suggesting an increased bacterial load. Given that such irradiation effects on gut bacterial communities have not yet been investigated in other tephritids, our findings warrant further investigation of bacterial population dynamics in irradiated flies. Despite the damage caused by irradiation on a tephritid gut (Lauzon and Potter 2012), previous research demonstrated that irradiated tephritids still display normal proteolytic activity (San Andres et al. 2007). Therefore, the damage caused by irradiation may enable some bacteria to exploit newly available resources, and/or compensate for the damage. Alternatively, we can also postulate that the damage caused by irradiation allows some bacteria to proliferate in the gut due to an inability of irradiated fruit flies to regulate their bacterial load. However, this increased bacterial load did not impact the total bacterial diversity and relative abundance of OTUs.

Although the effects of adult diets on tephritids have been well characterised (Fanson et al. 2009; Kaspi and Yuval 2000; Reynolds et al. 2014), to date little is known about the impact of diets on their microbiome (Wang et al. 2011). The comparison of OTU diversity between teneral and mature adults revealed a clear distinction between flies with different colony origins (at FFPF and HIE flies were reared on different larval diets in different environments and were sourced from different field populations in different years). This is consistent with the PCoA plots (Fig. 2.4 A, B, C, D, and E) where an emerging separation was visible between samples from different colony origins.

The colony origin significantly influenced the number of sequence reads in tenerals and the number of OTUs in both tenerals and matures. The flies from the FFPF and HIE were derived from different lines. Further, the FFPF line had been captive for under two years, while the HIE colony had been established for six years. Fruit flies are known to lose their field characteristics in as little as three generations (Gilchrist et al. 2012; Hoffmann et al. 2001) as they become domesticated. Further to this, FFPF flies were reared at higher densities compared to the HIE flies and this may have impacted the stress of the environment for developing larvae, and prior generations of adult flies. Therefore, different host genotypes

and environmental influences such as larval densities could play a part in the different bacterial community composition observed between flies originating from the FFPF and HIE. Although we cannot entirely separate the effects of larval diet, larval environment (including density of larvae in the diet) and domestication history of the two fly colonies, it remains likely that the different bulking agents used in the larval diets of FFPF and HIE were probably important contributors to the observed differences in the bacterial community in tenerals. Regardless of the pupal origin, as the adult flies matured within the same environment, the bacterial communities became increasingly similar; therefore, the adult environment impacted the bacterial communities of flies as they developed.

Besides this effect of colony origin, our study of captive *B. tryoni* indicates that, within diet treatments, the bacterial communities were similar in composition between male and female flies. Therefore, we can exclude any sex effects on bacterial community composition in captive flies.

Based on the short 16S rRNA gene amplicons, the genera of the dominant OTUs in the reared populations for *B. tryoni* were *Enterobacter*, *Pluralibacter/Klebsiella, Proteus*, *Providencia* and *Serratia* (Enterobacteriaceae) and *Asaia* (Acetobacteraceae). The dominance by Enterobacteriaceae supports previous findings from microbiome studies of *B. tryoni* (Morrow et al. 2015b; Thaochan et al. 2009), and other tephritids of the *Bactrocera* genus including *B. cacuminata* (Morrow et al. 2015b; Thaochan et al. 2009), *B. carambolae* (Yong et al. 2017b), *B. cucurbitae* (Hadapad et al. 2015; Sood and Nath 2005; Thaochan et al. 2010), *B. dorsalis* (Andongma et al. 2015; Pramanik et al. 2014; Thaochan et al. 2013; Wang et al. 2014b; Yong et al. 2017b), *B. jarvisi* (Morrow et al. 2015b), *B. neohumeralis* (Morrow et al. 2015b), *B. minax* (Wang et al. 2014a), *B. oleae* (Ben-Yosef et al. 2010; Kounatidis et al. 2009), *B. tau* (Khan et al. 2014; Prabhakar et al. 2013; Sood and Nath 2005) and *B. zonata* (Reddy et al. 2014).

Teneral *B. tryoni* originating from FFPF pupae were dominated by Acetobacteraceae (mostly *Asaia*) but, in the mature stage, these flies had a lower proportional representation of this bacterial family than Enterobacteriaceae, and provision of a full adult diet exacerbated this effect. This may suggest that the ratio of carbohydrates and proteins in the adult diet may shift bacterial community structure. Nitrogen, the key element in proteins, is considered to be a limiting factor in the reproductive success of both male and female *C. capitata* (Yuval

and Hendrichs 2000; Yuval et al. 1998). Despite the provision of yeast as a protein source, nitrogen is paradoxically limited (Galloway et al. 2004). Enterobacteriaceae are known to contain diazotrophic species (Behar et al. 2005) which would assist in providing more or specifically required forms of nitrogen. This would explain the abundance of Enterobacteriaceae in mature adult *B. tryoni*. Enterobacteriaceae species have also been credited for improving egg production in female *B. oleae* (Ben-Yosef et al. 2010; Sacchetti et al. 2014) and improved mating performance in male *C. capitata* (Ben-Yosef et al. 2008; Niyazi et al. 2004). These studies have sparked the research interest into the use of Enterobacteriaceae candidates to enhance performance of *B. tryoni* (Fitt and O'Brien 1985; Meats et al. 2009).

The high abundance of *Asaia* in *B. tryoni* adult flies reared from FFPF pupae (but not seen in adult HIE flies) is a novel finding as previous studies found *Asaia* only at low abundance in adult *B. tryoni* (Morrow et al. 2015b) and *B. oleae* (Sacchetti et al. 2008). The role of *Asaia* sp. in tephritids is still unknown, however, bacteria of this genus are dominant taxa in the microbiota of larvae and several adult mosquitoes (*Anopheles gambiae*, *A. maculipennis* and *A. stephensi)* (Damiani et al. 2010; Favia et al. 2007). *Asaia* spp. have been found to be important in the development of *A. stephensi* as when deprived of it, larval development was delayed (Chouaia et al. 2012).

2.5: Conclusion

Our study has shown that the microbiome of *B. tryoni* during adult development is impacted by irradiation, the environment and the adult diet, with a very similar microbiome shared between male and female captive and domesticated *B. tryoni*. Symbiotic bacteria have previously been supplemented to larval and adult diets of other tephritid pest species with the aim to improve the performance of mass-reared flies in SIT programmes (Yuval et al. 2010). Our findings demonstrate that colony origin (in our study, compounded by differences in larval diets, rearing environments, field source populations and duration of domestication) and adult diets impact mature *B. tryoni* gut microbiota. However, diet composition (such as the ratio of carbohydrates and protein) is evidently an important factor for the application of fruit fly probiotics. Importantly, our work also suggests that the ideal time to introduce a

probiotic to impact the mature adult tephritids' microbiota is from the teneral stage, which is consistent with the 2 to 3 days pre-release holding period for sterile adult *B. tryoni* (Reynolds et al. 2012). The dominance of the bacterial families Enterobacteriaceae and Acetobacteraceae, specifically *Asaia* sp., warrants more research into the association of these bacteria with *B. tryoni*, particularly in understanding the role they currently play in mass-rearing and performance of the sterile individuals released in SIT programmes.

Chapter 3: Gut bacterial communities of Queensland fruit fly, *Bactrocera tryoni*, across native and invasive ranges in tropical, sub-tropical and temperate Australia and tropical New Caledonia.

Abstract

Across Australia and the Pacific, Queensland fruit fly, *Bactrocera tryoni*, is a serious pest that impacts horticultural production and trade. It has the ability to adapt and thrive in new habitats. The gut of *B. tryoni* harbours a diverse array of bacteria that may play important roles in host biology and hold the key to understanding why it is a successful pest. Such information can be used in the management of *B. tryoni*. In this study, the gut bacteria of field-collected *B. tryoni* of native and invasive populations from across tropical, sub-tropical and temperate Australia and New Caledonia were investigated using next-generation sequencing of a short 16S rRNA gene amplicon. There was a significant difference in the bacterial communities between sexes with the bacterial community composition being more diverse in males. The bacterial community composition in females was somewhat similar across habitats. Across the entire field population, Enterobacteriaceae is the most dominant bacterial family. The endosymbiont *Wolbachia* was detected in the male samples collected from tropical rainforest in the Atherton Tablelands.

3.1: Introduction

Insects have diverse associations with bacteria, some of which are important for insect survival and success. The benefits of bacterial associations with insects include digestion of food and provisioning of nutrients (Akman Gündüz and Douglas 2009), host defence against pathogens (Dillon et al. 2005), increased resistance to insecticides (Cheng et al. 2017) and reproduction (Ben Ami et al. 2010). Thus, symbiotic bacteria can help insect hosts survive and thrive in diverse environments (Russell et al. 2009). Conversely, the environment that host insects inhabit, and the food that they consume, may impact their bacterial associations, in particular in the digestive system. The analysis of insect gut bacteria across different individuals and populations of a host species may enable us to better understand the dynamics of insect-microbe interactions, and potentially reveal bacterial associations that are important for host biology and behaviour. More specifically, the characterisation of the gut microbiome and its variability across different populations of a widely distributed pest species may provide insights into the dynamics and drivers of microbial community

composition and structure and reveal important microbiome constituents that may contribute to host fitness.

The Queensland fruit fly, *Bactrocera tryoni* (Froggatt), is native to tropical and sub-tropical Australia where it was initially recorded from fruit of coastal and rainforest plants (Froggatt 1897; 1910). In the early 20th century, it was reported to infest fruits in orchards across large parts of the east coast of Australia, including temperate regions where it was believed to have been accidentally introduced through infested fruits (Lea 1899). It has since been established in several temperate regions of Australia and is now considered the most economically important horticultural pest in Australia (Dominiak and Daniels 2012) that can infest fruit of over 40 plant families (Hancock et al. 2000). The first detection of *B. tryoni* in New Caledonia was in 1969 (Cochereau 1970). It was believed that *B. tryoni* was unintentionally introduced there by movement of infested fruits from Australia, and it has since become established as an invasive horticultural pest in New Caledonia (Amice and Sales 1997). It has also established on other Pacific islands (Drew et al. 1978; White and Elson-Harris 1992) and is a major biosecurity threat within and outside Australia (Sutherst et al. 2000).

Like many other invasive species, *B. tryoni* can adapt to new environments and has been known to outcompete other tephritid species. In temperate Australia, *B. tryoni* is believed to have outcompeted invasive Mediterranean fruit fly *Ceratitis capitata* (Wiedemann) populations in parts of New South Wales after years of coexistence (Allman 1939; Anonymous 1947). It was even hypothesised that *C. capitata* could have established in Queensland if not for the presence and dominance of *B. tryoni* in the region (Vera et al. 2002). Since the introduction into New Caledonia, *B. tryoni* has been observed to suppress populations of native South Sea guava fruit fly, *Bactrocera psidii* in urban areas restricting them to native forests, and also to outcompete and replace the native fruit fly, *Bactrocera curvipennis* in some areas (Amice and Sales 1997).

A diversity of control strategies are available for *B. tryoni* (Clarke et al. 2011). This includes the sterile insect technique (SIT) that requires the release of mass-reared flies that have been sterilised through irradiation. However irradiation impacts performance of mass-reared flies (Lance et al. 2000) and their microbiota (as discussed in Chapter 2). It has been hypothesised that the restoration of the gut microbiome of mass-reared and irradiated flies can improve the success of SIT (Cai et al. 2018). Therefore, this requires a deep understanding of the *B. tryoni*

gut microbiome composition and structure as it can be obtained through high throughput 16S rRNA gene amplicon sequencing analyses. Previous studies on three pools of laboratory-reared and one pool of field-collected *B. tryoni* females revealed their guts were dominated by Proteobacteria (Morrow et al. 2015b). However, so far, it is not known how the gut bacterial community of *B. tryoni* varies between individuals and sexes, and across populations of its wide range, including native and invasive populations.

Utilising next generation sequencing, the gut bacterial community of *B. tryoni* was investigated to reveal insights into the gut bacterial diversity and abundance. Of particular interest were the effects of host fruit and habitat within regions across varying climatic conditions in Australia and New Caledonia. *Bactrocera tryoni* is highly polyphagous (Hancock et al. 2000) and occurs in diverse environments. Therefore, its gut bacterial community is likely to be influenced by the wide array of food sources and habitats.

We expected that *B. tryoni* has a varied bacterial microbiome across different populations due to exposure of variable environments, hosts and climates. In contrast, the bacterial species that are common (i.e. core) to all or most individuals across populations will be few. Such a finding would indicate that the large proportion of the bacterial diversity in fruit fly guts is transiently there without being essential for the development of the flies, or that important functions of bacteria in *B. tryoni* can be performed by a number of bacteria. Furthermore, we expected that bacterial diversity also differs between females and males due to their different physiological requirements and foraging behaviours.

3.2: Methods

3.2.1: Sampling of Queensland fruit fly, *Bactrocera tryoni*

Adult female and male *B. tryoni* were sampled from four Australian regions of Atherton Tablelands, Brisbane and Sydney Basin, and from Grande Terre, New Caledonia's main island (Table 3.1). In Australia, *B. tryoni* were sampled from different habitats comprising of either introduced fruit-bearing trees, or Australian native rainforest plants, between February

and May of 2015. In New Caledonia, *B. tryoni* were sampled from habitats dominated by introduced fruit-bearing trees between May and July 2016.

All habitats sampled within the same region were approximately 50km from each other. We considered samples from the tropical Atherton Tablelands and the sub-tropical Brisbane region as belonging to native populations. Samples collected from the temperate Sydney Basin and tropical New Caledonia were introduced and therefore classified as samples of invasive populations.

The insects were collected using two methods. One method utilised a modified, entry-only Lynfield trap baited with cue-lure, but no toxicant so that the trapped insect remained alive in the trap until collection. The cue-lure was contained within the trap so that the trapped flies were not able to come into contact with the lure. Before deployment in the field, the traps were wiped with 70% ethanol (to reduce contamination with environmental bacteria) and then checked every 24 hours for live insect collection. Sampled insects were collected into 250ml specimen vials that had been wiped with 70% ethanol. Cue-lure only attracts male *B. tryoni* (Drew 1989), therefore an alternative method was employed, and live female and male adults were individually collected directly from the host plant into specimen vials. Vials containing sampled flies were kept in an insulated container with ice to reduce insect metabolic rates during collection and transportation to the laboratory, where sample processing occurred.

Table 3.1: Population samples of *Bactrocera tryoni*. First letter of the population label indicates the region, the second letter the habitat and the third letter the sex.

Population	Country	Region	Location	Habitat	Climate	Origins	Sex	Number of samples (*n*)
AMF	Australia	Atherton Tablelands	Walkamin (-17.133767, 145.427046)	Mango (research orchard)	Tropical	Native	Female	6
AMM	Australia	Atherton Tablelands	Walkamin (-17.133767, 145.427046)	Mango (research orchard)	Tropical	Native	Male	5
ARF	Australia	Atherton Tablelands	Koah (-16.872813, 145.580178)	Native rainforest	Tropical	Native	Female	2
ARM	Australia	Atherton Tablelands	Koah (-16.872813, 145.580178)	Native rainforest	Tropical	Native	Male	5
BMM	Australia	Brisbane	Redlands (-27.526987, 153.250875)	Mango (mixed research orchard)	Sub-tropical	Native	Male	10
BRM	Australia	Brisbane	Mt Coot-tha (-27.476991, 152.974465)	Botanical gardens, native rainforest collection	Sub-tropical	Native	Male	10
SMM	Australia	Sydney Basin	Ourimbah (-33.357794, 151.382673)	Mango (backyard)	Temperate	Invasive	Male	3
SCF	Australia	Sydney Basin	Richmond (-33.610681, 150.747221)	Cherry guava (backyard)	Temperate	Invasive	Female	5
SCM	Australia	Sydney Basin	Richmond (-33.610681, 150.747221)	Cherry guava (backyard)	Temperate	Invasive	Male	5
GAM	New Caledonia	Grande Terre	Bourail (-21.567204, 165.498438)	Avocado (backyard)	Tropical	Invasive	Male	6
GCM	New Caledonia	Grande Terre	Pocquereux (-21.748831, 165.921469)	Carambola (backyard)	Tropical	Invasive	Male	6

3.2.2: Extraction of guts from Queensland fruit fly, *Bactrocera tryoni*

Within 8 hours of sampling in Australia, adult flies were placed in a -5°C freezer for 5 minutes for immobilisation. The adults were then taken from the freezer and surface treated by sequentially immersing for 1 minute in each of 70% ethanol, sterile distilled water, 0.05% sodium hypochlorite and sterile distilled water. Individuals were then placed on a sterile concave glass slide that had been surface treated by wiping with 70% ethanol and 0.05% sodium hypochlorite. The glass slide was placed under a stereomicroscope and two pipette drops of sterile phosphate-buffered saline (PBS) were placed on top of the insect before its dissection with sterile forceps. After softening by immersion in PBS for 1 minute, the flies were dissected by removing first the wings, then the legs and exoskeleton. During dissection male flies were checked for fully developed testes and females were checked for presence of matured eggs, i.e. if they were gravid. The intact gut of the insects was then gently removed and placed in a sterile 1.5mL microcentrifuge tube which contained 0.8mL solution of brain heart infusion broth and 20% glycerol (BHIB+20%gly). The microcentrifuge tubes were immediately transferred to a freezer (-20°C) for short-term storage of up to 1 hour. Afterwards, the gut samples were stored at -80°C until required.

Adult *B. tryoni* from New Caledonia were also placed in a -5°C freezer for 5 minutes before being surface treated and then placed individually in sterile 1.5ml tubes with 1ml of absolute ethanol for shipment to Australia for dissection. Upon arrival in Australia, the samples were dissected immediately after repeating the surface treatment process. Once dissected, the gut samples were also placed in 0.8mL BHIB+20%gly solution and stored in -80°C until required.

3.2.3: DNA extraction and amplicon sequencing

A subsample for each of the 50 Australian individual gut samples stored at -80°C was DNA extracted, and the remainder of each sample was kept in the freezer for later microbiological isolation (see Chapter 5). Microbiological isolation was not possible for the 12 New Caledonian samples that were shipped to Australia in pure ethanol, but these samples were still ok for DNA extraction. DNA was extracted from the 62 individual gut samples using the

QIAmp DNA mini kit (Qiagen), including RNase treatment, and eluted in 50µL nuclease-free water. Using a vacuum concentrator, the DNA solutions were reduced to a volume between 15 and 20µL. DNA concentration was assessed using Qubit 2.0 Fluorometry.

About 7ng DNA for each sample was submitted to the HIE Next-Generation Sequencing Facility for 16S rRNA gene amplicon sequencing using primers 341F – 5' CCTACGGGNGGCWGCAG 3' and 805R – 5' GACTACHVGGGTATCTAATCC 3', which span the variable V3 and V4 regions of the 16S rRNA gene, producing a fragment of approximately 464bp.

In addition to the 62 *B. tryoni* gut samples, four control samples consisting of two mock bacterial community samples and two blanks of ultra-pure water, were also submitted for amplicon sequencing. The first mock sample contained 100% *Leuconostoc* sp. DNA and the second mock sample was a community of bacterial DNA, which was mixed based on DNA concentrations as estimated using Nanodrop. Four individual bacterial isolates, each from a different bacterial family were included in the proportions of 50% *Asaia* sp. (Acetobacteraceae), 35% *Enterobacter* sp. (Enterobacteriaceae), 10% *Leuconostoc* sp. (Leuconostocaceae), and 5% *Lactobacillus plantarum* (Lactobacillaceae). These DNA extracts were from bacteria that had been isolated from *B. tryoni* by using selective media in a separate study undertaken by L. Shuttleworth, 2017 (personal communication).

Library preparation for 62 gut samples and the four control samples was performed with the Nextera XT kit, and sequencing of 2 x 300bp paired ends was performed on a 384-multiplexed Illumina MiSeq run.

3.2.4: Sequence analysis

Bioinformatics processing of the MiSeq data was conducted in QIIME v1.8.0. The data received in fastq format was examined using FastQC v0.11.5 and trimmed using the trimfq command of seqtk removing the primer and the final 10bp (-b 17 –e 10) from the forward (R1) reads, as well as the primer and final 90bp from the reverse (R2) reads (-b 21 –e 90). The trimmed paired reads were joined using FLASH v1.2.11 into single sequences with a

minimum overlap of 10bp. Standardised trimming parameters were applied across all samples in order to minimise bias in merging the paired reads.

The *pick_open_reference_otus.py* command was used to assign operational taxonomic units (OTUs) at 97% identity, which also removed singletons. Chimeric sequences were detected and removed using the Blast_fragments approach.

The control libraries were used to assess the quality of the sequencing run. The first mock sample, which contained DNA from a *Leuconostoc* sp. isolate, resulted in 33 OTUs with 99.6% of its reads from OTU 1108007 with the expected BLAST search hit (i.e. a *Leuconostoc* sp.). The second mock sample, which was a known mix of bacterial DNA extracts, returned 82 OTUs with 98.7% of the reads represented by 6 OTUs: OTU 814266 was an *Asaia* sp. with 41.6% of the reads; OTUs 4318935, 2529285, 4423027 were highly similar *Enterobacter* spp., with 24.8%, 11.3 % and 11.3% of the reads respectively and a combined total of 47.4% of the reads; OTU 1108007 was a *Leuconostoc* sp. with 3.2% of the reads; and OTU 4305372 was *Lactobacillus plantarum* which had 6.9% of the reads. These proportions of sequence reads per OTUs were considered acceptable as they were very similar to the initial mixture of DNA used to make the mock community. The blank samples had 32 OTUs with 103 reads, of which the most dominant OTU was OTU 241441 with 31 reads whilst the average reads for the other OTUs was 2.3 reads. OTU 241441 was present in 43 samples at an average of 3.0 reads per sample. According to BLAST search, OTU 241441 was an *Agrobacterium* sp. sequence. By using the mock bacterial communities and blank samples, this identified potential contaminant, *Agrobacterium* sp. (OTU 241441), was removed. Following removal of the control samples, the sequence reads for all 62 samples ranged from 11,783 to 131,145 and were normalised to 5,500 using the command *single_rarefaction.py.*

3.2.5: Statistical analysis

All statistical analyses were conducted in R language and environment for statistical computing and graphics (R Core Team 2018). The rarefaction curves to assess coverage were created by the *rarecurve* command of the Vegan package (Oksanen et al. 2018). The stacked abundance of the OTU bar plot was created using *base* R commands. The OTU heatmap

plots were created using the *levelplot* command of the Lattice package (Sarkar 2008). The *Adonis* function of the Vegan package was used to calculate the permutation multivariate analysis of variance (PERMANOVA) which was used to investigate the influence of treatment factors in the entire dataset. The interaction between treatment factors and microbial communities was visualised using constrained analysis of principal co-ordinates (CAP). Prior to ordination, a Bray-Curtis dissimilarity matrix of relative abundance was standardised by a *Hellinger* transformation to reduce the impact of rarer OTUs (Legendre and Gallagher 2001). CAP was fitted using the function *capscale* from the Vegan package and constrained by treatment factors. The CAP plot was created in R and the 95% confidence ellipses were created using *ordihull* function of Vegan. The Venn diagram of OTUs was calculated using the *vennDiagram* command of the Limma package (Ritchie et al. 2015).

3.3: Results

3.3.1: Alpha diversity and abundance

The microbial communities of most adult *B. tryoni* collected from the field across a wide geographic range were well captured by the sequencing coverage as indicated by the rarefaction curves (Fig. 3.1) and Good's coverage (Table 3.2). Rarefaction of the sequence reads to 5,500, which is just below the lowest sequence read for one sample (i.e. 5,560), resulted in a total of 341,000 reads from 1,653 OTUs. The OTU diversity ranged from 36 OTUs to 174 OTUs per sample (Fig. 3.1 and Table 3.2).

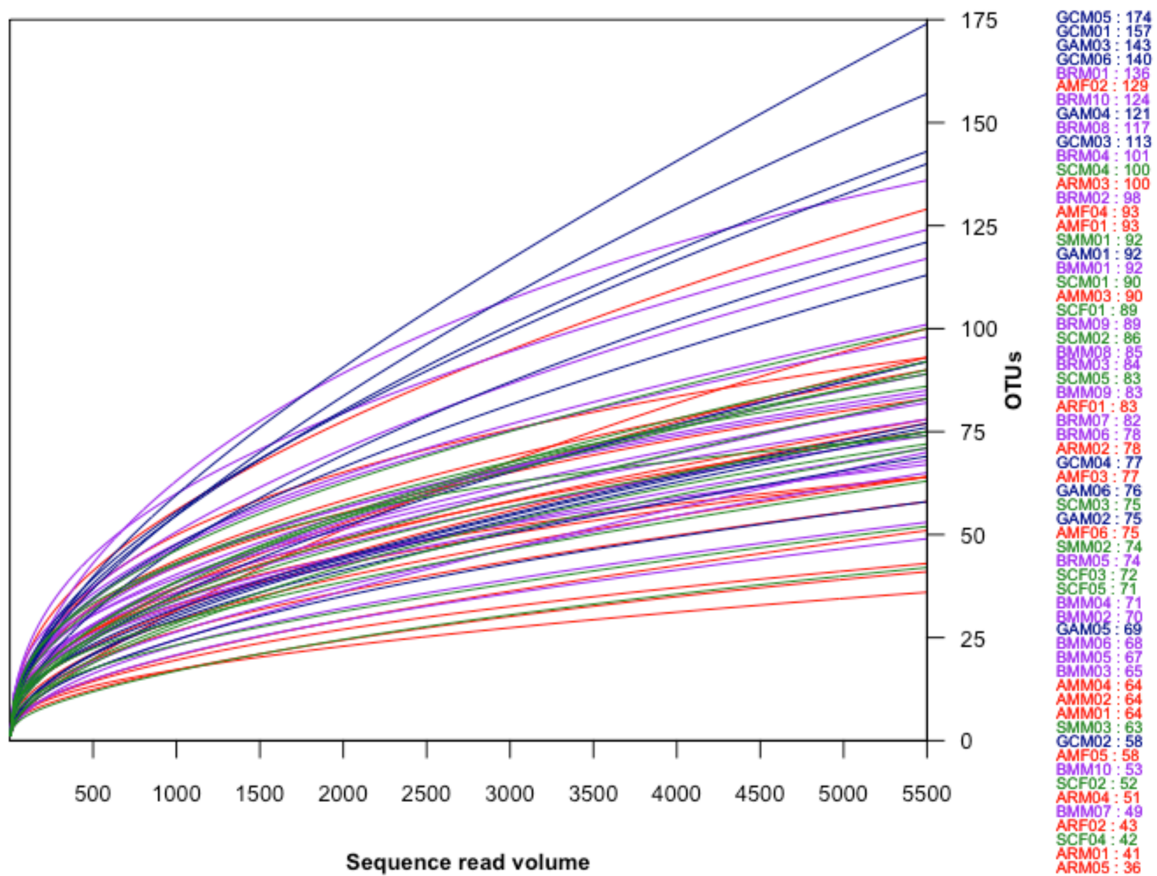


Figure 3.1: Rarefaction curves for field-collected *Bactrocera tryoni* gut samples from Australia and New Caledonia rarefied to 5,500 sequence reads. Labels to the right of the graph indicate the sampled individuals as sorted by number of OTUs. The sample name of individuals is made from combining the population (as described in Table 3.1) and the sample number within the group. Colour of lines and labels represent regions with Atherton Tablelands in red, Brisbane in purple, Sydney in green and Grande Terre, New Caledonia in dark blue.

Table 3.2: Alpha diversity metrics of 62 *Bactrocera tryoni* gut samples from Australia (n = 50) and New Caledonia (n = 12), calculated at 97% identity level, after rarefaction to 5,500 sequence reads. The sample name of individuals includes the population ID (as described in Table 3.1) and the sample number within this population. *N* = number of 16S rRNA gene sequences isolated from the host sample following filtering and chimera removal; OTUs = number of operational taxonomic units calculated at 97 % similarity; Chao1= estimate of OTU richness; Shannon and Simpson indices estimate diversity; Good's equation measures coverage.

Sample Name	N	OTUs	Chao1	Simpson	Shannon	Good
AMF01	29756	89	156.5714	0.7744	2.9456	0.992
AMF02	33336	114	155	0.81	3.4693	0.9924
AMF03	46323	70	121	0.6649	2.4283	0.9938
AMF04	49060	98	180	0.6742	2.6486	0.9925
AMF05	21186	64	154	0.6261	2.0762	0.9935
AMF06	34853	66	126	0.7313	2.6544	0.9955
AMM01	48267	64	111.25	0.7776	2.7973	0.9949
AMM02	26286	72	167.6667	0.7296	2.4759	0.9924
AMM03	17130	82	177.1429	0.7865	2.9007	0.9933
AMM04	63249	63	94.625	0.8131	2.9986	0.9958
ARF01	93804	94	219.3333	0.7777	3.0908	0.9913
ARF02	35419	45	66.375	0.2894	1.0908	0.9965
ARM01	41353	51	99.8571	0.8195	2.9761	0.9955
ARM02	40099	57	195.3158	0.6318	1.9681	0.9889
ARM03	25027	99	104.5	0.4916	1.5411	0.9951
ARM04	38162	46	54	0.5959	1.6839	0.9973
ARM05	70842	33	82.9091	0.6861	1.9884	0.9951
BMM01	63044	56	122.4286	0.5329	1.4723	0.9944
BMM02	29948	64	346	0.66	2.0541	0.9913
BMM03	35483	79	133.6667	0.6866	2.3761	0.9925
BMM04	32145	80	195	0.8782	3.4475	0.9916
BMM05	32959	71	137.1111	0.8036	2.9523	0.9936
BMM06	26876	73	136	0.8151	3.0442	0.9935
BMM07	33479	47	78.625	0.6828	2.0691	0.9958
BMM08	36323	85	127	0.8233	3.2659	0.9935
BMM09	36415	81	204.75	0.8281	3.1206	0.9918
BMM10	33426	103	265.75	0.8001	3.0021	0.9885
BRM01	83882	128	288	0.889	3.9112	0.9882
BRM02	42273	86	197	0.8345	3.3802	0.9933
BRM03	60670	95	248.1111	0.8632	3.5236	0.9904
BRM04	31620	102	200.0769	0.8868	3.7164	0.9907
BRM05	45403	87	228	0.8368	3.2961	0.9913
BRM06	131145	75	485	0.7797	2.9636	0.9925
BRM07	32403	71	125.375	0.9001	3.7888	0.9945
BRM08	35802	115	332	0.8523	3.6797	0.9885
BRM09	41276	96	202.9091	0.8426	3.432	0.9911

BRM10	25161	140	254.375	0.7214	2.8302	0.9889
SMM01	53015	78	148.3	0.8753	3.3723	0.9931
SMM02	52485	80	132.8	0.7853	3.1064	0.994
SMM03	42354	60	200.25	0.7209	2.5146	0.9938
SCF01	25170	96	194.0769	0.8089	2.9784	0.9907
SCF02	65683	52	82.6667	0.5921	2.0651	0.9956
SCF03	45173	71	149.2727	0.3704	1.3389	0.9924
SCF04	43213	38	55	0.565	1.4948	0.9967
SCF05	11783	74	215.4286	0.7015	2.0888	0.9918
SCM01	38299	75	160	0.8351	3.1245	0.9936
SCM02	50299	99	258	0.8483	3.4469	0.9902
SCM03	50529	72	142	0.734	2.4733	0.9935
SCM04	74414	98	196.0769	0.8328	3.3564	0.9907
SCM05	52364	67	162.1429	0.8223	2.9857	0.9933
GAM01	61486	81	198.6	0.8337	2.9949	0.9911
GAM02	45169	71	163.625	0.5078	1.7553	0.9929
GAM03	70096	150	403.3333	0.6715	2.685	0.9825
GAM04	45038	127	435.1	0.5319	2.1161	0.9856
GAM05	50513	68	168.4286	0.5538	1.7738	0.9931
GAM06	45497	74	144.9091	0.4749	1.8715	0.9927
GCM01	19549	186	549.6818	0.5452	2.3563	0.9769
GCM02	34551	76	187.3636	0.3471	1.264	0.9909
GCM03	68417	111	233.2143	0.8389	3.3276	0.9893
GCM04	46641	77	202.3333	0.814	2.7978	0.9913
GCM05	13485	169	387.4	0.6413	2.5759	0.9809
GCM06	11801	185	572.5	0.738	3.0171	0.9773

OTU richness varied between populations, habitats, regions and countries, and did not vary between sex and climatic regions (Table 3.3). Comparing individual *B. tryoni* collected across populations, there was a large variation in OTU abundance from samples collected from the habitats of backyard avocado and carambola in Grande Terre (New Caledonia - see populations GAM and GCM in Fig. 3.2A and avocado and carambola in Fig. 3.2D). This can be explained by the high OTU abundance of some individuals (Fig 3.1). These outliers may have also influenced the mean OTU richness grouped by sex (Fig. 3.2B) and climate (Fig. 3.2C). However, only when the Grande Terre flies were grouped as a region (or country) and then compared with other regions in Australia were their OTU abundance significantly higher (Table 3.3).

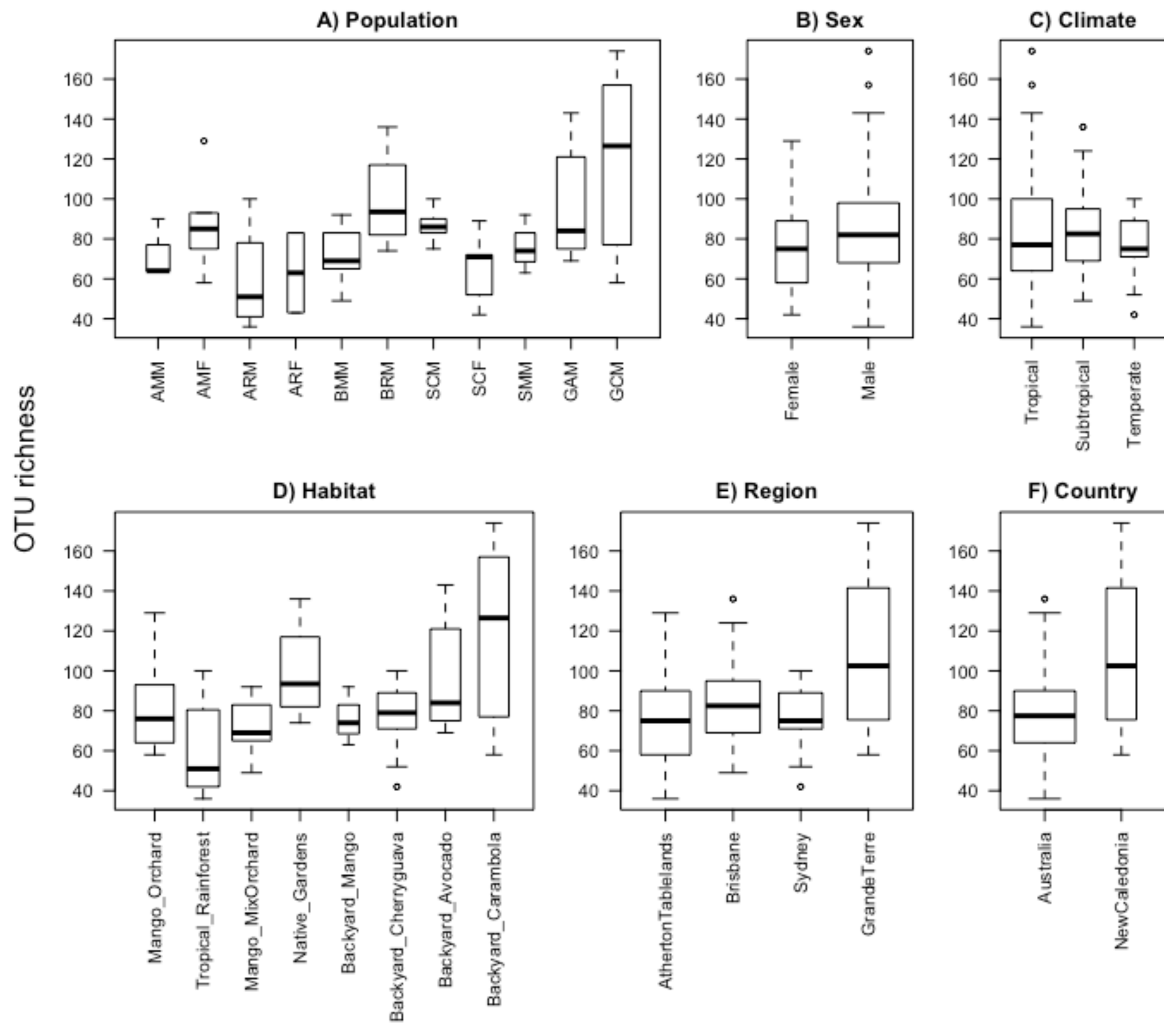


Figure 3.2: *Bactrocera tryoni* gut bacterial OTU richness by A) populations; B) sex C) climate D) habitat E) region; and F) country

Table 3.3: Analysis of variance (ANOVA) of *Bactrocera tryoni* gut bacterial OTU richness

Factor	Df	Sum Sq	Mean Sq	F value	Pr(>F)
Population	10	18756	1875.6	3.212	<0.005
Sex	1	1292	1291.9	1.641	0.205
Climate	2	1149	574.5	0.715	0.493
Habitat	7	16892	2413	4.118	<0.005
Region	3	9783	3261	4.881	<0.005
Country	1	8500	8500	12.740	<0.001

3.3.2: Taxonomic groups and phylogenetic representation

Of the 1,653 OTUs, 36 OTUs (0.02%) were not assigned to any taxonomic lineage while the remaining 1,617 OTUs were grouped into 23 phyla, 51 classes, 79 orders and 280 families. 98% of the reads came from three phyla: Proteobacteria (64% of reads), Firmicutes (20% of reads) and Bacteroidetes (14% of reads). Proteobacteria was the most diverse phylum with 125 families and contained Enterobacteriaceae, which was the most abundant bacterial family with 123,848 reads (36% of reads) and was the second most diverse bacterial family with 194 OTUs (18% of OTUs). The dominant families in Firmicutes included Enterococcaceae (16% of total reads), which was the most diverse bacterial family with 374 OTUs (23% of OTUs); an unidentified family from the order Pasteurellales (14% of reads); and Desulfovibrionaceae (7% of total reads). Porphyromonadaceae (13% of reads) was the most abundant family in the phylum Bacteroidetes.

There was also a marked difference between the sexes when comparing microbial community composition. At the phylum level, apart from similar proportions of Bacteroidetes, the male samples contained a larger proportion of Firmicutes (23%) than females (3.5%) and the females had proportionately more Proteobacteria (76.1%) than males (56.4%) (Figure 3).

At a bacterial family level, males had greater gut bacterial diversity with seven dominant bacterial families compared to females with five dominant bacterial families. The relative abundance of these families varied within each population. The male samples had four dominant Proteobacteria families: Enterobacteriaceae, two unidentified Pasteurellales families and Desulfovibrionaceae. The females had only two dominant Proteobacteria families: Acetobacteraceae and Enterobacteriaceae. Enterobacteriaceae dominated over 60% of relative abundance in females of three populations. Such dominance by any family was not observed for males where the dominant bacterial family in each population changed between each habitat. Males also had two dominant families of Firmicutes; Streptococcaceae and Enterococcaceae. These were, relatively, the second and third most abundant families in males. With Enterococcaceae being absent, females only had Streptococcaceae as a family of Firmicutes. Streptococcaceae in females was similarly abundant as in males. Both females and males had similar proportions of Bacteroidetes which in females consisted of Porphyromonadaceae and a small proportion of Weeksellaceae, while males only contained

Porphyromonadaceae at a similar abundance as in females but lacked Weeksellaceae as a dominant OTU. OTUs that were represented by fewer than 1% of reads were grouped under "others" with male samples having a higher number of OTUs that were less than 1% compared with females.

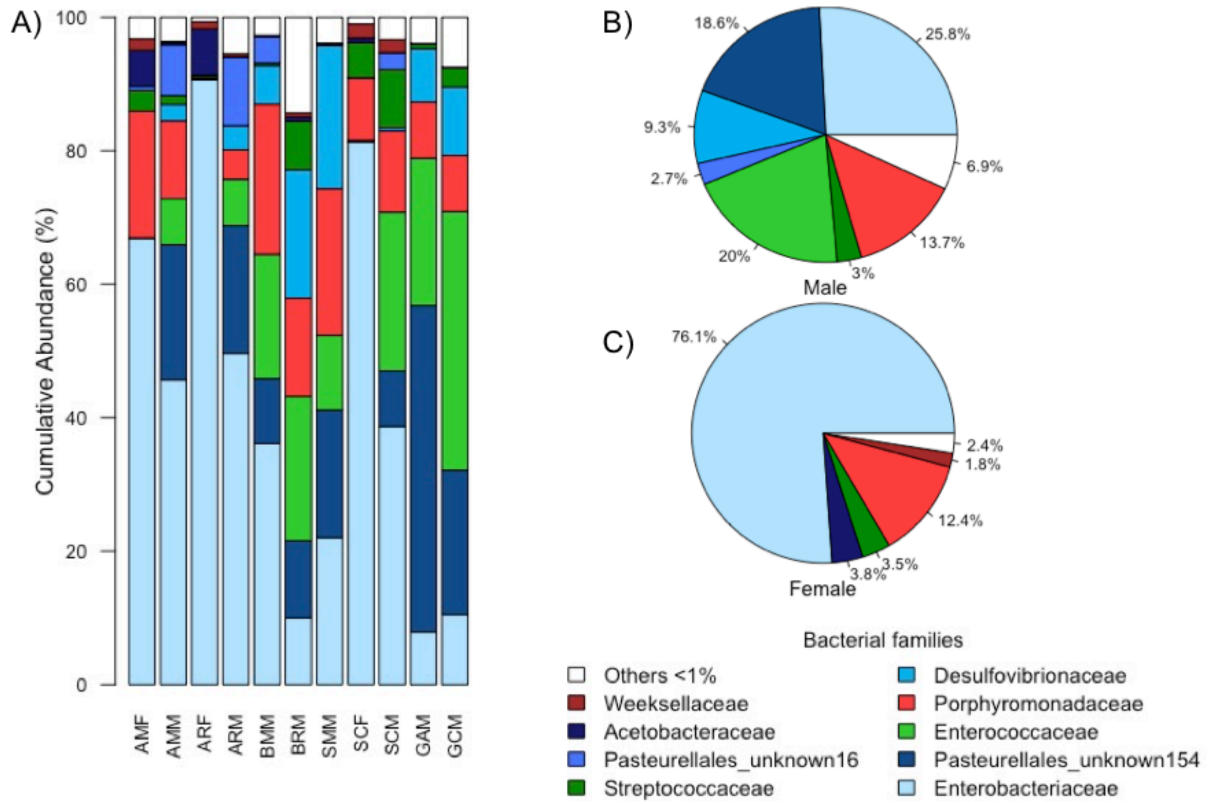


Figure 3.3: OTU abundance of bacterial families in field-collected *Bactrocera tryoni* represented by 16S rRNA gene sequences. (A) Cumulative abundance of bacterial families in populations. Distribution of bacterial families in *B. tryoni* males (B) and females (C). The colour codes represent the bacterial phyla with Proteobacteria in shades of blue, Firmicutes in shades of green, Bacteroidetes in shades of red, and phyla that were less than 1% of total abundance were grouped under “Others” in white.

Also observed was a pattern of co-occurrence and exclusion of particular bacterial families. In females where Enterobacteriaceae was relatively abundant, Enterococcaceae, Desulfovibrionaceae and unidentified Pasteurellales families were at very low abundance, and in some cases absent (Fig. 3.4). Further, Enterococcaceae OTUs and an unknown Pasteurellales OTU154 consistently co-occurred in males, and together made up a similar-sized fraction across male fly groups whereby both families were complementary in their abundance (i.e. more Enterococcaceae occurred with fewer Pasteurellales and vice versa).

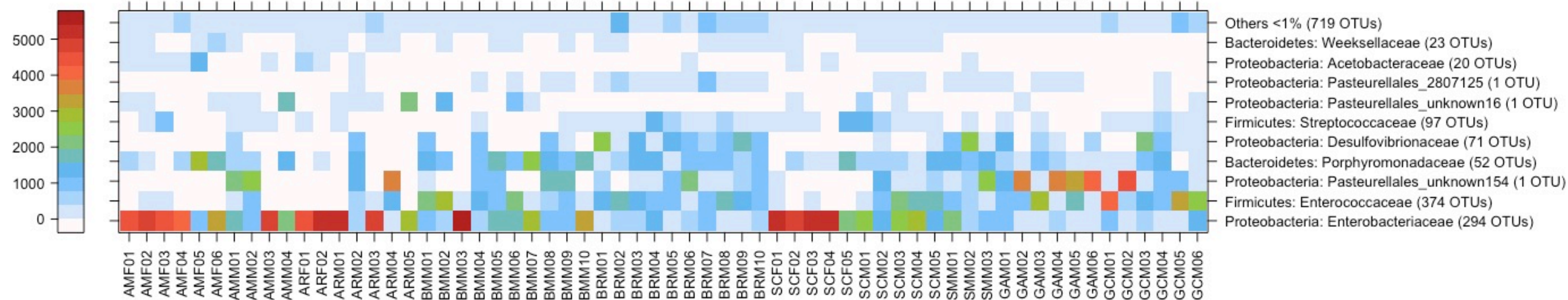


Figure 3.4: Heatmap of bacterial families based on 16S rRNA gene amplicons (rarefied to 5,500 reads) of individual *Bactrocera tryoni* samples from field collections in Australia and New Caledonia.

3.3.3: Core Microbiome

The rarefied OTU table (Appendix Table 3.1) contained 1,653 OTUs that were represented with 341,000 reads. Of these, 1,170 OTUs (70.8% of OTUs) totalled 1,440 reads (0.4% of total reads) and were present with one to two reads per OTU. 483 OTUs (29.2% of OTUs; 339,560 reads; 99.6% of total reads) were present with three to 51,474 sequence reads. Further, there were 1,331 OTUs (80.5% of OTUs) which were present in only one or two samples. To investigate the core microbiome, OTUs with 1 to 2 reads and those with a presence count of 1 to 2 (i.e. only present in 1 to 2 individual samples) were excluded. This correction resulted in 322 OTUs with 337,964 reads. This OTU table subset was then be used to identify the core microbiome.

107 of the 322 core OTUs were shared by all samples, which was higher than the number of OTUs either shared between regions or that were unique to each region (Fig. 3.5A). The *B. tryoni* regional group that contained the largest number of core OTUs were the flies from Brisbane, followed by Grande Terre (New Caledonia), Atherton Tablelands and then Sydney.

When grouped by climatic regions (Fig. 3.5B), the *B. tryoni* samples collected from the tropical habitats contained more core OTUs (92.2%) than sub-tropical and temperate regions, including 45 OTUs that were found only in the tropical samples. In contrast, temperate samples did not harbour any bacterial OTUs that were not also found in sub-tropical or tropical climates. Apart from 164 shared core OTUs (50.9%) between all climatic regions, the next largest shared number of core OTUs was 57 between tropical and sub-tropical habitats, indicating a large similarity between these habitats. Notably, the number of shared OTUs between samples from sub-tropical and temperate habitats was less than the number of shared core OTUs between samples from tropical and temperate habitats (Fig. 3.5B).

The core microbiome was strongly influenced by sex as displayed in Fig. 3.5C. Despite females and males sharing 184 OTUs (57% of the core OTUs), the females only had two additional OTUs that were not found in males, whilst the males had a further 136 unique core OTUs.

Figure 3.5: Venn diagram of core microbiome within *Bactrocera tryoni* gut samples by A) regions B) climate; and C) sex collected from the field in Australia and New Caledonia.

3.3.4: Presence of *Wolbachia*

Three OTUs of the endosymbiont *Wolbachia* sp. were detected in all five male *B. tryoni* collected from the rainforest in the Atherton Tablelands. One OTU was relatively most abundant and accounted for 696 of the total 700 rarefied *Wolbachia* sp. sequence reads. It also appears that these flies with *Wolbachia* had the lowest OTU richness (Fig. 3.2A and 3.2D)

3.3.5: Beta diversity

According to permutational multivariate analysis of variance (PERMANOVA) habitat, region, sex and climate were significant drivers for microbial differences in the composition and structure of gut bacterial communities in individual *B. tryoni* (Table 3.3). Habitat, with the highest R^2 value, was the strongest driver ($P < 0.01$, $R^2 = 0.292$). This was followed by region ($P < 0.001$, $R^2 = 0.164$), sex ($P < 0.001$, $R^2 = 0.121$), countries ($P < 0.001$ $R^2 = 0.061$) and then climate ($P < 0.005$, $R^2 = 0.079$). While there was a significant differentiation between Australia and New Caledonia, there was no differentiation between native and invasive ranges if the Sydney region was considered part of the invasive range.

Table 3.4: Permutational multivariate analysis of variance (PERMANOVA) of OTU reads

	Df	F Model	R^2	Pr (>F)
Habitat	7	3.1836	0.29213	<0.001
Region	4	3.8027	0.16436	<0.001
Sex	1	8.3247	0.12184	<0.001
Country	1	3.9242	0.06139	<0.001
Climate	2	2.5148	0.07855	<0.005
Origins (native vs. invasive)	1	1.3828	0.02253	0.124

Constrained analysis of principal coordinates (CAP; Fig. 3.6) supports the PERMANOVA findings (Table 3.4) and showed patterns of grouping by habitat, region, country and sex. Females grouped tightly together on the first principle coordinate axis (which explained most of the variation) while males were more scattered across the first principle coordinate axis and second principle coordinate axis. There was also some separation between the Australian and New Caledonian samples with Australian flies grouping at the bottom of the second principle coordinate axis and spreading along the first principle coordinate axis, and New Caledonian flies grouping on the top of the second principle coordinate axis with a narrow spread along the first principle coordinate axis.

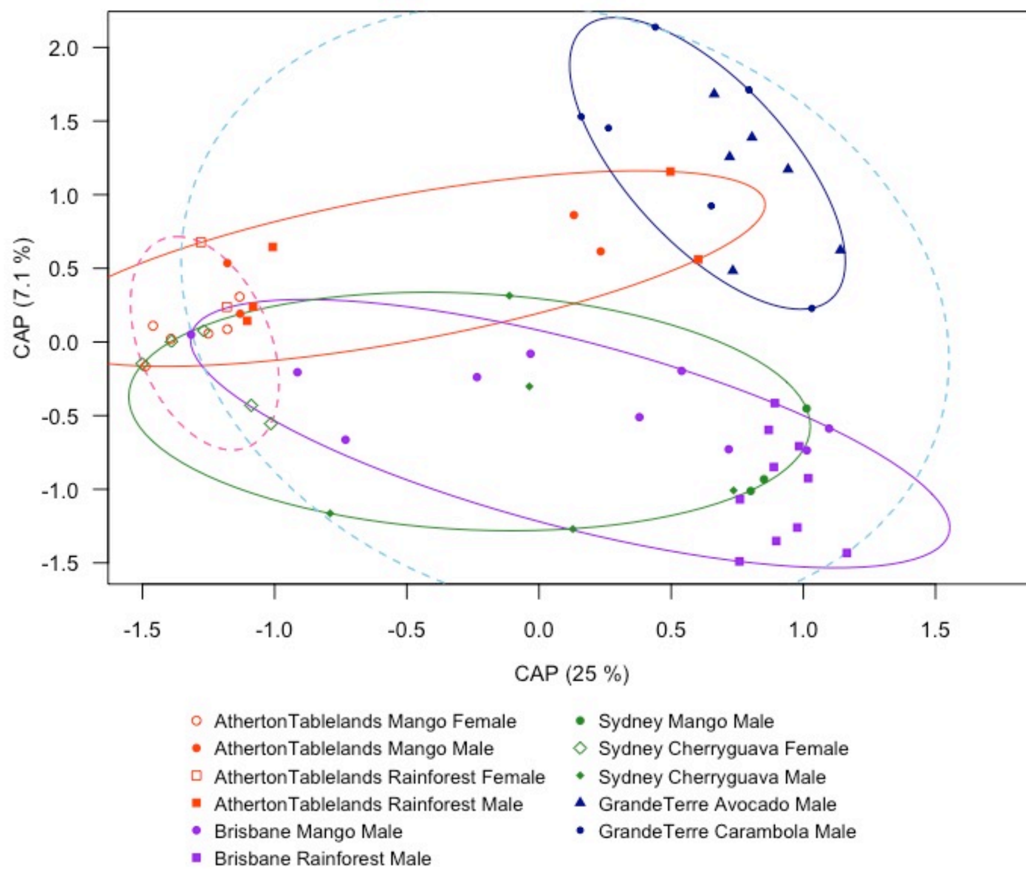


Figure 3.6: Constrained analysis of principal coordinates (CAP) of field-collected *Bactrocera tryoni* from regions in Australia and New Caledonia. Ellipses contain samples at 95% confidence intervals with colours corresponding to each region and symbols corresponding to host plant habitat. The dotted lines are 95% confidence ellipses grouping samples by sex with males in light blue and females in pink.

3.4: Discussion

Gut bacteria are known to assist invasive insect species in the adaptation to and colonisation of new territories (Asiimwe et al. 2014; Hendry et al. 2014; Himler et al. 2011; Russell et al. 2009). Previous research has demonstrated that gut bacteria of tephritids differs across sampling locations (Liu et al. 2016a). However, the dynamics that shape gut bacterial communities across geographical regions, habitats and between native and invasive insects are little understood. Our study is first to investigate the diversity of gut bacteria within individuals of a significant tephritid pest, across both the native and invasive range. For this we have used a non-culture based, molecular method. We demonstrate that the gut bacterial microbiome of *B. tryoni* is significantly influenced by habitat, region, climate and sex.

Comparing bacterial OTU richness of *B. tryoni* between Australia and New Caledonia, the latter were more OTU rich than Australian samples. Since bacteria have been linked to assist insects in processing new food types in new habitats and *B. tryoni* is not native to New Caledonia, it is possible that the invasive *B. tryoni* population that is in New Caledonia has acquired a large diversity and abundance of gut bacteria to assist it in processing the large array of tropical food sources that are different from its native range in Australia.

The bacterial community from field-collected, individually analysed *B. tryoni* was dominated by the phyla Proteobacteria, Firmicutes and Bacteroidetes. Previous findings of pooled samples of closely related species including *Bactrocera neohumeralis, Bactrocera cacuminata, Bactrocera dorsalis, Bactrocera latifrons*, *Bactrocera carambolae* and *Dirioxa pornia* (Liu et al. 2016a; Morrow et al. 2015b; Yong et al. 2017a), as well as of various field-collected insects (Colman et al. 2012; Yun et al. 2014b).

Enterobacteriaceae is the most dominant and diverse bacterial family in both males and female *B. tryoni* as was also evidenced in previous studies on this species (Drew and Lloyd 1987; Morrow et al. 2015b; Thaochan et al. 2009). Our study showed that the relative abundance of Enterobacteriaceae was almost three times higher in females than in males. Similarly, Acetobacteraceae were relatively more abundant in females than in males. This suggests that Enterobacteriaceae and Acetobacteraceae may play different roles in sexes, and/or are more important in females than in males (for example in female reproductive development). The female gut may provide a more conducive environment for

Enterobacteriaceae and Acetobacteraceae to thrive in comparison to the male gut environment. Furthermore, males and females have different foraging behaviours and physiological requirements (e.g. for reproductive maturation). Another sex-specific difference was, that in contrast to females, males had more Desulfovibrionaceae, Enterococcaceae and an unidentified bacterium of Pasteurellales. Porphyromonadaceae and Streptococcaceae were bacterial families with similar relative abundance in male and female B. *tryoni*.

As shown in Chapter 2, diet is known to impact the microbiome of adult *B. tryoni*. Findings from this study indicate that the gut bacterial communities of field-collected male *B. tryoni* differ between habitats and climates, with a sex-effect within. It is feasible that this is linked with the foraging and dispersal of male flies and the variation found between the differing regions. Alternatively, this could support the idea that males disperse further from their site of emergence to locate a suitable mate and are therefore exposed to more diverse bacteria.

Some of the samples from different sites within the same region clustered together (Fig. 3.6). Given all sampling locations within a given region were separated by about 50km, this association could be due to two reasons: similar environmental conditions (similar food sources and climate) and close genetic relationship of populations within a region.

The analysis of the core microbiome revealed a reduction of bacterial species diversity between climatic regions. Notably, there was a reduction of unique OTUs particularly in the temperate region compared with the tropical and sub-tropical regions. This is likely due to the reduced diversity of host plants and suitable habitat for *B. tryoni* in temperate regions as compared to the native climatic regions of tropical and sub-tropical climates.

A strong sex effect was detected when comparing the core microbiome of individual *B. tryoni* samples. The male samples shared almost all the OTUs with females and also contained over 42% more unique OTUs (Fig. 3.5). This large bacterial diversity in the male gut further supports the theory that males disperse further as they search for females, with a more opportunistic feeding habit as they forage on available foods across a range of environments where they are exposed to a larger array of bacteria.

From the eight sampling sites across Australia and New Caledonia, three OTUs of the endosymbiont *Wolbachia* was detected exclusively samples from the rainforest of the

Atherton Tablelands. The short 16S rRNA gene sequences of *Wolbachia* appeared to be close to supergroup B strains (it needs to be noted that short sequence information for a single conserved gene as the 16S rRNA gene provides insufficient resolution for strain identification). Interestingly, the three tested males had more *Wolbachia* sequence reads than the two tested females. Some of this supports previous findings of restriction of *Wolbachia* in *B. tryoni* to tropical northern Australia, however in these earlier studies more detailed characterisation of *Wolbachia* revealed presence of *Wolbachia* strains belonging to the A supergroup (Morrow et al. 2015a; Morrow et al. 2014). These previous studies only had access to male samples collected by cue-lure and did not assess field-collected females. These findings warrant future research efforts to explore the association of *Wolbachia* with *B. tryoni* and its potential as a biocontrol agent (Raphael et al. 2014).

According to CAP, there was a separation between flies from Australian regions (Atherton Tablelands, Brisbane, Sydney Basin) and New Caledonia (Grande Terre) except for some overlap with flies from the Atherton Tablelands and Grande Terre. Habitat, diet, developmental stage and genetics have previously been found to influence microbial diversity in flies (Yun et al. 2014b). It appears that all Australian *B. tryoni* share a similar bacterial community composition despite the gradual climatic and host plant variation across Australia. This may be due to the flies sharing a close genetic relationship (Gilchrist and Meats 2012) which may also impact their bacterial associations. The similar bacterial community of flies from New Caledonia (Grande Terre) to flies from the Atherton Tablelands might suggest a close genetic relationship of flies. The Atherton Tablelands could be the source from which the New Caledonia flies derived, and, thus, the flies obtained a similar microbiome composition, and/or the exposure of flies to similar climatic conditions as both Atherton Tablelands and Grande Terre are within the tropics. There was no significant statistical support to suggest a difference between native and invasive populations when populations from temperate Australia were included as invasive range in the analysis, supporting the idea that the tropical climate may also contribute to similarities between the Atherton Tablelands and New Caledonia. The relatively low p value ($p = 0.124$) from the PERMANOVA (Table 3.3) and an emerging pattern of separation in the CAP plot (Fig. 3.2) seem to suggest that native and invasive *B. tryoni* tend to have differentiated gut microbiomes. Female samples collected from the Atherton Tablelands and the Sydney Basin contained a gut bacterial composition that varied extensively from the males from those

regions. Further, the CAP diagram illustrated that the females had very similar gut bacterial communities across all regions and habitats compared to the male samples which were more spread out. This increased compositional variation in male *B. tryoni* could have multiple explanations. Field males might require diverse bacteria based on their individual needs, search less for specific foods and therefore are exposed to more diverse diets or disperse more than females.

3.5: Conclusion

This study has provided insights into the dynamics of gut bacterial communities in a tephritid pest species by investigating female and male individuals and across invasive and native populations in different climatic regions and habitats. The gut bacterial communities were more similar in females than males across habitats, climatic regions and between native and invasive regions. Therefore, we concluded that either field males had more varying diets or dispersed further from their natal habitat, possibly in search for mate or food. It is unclear whether males simply have more diverse bacterial communities because of their behaviour, or whether they actually profit from this increased diversity of bacteria. Our findings warrant further research into this. If the assumption is correct that males do require this higher bacterial diversity, then this could suggest for the management of tephritid fruit fly pests, and in particular the SIT, releasing adult male tephritids exposed to a pre-release diet might be a better option than releasing pupae or unfed males, so the male tephritids utilise their energy primarily to seek and mate with wild females, thus increasing the success of a SIT programme.

Despite the difference in bacterial composition at OTU, the bacteria are similar at the higher classifications of genus and family. This indicates that bacteria are being substituted for closely related species across populations. This finding also has direct implications in tephritid fruit fly pest management, particularly SIT. Bacteria have been known to improve the performance of sterile male tephritids in laboratory tests (Ben Ami et al. 2010; Gavriel et al. 2010; Hamden et al. 2013). However, the challenge has been in identifying a candidate bacterium or a combination (consortium) of bacteria for application in the field. Our work provides insights into the level of substitution of closely related bacteria.

Chapter 4: Comparing the gut bacteria of field-collected and laboratory-reared Queensland fruit fly, *Bactrocera tryoni*

Abstract

The Queensland fruit fly, *Bactrocera tryoni* (Diptera: Tephritidae) is a serious horticultural pest in Australia and on several Pacific Islands. The area wide management of *B. tryoni* includes the use of the sterile insect technique (SIT) that involves the release of mass-reared fruit flies after they have been sterilised by irradiation. However, irradiation damages the gut of fruit flies. Furthermore, mass-reared sterilised male tephritids used in SIT are known to be less competitive than wild males. It has been hypothesised that supplementing mass-reared and irradiated flies with gut bacteria may improve performance of tephritids in SIT programs. For this a good understanding of bacterial community composition and structure across flies from field populations and fly production facilities is required. Using next-generation sequencing, we compared the gut bacterial communities of *B. tryoni* of field-collected individuals and individuals reared in controlled environments in Australia and New Caledonia in order to identify core bacteria of field collected flies that either are lacking or variable in abundance in flies reared in controlled environments. These bacterial taxa could be potential bacterial candidates to use as probiotics in improving mass-reared irradiated flies to be used in SIT. We found that, at the level of operational taxonomic units (OTUs), the field flies were more diverse than flies from controlled environments. However, at the bacterial family level, the flies had very similar bacterial communities, indicative of substitution of some bacterial taxa for related taxa across sampling locations. Furthermore, we found that the laboratory flies contained the same bacterial genera as the field flies but at different abundance. In the instance of particular bacteria being important for performance of *B. tryoni* we conclude that the challenge to improve the sterile *B. tryoni* microbiome for increased SIT success will not necessarily require the supplementation of bacteria but rather the facilitation of the development of existing microbiota so that released sterile flies, have a similar bacterial abundance to that of field fly population microbiome.

4.1: Introduction

The Queensland fruit fly, *Bactrocera tryoni* (Froggatt) (Diptera: Tephritidae) is a serious horticultural pest in Australia and several Pacific Islands. It is also a key biosecurity threat to

horticultural industries within Australia and across the world. For the control of *B. tryoni,* area wide management programmes have been developed which incorporate the use of the sterile insect technique (SIT) whereby *B. tryoni* are mass-produced and exposed to gamma irradiation to induce reproductive sterility (Clarke et al. 2011; Dominiak and Ekman 2013; Jessup et al. 2007). The success of SIT relies on sterile male insects that, once released into the wild pest population, outcompete field males in successfully mating with wild fertile females. Such sterile matings of irradiated males and field females result in embryonic mortality, and with repeated releases this can result in a reduction or extinction of the local pest population over time (Knipling 1955).

However, sterile male tephritids released in SIT programmes are not as competitive as wild males in mating with wild females (Lance et al. 2000). Mass-production and the exposure to gamma irradiation are known to affect the quality and performance of *B. tryoni* used in SIT (Collins et al. 2008; Gilchrist and Meats 2012). Furthermore, the exposure to gamma irradiation affects the gut bacterial community of *B. tryoni* (Morrow et al. 2015b). The concept of using bacteria as probiotics to improve the performance (including the mating performance) of sterile male tephritids used in SIT has gained momentum in recent times (Estes et al. 2012; Yuval et al. 2010). At the same time, the advancement of sequencing technology allows extensive microbiome characterisation of many flies at cheaper costs. A fundamental question in the search for probiotic candidates is whether flies mass-reared for many generations in controlled environments of fruit fly production facilities have a different bacterial community composition and structure than target pest flies in field populations, and this requires a deeper understanding of the existing microbiome.

In this study, we used next generation 16S rRNA gene amplicon sequencing to compare the bacterial microbiome of *B. tryoni* adult flies collected from the field in Australia and New Caledonia with irradiated and unirradiated *B. tryoni* from captive populations kept on different diets in three different controlled environments. First, we aimed to identify the core microbiome of *B. tryoni* across the different populations and then we identified core bacteria of field fly populations that were either missing in laboratory populations or varied in abundance. This is important step towards the identification of candidate bacteria for further testing and eventual application in the probiotic improvement of *B. tryoni* in SIT programmes.

4.2: Methods

4.2.1: *Bactrocera tryoni* sampling and gut extraction

This study involved the analysis of 145 adult male and female *B. tryoni* samples that were collected from three controlled environments (two in Australia and one in New Caledonia) and eight field populations from tropical, subtropical and temperate Australia and tropical New Caledonia between 2015 and 2016 (see Table 4.1). The analysis of the 145 flies in this chapter includes data obtained from flies used in previous chapters as well as flies not included in any previous chapters. For field-collected flies, 62 mature adult *B. tryoni* were collected and processed as described in Chapter 3.

For controlled environment flies, individuals from four categories of populations were sampled from three controlled environments. The first controlled-environment population comprised 39 mature adults kept at the insect laboratory at the Hawkesbury Institute for the Environment (HIE) as processed and described in Chapter 2. This category also includes groups of flies that were reared on larval diets at the NSW Department of Primary Industries' Elizabeth Macarthur Agricultural Institute (EMAI) entomology laboratory and brought to HIE for adult development after half had been irradiated.

The second controlled-environment category was from the New Caledonian Agronomical Institute (Institut Agronomique Néo-Calédonien, IAC) entomology laboratory. This population consisted of eight mature adult *B. tryoni* that were reared out in a laboratory environment from field-infested fruits of carambola, *Averrhoa carambola* (Oxalidaceae). The infested fruits were placed on sterilised vermiculite in 30cm x 30cm x 30cm insect rearing cages and emerged flies were provided with water but no alternative food sources other than the infested fruits from which they eclosed. The emerged flies were maintained in the laboratory for 14 days. The mature adults were then individually collected in clean specimen jars and individually surface sterilised by sequentially immersing for 1 minute in each of 70% ethanol, sterile distilled water, 0.05% sodium hypochlorite and lastly sterile distilled water. After surface sterilization, individual insects were preserved in 1.5ml centrifuge tubes containing absolute ethanol and shipped to Australia for processing. Upon receipt of the IAC

samples, the insects were surface treated using the same procedure and the insect gut removed and preserved in 1ml of brain heart infusion broth plus 20% glycerol (BHIB+20%Gly) and stored in -80°C as described for samples in Chapter 3.

The third controlled-environment category comprised mature adult *B. tryoni* flies collected from the EMAI entomology laboratory. This population was 50 generations in captivity and was reared as larvae on a gel diet (Moadeli et al. 2017) at EMAI's Fruit Fly Production Facility. The pupae were subdivided into two groups with one half irradiated and the other half unirradiated. This EMAI population contained 12 *B. tryoni* which were reared out at EMAI in small insect rearing cages (30cm x 30cm x 30cm) and were provided yeast hydrolysate, sugar and water as adult diet. Separate cages were set up for irradiated and unirradiated flies. After 14 days, mature adult *B. tryoni* were individually collected using clean specimen jars, surface sterilized, the gut extracted and preserved in BHIB+20%Gly and stored in -80°C as described in Chapter 3.

The fourth controlled-environment population consisted of 24 mature adult *B. tryoni* from the same EMAI source population and was also reared on a gel diet. In the pupal stage, half of the individuals were irradiated, and the other half remained unirradiated. Then, the insects were reared out in a laboratory setup at EMAI designed to imitate a field environment. Therefore, this population was referred to as "EMAI wild". The flies were kept in two large cages (1.5m x 1.5m x 1.5m) within a laboratory room with controlled conditions for fruit fly rearing. One cage contained the irradiated flies and the other cage contained unirradiated flies. A potted orange tree (approximately 1m high) was placed in the middle of each cage. The trees were cleared of any insects before *B. tryoni* pupae were placed inside the cages. The emerged flies were provided with a "wild" diet and consisted of cut oranges and free-range backyard chicken faeces and water which was replenished every 4 days. After 14 days, the mature adult *B. tryoni* were individually collected in sterilised specimen jars, surface sterilised, gut extracted and preserved in BHIB+20%Gly and stored in -80°C as described in Chapter 3.

Table 4.1: *Bactrocera tryoni* experimental treatment groups. For field-collected samples, the first letter indicates the region from which the fly was sampled, the second letter the habitat, and the third letter is the sex. For the controlled environment (laboratory reared) flies, the first letter represents the laboratory or facility of pupae origin (E, EMAI is Elizabeth Macarthur Agriculture Institute; H, HIE is Hawkesbury Institute for the Environment; NC, IAC is Institut Agronomique Néo-Calédonien), the second letter represents the larval diet (C, carrot; L, lucerne; G, gel), the third letter represents the adult diet (Y, yeast and sugar; S, sugar only; W adult diet more similar to wild environments), the fourth letter indicates if they were exposed to irradiation or not (I, irradiated; U, unirradiated), and the fifth letter is the sex (F, female; M, male).

Treatment Group	**Country**	**Region or controlled adult environment**	**Location**	**Habitat**	**Population**	**Sex**	**Irradiation**	**Larval diet**	**Adult diet**	**Number of samples (*n*)**
AMF	Australia	Atherton Tablelands	Walkamin (-17.133767, 145.427046)	Mango (research orchard)	Field	Female	Unirradiated	Field	Field	6
AMM	Australia	Atherton Tablelands	Walkamin (-17.133767, 145.427046)	Mango (research orchard)	Field	Male	Unirradiated	Field	Field	4
ARF	Australia	Atherton Tablelands	Koah (-16.872813, 145.580178)	Native rainforest	Field	Female	Unirradiated	Field	Field	2
ARM	Australia	Atherton Tablelands	Koah (-16.872813, 145.580178)	Native rainforest	Field	Male	Unirradiated	Field	Field	5
BMM	Australia	Brisbane	Redlands (-27.526987, 153.250875)	Mango (backyard)	Field	Male	Unirradiated	Field	Field	10
BRM	Australia	Brisbane	Mt Coot-tha (-27.476991, 152.974465)	Native rainforest collection	Field	Male	Unirradiated	Field	Field	10
SCF	Australia	Sydney	Richmond (-33.610681, 150.747221)	Cherry guava (backyard)	Field	Female	Unirradiated	Field	Field	5
SCM	Australia	Sydney	Richmond (-33.610681, 150.747221)	Cherry guava (backyard)	Field	Male	Unirradiated	Field	Field	5
SMM	Australia	Sydney	Ourimbah (-33.357794, 151.382673)	Mango (mixed research orchard)	Field	Male	Unirradiated	Field	Field	3
GAM	New Caledonia	Grand Terre	Bourail	Avocado (backyard)	Field	Male	Unirradiated	Field	Field	6

			(-21.567204, 165.498438)							
GCM	New Caledonia	Grand Terre	Pocquereux (-21.748831, 165.921469)	Carambola (backyard)	Field	Male	Unirradiated	Field	Field	6
NCCUF	New Caledonia	IAC	IAC (-22.102436, 166.318993)	Field infested carambola in lab	IAC F1	Female	Unirradiated	Carambola	Carambola	4
NCCUM	New Caledonia	IAC	IAC (-22.102436, 166.318993)	Field infested carambola in lab	LIAC F1	Male	Unirradiated	Carambola	Carambola	4
EGWIF	Australia	EMAI	EMAI (-34.117459, 150.716986)	Lab	EMAI Wild	Female	Irradiated	Gel	Wild	6
EGWIM	Australia	EMAI	EMAI (-34.117459, 150.716986)	Lab	EMAI Wild	Male	Irradiated	Gel	Wild	6
EGWUF	Australia	EMAI	EMAI (-34.117459, 150.716986)	Lab	EMAI Wild	Female	Unirradiated	Gel	Wild	6
EGWUM	Australia	EMAI	EMAI (-34.117459, 150.716986)	Lab	EMAI Wild	Male	Unirradiated	Gel	Wild	6
EGYIF	Australia	EMAI	EMAI (-34.117459, 150.716986)	Lab	Lab	Female	Irradiated	Gel	Yeast & sugar	3
EGYIM	Australia	EMAI	EMAI (-34.117459, 150.716986)	Lab	Lab	Male	Irradiated	Gel	Yeast & sugar	3
EGYUF	Australia	EMAI	EMAI (-34.117459, 150.716986)	Lab	Lab	Female	Unirradiated	Gel	Yeast & sugar	3
EGYUM	Australia	EMAI	EMAI (-34.117459, 150.716986)	Lab	Lab	Male	Unirradiated	Gel	Yeast & sugar	3
ELSIF	Australia	HIE (larval development at EMAI)	HIE (-33.611206, 150.745239)	Lab	Lab	Female	Irradiated	Lucerne	Sugar gel	3
ELSIM	Australia	HIE (larval development at EMAI)	HIE (-33.611206, 150.745239)	Lab	Lab	Male	Irradiated	Lucerne	Sugar gel	4

ELSUF	Australia	HIE (larval development at EMAI)	HIE (-33.611206, 150.745239)	Lab	Lab	Female	Unirradiated	Lucerne	Sugar gel	4
ELSUM	Australia	HIE (larval development at EMAI)	HIE (-33.611206, 150.745239)	Lab	Lab	Male	Unirradiated	Lucerne	Sugar gel	3
ELYIF	Australia	HIE (larval development at EMAI)	HIE (-33.611206, 150.745239)	Lab	Lab	Female	Irradiated	Lucerne	Yeast-sugar gel	3
ELYIM	Australia	HIE (larval development at EMAI)	HIE (-33.611206, 150.745239)	Lab	Lab	Male	Irradiated	Lucerne	Yeast-sugar gel	3
ELYUF	Australia	HIE (larval development at EMAI)	HIE (-33.611206, 150.745239)	Lab	Lab	Female	Unirradiated	Lucerne	Yeast-sugar gel	3
ELYUM	Australia	HIE (larval development at EMAI)	HIE (-33.611206, 150.745239)	Lab	Lab	Male	Unirradiated	Lucerne	Yeast-sugar gel	3
HCSUF	Australia	HIE (larval development at HIE)	HIE (-33.611206, 150.745239)	Lab	Lab	Female	Unirradiated	Carrot	Sugar gel	3
HCSUM	Australia	HIE (larval development at HIE)	HIE (-33.611206, 150.745239)	Lab	Lab	Male	Unirradiated	Carrot	Sugar gel	3
HCYUF	Australia	HIE (larval development at HIE)	HIE (-33.611206, 150.745239)	Lab	Lab	Female	Unirradiated	Carrot	Yeast-sugar gel	4
HCYUM	Australia	HIE (larval development at HIE)	HIE (-33.611206, 150.745239)	Lab	Lab	Male	Unirradiated	Carrot	Yeast-sugar gel	3

4.1.1: DNA extraction and amplicon sequencing

The extraction of DNA from *B. tryoni* gut tissue and 16S rRNA gene amplicon sequencing were undertaken as outlined in Chapters 2 and 3. The DNA of individual *B. tryoni* samples was extracted using the QIAmp DNA mini kit (Qiagen), including RNase treatment, and elution in 50µL of nuclear-free water. The volume of the DNA solution was then reduced to approximately 15µL to increase the DNA concentration by using a vacuum concentrator. DNA quality and concentration were assessed using the Qubit 2.0 Fluorometry.

The DNA was submitted to the Hawkesbury Institute for the Environment (HIE) Next-Generation Sequencing Facility for 16S rRNA gene amplicon sequencing using primers 341F and 805R, which span the variable V3 and V4 regions. The Nextera XT kit was used for the library preparation of the 145 *B. tryoni* gut DNA samples. Sequencing of 2 x 300bp paired ends was performed on a 384-multiplexed Illumina MiSeq run.

4.1.2: Sequence analysis

The analysis of the MiSeq data was performed in QIIME 1.8 (Caporaso et al. 2010) and followed the same protocols and tools as outlined in Chapters 2 and 3. After quality control checks, the *pick_open_reference_otus.py* command was used to assign operational taxonomic units (OTUs) at 97% identity. This also removed singletons. Chimeric sequences were detected and removed using the *Blast_fragments* approach.

4.1.3: Statistical analysis

The statistical analyses were done in R language and environment for statistical computing and graphics (R Core Team 2018) by using the same tools as outlined in Chapters 2 and 3. The rarefaction curves were created by the *rarecurve* command of the Vegan package (Oksanen et al. 2018). The stacked abundance of the OTU bar plot was created using *base R* plot and commands. The heatmaps were created by using the *levelplot* command of the Lattice package (Sarkar 2008). To investigate the influence of treatment factors in the entire

dataset, the permutation multivariate analysis of variance (PERMANOVA) was calculated using the *Adonis* function of the Vegan package. The interaction between treatment factors and microbial communities was visualised using constrained analysis of principal co-ordinates (CAP). Prior to ordination, a Bray-Curtis dissimilarity matrix of relative abundance was standardised by a *Hellinger* transformation to reduce the impact of rarer OTUs (Legendre and Gallagher 2001). CAP was fitted using the function *capscale* from the Vegan package and constrained by treatment factors. The CAP plot was created in R and the 95% confidence ellipses were drawn using *ordihull* function of Vegan. The Venn diagram of OTUs was calculated using the *vennDiagram* command of the Limma package (Ritchie et al. 2015).

4.2: Results

4.2.1: Alpha diversity measures

A total of 145 libraries from individual fly guts were high-throughput amplicon sequenced for their 16rRNA gene using primers 341R and 805R (approximately 460bp). After filtering, OTU picking, singleton and chimera removal, a total of 5,364,884 reads remained with a range from 1,460 to 130,342 per fly library (Table 4.2).

Table 4.2: Alpha diversity metrics of 145 *Bactrocera tryoni* gut samples from cultured and wild, sterile and fertile adults from Australia and New Caledonia calculated at 97% identity level, after rarefaction to 1450 sequence reads. Sample IDs are as per Table 4.1. *N* = number of 16S rRNA gene sequences isolated from the host sample following filtering and chimera removal; OTUs = number of operational taxonomic units calculated at 97 % similarity; Chao1= estimate of species richness; Shannon and Simpson indices estimate diversity; Good's equation measures coverage.

Sample Name	*N*	OTUs	Simpson	Shannon	Chao1
AMF01	29,788	49	0.7743	2.9164	143.5000
AMF02	33,375	69	0.8041	3.4040	159.0000
AMF03	46,591	34	0.6674	2.4366	47.0000
AMF04	49,107	59	0.6795	2.6354	86.0833
AMF05	21,216	31	0.6203	2.0333	49.2000
AMF06	34,932	42	0.7240	2.6127	48.1111
AMM01	48,306	44	0.7795	2.8347	79.0000
AMM02	26,380	34	0.7397	2.4699	58.0000
AMM03	17,130	44	0.7916	2.9340	52.2500
AMM04	63,289	39	0.8158	3.0235	46.3333
ARF01	93,908	51	0.7688	2.9838	70.0000
ARF02	35,413	28	0.2979	1.1248	54.2500
ARM01	41,351	21	0.6818	1.9675	34.7500
ARM02	40,161	41	0.8165	2.9801	66.5000
ARM03	25,034	38	0.6412	1.9776	113.0000
ARM04	38,257	23	0.4885	1.4872	45.0000
ARM05	70,555	20	0.5921	1.6837	27.0000
BMM01	33,521	47	0.7976	3.0064	131.3333
BMM010	63,076	28	0.5310	1.4845	54.0000
BMM02	30,113	28	0.6574	2.0370	54.2500
BMM03	35,486	40	0.6949	2.4129	95.2000
BMM04	30,228	47	0.8647	3.2926	122.0000
BMM05	33,009	43	0.7985	2.9501	85.7500
BMM06	27,009	45	0.8105	3.0462	60.1111
BMM07	33,483	33	0.6833	2.1090	110.0000
BMM08	36,344	54	0.8314	3.3086	108.1667
BMM09	36,482	38	0.8270	3.0568	46.2500
BRM01	25,180	80	0.7404	2.9192	132.9286
BRM010	83,108	68	0.8892	3.8881	98.0000
BRM02	42,436	52	0.8234	3.3231	71.0000
BRM03	60,720	63	0.8676	3.6071	138.4286
BRM04	31,605	58	0.8839	3.6897	98.6250
BRM05	45,473	52	0.8404	3.3494	88.1429
BRM06	130,342	41	0.7692	2.8784	65.0000
BRM07	32,382	44	0.8988	3.7664	72.5000

BRM08	35,780	63	0.8469	3.6346	150.0000
BRM09	41,492	46	0.8545	3.4759	65.1250
SMM01	53,055	47	0.8728	3.3336	89.1667
SMM02	51,511	47	0.7813	3.0982	64.0000
SMM03	42,878	26	0.7228	2.5290	35.3333
GAM01	61,531	40	0.8509	3.1588	87.5000
GAM02	45,246	33	0.5000	1.7138	48.1667
GAM03	70,471	65	0.6899	2.7853	170.0000
GAM04	45,062	46	0.5214	2.0256	63.2727
GAM05	50,669	37	0.5314	1.6469	94.7500
GAM06	45,539	37	0.4333	1.7376	100.3333
GCM01	19,730	80	0.5426	2.3352	1,565.0000
GCM02	34,623	31	0.3552	1.2627	88.0000
GCM03	68,556	54	0.8606	3.4521	104.1429
GCM04	46,546	33	0.8226	2.8519	54.0000
GCM05	13,559	76	0.6572	2.5703	217.4286
GCM06	11,827	69	0.7308	2.9438	156.8750
SCF01	25,175	40	0.8150	2.9411	61.3750
SCF02	65,690	30	0.6181	2.1331	60.0000
SCF03	45,180	36	0.3728	1.3455	106.0000
SCF04	43,219	16	0.5620	1.4505	26.5000
SCF05	11,840	38	0.7057	2.1047	93.2000
SCM01	38,544	41	0.8394	3.0971	49.2727
SCM02	50,685	52	0.8483	3.4348	85.0000
SCM03	50,719	50	0.7346	2.5271	90.6250
SCM04	74,581	46	0.8387	3.3119	57.3750
SCM05	52,532	41	0.8232	3.0257	88.5000
NCCUF01	43,833	35	0.3516	1.3196	48.3333
NCCUF02	18,551	53	0.7692	2.8639	93.6250
NCCUF03	6,608	90	0.7813	3.1612	273.2727
NCCUF04	14,782	25	0.7002	1.9835	55.3333
NCCUM01	9,000	80	0.8066	3.3400	237.6667
NCCUM02	10,667	71	0.6539	2.5354	134.0769
NCCUM03	18,289	34	0.2466	1.0694	59.5000
NCCUM04	5,599	175	0.8589	4.6056	334.4643
EGWIF01	70,940	46	0.8943	3.6827	98.5000
EGWIF02	32,560	43	0.6650	2.4755	60.0000
EGWIF03	42,824	64	0.8728	3.5146	138.3750
EGWIF04	26,331	37	0.6983	2.4700	79.7500
EGWIF05	26,272	55	0.8541	3.1953	103.3333
EGWIF06	72,402	40	0.8298	2.9252	71.6667
EGWIM01	49,421	58	0.9249	4.1936	77.0000
EGWIM02	34,563	81	0.8543	3.7666	163.0000

EGWIM03	52,521	52	0.7806	3.0403	117.0000
EGWIM04	46,883	49	0.7380	2.5216	74.6667
EGWIM05	62,264	53	0.7591	2.9815	84.6250
EGWIM06	26,539	68	0.8167	3.4837	135.6667
EGWUF01	38,387	39	0.8472	3.1461	69.6000
EGWUF02	32,892	55	0.6859	2.4137	148.5000
EGWUF03	48,871	28	0.6858	2.3045	33.1429
EGWUF04	48,076	35	0.6105	2.1835	80.3333
EGWUF05	38,986	44	0.8055	3.0104	80.1429
EGWUF06	50,411	41	0.8587	3.2075	66.5000
EGWUM01	26,277	69	0.9236	4.3634	92.0000
EGWUM02	38,723	82	0.7929	3.3635	192.0000
EGWUM03	53,802	63	0.8231	3.4712	113.1429
EGWUM04	54,031	56	0.8238	3.3623	73.1000
EGWUM05	23,528	77	0.8860	4.0659	112.7692
EGWUM06	31,421	63	0.7707	3.0024	99.1111
EGYIF01	41,317	31	0.6323	2.2918	46.6000
EGYIF02	1,460	51	0.8683	3.5283	93.1667
EGYIF03	42,784	57	0.7932	3.2760	74.2727
EGYIM01	34,476	47	0.7545	2.9654	139.0000
EGYIM02	53,353	64	0.9077	4.1762	145.2000
EGYIM03	69,460	64	0.8798	3.7088	152.0000
EGYUF01	19,684	40	0.7772	2.7041	78.2500
EGYUF02	16,537	60	0.7670	3.0145	122.1429
EGYUF03	39,875	39	0.8405	3.2585	79.0000
EGYUM01	56,323	44	0.8286	2.9797	82.5000
EGYUM02	49,294	55	0.9297	4.1955	115.0000
EGYUM03	81,139	43	0.6122	2.2608	74.6667
ELSIF01	32,709	21	0.8103	2.7029	30.3333
ELSIF02	25,560	21	0.7462	2.3054	30.3333
ELSIF03	57,820	19	0.6285	1.9128	26.0000
ELSIM01	20,812	24	0.6082	2.0908	39.0000
ELSIM02	46,186	20	0.5093	1.5489	25.2500
ELSIM03	41,473	24	0.7328	2.4362	45.0000
ELSIM04	12,549	28	0.7382	2.3260	37.1667
ELSUF01	31,329	13	0.0950	0.4064	18.0000
ELSUF02	22,512	24	0.5410	1.4200	42.2000
ELSUF03	22,602	20	0.6168	1.7569	30.5000
ELSUF04	13,825	19	0.5629	1.6857	31.0000
ELSUM01	22,733	14	0.3759	1.1276	15.5000
ELSUM02	19,646	12	0.6427	1.8759	13.5000
ELSUM03	18,796	15	0.4669	1.4578	20.0000
ELYIF01	15,870	15	0.7499	2.3070	30.0000

ELYIF02	26,319	20	0.7390	2.3756	22.5000
ELYIF03	18,922	17	0.6365	1.8627	27.5000
ELYIM01	42,804	23	0.7778	2.5510	30.0000
ELYIM02	29,958	26	0.7416	2.4543	28.5000
ELYIM03	37,026	25	0.7263	2.3239	43.3333
ELYUF01	14,510	22	0.7901	2.5175	26.2000
ELYUF02	20,553	47	0.6919	2.1555	97.7500
ELYUF03	30,794	19	0.7038	2.1125	26.0000
ELYUM01	14,053	26	0.7931	2.5893	33.5000
ELYUM02	31,882	20	0.7478	2.4289	29.3333
ELYUM03	27,429	19	0.7612	2.4661	29.5000
HCSUF01	14,748	10	0.1340	0.4691	11.5000
HCSUF02	16,559	10	0.0804	0.3276	12.0000
HCSUF03	26,973	13	0.3809	1.1810	18.0000
HCSUM01	14,144	18	0.6131	1.7587	18.7500
HCSUM02	25,463	10	0.0569	0.2614	11.0000
HCSUM03	19,835	14	0.4594	1.3605	29.0000
HCYUF01	32,549	13	0.2251	0.7252	28.0000
HCYUF02	25,065	4	0.0041	0.0247	7.0000
HCYUF03	31,277	16	0.3801	1.1953	23.5000
HCYUF04	7,850	9	0.3451	1.0285	15.0000
HCYUM01	24,828	9	0.3875	1.1273	15.0000
HCYUM02	31,924	9	0.1279	0.4465	15.0000
HCYUM03	12,011	8	0.0206	0.1067	18.0000

Sequence reads were rarefied to 1,450 which was just below the lowest sequence read number of 1,460 from sample EGYIF02 (Table 4.2). The rarefaction captured all samples as indicated by the rarefaction curve (Fig. 4.1) and Good's coverage analysis (Table 4.2). A total of 1,301 operational taxonomic units (OTUs) were observed (Appendix Table 4.1). The highest number of OTUs was found in a sample belonging to treatment group NCCUMN, which comprised adult males reared out in the IAC laboratory from field-infested carambola fruits, collected at Pocquereux in New Caledonia. This treatment group also has the largest number of OTUs (Figs 4.2A, 2B and 2C).

Treatment groups ELSIF, ELSIM, ELSUF, ELSUM, ELYIF, ELYIM, ELYUF, ELYUM, HCSUF, HCSUM, HCYUF and HCYUM were least diverse with low number of OTUs (Figs. 4.2A, B and C). All of these treatment groups were reared as adults at the HIE laboratory, with prior larval rearing of some flies at HIE, while others were reared as larvae at EMAI, followed by irradiation of one part of these EMAI flies (Chapter 2).

In comparison, treatment groups EGYIF, EGYIM, EGYUF and EGYUM, which developed at the EMAI both in larval and adult stages, were more OTU diverse and had similar OTU richness to the field-collected flies from Australia and New Caledonia and the controlled environment flies which were fed the wild diet (Figs. 4.2A, B, and C). There were not differences in OTU richness between the irradiated and unirradiated flies (Fig. 4.2D)

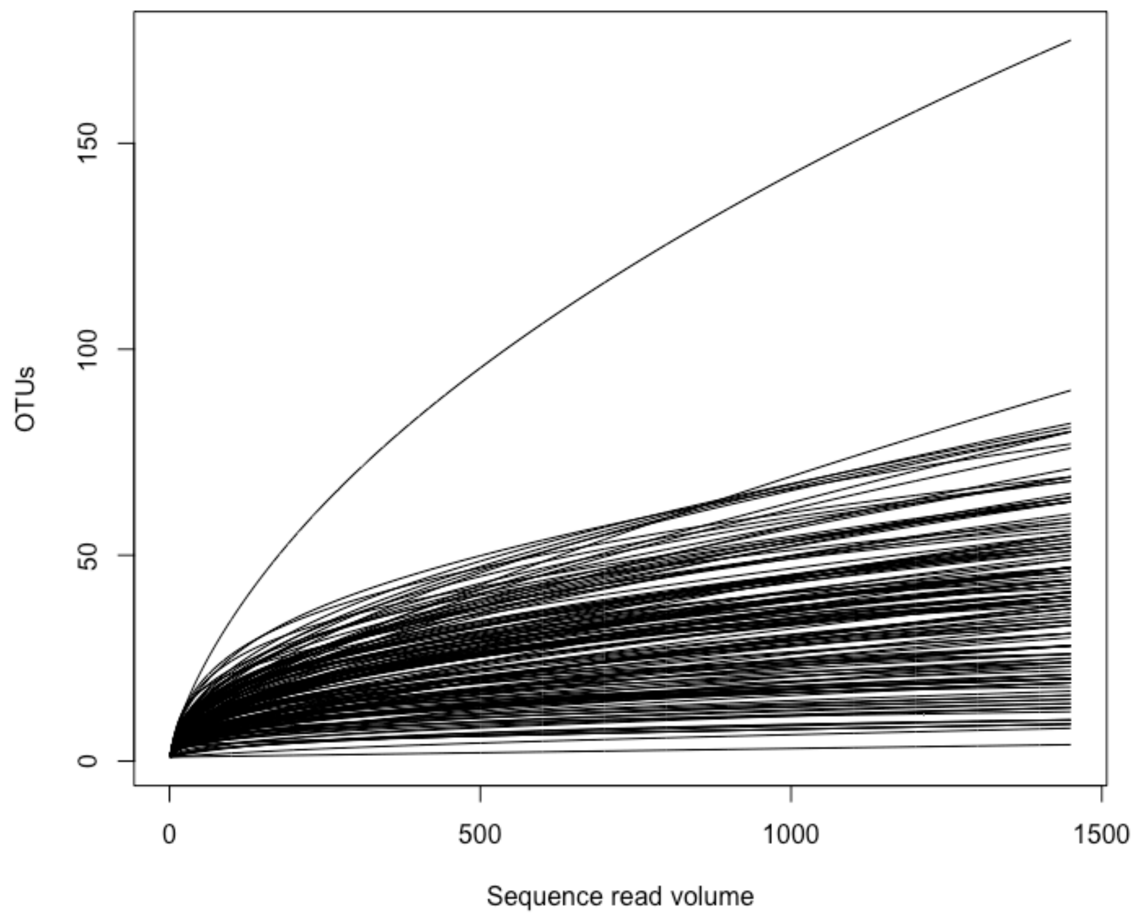


Figure 4.1: Rarefaction curves showing OTU coverage of *Bactrocera tryoni* samples.

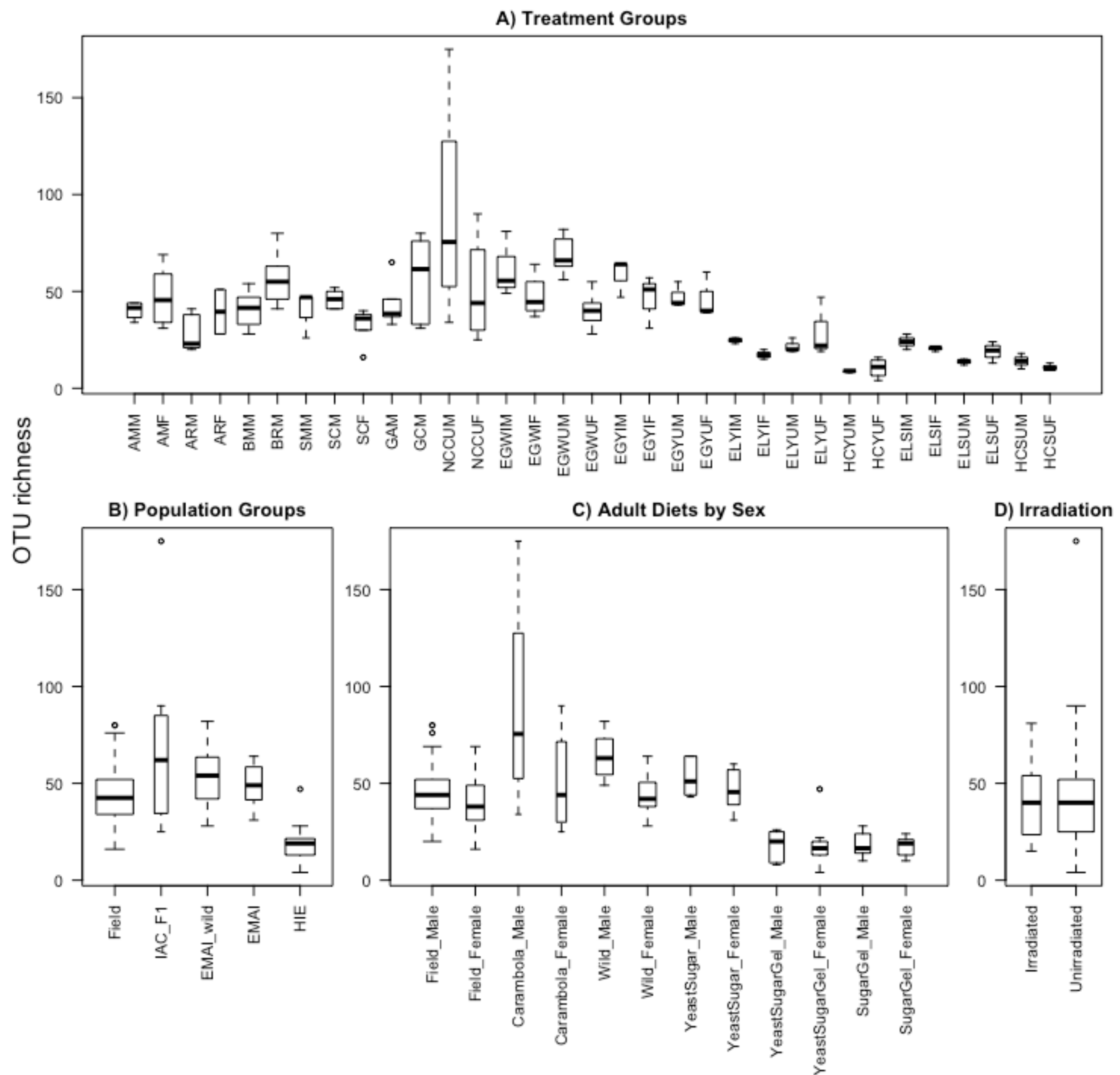


Figure 4.2: Bacterial OTU richness of *Bactrocera tryoni* by A) treatment groups, B) population groups (note that HIE here includes all flies that were kept as adult flies at HIE, and also included the flies obtained from EMAI with or without irradiation), C) adult diets and sex, and D) exposure to irradiation.

Comparing the OTU distribution across field-collected and controlled-environment populations, the most OTU-rich population group was the field collected flies which had 784 OTUs (60% of total number OTUs) of which 535 were unique (41% of total number OTUs) (Fig. 4.3 and Table 4.3). The IAC F1 and EMAI-wild had similar OTU richness of 332 and 362 OTUs respectively while flies kept in adult stage at HIE were least diverse (i.e. flies with larval development at EMAI had 213 OTUs and flies with both larval and adult development at HIE had 158 OTUs). The largest number of shared OTUs was 54 OTUs shared by the field, IAC, EMAI-wild and EMAI. With 249 OTUs, field flies had the largest number of shared OTUs between one or multiple population groups, although this is not too different from the IAC, EMAI-wild and EMAI flies which each shared 177, 202 and 147 OTUs, respectively. However, the HIE population only shared 66 OTUs with all the other sample groups.

All sampling groups shared a total of 16 OTUs (Fig 4.3). 14 of these OTUs were Enterobacteriaceae with the majority of the isolates likely belonging to the genera *Providencia*, *Morganella* and *Enterobacter* (Table 4.4). The shorter fragments of DNA used in the analysis do not allow for a more accurate identification of the OTUs. There were two Acetobacteraceae OTUs shared between all sampling groups and they are likely to belong to *Asaia* and *Commensalibacter* species.

Figure 4.3: Venn diagram of bacterial OTUs within *Bactrocera tryoni* by population groups.

Table 4.3: Number of OTUs of *Bactrocera tryoni* present in population groups.

OTUs	Field	IAC-F1	EMAI-wild	EMAI	HIE
Unique	535	155	160	66	92
Shared	249	177	202	147	66
Total	784	332	362	213	158

Table 4.4: The 16 OTUs of *Bactrocera tryoni* present in all population groups. All listed BLAST search results were at ≥ 98% sequence identity.

OTU	Family	MiSeq Identification	BLAST search of closest match
814266	Acetobacteraceae	unidentified	*Asaia* sp.
2499164	Acetobacteraceae	unidentified	*Commensalibacter* sp.
2529285	Enterobacteriaceae	*Enterobacter* sp.	*Providencia* sp. / *Enterobacter* sp.
4439606	Enterobacteriaceae	*Morganella morganii*	*Morganella morganii*
922761	Enterobacteriaceae	*Morganella* sp.	*Morganella* sp.
1122622	Enterobacteriaceae	*Providencia* sp.	*Providencia* ps.
3101394	Enterobacteriaceae	*Providencia* sp.	*Providencia* sp.
572750	Enterobacteriaceae	*Trabulsiella* sp.	*Citrobacter* sp.
676211	Enterobacteriaceae	unidentified	*Citrobacter* sp.
581021	Enterobacteriaceae	unidentified	*Klebsiella* sp. / *Enterobacter* sp.
720489	Enterobacteriaceae	unidentified	*Morganella* sp.
4418165	Enterobacteriaceae	unidentified	*Pluralibacter* sp. / *Enterobacter* sp.
329096	Enterobacteriaceae	unidentified	*Pseudocitrobacter* sp./ *Cronobacter* sp. / *Salmonella* sp.
119010	Enterobacteriaceae	unidentified	Uncultured bacterium
3232397	Enterobacteriaceae	unidentified	Uncultured bacterium
New.ReferenceOTU209	Enterobacteriaceae	unidentified	Uncultured bacterium

4.2.2: Taxonomic groups and phylogenetic distribution

Across all samples, the gut bacterial community of mature *B. tryoni* is dominated by the three phyla of Proteobacteria (74%), Firmicutes (18%) and Bacteroidetes (7%). Grouping the OTUs by family, seven bacterial families were dominant in treatment groups (Fig. 4.4). Dominant families were Enterobacteriaceae, Pasteurellaceae, Acetobacteraceae, Desulfovibrionaceae (all four Proteobacteria), Enterococcaceae, Streptococcaceae (both Firmicutes) and Porphyromonadaceae (Bacteroidetes). The remaining bacterial families each accounted for less than 1% of the total OTUs.

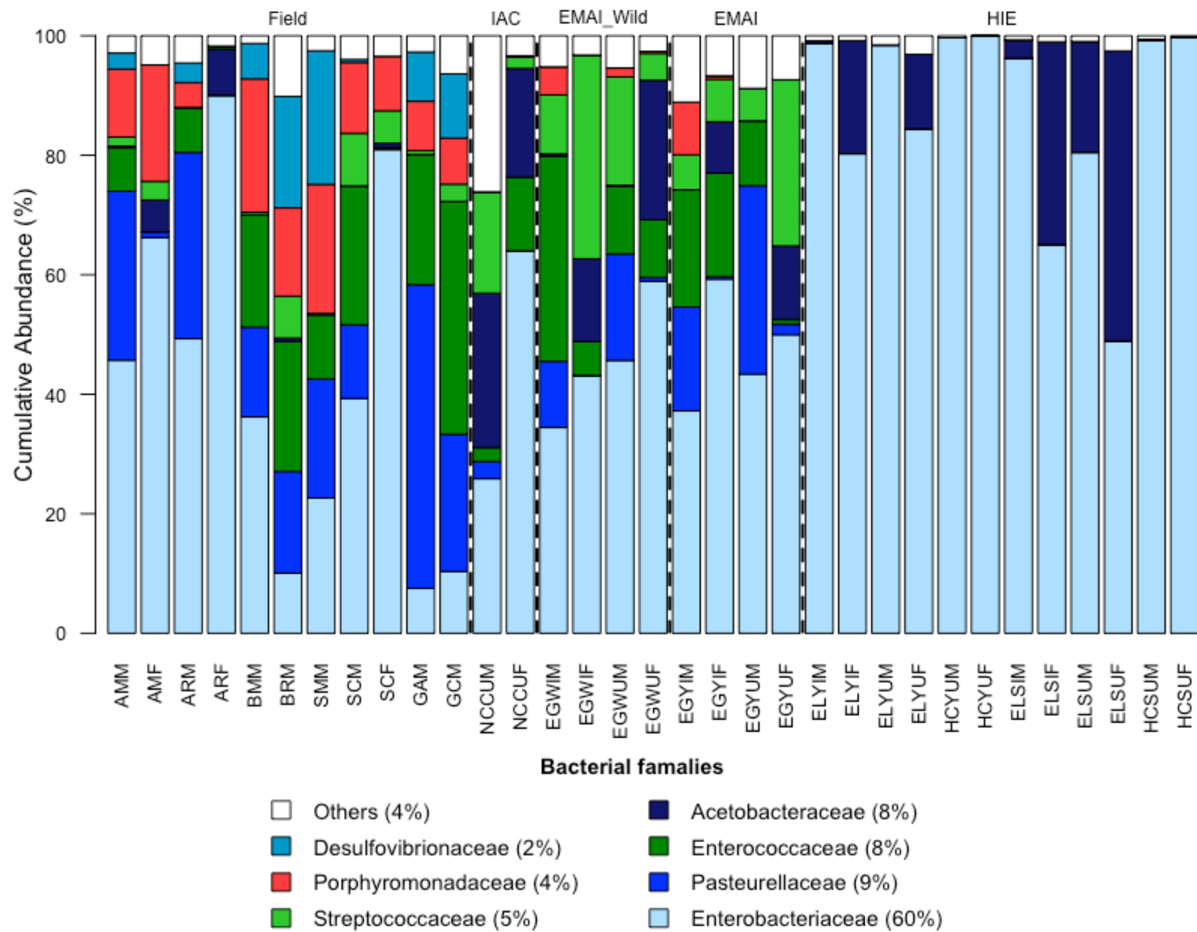


Figure 4.4: Abundance of bacterial families of *Bactrocera tryoni* by treatment groups. The colour codes represent the bacterial phyla with Proteobacteria in shades of blue, Firmicutes in shades of green, and Bacteroidetes in shades of red. Bacterial families that were less than 1% of total abundance were grouped as "Others" in white. As labelled on the top of the graph, the dashed lines separate categories of flies depending on where adult flies were sampled. The legend also shows percentage of total abundance of major bacterial families across all treatment groups.

The presence and abundance of the bacterial families differed between populations and within treatment groups. The HIE population group had a distinct composition of bacterial populations which was predominantly Enterobacteriaceae (for flies reared at HIE at both larval and adult stages), and Enterobacteriaceae as well as Acetobacteraceae (for flies reared at EMAI at the larval stage and at HIE in adult stage). The EMAI adult fly population was more diverse and had similar bacterial family constituents as the EMAI-wild, IAC and field populations.

A notable sex effect was observed with Enterobacteriaceae being more abundant in females than males. Enterococcaceae and Desulfovibrionaceae also demonstrated a strong sex effect with higher abundance in males than in females. In comparing the controlled environment flies, irradiated flies had proportionately less Enterobacteriaceae than unirradiated flies.

4.2.3: Core microbiome of field-collected *Bactrocera tryoni* and its distribution in controlled environment populations

In order to understand how the microbiome of adult flies from controlled environment colonies differs from the microbiome of field colonies, the core microbiome of the field flies was determined and compared across different populations. The core microbiome is comprised of the members common to multiple microbial assemblages associated with a habitat (Hamady and Knight 2009; Shade and Handelsman 2012). To determine the core microbiome of the field flies, the 784 OTUs of field-collected samples (Table 4.3) were grouped by region. This revealed that all field colonies shared 72 OTUs (Fig. 4.5).

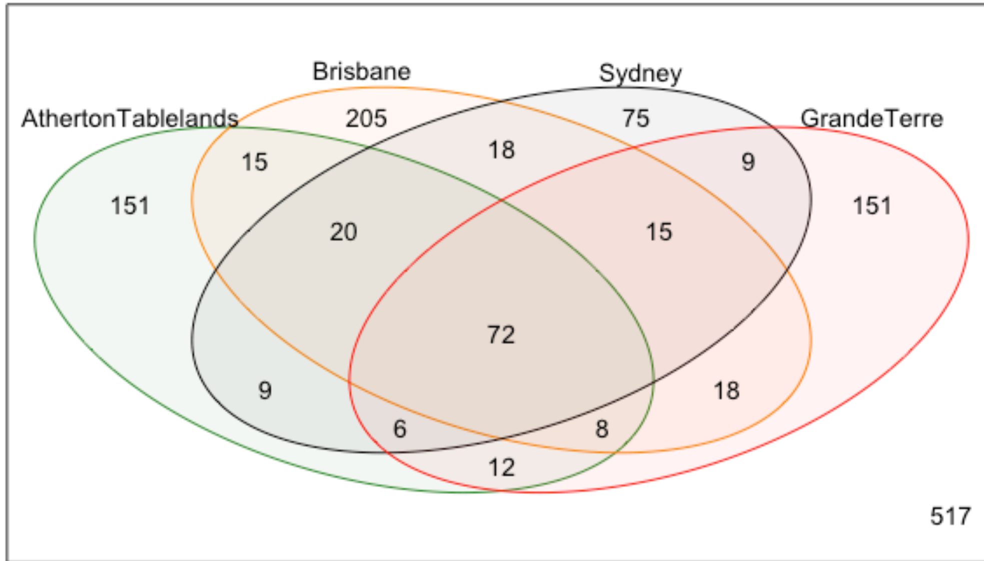


Figure 4.5: Venn diagram of bacterial OTUs within adult *Bactrocera tryoni* collected from the field grouped by regions.

To investigate the distribution of the 72 shared OTUs of the field-collected samples in the controlled environment colonies, the shared OTUs were BLAST searched to identify genus and species. Then their distribution in controlled environment treatment groups was analysed using a heatmap (Fig. 4.6). Bacterial communities of *B. tryoni* samples were visibly different between the controlled environments where the samples were reared as adults. The dominant OTUs from the field samples were more abundant in samples reared as adults in the IAC and EMAI laboratories compared to samples reared as adults at the HIE laboratory. The presence of OTUs shared with field-collected flies ranged from 4 to 10 OTUs in adults kept at HIE, compared to 33 to 48 OTUs in adults kept at EMAI, and 43 to 47 OTUs in IAC adults.

There were no dominant OTUs that were present in all samples and sample groupings. However, the heatmap analysis (Fig. 4.6) revealed a trend of substitution of bacteria from the same genus or similar genera in more dominant OTUs. For example, closely related OTU 581021 (Enterobacteriaceae: *Enterobacter* sp.) and OTU 2529285 (Enterobacteriaceae: *Enterobacter* sp.) were dominant in adult *B. tryoni* samples from the field, IAC and EMAI populations but were almost absent in the HIE population (Fig. 4.6). However, there was a dominant presence of a close relative, OTU 4418165 (Enterobacteriaceae: *Enterobacter* sp.), in the HIE samples which was not very abundant in the other laboratory and field populations.

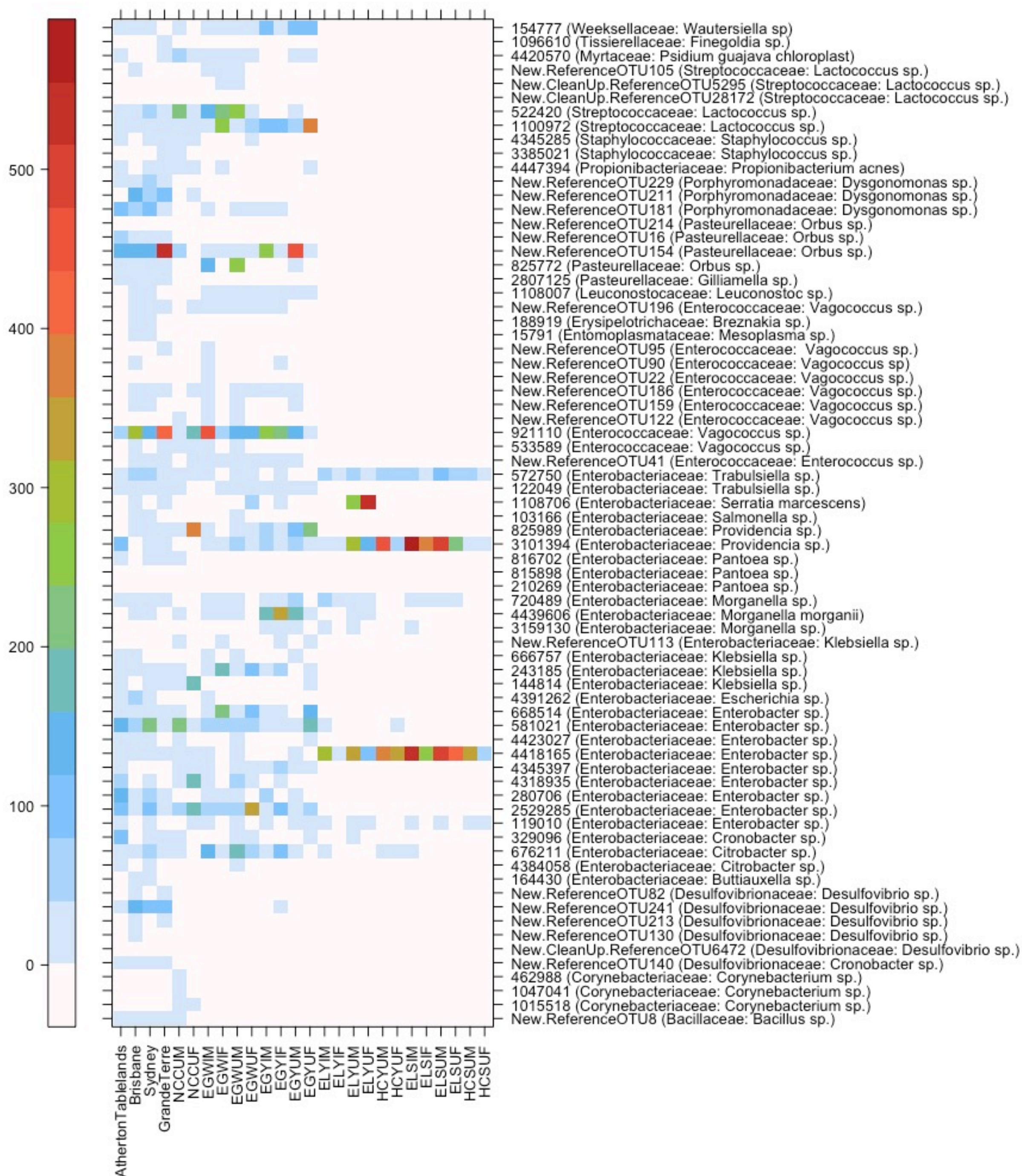


Figure 4.6: Heatmap of the shared OTUs of field-collected adult *Bactrocera tryoni* by regions and their distribution in controlled environment treatment groups. The x-axis shows field-collected *B. tryoni* by region and controlled environment treatment groups, and y-axis shows the OTU number with the BLAST identity by family and species.

Therefore, to further understand the dynamics of the core microbiome of adult *B. tryoni*, the OTUs were grouped by genera, a suggested method for investigating core microbiome (Hamady and Knight 2009). This also absorbed a large number of less abundant OTUs of the same genus. From the rarefied OTU table, 390 OTUs (30%) had between 3 and 21,054 reads while the remaining 911 OTUs (70%) had 1 to 2 reads. The heatmap was used to analyse the distribution of the core gut genera of *B. tryoni* (Fig. 4.7). Bacterial families which individually account for less than 1% of the total reads were grouped together under "Others".

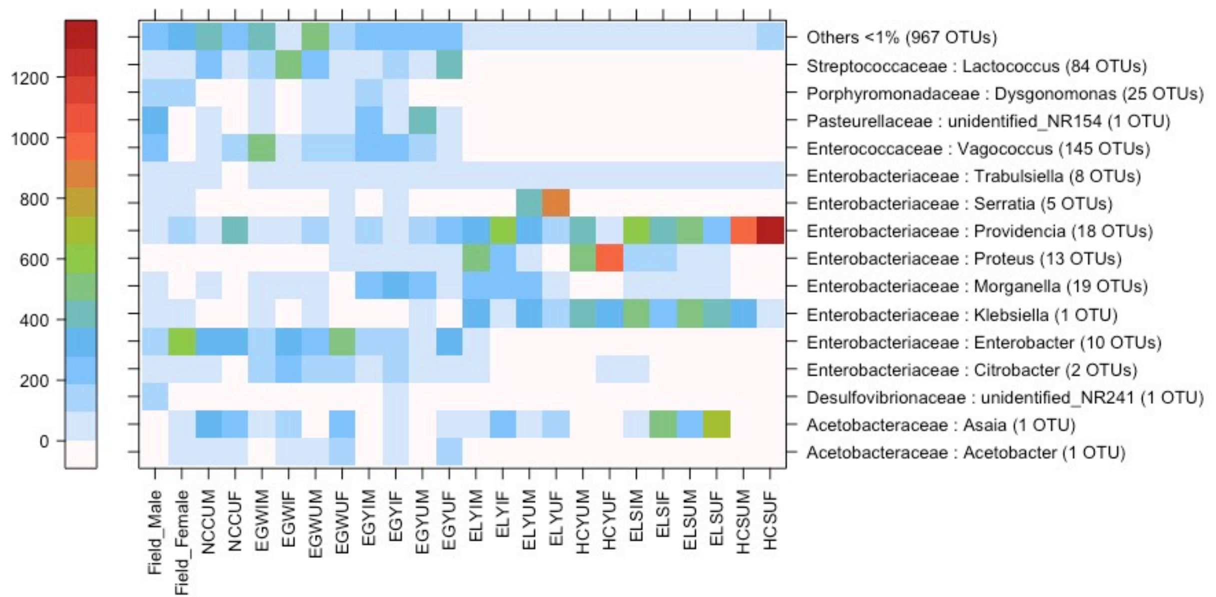


Figure 4.7: Heatmap of the core genera of field-collected adult *Bactrocera tryoni* grouped by sex and their distribution in controlled environments treatment groups. The x-axis shows field-collected *B. tryoni* by sex and controlled environment treatment groups, and y-axis shows the core genera with the number of OTUs.

The core microbiome of adult *B. tryoni* was dominated by Enterobacteriaceae with eight genera totalling 76 OTUs and representing 53% of the total core microbiome abundance. The other bacterial families that contributed to the core microbiome were Enterococcaceae (13 OTUs and 8% of abundance), Acetobacteraceae (2 OTUs and 8% of abundance), Pasteurellaceae (1 OTU and 7% of abundance), Streptococcaceae (84 OTUs and 5% of abundance), Porphyromonadaceae (25 OTUs and 4% of abundance) and Desulfovibrionaceae (1 OTU and 2% of abundance).

The most diverse core genus was *Vagococcus* from Enterococcaceae with 145 OTUs and it was the third most abundant genus. The most abundant bacterial genus was *Providencia* with 18 OTUs with a very strong presence in laboratory flies reared in HIE as adults. The second most abundant genus was *Enterobacter* with a strong presence in the field-collected *B. tryoni* samples and also laboratory samples reared at EMAI as adults.

4.2.4: Beta Diversity

Permutational multivariate analysis of variance (PERMANOVA) revealed that the factors of habitat, adult diet, location, population, sex and irradiation were all significant drivers of microbiome of the sampled *B. tryoni* (Table 4.5).

Table 4.5: PERMANOVA of 16S rRNA gene sequence reads of adult *Bactrocera tryoni* sampled from the field and laboratories in Australia and New Caledonia.

Factors	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)
Habitat	11	20.714	1.88307	8.1898	0.40382	<0.001
Adult Diet	5	16.083	3.2166	12.698	0.31354	<0.001
Population	4	15.111	3.7777	14.616	0.29459	<0.001
Sex	1	2.844	2.84379	8.3934	0.05544	<0.001
Irradiation	1	1.691	1.69054	4.8736	0.03296	<0.001

The constrained analysis of principal coordinates (CAP) of the samples (Fig. 4.8) confirmed the PERMANOVA findings. When grouped by population groups, the diversity of some of the controlled environment populations was observed to sit within a subsection of the field-collected flies: EMAI-wild and the IAC F1 flies from field-collected infested New Caledonian carambola grouped within the field flies.

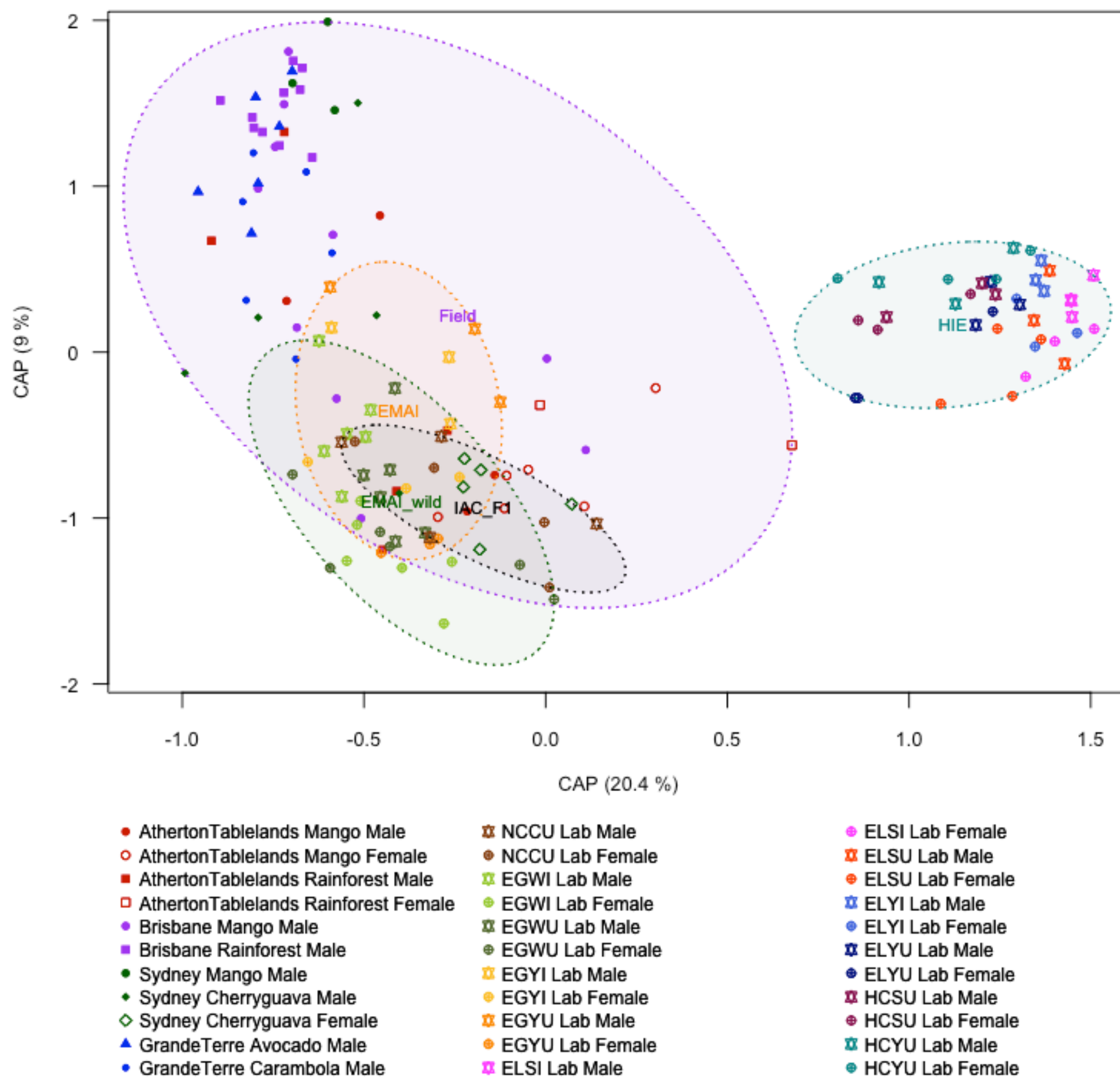


Figure 4.8: Constrained analysis of principal coordinates of adult *Bactrocera tryoni* gut bacterial_samples from Australia and New Caledonia. 95% confidence ellipse show population groups.

4.2.5: Controlled environment *Bactrocera tryoni*

The CAP analysis of the controlled environment flies showed a distinct separation between different adult diets (Fig. 4.9). The HIE reared flies were grouped together with an emerging pattern of separation between the adult diets of sugar only, and yeast and sugar.

Although the EMAI-wild and EMAI populations were grouped together on the CAP graph, an emerging pattern of separation was observed. The microbiome of the EMAI flies reared as adults on yeast and sugar had a similar pattern of spread as the HIE reared adults. The EMAI-wild grouped with the flies emerging from field-collected infested carambola in New Caledonia. This indicated that the gut microbiome of the laboratory *B. tryoni* fed a "wild" diet was similar to the IAC F1 samples from New Caledonia, however had greater variation than flies reared on a standard laboratory diet or yeast and sugar.

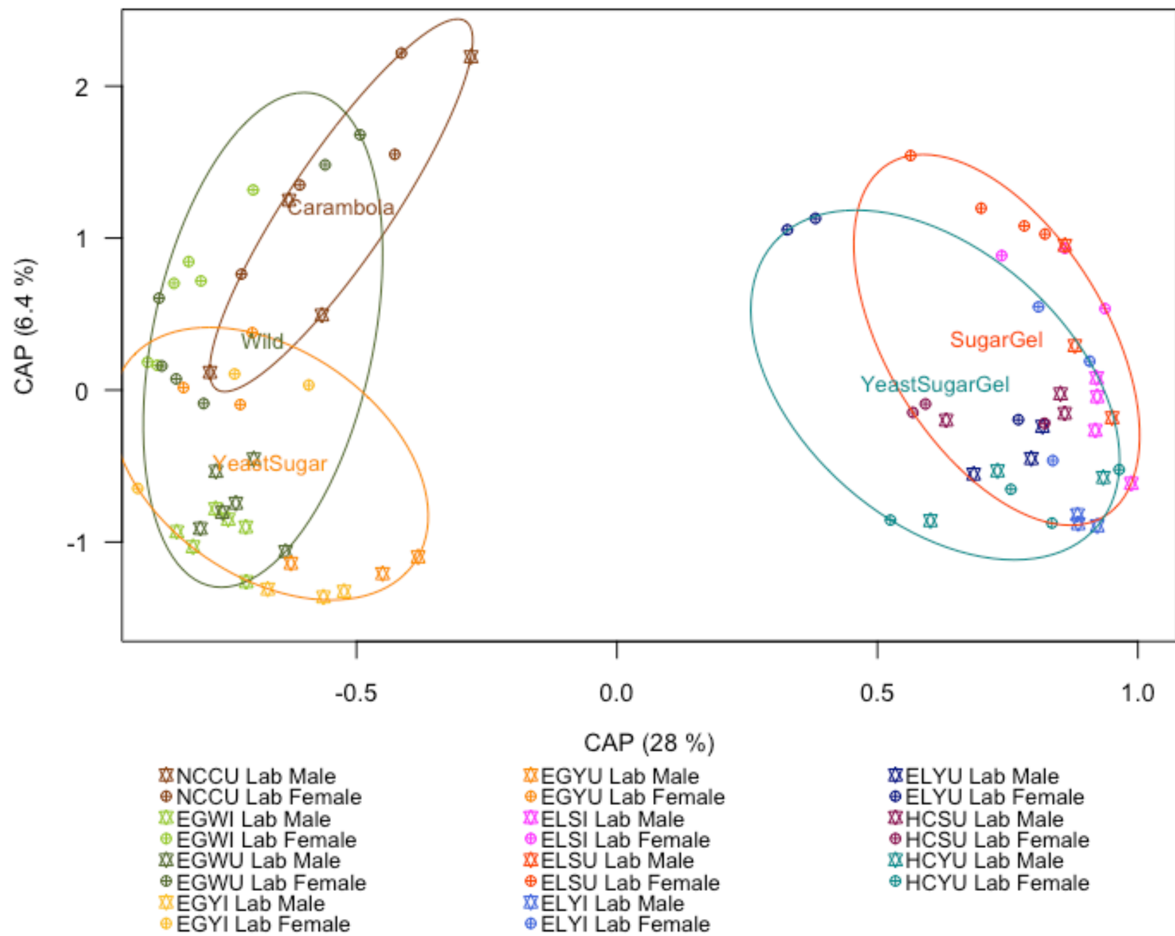


Figure 4.9: Constrained analysis of principal coordinates (CAP) of adult *Bactrocera tryoni* reared from EMAI, HIE and IAC laboratories. Ellipses represent 95% confidence intervals of controlled environment samples grouped by adult diet types.

4.2.6: The "wild" diet vs. standard laboratory diet of *Bactrocera tryoni*

The flies from the EMAI controlled environment presented a good opportunity to further investigate the effects of sex, irradiation and diets of 'wild' and standard laboratory adult diets. The PERMANOVA of the EMAI *B. tryoni* flies showed that sex and adult diet were strong drivers for differentiation of bacterial communities (Table 4.6). Exposure to irradiation did not influence bacterial diversity, although the low *p*-value ($p = 0.082$) might suggests a small non-significant effect.

Table 4.6: PERMANOVA of 16S rRNA gene sequence reads of adult *Bactrocera tryoni* sampled from the EMAI laboratory.

Treatments	Df	SumsOfSqs	MeanSqs	F.Model	R^2	Pr(>F)
Sex	1	1.4071	1.40715	8.2155	0.19461	<0.001
Adult Diet	1	1.0593	1.05932	5.8362	0.1465	<0.001
Irradiation	1	0.3178	0.31775	1.5628	0.04395	0.082

The CAP analysis of the EMAI controlled environment *B. tryoni* (Fig. 4.10) concurred with the PERMANOVA (Table 4.5) and showed that adult diet and sex were strong drivers for differences in the gut bacterial communities of *B. tryoni* in controlled environments. The adult flies exposed to the standard laboratory diet of yeast and sugar were grouped on one side along the principle coordinate 1 while the adults exposed to the "wild" diet grouped on the other side. Within adult diet groups there was a strong sex differentiation. An irradiation effect was also observed with flies of the same diets and sex grouping together with an emerging trend of separation between those exposed to irradiation and those unexposed.

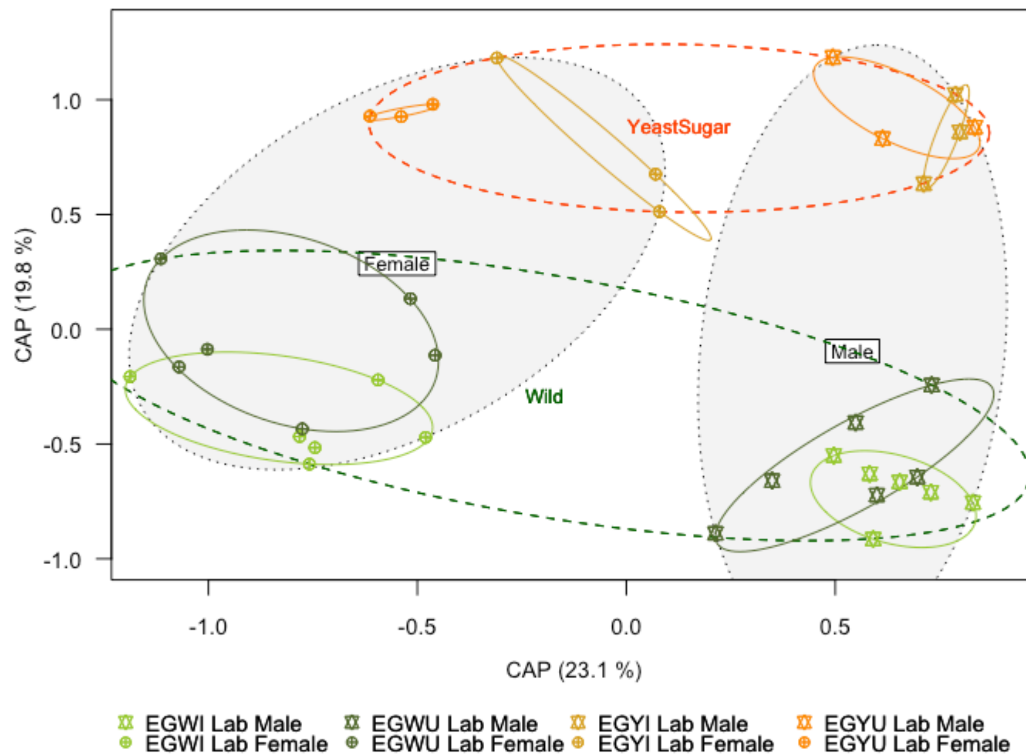


Figure 4.10: Constrained analysis of principal coordinates (CAP) of sterile and fertile adult *Bactrocera tryoni* from EMAI fed different adult diets. Ellipses represent 95% confidence intervals with dotted ellipses showing samples grouped by adult diet types, lined ellipse showing treatment groups as per inset legend, and shaded ellipses showing grouping by sex.

4.3: Discussion

This study is the first to compare the microbiome of different controlled environment populations with field-collected samples of a major tephritid pest species from diverse regions. Using next-generation sequencing, this study comprehensively examined the gut bacterial communities of 145 individual adult *B. tryoni* from different field and laboratory populations.

The aim of this study was to identify the core bacteria of the field collected flies and investigate their presence and abundance in the captive flies maintained in controlled environments. The field flies had more bacterial diversity than the controlled environment flies. However, despite the controlled environment flies not having the same amount of diversity at an OTU level, when the same genera of bacteria were amalgamated at the genus level, the controlled environment had all the dominant genera found in the field flies. Further, the bacterial diversity of the controlled environment flies responded strongly to environmental factors, and therefore, it may be possible to manipulate it so that it is similar to the field flies within just one life-stage (tenerals to adults) of a generation.

4.3.1: Low bacterial diversity associated with standard controlled environment populations compared to field populations of *Bactrocera tryoni*

Comparing all treatment groups, the field populations of *B. tryoni* had the highest number of OTUs. This is expected due to the vast array of nutritional sources available to the flies in the field. This diversity of bacteria could also be a due to the wide climatic (tropical, subtropical and temperate) and geographic (distances up to 2,500 km) range covered in field sampling.

Tephritids are known to require different types of bacteria to assist them in processing different nutrients (Ben-Yosef et al. 2010). While the IAC-F1 and EMAI-wild populations have a similar number of OTUs, the flies were originally from different sources with the IAC reared from field-collected infested fruit in New Caledonia while the EMAI flies were 50 generations in captivity and reared on the mass production facility diet throughout this period

(while being reared on gel larval diets for the experimental generation). The population kept at HIE harboured the least number of OTUs. This could be due to a number of reasons including the fly line is oldest in captivity and the flies have lost bacterial diversity over generations, or the fly population is relatively smaller and thus creating a bottleneck for diversity, or the laboratory conditions at HIE could be more cleaner and there were less bacteria in the environment for the flies to pick up. The IAC-F1 flies emerged from field infested carambola fruits and matured in captivity with no other food sources but the host fruit from which they emerged. The bacterial species they possessed would be from the host fruit and any residual bacteria they could carry through from their exposure as eggs or larvae in the fruit, prior to being reared out in captivity. It is likely that their gut bacterial population would be a reduction of the bacteria that is found in the field adults collected from the same habitat. The greater bacterial diversity observed in the field adults is likely due to their wider exposure to available food sources.

The HIE population used in this analysis were reared for over six years and had gone through multiple generations (more than 50 generations but not much more than the number of generations at EMAI). This could mean that the HIE flies were most adapted to being reared in the controlled laboratory environment and therefore require a less diverse bacterial community. There are several alternative non-exclusive potential reasons. HIE flies may be able to cope with the limited diversity given they receive the full diet. Further, as these samples were taken from a much smaller population, there are likely to have been drift effects for bacterial populations (with some bacteria being lost in random processes), or may have been reared in a generally cleaner environment (with some bacteria also being lost because of this additional selection pressure). The difference in OTU diversity between the EMAI population and the field population is most likely due to the reduction of microbiome and adaptation of the flies to the standard laboratory larval and adult diets and conditions. Interestingly, when the EMAI flies were exposed to a “wild” adult diet under laboratory conditions, their gut bacterial community quickly becomes more diverse within the same generation. This finding is a testament to the strong contribution of environmental factors and highly dynamic processes that shape *B. tryoni* bacterial communities. While some gut bacteria may be transient in digestive systems of insects, many gut bacteria are known to help insects feed on new types of food materials (Russell et al. 2009), process nutrients (Behar et al. 2005; Ben-Yosef et al. 2010), protect against pathogens (Dillon et al. 2005) to even

increase resistance to insecticides (Cheng et al. 2017). Such a trait is essential for an organism's processing of new food materials, thus enabling the organism to colonise new environments. Therefore, this ability of *B. tryoni* to quickly change its gut bacterial community may be an important reason it is a successful pest species.

4.3.2: Gut bacterial taxa of *Bactrocera tryoni* changes with habitat

The gut bacterial communities of IAC-F1, EMAI and EMAI-wild flies were dominated by three main phyla, Proteobacteria (74%), Firmicutes (18%) and Bacteroidetes (7%). This finding concurs with a similar composition observed in a previous study of 305 field-collected insects representing 21 taxonomic orders (including Diptera) which found that Proteobacteria and Firmicutes represent almost 83% of total sequences (Yun et al. 2014a). Similarly, in a study of 14 *Drosophila* species from field and wild populations, the dominant phyla were Proteobacteria (69%) and Firmicutes (21%) (Chandler et al. 2011). And in the more closely related *B. dorsalis* of wild and laboratory populations, Proteobacteria was found to constitute over 90% of the gut bacterial phyla (Liu et al. 2016b). These studies indicate that the gut bacteria of *B. tryoni* consists of the main phyla that are similar across insects, and indeed dipterans. However, across different populations and treatments, we found bacterial abundance at a family level varies and is defined by location and diet. The difference in gut microbiome across locations and populations is also known from closely related *B. dorsalis* (Liu et al. 2016b; Wang et al. 2011).

In contrast to the other investigated fly populations, the HIE population had a gut bacterial community that was dominated mostly by Enterobacteriaceae when reared at HIE also in the larval stage, and both Acetobacteraceae and Enterobacteriaceae when reared at EMAI in larval stage and at HIE in adult stage (also see Chapter 2). This might imply that these bacterial families are adequate for the insect host to process the available diet under the given conditions.

Within population groups, a sex effect was observed indicating that males and females of the same environment have slightly different proportional abundance of bacteria, and this may be a consequence of male behaviour and/or physiology that differs from females. This difference

of microbiome by sex, as also observed in Chapters 2 and 3, is a novel finding not only for *B. tryoni*, but for tephritids as well.

It also appears that there was an emerging trend that irradiation might have an effect. Irradiation may shift the bacterial community and perhaps suppress some microbiota, and thus leading to the other bacteria becoming more prevalent. Alternatively, as discussed in Chapter 2, the effect of irradiation increasing the bacterial load with some bacteria thriving more than others and thus shifting the microbiome.

4.3.3: Core microbiome of field-collected *Bactrocera tryoni* is present, but distributed differently, in controlled environment populations

The analysis of the core microbiome of field collected flies did not reveal any OTUs that were present across all individuals. When the samples were grouped by population, we see a trend of different (but closely related) OTUs and these may substitute each other in function across individuals and populations. Despite these differences, we observed a pattern of similar composition in treatment groups as the OTUs clustered at the higher levels of families and phyla. This led us to hypothesise that the bacterial composition is driven by a functional need of the individual insect. Bacteria are known to perform multiple roles in insects. Thus, an insect would encourage certain bacteria which might perform certain roles better another type of bacteria. This hypothesis could explain how in some cases, a particular bacterium which was fed to a fruit fly affected their performance (Ben-Yosef et al. 2010; Gavriel et al. 2010) and in some cases the bacterium did not (Meats et al. 2009). For a positive effect, it may be that the bacteria established itself in the insect and in the process changed the microbial composition, allowing some bacteria which have the potential to be beneficial to proliferate and impact insect behaviour.

The diversity in the captive population contains almost all the genera of field-collected invasive and native populations, demonstrating that it is likely that the bacteria of interest for use in improving flies for SIT are already present in the lab-reared populations. However, they occur at a lower, or reduced abundance. For use of bacteria to improve SIT, the

challenge may be to manipulate existing gut bacterial abundance to resemble that of field populations, rather than necessarily providing bacteria as a supplement.

4.3.4: Bacterial diversity influenced by diet and sex

The beta diversity analyses confirmed that adult diet is a significant driver for gut bacterial diversity, on top of variation across habitats and populations. Within each population, a sex effect was observed. This was expected as males and females have different behaviours, physiology and biological needs, and these would largely affect abundance and, in some cases, the presence of bacteria. It was also found that irradiation affected the bacterial communities' structure but not as much as the diet and sex effects.

Interestingly, this study has proven that even a controlled environment *B. tryoni* that has been reared for around 50 generations can quickly change its microbiome to be as close to if not better than the F1 populations and even be like the field flies' microbiome.

4.4: Conclusion

This study has identified the core microbiome of field-collected *B. tryoni* and the presence of these bacteria in the laboratory populations. It has become evident that the core bacteria are being substituted within populations. Thus, it is highly likely that the bacteria required to improve irradiated flies reared in controlled environments might already be present but at low abundance. Further, it was evident that *B. tryoni* is able to quickly change its gut bacterial community and this could be a trait for its success as a pest species. This study shows that to change the diversity and abundance of bacteria, the influence of diets and environment are the key factors to consider in rearing and release programs for SIT as they greatly influence the tephritid bacterial microbiome.

Chapter 5: Microbiological isolation and near full length 16S rRNA gene characterisation of Enterobacteriaceae from Queensland fruit fly, *Bactrocera tryoni*, across tropical, subtropical and temperate regions of Australia

Abstract

Recent advances in DNA sequencing technology have enabled more insights into the diversity and dynamics of gut bacterial communities of insects and the roles they play in insect development. Gut bacteria have been known to improve the performance of tephritid fruit flies and thus is a promising prospect for use in improving the sterile insect technique used in tephritid fruit fly management. In this work, gut bacterial isolates of major Australian horticultural tephritid pest, Queensland fruit fly, *Bactrocera tryoni,* were isolated, cultured and identified using near full length Sanger sequencing of 16s rRNA gene. The flies from the field had more diverse bacteria than flies reared in the controlled environment. The most common bacteria isolated were *Citrobacter* followed by *Enterobacter, Klebsiella*, *Providencia* and *Kluyvera.*

5.1: Introduction

The guts of insects harbour a diverse array of bacteria, some of which are in a symbiotic association with the host insect (Dillon and Dillon 2004). This symbiotic relationship between bacteria and their insect host has become of interest in recent times because of its potential application in insect pest management (Crotti et al. 2012). This has also been the case in the research of new management options of tephritid fruit fly pests where a key focus has been in using beneficial bacteria as probiotics in improving sterile males deployed in the sterile insect technique (SIT) (Estes et al. 2012; Yuval et al. 2010). SIT is an environmentally friendly and reliable insect pest management strategy that involves the release of mass-produced individuals (preferably males) who have been exposed to gamma irradiation to make them sterile (Knipling 1955). The success of SIT depends on the sterile males seeking and mating with the wild females thus resulting in embryonic mortality, and with continuous sterile male releases over time this will result in pest population decline. However, sterile male tephritids are not as competitive as wild males (Lance et al. 2000) because of mass production, loss of genetic diversity (Gilchrist and Meats 2012) and the exposure to irradiation (Collins et al. 2008). Gut bacteria are known to improve the performance of sterile

male tephritids (Ben Ami et al. 2010; Cai et al. 2018; Gavriel et al. 2010; Hamden et al. 2013).

Interestingly, so far almost all research of bacteria in tephritid pest management has found the bacterial family Enterobacteriaceae to be an important constituent of the tephritid microbiome, with some (but not all) bacteria within this family having beneficial effects on hosts. Enterobacteriaceae species were found to persist in all life stages of the Mediterranean fruit fly *Ceratitis capitata* for over 20 generations (Lauzon et al. 2009) thus suggesting their importance in host fly development. In *C. capitata,* a shift in the Enterobacteriaceae species was observed in irradiated males and by correcting this imbalance by providing *Klebsiella oxytoca* (Enterobacteriaceae), the mating success of sterile males improved (Ben Ami et al. 2010). Diazotrophic Enterobacteriaceae, culturally identified as *Klebsiella* and *Enterobacter*, were believed to assist in nitrification in Queensland fruit fly *Bactrocera tryoni* (Murphy et al. 1988; Murphy et al. 1994) and *C. capitata* (Behar et al. 2009). In the apple maggot *Rhagoletis pomonella*, Enterobacteriaceae species, microbiologically isolated and biochemically identified as *K. oxytoca* and *Enterobacter cloacae*, were responsible for the degradation of polysaccharides, cellulose and pectin into forms which *R. pomonella* larvae can utilise (Rossiter et al. 1982). In the quest to identify probiotic candidates to improve sterile *B. tryoni* used in SIT, we expected that Enterobacteriaceae species are important in this fly species also and that they should be further investigated and considered as probiotic candidates to improve performance of flies in SIT programs. Findings from Chapters 2, 3 and 4 have displayed the significant presence and abundance of Enterobacteriaceae in adult *B. tryoni*. Enterobacteriaceae species are the most prevalent in the gut of many tephritid fruit fly species (Aharon et al. 2013; Behar et al. 2009; Behar et al. 2008a; Behar et al. 2008b; Morrow et al. 2015b). However, the diversity of Enterobacteriaceae in a tephritid species across ranges of habitat, region and climate has so far not been investigated.

The aim of this chapter was to isolate Enterobacteriaceae from the gut of adult *B. tryoni* collected from the regions around Cairns, Brisbane, Sydney and a captive sterile population as used in SIT and then characterise these using a large section of the 16S rRNA gene. Another part of the same adult *B. tryoni* individuals has previously been used for MiSeq amplicon sequencing of a shorter fragment of the 16S rRNA gene as discussed in Chapters 3 and 4. The near full length 16S rRNA gene sequences of the bacterial isolates were then compared across populations and also with the sequences from the MiSeq 16S rRNA

amplicon sequencing to identify Enterobacteriaceae isolates that are unique or shared across populations.

5.2: Methods

5.2.1: Processing of *Bactrocera tryoni* guts

Adult *B. tryoni* (*n* = 23) were selected from within 145 field-collected flies from various habitats, regions and climates for the isolation and culturing of gut bacteria (Table 4.1). Most populations were only represented by males (which are the targeted sex of interest with regard to SIT applications) while we included both males and females from the population of the Atherton Tableland (which based on previous analyses had a higher bacterial diversity than subtropical and temperate populations). In the field, individual *B. tryoni* were placed into sterile 250mL specimen jars and, within 30 minutes of sampling, were transferred into a freezer (-20°C) for at least 5 minutes. Individual insects were then surface sterilised by sequentially immersing for 1 minute in each of 70% ethanol, sterile distilled water, 0.05% sodium hypochlorite and lastly sterile distilled water. Then individuals were placed on a sterile concave glass slide that had been surface treated by wiping with 70% ethanol and 0.05% sodium hypochlorite. The glass slide was placed under a stereomicroscope and two pipette drops of sterile phosphate-buffered saline (PBS) were placed on top of the insect before dissection with sterile forceps. The dissection involved the removal of the wings, the legs and the exoskeleton after softening by immersion in PBS for 1 minute. During dissection, the adult insects were checked for fully developed testes as an indication of male sexual maturity, and full ovaries as an indication of female maturity. All insects collected were fully matured. The intact gut of the insects was then gently removed and placed in a 1.5mL microcentrifuge tube which contained 0.8mL solution of brain heart infusion broth and 20% glycerol (BHIB+20%gly). The microcentrifuge tubes were immediately transferred to a freezer (-20°C) and held for a maximum of 1 hour while more dissections were conducted. Afterwards, samples were stored at -80°C until required.

Table 5.1: Gut bacterial isolates cultured from adult male *Bactrocera tryoni* collected from the field and from an irradiated (sterile) captive population reared on a standard laboratory diet and in controlled environment at Elizabeth Macarthur Agricultural Institute (EMAI).

Qfly ID	Region	Location	Habitat	Sex	Origins	Isolates used	Total isolates
AMF04	Atherton Tablelands	Walkamin (-17.133767, 145.427046)	Mango (research orchard)	Female	Native	1,2,3,5,6,7,8,9,10	9
AMF05	Atherton Tablelands	Walkamin (-17.133767, 145.427046)	Mango (research orchard)	Female	Native	1,6,8,9	4
AMF06	Atherton Tablelands	Walkamin (-17.133767, 145.427046)	Mango (research orchard)	Female	Native	1,2,3,5,6,7,8,9,10	9
AMM03	Atherton Tablelands	Walkamin (-17.133767, 145.427046)	Mango (research orchard)	Male	Native	1,3,4,5,6,7,8,9,10	9
AMM04	Atherton Tablelands	Walkamin (-17.133767, 145.427046)	Mango (research orchard)	Male	Native	4,5,8,9,10	5
ARF01	Atherton Tablelands	Koah (-16.872813, 145.580178)	Native rainforest	Female	Native	1,3,4,5,6,7,9,10	8
ARF02	Atherton Tablelands	Koah (-16.872813, 145.580178)	Native rainforest	Female	Native	1,2,7,8,9,10	6
ARM01	Atherton Tablelands	Koah (-16.872813, 145.580178)	Native rainforest	Male	Native	1,5,8,10	4
ARM03	Atherton Tablelands	Koah (-16.872813, 145.580178)	Native rainforest	Male	Native	1,2,3,5,6,7,8,9,10	9
ARM04	Atherton Tablelands	Koah (-16.872813, 145.580178)	Native rainforest	Male	Native	1,2,4,5,7,8,9,10	8
BMM01	Brisbane	Redlands (-27.526987, 153.250875)	Mango (backyard)	Male	Native	1,2,3,4,5,7,8,9,10	9
BMM02	Brisbane	Redlands (-27.526987, 153.250875)	Mango (backyard)	Male	Native	1,2,3	3
BMM05	Brisbane	Redlands (-27.526987, 153.250875)	Mango (backyard)	Male	Native	1,2,4,5,6,7,8,9,10	9
BMM10	Brisbane	Redlands (-27.526987, 153.250875)	Mango (backyard)	Male	Native	1,2,3,5,6,8,10	7
BRM02	Brisbane	Mt Coot-tha (-27.476991, 152.974465)	Native rainforest collection	Male	Native	1,3,4,5,7,8,9	7
EGYIM01	EMAI Lab	EMAI (-34.117459, 150.716986)	Lab	Male	Captive	2,3,7	3
EGYIM02	EMAI Lab	EMAI (-34.117459, 150.716986)	Lab	Male	Captive	1,2,3,4,5,6,7,8,9,10	10
EGYIM03	EMAI Lab	EMAI (-34.117459, 150.716986)	Lab	Male	Captive	1,2,5,7,8,9,10	7
SCM01	Sydney	Richmond (-33.610681, 150.747221)	Cherry guava (backyard)	Male	Invasive	8,9	2
SCM02	Sydney	Richmond (-33.610681, 150.747221)	Cherry guava (backyard)	Male	Invasive	1,2,3,5	4
SMM01	Sydney	Ourimbah (-33.357794, 151.382673)	Mango (mixed research orchard)	Male	Invasive	1,3,5,6,7,8,9,10	8
SMM02	Sydney	Ourimbah (-33.357794, 151.382673)	Mango (mixed research orchard)	Male	Invasive	2,3,4,6	4
SMM03	Sydney	Ourimbah (-33.357794, 151.382673)	Mango (mixed research orchard)	Male	Invasive	6,7,8,9	4

5.2.2: Isolation of bacteria from *Bactrocera tryoni* guts

Under a biological safety cabinet, five sterilised 2mm ∅ glass beads were placed inside individual microcentrifuge tubes containing the frozen dissected insect gut tissue in BHIB+20%gly solution. After the tubes had been thawed the microcentrifuge tubes were individually homogenised using a vortex mixer at high speed for three minutes. 10µL of the homogenised gut in BHIB+20%gly solution was then pipetted onto individual Petri dishes containing MacConkey agar (Mossel et al. 1962), an Enterobacteriaceae-selective medium, and streaked using sterile disposable plastic loops before being incubated at 26°C. The remaining gut solutions were pipetted into new microcentrifuge tubes and used in DNA extraction for next generation 16S rRNA gene amplicon sequencing (Chapters 3 and 4). The inoculated Petri dishes were observed daily for three to seven days, and from each original plate, up to ten isolates were individually sub-cultured onto new MacConkey agar plates in order to obtain pure cultures. A total of 230 bacterial isolates were obtained.

5.2.3: DNA extraction from bacterial isolates

Bacterial DNA was extracted from the 230 sub-cultured isolates using a modified "pick and swizzle" method adapted from Michael (2006). This involved picking of approximately 5µL of sub-cultured bacterial isolates using a sterile disposable loop and suspending the bacteria in a PCR strip tube (0.2mL capacity) containing 50µL of a solution of 50% sterile DNase and RNase free ultrapure water and 50% 5x colourless GoTaq Flexi Buffer. PCR strips containing the bacterial suspension were then incubated in a PCR machine at 95°C for 5 minutes. The suspension was then centrifuged at maximum speed for 5 minutes, and 1µL of the supernatant was used as DNA template in the PCR which used the primers 27F and 1492R in order to amplify a near full length amplicon of the bacterial 16S rRNA gene. The PCR products were prepared for direct sequencing using the ExoSAP method (Dugan et al. 2002) and sent to Macrogen Inc. in South Korea for Sanger sequencing.

5.2.4: Sequence alignments and analyses

The forward and reverse sequences for individual isolates were assembled, trimmed and consensus sequences determined using the Geneious software version 10.2 (Kearse et al. 2012). These were then imported into the Mega software version 7 (Kumar et al. 2016) for sequence alignment using the MUSCLE algorithm (Edgar 2004). Three bacterial isolate sequences, KR232639.1, KR232639.1 and KR232639.1 from the microbiome study of *C. capitata* by Augustinos et al. (2015), and five reference Enterobacteriaceae samples, CP007592.1, JF772064, KOU78183, LC060916.1, and MF455197 from the National Centre for Biotechnology Information's (NCBI 2011) GenBank were included in the alignment with near-full-length 16S rRNA gene sequences from the Enterobacteriaceae bacterial isolates. The bacterial isolate sequences were BLAST searched on NCBI's GenBank for their identification (see OTU table, Appendix Table 3.1).

A second alignment was generated that combined the 148 near full-length 16S rRNA gene sequences from cultured isolates with Enterobacteriaceae sequences from the MiSeq 16S rRNA gene amplicon sequences described in Chapters 3 and 4. These shorter MiSeq 16S rRNA gene amplicon sequences of approximately 430bp, were clustered into OTUs at 97% similarity and classified with reference to the greengenes database version 13.8 (DeSantis et al. 2006). Using the *SeqinR* package (Charif and Lobry 2007), the 16S rRNA gene amplicon sequences were imported into R (R Core Team 2018) where a subset of Enterobacteriaceae OTUs was generated using *base R* commands. The Enterobacteriaceae 16S rRNA gene Sanger sequences subset contained 258 OTUs, which were included in this second alignment.

Maximum Likelihood phylogenetic trees were calculated for both alignments by using the Find Best DNA Models command providing the lowest BIC scores, and supported by 100 bootstrap replicates. The phylogenetic relationship of the isolates (Fig. 5.1) was used to create the OTU table (Appendix Table 5.1). The Venn diagrams (Fig. 5.2) were drawn in R using the *vennDiagram* command of the Limma package (Ritchie et al. 2015).

5.3: Results

After quality control checks of the 230 isolates sequences, 82 were of poor quality or with less than 400 base pairs and were excluded from further analyses. Therefore 148 isolates remained for phylogenetic sequence analyses (Table 5.1). The threshold of 400 base pairs was chosen as these sequences were to be aligned with sequences from MiSeq which were ≈430 base pairs. The available sequence information for the 148 isolates was on average 790 base pairs per sequence. These isolates represented 94 OTUs with an average count of 1.57 and ranged from 1 to 15 counts per OTU.

An OTU table (Appendix Table 5.1) was generated based on the phylogenetic clustering and then used to create the Venn diagrams (Fig. 5.1) which displayed the sharing of OTUs between samples by region, and this included native (from tropical and sub-tropical regions), invasive (temperate regions) and captive (controlled environment) populations. For Cairns we also had both females and males, allowing the analysis of any sex effects. By comparing the number of bacterial isolates to the number of OTUs present in each group of flies (habitat, region, sex), a diversity index was calculated as percentage of sequence types contained within the number of isolates obtained from this group (Table 5.2). A large diversity index would therefore indicate that the full diversity within these populations has not yet been fully represented, indicating that these populations contain more diversity, and therefore may be more OTU diverse host populations.

Comparing by region, the Sydney population and the controlled environment EMAI population had lower diversity indices compared to Atherton Tablelands and Brisbane regions (Table 5.2). This indicates that populations from the invasive range and the controlled environment were less diverse than the populations of the native range. There were 20 isolates from EMAI which were grouped into 11 OTUs of which seven were unique to that population but four of those isolates were the same as those found in samples from the Atherton Tablelands (Fig 1A).

For the comparison of OTUs between sexes, only the Atherton Tablelands population was used as this was the only region from which isolates were obtained from both males and females. The five adult males had 35 bacterial isolates and the five adult females had 36 bacterial isolates. Both male and female samples from the Atherton Tablelands region had

high diversity within their population as indicated by their large diversity index, however, despite their abundance in diversity, both males and females only shared two OTUs indicating that across the sexes, the culturable Enterobacteriaceae isolates were distinctively diverse.

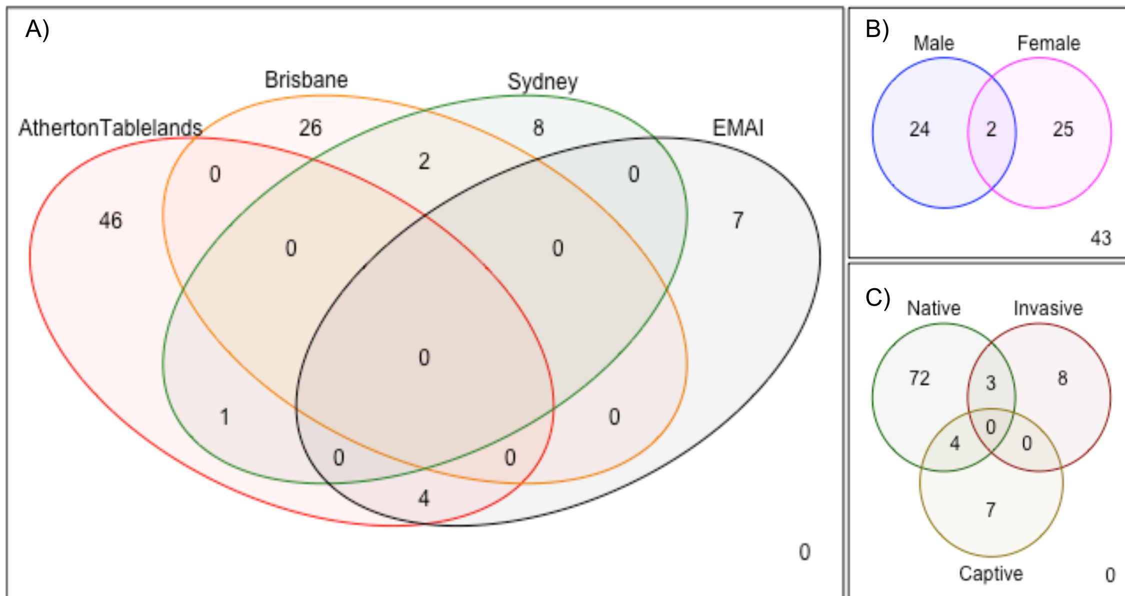
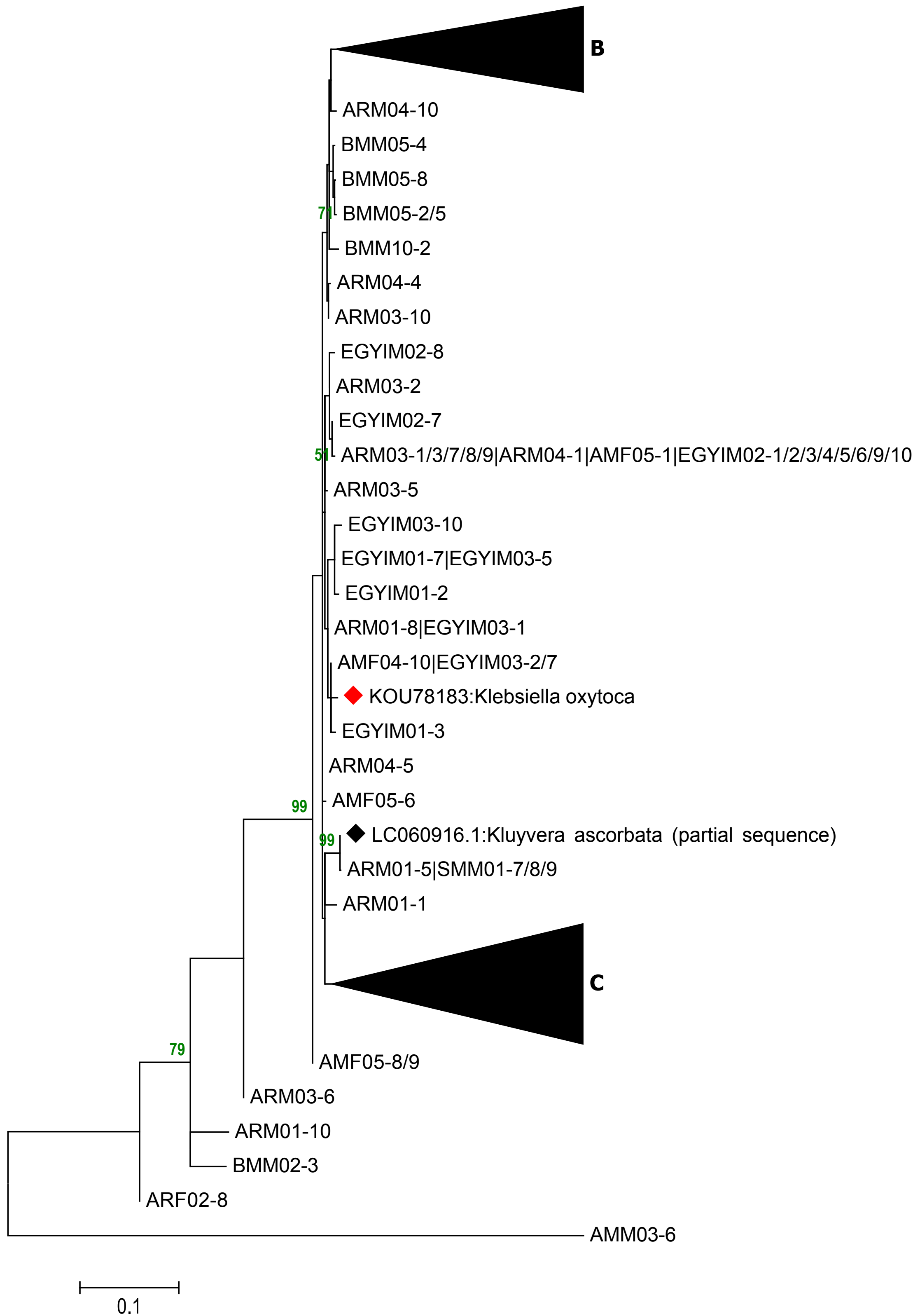


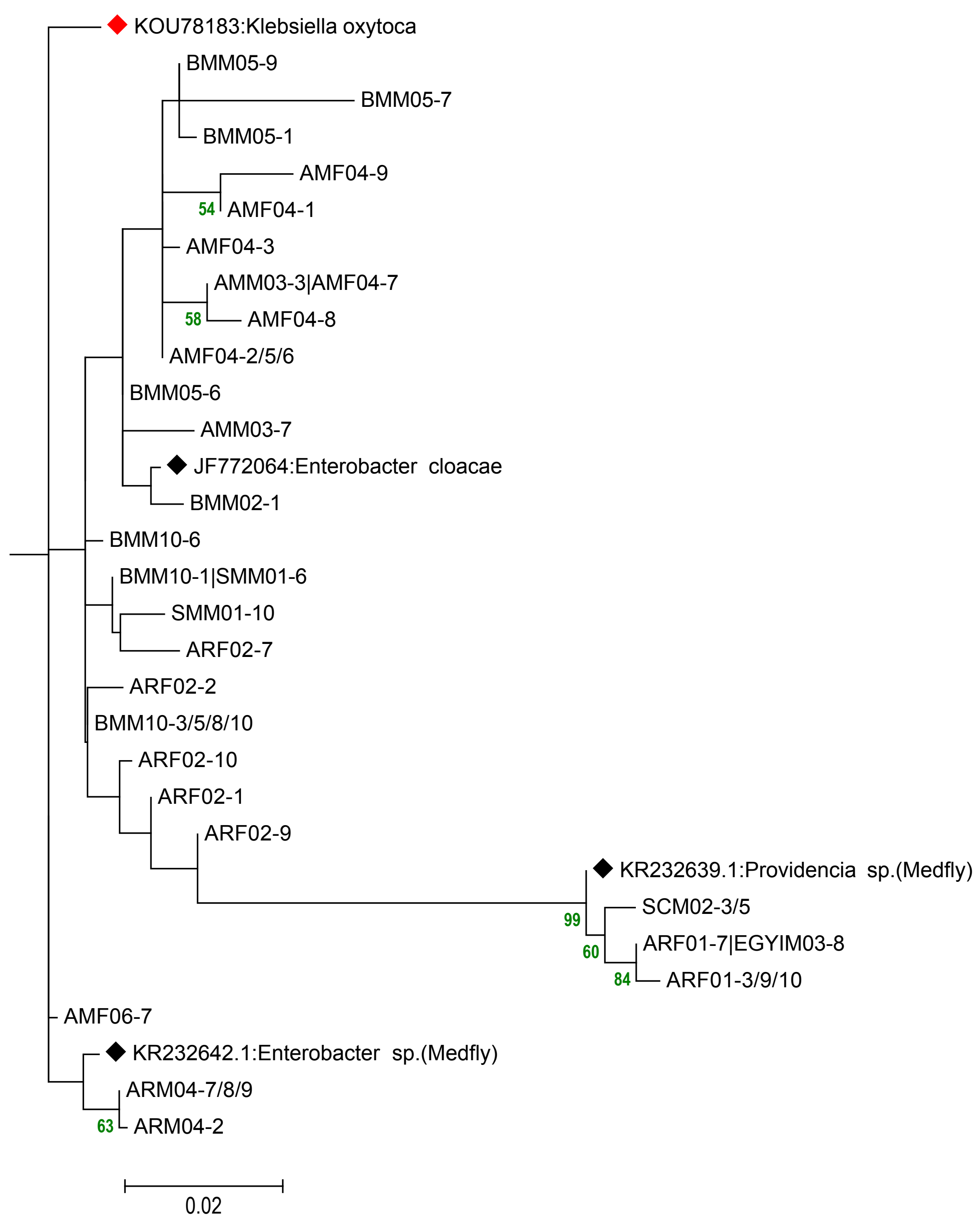
Figure 5.1: Venn diagrams of OTUs from *Bactrocera tryoni* gut isolates by A) region; B) sex from Atherton Tablelands samples only, and; C) population origin types of native (tropical and subtropical), invasive (temperate) and captive.

Table 5.2: Gut bacterial isolates of adult *Bactrocera tryoni* grouped by OTUs. The diversity index was calculated as a percentage of new sequence types contained within the sequences obtained from populations (number of OTUs obtained per isolate).

	Samples	Isolates	OTUs	Diversity index
Region				
Atherton Tablelands	10	71	51	72%
Brisbane	5	35	28	80%
Sydney	5	22	11	50%
EMAI	3	20	11	55%
Sex (Atherton Tablelands only)				
Male	5	35	26	74%
Female	5	36	27	75%
Population types				
Native (tropical and subtropical)	15	106	79	74%
Invasive (temperate)	5	22	11	50%
Captive	3	20	11	55%

A)




B)
KOU78183:Klebsiella oxytoca
BMM05-9
BMM05-7
BMM05-1
AMF04-9
54
AMF04-1
AMF04-3
AMM03-3|AMF04-7
58
AMF04-8
AMF04-2/5/6
BMM05-6
AMM03-7
JF772064:Enterobacter cloacae
BMM02-1
BMM10-6
BMM10-1|SMM01-6
SMM01-10
ARF02-7
ARF02-2
BMM10-3/5/8/10
ARF02-10
ARF02-1
ARF02-9
KR232639.1:Providencia sp.(Medfly)
99
SCM02-3/5
60
ARF01-7|EGYIM03-8
84
ARF01-3/9/10
AMF06-7
KR232642.1:Enterobacter sp.(Medfly)
ARM04-7/8/9
63
ARM04-2
0.02

C)

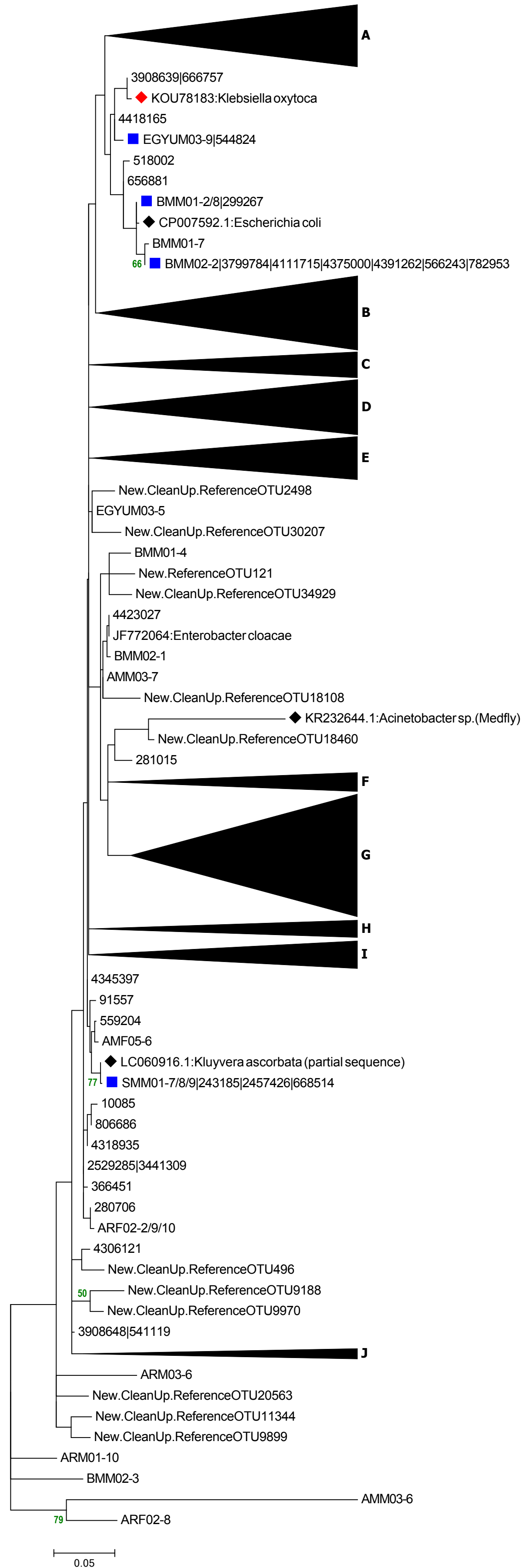
Figure 5.2: Phylogenetic trees (A, B and C) of near full-length 16S rRNA gene sequences of bacteria isolated from *Bactrocera tryoni* gut and from reference samples (◆), inferred by using the Maximum Likelihood method based on the Jukes-Cantor model and supported by 100 bootstrap replicates (with only bootstrap values of 50% and above shown). KOU78183: *Klebsiella oxytoca* (◆) was used as reference across all phylogenetic trees. The analysis involved 102 sequences after similar sequences were collapsed from an original number of 156 sequences. All nucleotide positions with less than 35% site coverage across all sequences were eliminated. There were a total of 1337 positions in the final dataset.

The phylogenetic tree of the bacterial isolates and reference samples (Fig. 5.2 A, B and C) showed that all the bacterial isolates were somewhat closely related, with many nodes having very low bootstrap values and therefore remaining unresolved. A noteworthy find was that the 11 bacterial isolates from the EMAI sterile male B. tryoni reared in captivity grouped within clades that contained the field-collected samples albeit they were distinct OTUs (Fig. 5.1).

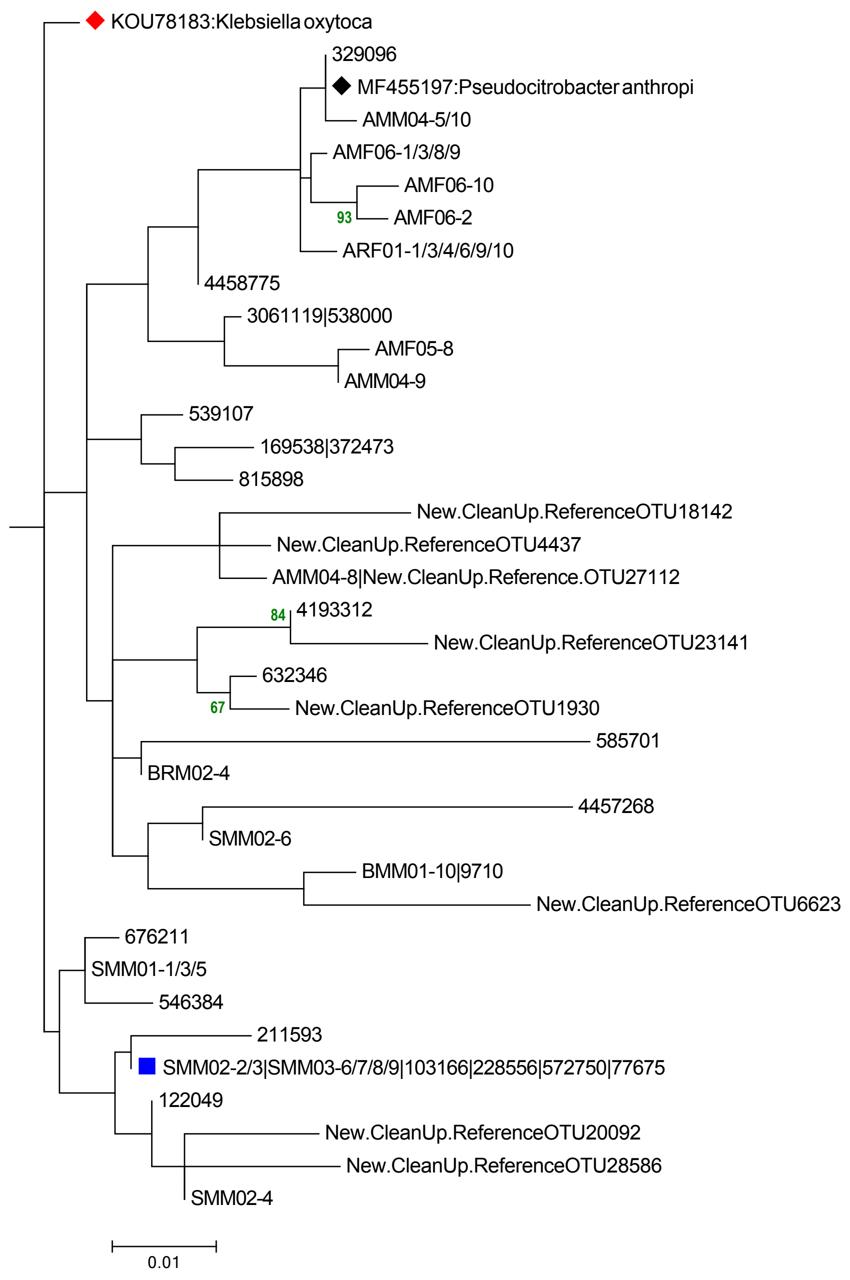
The isolates grouped into 94 clades which were grouped as OTUs. From NCBI (2011) BLAST search, the most abundant OTUs genera were *Citrobacter* with *Enterobacter, Klebsiella*, and *Kluyvera* also being present. The less abundant OTUs include *Providencia*, *Pantoea*, *Pseudocitrobacter* and *Escherichia*.

When the 258 Enterobacteriaceae sequences from the Miseq run (discussed in Chapters 3 & 4) were incorporated into the phylogenetic analysis of the isolates, all the MiSeq sequences grouped with the sequences of the cultured isolates (Fig. 5.3 A, B, C, D, E, F, G, H and I). The phylogenetic trees were made from 414 sequences (including references) and grouped into 315 clades. Within these, there were 12 clades where the isolate sequences were identical to the MiSeq sequences (represented by ■ in Fig. 5.3 and listed in Table 5.3) indicating a successful culturing of the bacteria identified in the MiSeq runs. The results from BLAST search of these isolate sequences identified almost all of the bacteria to the genus level, however, the MiSeq sequences identification only identified the bacterial sequences to family level for all but one sequence.

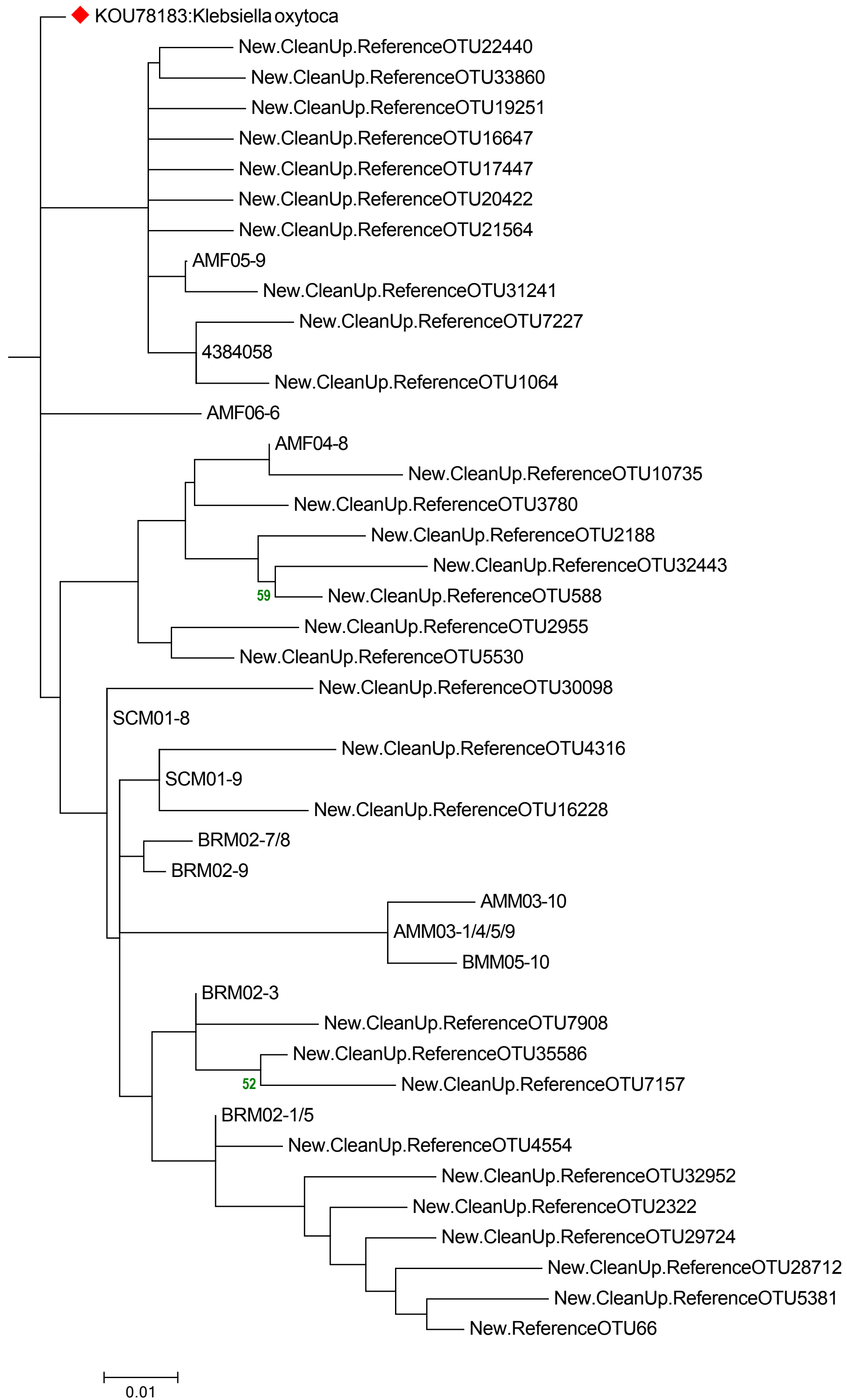
A)



B)



C)



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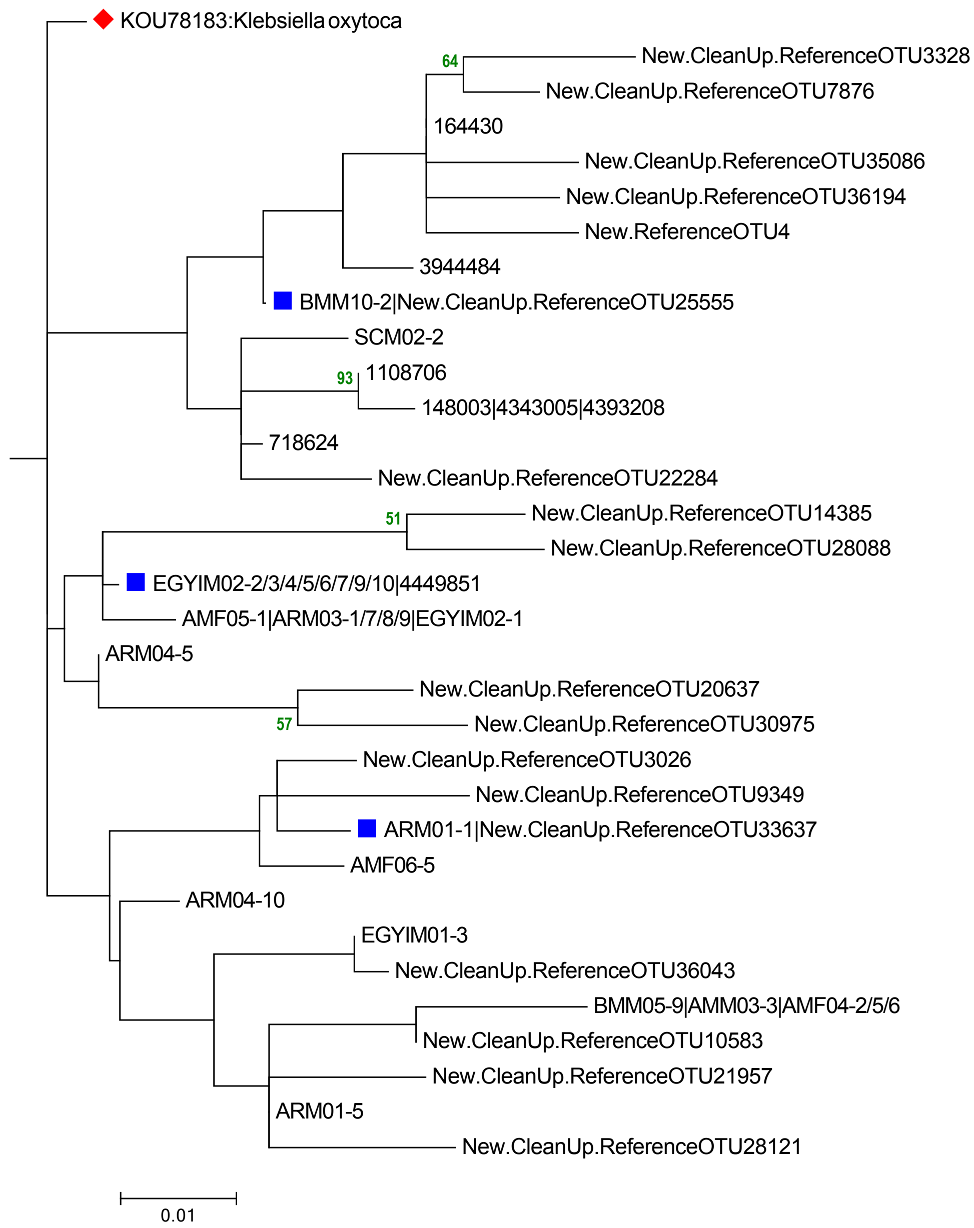

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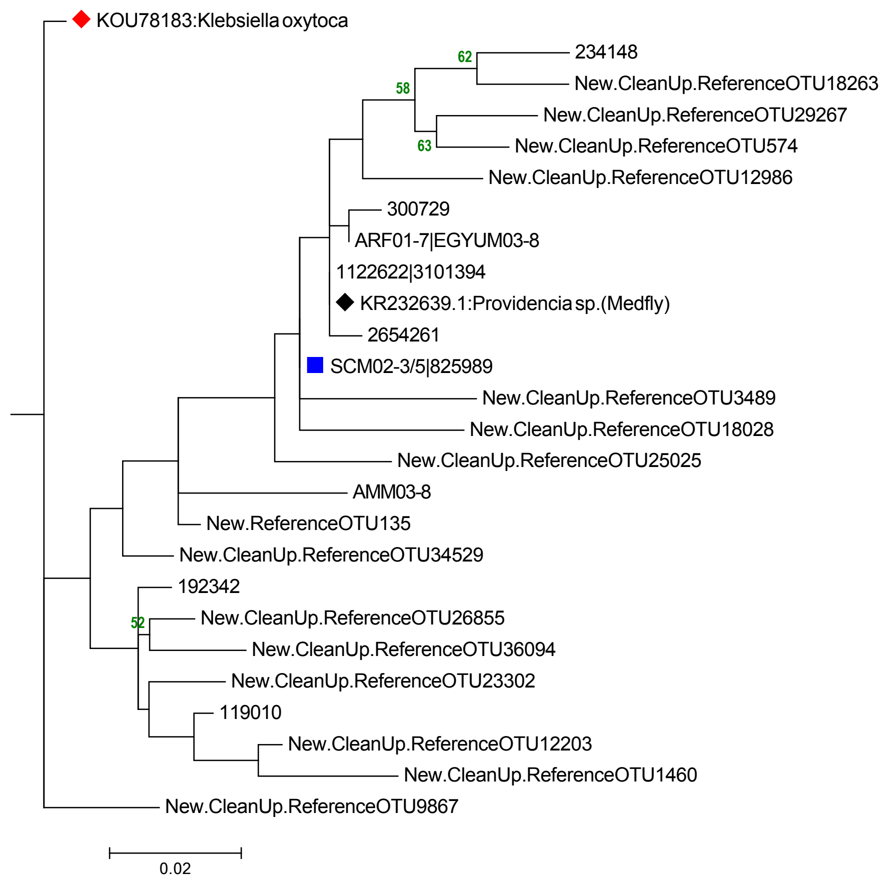

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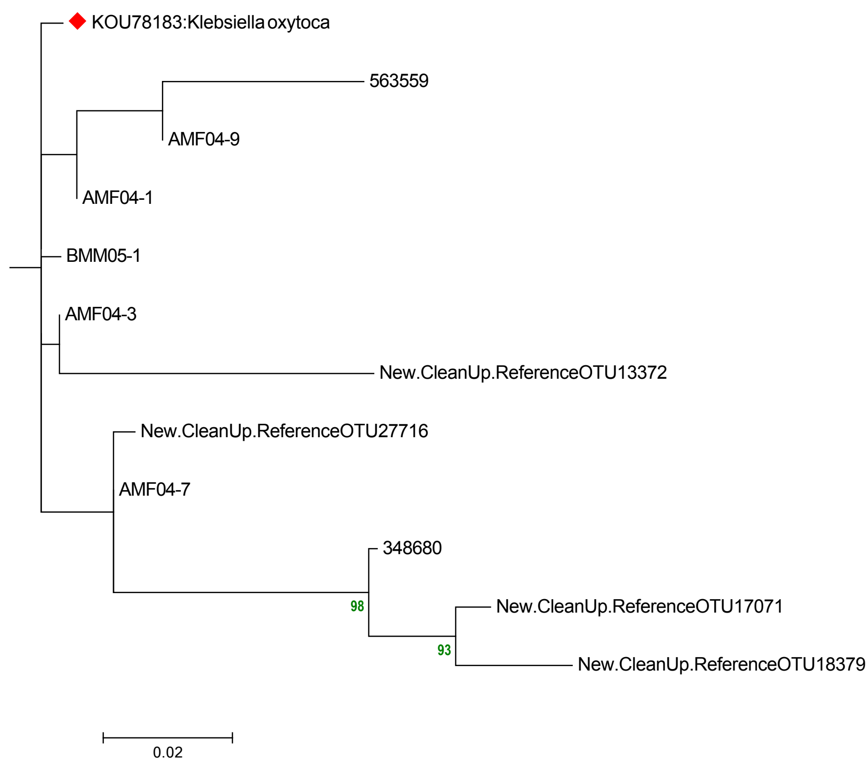

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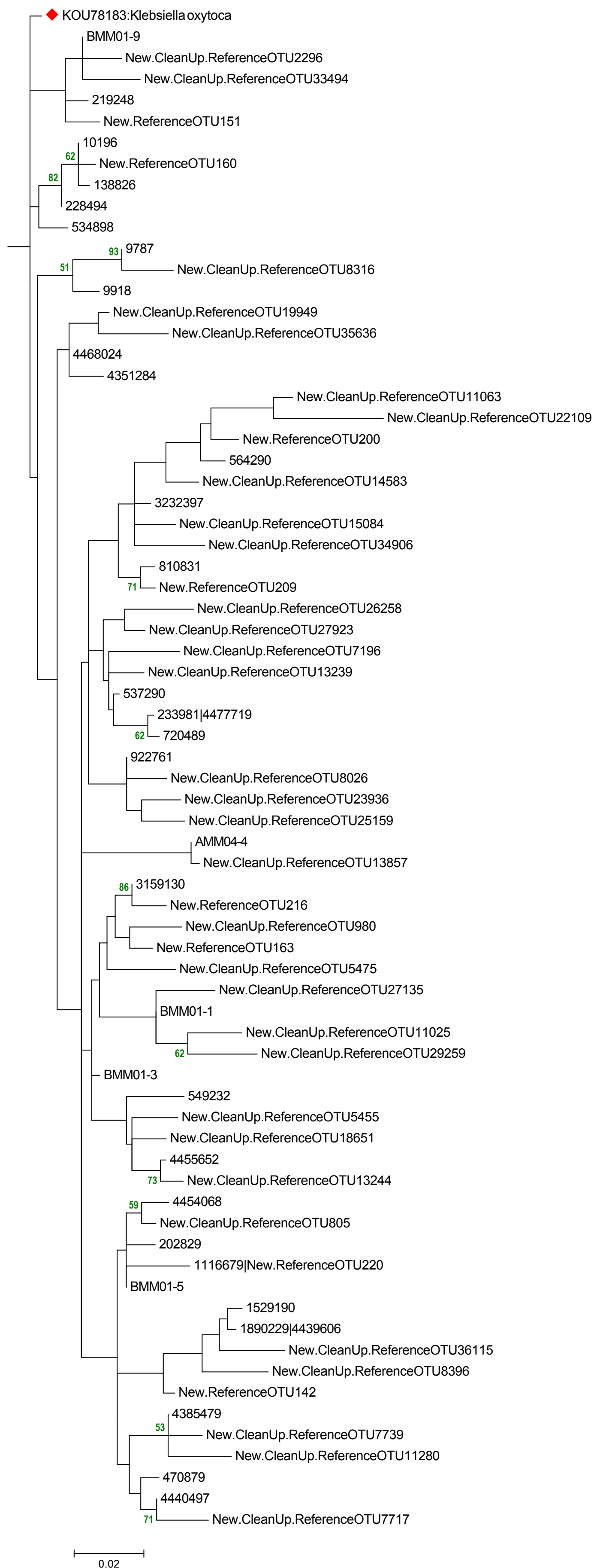

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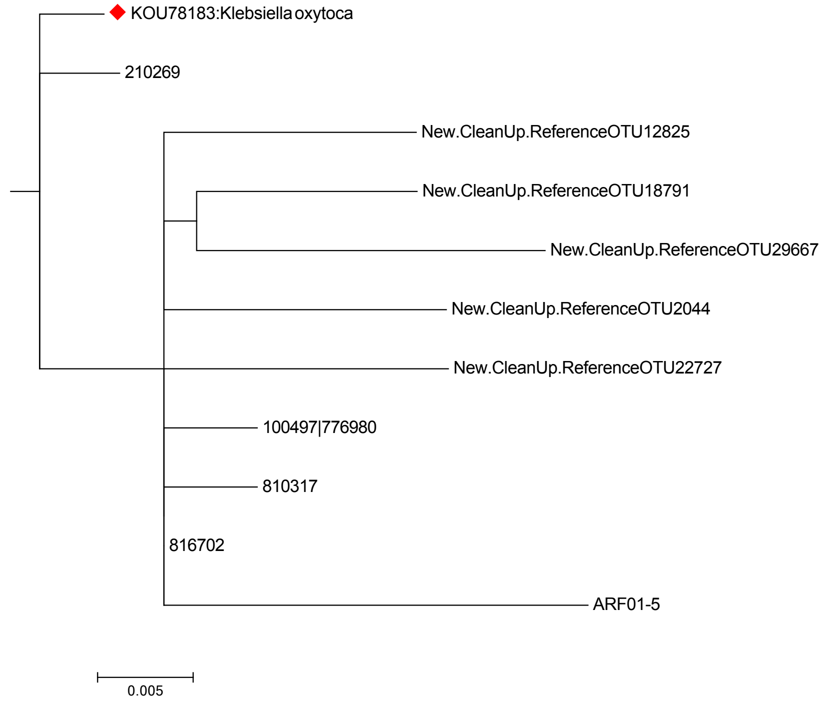

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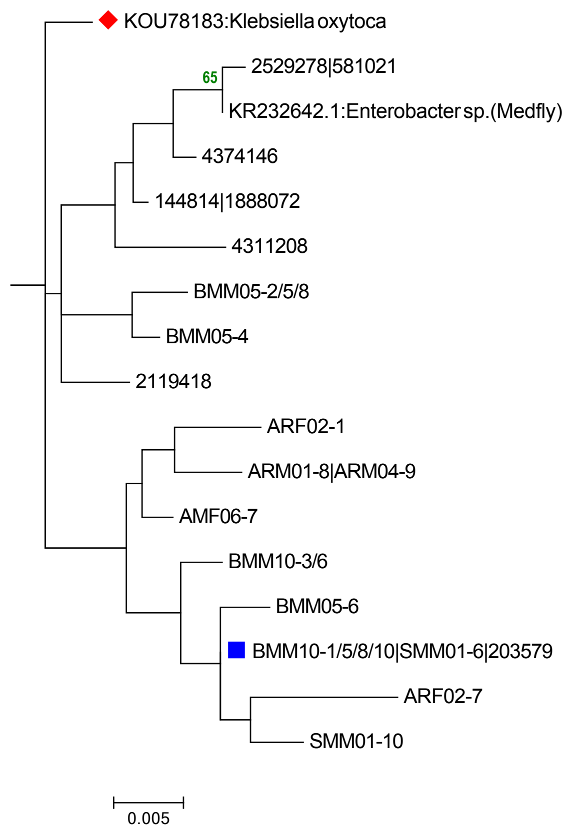

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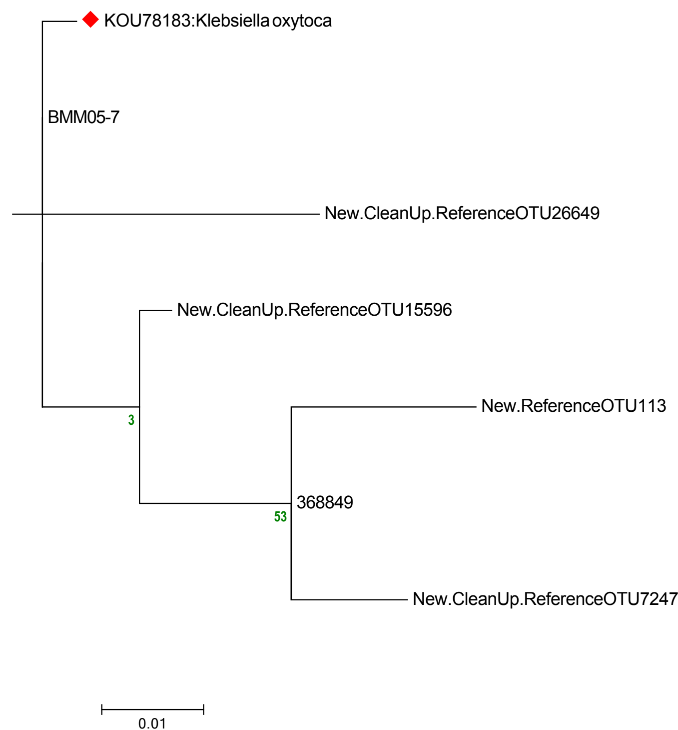

Figure 5.3: Phylogenetic trees (A, B, C, B, E, F, G, H and I) of near full-length 16S rRNA gene sequence from bacteria isolated from *Bactrocera tryoni* gut, 16S rRNA gene sequences obtained from MiSeq analysis of *Bactrocera tryoni* guts, and reference bacterial samples (◆), inferred by using the Maximum Likelihood method based on the Tamura-Nei model and supported by 100 bootstrap replicates (with only bootstrap values of 50% and above are shown). KOU78183: *Klebsiella oxytoca* (◆) was used as reference across all phylogenetic trees. Clades where the isolate sequences were identical to the MiSeq sequences are represented by ■. The analysis involved 315 sequences after similar sequences were collapsed from an original number of 414 sequences. The trees are drawn to scale, with branch lengths measured in the number of substitutions per site. All nucleotide positions with less than 20% site coverage across all sequences were eliminated.

Table 5.3: Identical clades of bacterial isolate and MiSeq sequences of DNA extracted from the guts of *Bactrocera tryoni*. All listed BLAST search results used were at ≥ 98% sequence identity.

	Identical clades	Closest BLAST search	MiSeq.ID
1	AMM04-8|New.CleanUp.Reference.OTU27112	*Citrobacter* sp. / *Klebsiella oxytoca*	Enterobacteriaceae unknown
2	ARM01-1|New.CleanUp.ReferenceOTU36043	*Kluyvera* sp. / *Citrobacter* sp.	Enterobacteriaceae unknown
3	BMM01-2/8|299267	*Escherichia coli*	*Escherichia coli*
4	BMM01-10|9710	*Escherichia coli*	*Escherichia coli*
5	BMM02-2|3799784|4111715|4375000|4391262|566243|782953	*Escherichia coli* / *Shigella* sp.	Enterobacteriaceae unknown
6	BMM10-1/5/8/10|SMM01-6|203579	*Enterobacter* sp.	Enterobacteriaceae unknown
7	BMM10-2|New.CleanUp.ReferenceOTU25555	*Raoultella terrigena* / *Klebsiella* sp.	Enterobacteriaceae unknown
8	EGYIM02-2/3/4/5/6/7/9/10|4449851	*Citrobacter* sp.	Enterobacteriaceae unknown
9	EGYUM03-9|544824	*Citrobacter farmeri*	Enterobacteriaceae unknown
10	SCM02-3/5|825989	*Providencia* sp.	*Providencia* sp.
11	SMM01-7/8/9|243185|2457426|668514	*Kluyvera ascorbata*	Enterobacteriaceae unknown
12	SMM02-2/3|SMM03-6/7/8/9|103166|228556|572750|77675	*Providencia* sp.	Enterobacteriaceae unknown

5.4: Discussion

The microbiological isolation using Enterobacteriaceae-selective media returned a good diversity of Enterobacteriaceae that was also found in the next generation 16S rRNA gene amplicon sequencing analysis. Based on the analysis of the Sanger sequenced near full length 16S rRNA gene amplicons, there was a lot of diversity in the Enterobacteriaceae found in the gut of adult *B. tryoni* within and across populations. It also appeared that near full length 16S rRNA gene might still not be adequate to discriminate different clades of Enterobacteriaceae given that BLAST searches revealed matches of the same sequence to different bacteria in the database, and given that the clades were not well resolved as can be seen by the low boot strap values (Figs. 2 and 3). Alternative approaches such as multi locus sequence typing and full genome sequencing and characterisation can be utilised to investigate Enterobacteriaceae further, as they might be able to differentiate closely related OTUs (Martens et al. 2008).

The OTU table generated from the phylogenetic alignment indicated that the bacterial isolates from flies from the native populations of the Atherton Tablelands and Brisbane regions were more diverse than bacterial isolates from the invasive population of the Sydney region and the captive laboratory population. This finding supports findings of Chapters 3 and 4 using the 16S rRNA gene amplicon sequencing data. The native populations from the tropical and subtropical regions would have access to a more diverse food sources which could contribute to the diversity in gut microbiome.

The next steps will be to find out how these isolates influence *B. tryoni* fitness and performance. Once this is established the recommended focus should be on how to either encourage the proliferation of those bacteria in the gut of SIT flies, or how to provide it to them as probiotics, so their microbiome are similar or better than the field-collected *B. tryoni*.

Chapter 6: Thesis discussion

6.1: Summary of objectives

The overall objective of this thesis was to investigate the relationship between the tephritid pest Queensland fruit fly, *Bactrocera tryoni* and its gut bacteria in the context of the sterile insect technique (SIT). To achieve the main objective, the following research questions were addressed:

vii) What is the abundance and diversity of the gut bacteria in teneral and mature adult Queensland fruit fly reared in captivity?

viii) How does gamma irradiation (to render the flies sterile) affect teneral and mature adult Queensland fruit fly?

ix) What is the impact of larval and adult rearing environments and diets on the gut bacteria of captive adult Queensland fruit fly?

x) What are the gut bacterial communities of field-collected populations of Queensland fruit fly from native and invasive populations within and across climatic regions and habitats?

xi) How does the gut bacterial community of Queensland fruit fly from captive populations, fertile and sterile, differ from field-collected populations and are there any missing or deficient bacteria?

xii) Is it possible to isolate and culture Enterobacteriaceae that are representative of the entirety of Enterobacteriaceae present in field collected individuals across climatic regions and habitats? Are these isolates similar or different across different climatic regions? Are these isolates similar to the bacteria that are lacking or deficient in captive and irradiated flies? If so, these are prime candidates for probiotic development to fill in any gaps in microbiomes of captive populations used for SIT.

6.2: Summary of Results

6.2.1: The abundance and diversity of gut bacteria in captive teneral and mature adult Queensland fruit fly

It was important to firstly understand the change in bacterial load and diversity during the adult developmental stages of the Queensland fruit fly. A key finding from this research was the low diversity and abundance of gut bacteria during the teneral stage compared to the mature adult stage indicating that only a small number of bacteria from the larval and pupal stages are transferred to the adult stage. This suggests a quantitative and potentially qualitative bottleneck of bacterial community during metamorphosis of the Queensland fruit fly, indicating that the bacterial community in the adult Queensland fruit fly is not only determined by the larval development but strongly by the environment that adults are exposed to where they can forage for, and obtain new bacteria. This also means that vertical transmission of gut bacteria may be fairly loose, with large potential for horizontal transmission of microbiota between genetically related and unrelated individuals in their adult environments, for example as shown in the Queensland fruit fly larval environment by Deutscher et al. 2018.

A key interest for use of bacteria in sterile insect technique (SIT) against tephritid pests is to introduce bacteria to change the gut bacterial community of the mass-reared sterile males (Estes et al. 2012; Yuval et al. 2010). This can occur at the different developmental stages, depending on the desired outcome. For the improved performance of released adult flies this could occur in the larval stage as this will generally increase the performance of developing males. My research supports findings of previous studies on tephritids and other insects, that manipulating the adult microbiome is likely the best time in order to observe increases in sterile fly performance, however my findings go further to show that the ideal time to introduce bacteria is at the teneral stage.

6.2.2: The diversity and abundance of gut bacteria between irradiated and unirradiated teneral and mature adult Queensland fruit fly in captivity

Gamma irradiation is known to cause physical damage to the gut of tephritids (Lauzon and Potter 2012) and also impact the mating performance of sterile males used in SIT (Lance et al. 2000). However, little is known about the impact of gamma irradiation on the gut bacterial community of a tephritid. My research is the first to compare the gut bacteria of an irradiated and unirradiated tephritid. I found that gamma irradiation did not affect gut bacterial diversity, but it did impact the total titre of gut bacteria. This leads to the conclusion that gamma irradiation might either affect the flies' ability to regulate their bacterial load or that gamma irradiation makes available new niches within the gut for bacteria to colonise.

6.2.3: The impact of larval and adult rearing environments and diets on the gut bacteria of mature adult Queensland fruit fly in captivity

A key factor within the environment of an adult tephritid that is most likely to impact gut bacterial community is the diet. The effect of diets, and particularly yeast hydrolysate fed to adults as a protein source, on mass-reared sterile adult tephritids used in SIT are well known (Reynolds et al. 2014; Taylor et al. 2013a). However, little is understood about the impact of yeast-containing adult diets on the gut of mass-reared tephritids.

Firstly, I showed that regardless of pupal origin, when the adult flies mature in the same environment, their gut bacterial community as mature adults are similar. This indicates that the environment is an important factor in determining the mature adult tephritid gut bacterial community. It was also found that the presence and absence of supplemented yeast hydrolysate in the adult diet shifted the gut bacterial community. These findings are fundamental in understanding how the environment and diets can be manipulated to impact the gut bacterial community of sterile male tephritids used in SIT.

The effects of the adult rearing environment and diets influencing the gut bacteria of adult Queensland fruit fly also provided a foundation for the next phase of the research which investigated the gut bacteria of mature adult flies across different environments.

6.2.4: The gut bacterial communities of individual field-collected Queensland fruit fly from native and invasive populations within and across climatic regions and habitats

The study investigated the gut bacterial communities of individual field-collected male and female Queensland fruit fly from Australia and New Caledonia. The samples were from various natural and managed habitats across climatic regions. The gut bacterial community was different for each population and region. There was a strong sex effect observed within population groups with the field males possessing a diverse community of bacteria. Interestingly, the gut bacterial community in field females was similar across all sampled environments while it varied in field males. This suggests that field males have a more variable diet, are perhaps less choosy, or disperse further from their natal habitat, possibly in search of mates or food. This also suggests that females have a core microbiome that is probably essential to their fitness and performance, including reproductive performance. The finding supports the use of a pre-release diet for the sterile males used in SIT so the sterile males' efforts are spent on seeking and mating with field females and not investing more energy in locating food to mature.

6.2.5: Comparing the gut bacterial communities of captive populations of Queensland fruit fly to field-collected populations to identify missing or deficient bacteria

A key question in the use of bacteria to enhance the performance of mass-reared captive fruit flies used in SIT is identifying bacteria that are found in field populations that are missing or deficient in sterile captive. To achieve this, the core gut bacteria of field flies were determined and compared to the bacteria from sterile and fertile captive flies.

The microbiota of captive flies were not as diverse as the microbiota of field flies, and they had different bacterial communities across different rearing facilities. This means that the rearing environment can shift bacterial gut communities in flies.

Interestingly, flies emerging in the laboratory from field-collected infested fruits and captive flies provided with a more natural diet such as cut oranges and chicken faeces, had gut bacteria which were almost as diverse as the gut microbiomes of field flies. This finding demonstrates the ability of the Queensland fruit fly to quickly adapt its microbiome in a changing environment, a trait that is important for the evolution and success of Queensland fruit fly as a pest species.

In microbiome analyses that clustered bacterial taxa at the higher taxonomic levels of genus and family, the controlled environment flies possessed the same bacterial genera and families as the field collected flies. This indicated that between populations bacterial species were being substituted thus suggesting that closely related bacteria may be substituting each other in function. Therefore, the bacteria that might improve mass-reared Queensland fruit fly adult performance may already be present in the mass-reared populations, but in lower or reduced abundance. This finding suggests that by manipulating the mass-rearing environment, the gut bacterial community of the mass-reared flies used in SIT can also be manipulated.

6.2.6: Culturing and identification of Enterobacteriaceae isolates from field-collected individual Queensland fruit fly across climatic regions and habitats as potential candidates for probiotic development

Enterobacteriaceae was the most common, and in most cases also the most abundant, bacterial family in adult Queensland fruit fly across populations, environments, climatic gradients and captive rearing facilities as shown in Chapters 2, 3 and 4. This also confirms previous work on this and closely related species (Morrow et al. 2015b). This suggests that the flies may maintain Enterobacteriaceae because of a symbiotic relationship. Therefore, Enterobacteriaceae was targeted for isolation from flies with the aim to obtain probiotic candidate bacteria that were also identified to be key components of the gut microbiome of the same individual fly by using next generation sequencing techniques. These may then be used in improving SIT. Bacterial DNA were extracted using a crude method and sent for Sanger sequencing.

Across both approaches, next generation sequencing analyses and microbiological isolation, the field flies were more diverse than the controlled environment flies. Both males and females were equally diverse but had different bacterial communities. Based on the isolation approach, the most abundant bacterial genera in males and females were *Citrobacter*, *Enterobacter, Klebsiella*, *Providencia* and *Kluyvera.*

However, the quality of the near full-length 16 S rRNA gene sequences were not as good as expected and, for some but not all isolates, were either unresolved or not long enough due to potential issues with impure subcultures (i.e. multiple isolates) and/or DNA concentration of isolation extracts. Also, for some bacteria even the near full-length 16S rRNA gene does not resolve the species ID status. These issues will need to be further investigated in the future, maybe with the use of multiple selective media to isolate a wider range of Enterobacteriaceae and also the use of other identification techniques such as the multilocus sequence typing (MLST) approach which might give better resolutions for isolate identification. Once identified, the isolates should then be tested on flies to assess their effect of Queensland fruit fly performance.

6.3: Implications for application

My PhD research has generated knowledge that is directly applicable to using, or manipulating bacteria to improve SIT for Queensland fruit fly. This application of bacteria can either be as a probiotic for improving the performance of the irradiated male released for SIT and also for improving the quality of mass-reared flies.

Firstly, this work has resolved some key concerns about tephritid gut bacteria and its application in SIT. This includes understanding that the male Queensland fruit fly is very diverse and is strongly influenced by its environment. The effect of irradiation on the bacterial abundance and diversity is also a new key understanding. Irradiated flies have the capacity to hold a higher bacterial load in comparison to the unirradiated flies but despite this, irradiation does not significantly impact gut bacteria diversity. Another key understanding from this thesis was the ability of Queensland fruit fly to quickly alter its microbiome within a generation when presented with a change of environment and diet.

These findings assist in understanding the dynamics of the relationship between the Queensland fruit fly and its gut bacteria.

The findings from this work will inform practical application to SIT includes the knowledge that the teneral Queensland fruit fly have very few gut bacteria, indicative of a bottleneck of bacterial abundance and diversity during fly metamorphosis. This means that efforts at introducing bacteria intended to affect the bacterial community of an adult fly, can focus from the teneral stage. Another direct application is the potential to manipulate the diet and rearing environment of the tephritid, which in turn will impact gut bacterial community diversity. A finding from this work supports the use of pre-release adult diets, possibly including beneficial bacteria for sterile male tenerals before they are released as part of a SIT program to manage Queensland fruit fly.

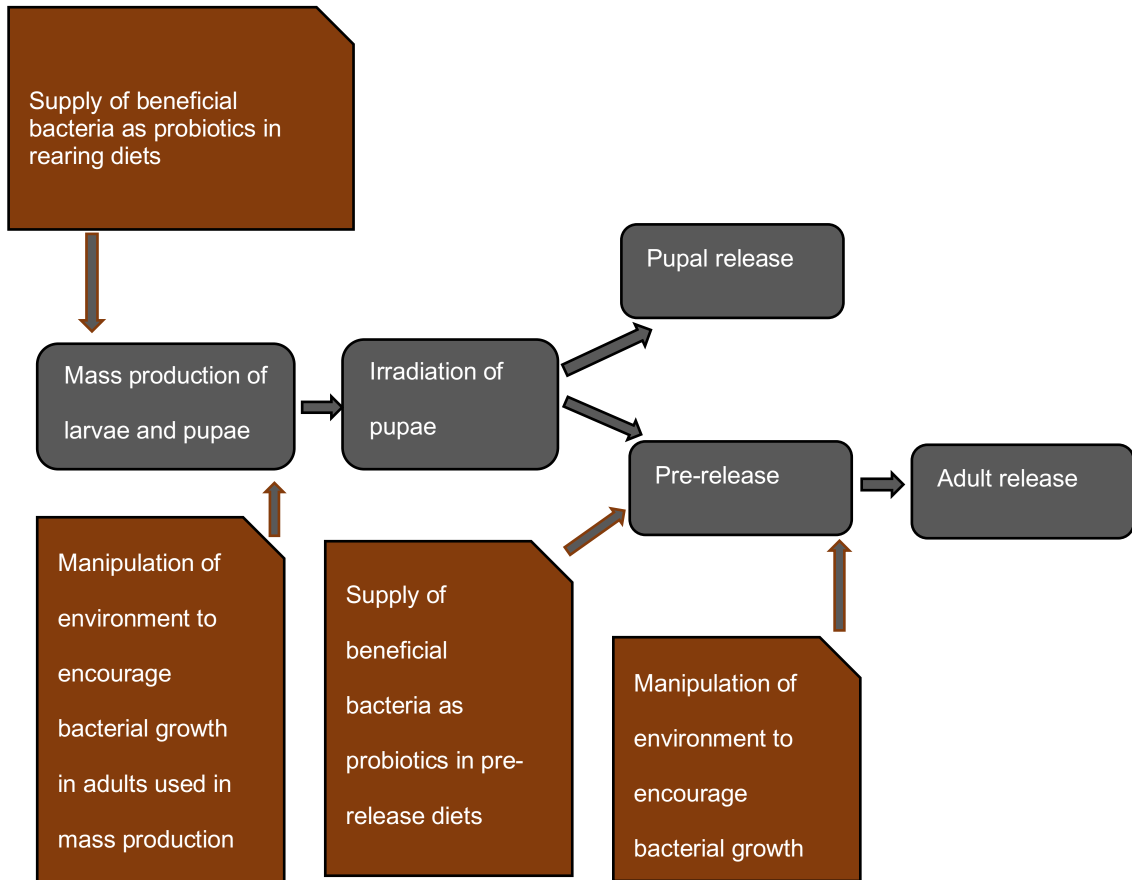

Figure 6.1: Potential areas for application of bacteria in Queensland fruit fly sterile insect technique

6.4: Concluding remarks

For this study the Queensland fruit fly was an ideal tephritid study species to investigate the diversity and dynamics of bacterial gut communities across native and invasive ranges. Its distribution across tropical, sub-tropical and temperate Australia (Dominiak and Daniels 2012), its introduction as an invasive pest species in a distant non-native island such as New Caledonia (Amice and Sales 1997), and large host range from native wild fruits to horticultural crops (Hancock et al. 2000), provided an ideal opportunity to sample across wide climatic and habitat gradients, including both native and invasive ranges, to understand the varying factors that influence the microbial gut communities of a significant pest tephritid. The findings from this thesis inform the use of beneficial gut bacteria in tephritid pest SIT.

Beyond the scope of the Queensland fruit fly SIT application, the findings of this thesis also demonstrate the evolution of the microbial community in an important and invasive fly species with an indication that these communities converge to similar composition and structure across development and different environments. As such, the significance of these findings sits beyond the application in pest control but contribute to progress in the research of gut bacteria, their biology and ecology in fruit fly species, and more widely in other insects and animals.

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Appendices

Appendix 1.1: Possible functions of identified *Bactrocera tryoni* gut bacteria

Acinetobacter calcoaceticus **(Beijerinck 1911) (NCBI 2011)**

Colonies of *Acinetobacter calcoaceticus* were isolated from the heads and eggs of *Bactrocera tryoni* by and identified using API-20E (Fitt and O'Brien 1985). The eggs were taken from field-collected peach, *Prunus persica,* and the heads were from adults reared from field-collected infected fruits. In this same work, this bacterium was isolated from the head of *Bactrocera neohumeralis,* reared from *P. persica* and the abdomen of adult *Bactrocera jarvisi,* reared from *Planchonia careya* (Fitt and O'Brien 1985). Records of *A. calcoaceticus* in other tephritid fruit flies include *Anastrepha ludens* (Martinez et al. 1994) and *Rhagoletis completa* (Howard et al. 1985). However, *A. calcoaceticus* has not been recorded in *B. tryoni* since then when molecular techniques have been deployed in identifying gut bacteria of insects. It is possible that this bacterium was misidentified. The role or association of *A. calcoaceticus* to tephritids is still unclear.

Aeromonas hydrophilia (Chester 1901) Stanier 1943 (NCBI 2011)

In the work by Fitt and O'Brien (1985) where bacteria were isolated from different parts of four *Bactrocera* species of Australia, the bacterium *Aeromonas hydrophilia* was isolated from the head of *B. tryoni* specimens. This bacterium has since not been recorded on *B. tryoni* and thus could have been misidentified. The role or association of *A. hydrophilia* with tephritids is still unclear.

Citrobacter freundii **(Braak 1928) Werkman and Gillen 1932 (NCBI 2011)**

Citrobacter freundii was a common bacterial species within cultured isolates from the crop and gut of *B. tryoni* (Lloyd et al. 1986; Thaochan et al. 2009). The bacterium was also commonly isolated from other tephritids, *Bactrocera cacuminata* (Thaochan et al. 2009),

Bactrocera dorsalis (Wang et al. 2014b) and *Bactrocera oleae* (Estes et al. 2012; Tsiropoulos 1983). When a strain of *C. freundii* was used in an Enterobacteriaceae consortium with strains of other bacteria *vis Pectobacterium cypripedi*, *Enterobacter* spp., *Klebsiella oxytoca*, and *Pantoea* spp., and fed to *Ceratitis capitata*, the consortium increased longevity of the fruit fly species (Behar et al. 2005). A *C. freundii* strain isolated from *B. dorsalis* was screened for its attraction potential and was found to be an effective attractant to the fruit fly species (Wang et al. 2014b). *Citrobacter freundii* should be investigated further for its potential role in a *B. tryoni* probiotic diet formulation.

Cronobacter sakazakii **(Farmer et al. 1980) Iversen et al. 2008 (NCBI 2011)**

To date, there is only one record of *Cronobacter sakazakii* isolated from *B. tryoni* (Thaochan et al. 2009). In that work, the bacterium was identified using the molecular tools and was recorded as the synonym, *Enterobacter sakazakii*. The role or significance of *C. saksazakii* in *B. tryoni* is still unknown.

Enterobacter aerogenes Hormaeche and Edwards 1960 (NCBI 2011)

The bacterium *Enterobacter aerogenes* was isolated from the mid gut of *B. tryoni* and *B. cacuminata* and identified using molecular techniques (Thaochan et al. 2009). The role or association of *E. aerogenes* to tephritids is still unclear.

Enterobacter cloacae **(Jordan 1890) Hormaeche and Edwards 1960 (NCBI 2011)**

Enterobacter cloacae were frequently recorded in the crop and gut of *B. tryoni* (Drew and Lloyd 1987; Fitt and O'Brien 1985; Lloyd et al. 1986; Thaochan et al. 2009). The bacterium was also isolated from *B. tryoni* faeces (Lloyd et al. 1986) and in infested fruit hosts (Fitt and O'Brien 1985). Records of this bacterium were also made as either a commonly isolated species from other tephritids such as *B. cacuminata* (Raghu et al. 2002; Thaochan et al. 2010; Thaochan et al. 2013), *B. dorsalis* (Thaochan et al. 2013; Wang et al. 2014b) *B. oleae* (Estes et al. 2012), and *Bactrocera zonata* (Reddy et al. 2014).

Enterobacter cloacae was the bacterial species most frequently isolated from the feeding surface of hosts fruits and leaves where the adult *B. tryoni* regurgitated and re-ingested fluids during feeding or oviposition (for females) (Drew and Lloyd 1987). *Enterobacter cloacae* was also the most frequently isolated species in this naturally-occurring attractive bacterial consortium which included *Pantoea agglomerans* (recorded as *Erwinia herbicola*) and *K. oxytoca* which was spread by the fliesand over time, were observed to make the host tree more attractive to other fruit flies (Drew and Lloyd 1987). *Enterobacter cloacae* was also isolated from the female *B. cacuminata* oviposition sites (Raghu et al. 2002). Drew and Lloyd (1987) put forward the possible role of *E. cloacae* as an attractant for other females to oviposit in the same infested fruit or other fruit flies to feed from the same plant surface.

The attractiveness potential of *E. cloacae* was also evaluated on *B. zonata* (Reddy et al. 2014). When compared to *Stenotrophomonas maltophilia*, *Klebsiella pneumoniae* and the control treatments used in this research, *E. cloacae* was the most frequently isolated attractive bacterium for different age groups of *B. zonata*. The attractiveness of *E. clocae* and fourteen other bacteria were evaluated on *B. dorsalis*, where it was found that *E. cloacae* was the most attractive bacterium to *B. dorsalis* (Wang et al. 2014b)

The inconsistency of finding *E. cloacae* in insects led Raghu et al. (2002) to suggest that the association of the *E. cloacae* and *B. cacuminata* could be more fortuitous than a highly specific symbiosis such as the release of vitamins as proposed by Fitt and O'Brien (1985). This suggestion dismisses the possibility of an internal nutrient-provisioning symbiotic relationship between *E. cloacae* and potential host fruit fly.

Apart from these symbiotic strains of *E. cloacae*, there is a known entomopathogenic strain of this bacterial species that was isolated from *Anastrepha fraterculus* and screened for its potential application as a biological control agent of the citrus pest, the leafminer *Phyllocnistis citrella* (Campos et al. 2007). This suggests that despite the potential of *E. cloacae* as a probiotic candidate, isolates of *E. cloacae* will need to be individually screened to understand the properties of each strain which could be beneficial or pathogenic to the potential host fruit fly.

***Escherichia coli* (Migula 1895) Castellani and Chalmers 1919 (NCBI 2011)**

Escherichia coli is common in animal and human guts and although most strains of this bacterium are harmless, a few have been known to cause serious illnesses in animals and humans (Velvez 2012). Fruit flies have been investigated as vectors of harmful *E. coli* strains (Sela et al. 2005). However, the record of *E. coli* strains detected in tephritid species have been few; *B. tryoni* (Lloyd et al. 1986; Thaochan et al. 2009), *B. cacuminata* (Thaochan et al. 2010; Thaochan et al. 2009) and *Rhagoletis pomonella* (Lauzon et al. 1998). In *B. tryoni*, *E. coli* has been isolated from the crop, midgut, oesophageal bulb and faeces (Lloyd et al. 1986; Thaochan et al. 2009). When *E. coli* was used in tephritid research, it was basically as a blank control for the isolation technique (Aharon et al. 2013; Lauzon et al. 1998). It is unknown what effect *E. coli* strains have on fruit flies but given the enormous genetic diversity and physiological versatility of this organism, it would be advisable to not use in probiotic research when *E. coli* has strain that could be or become pathogenic to humans.

***Flavobacterium* sp. Flavobacterium Bergey et al. 1923 (Approved Lists 1980) emend. Dong et al. 2013 (NCBI 2011)**

The only record of any bacterium in the *Flavobacterium* genus in *B. tryoni* were isolations of *Flavobacterium* sp. from cultures of adult *B. tryoni* head and abdomen (Fitt and O'Brien 1985). Traces of this bacterial genus were also found in more recent works on pumpkin fly *Bactrocera tau* (Walker) (Khan et al. 2014; Prabhakar et al. 2013). In all cases, the presence of *Flavobacterium* sp. was in small quantities and was deemed not significant. At this stage, the role of *Flavobacterium* sp. in tephritids is unclear.

***Hafnia alvei* Moller 1954 (NCBI 2011)**

Isolates of *Hafnia alvei* were identified on cultures of *B. tryoni* midgut and crop (Thaochan et al. 2009). Other records of this bacterium in tephritids include identifications from gut bacteria cultures of two different strains of *B. oleae* (Tsiropoulos 1983) and from *B. cacuminata* (Thaochan et al. 2009). The role of this bacterium in tephritids is unclear.

***Klebsiella oxytoca* (Flugge 1886) Lautrop 1956 (NCBI 2011)**

Klebsiella oxytoca is one of the dominant bacterial species in the gut of *B. tryoni* and other Australian *Bactrocera* species including *B. neohumeralis*, *B. cacuminata*, *and Bactrocera musae* (Lloyd et al. 1986; Murphy et al. 1994; Raghu et al. 2002; Thaochan et al. 2009). It has been identified in other tephritids such as *Bactrocera cucurbitae* (Coquillett) (Thaochan et al. 2010), *B. dorsalis* (Thaochan et al. 2013), *B. tau* (Khan et al. 2014), and *C. capitata* (Behar et al. 2008b; Ben Ami et al. 2010).

Drew and Lloyd (1987) classified the *K. oxytoca* as one of the bacteria commonly associated with fruit flies ("fruit-fly-type" bacteria) as it was part of the three dominant species which included *E. cloacae* and *P. agglomerans* (recorded as *Erwinia herbicola*) as identified by Lloyd et al. (1986). In that work, *K. oxytoca* was observed to be easily ingested and established well in the alimentary gut of *B. tryoni.* It was also observed as being one of the dominant species of bacteria that is spread by adult *B. tryoni* when feeding and regurgitating and when the female oviposit (Drew and Lloyd 1987). The bacteria was also identified as one of the dominant species suggested to increase the attractiveness of a host tree to other *B. tryoni* (Drew and Lloyd 1987). However, Meats et al. (2009) were not able to replicate the success of *B. tryoni* being attracted to *K. oxytoca*.

Drew and Lloyd (1987) suggested a possible role *K. oxytoca* and the "fruit-fly-type" bacteria might play in concentrating leachate nitrogen into suitable food source for *B. tryoni* and *B. cacuminata*, the two *Bactrocera* species that were used in that investigation. However, when *B. tryoni* were fed nitrogen-fixing strain of *K. oxytoca* and the nitrogenous activity measured using an acetylene reduction assay, no significant effect was observed (Murphy et al. 1988).

Pertaining to the conclusions by Drew and Lloyd (1987), relative research on bacteria associated with fruit fly was conducted on *B. cacuminata* by Raghu et al. (2002) which found the bacteria commonly present on host fruit surfaces but reached inconclusive evidence to associate a mutualistic relationship between bacteria and fruit fly.

In more recent work, *K. oxytoca* and *K. pneumoniae* were fed as food to *B. tryoni* and the effect on fecundity was assessed (Meats et al. 2009). Both bacteria were fed as single species

and in combination in standard diets or as bacterial cultures. *B. tryoni* were not attracted to any of the bacterial diets and the *K. oxytoca* and *K. pneumoniae* as a food source or symbiont, did not increase *B. tryoni* fecundity. However, origins of the isolates of used in the experiment were not clarified (Meats et al. 2009). It is possible that the isolates used could have been from non-effective strains of *K. oxytoca* and *K. pneumoniae.*

When a strain of *K. oxytoca* was fed to *C. capitata*, the mating competiveness of the sterile male was significantly improved (Gavriel et al. 2010). In addition, the male bacteria-enriched *C. capitata* inhibited female receptivity more efficiently than sugar-fed males and survived longer duration of starvation. Strains of *K. oxytoca* and *Providencia rettgeri* were used in mating efficiency trials of *B. tau*, however it did not enhance the female fecundity as expected (Khan et al. 2014). *Klebsiella oxytoca* was one of the species in a consortium of bacteria fed to *C. capitata* which resulted in increased longevity of the fruit fly species (Behar et al. 2008b).

It is very likely that *K. oxytoca* will be found in the guts of field collected *B. tryoni*. From the literature, this bacterium should be a key probiotic candidate. However, after reviewing the work of Meats et al. (2009) and Khan et al. (2014), it is possible that there might be different strains of *K. oxytoca* which could have different associations with *B. tryoni*.

***Klebsiella pneumoniae* subsp. *ozaenae* (Abel 1893) Orskov 1984 (NCBI 2011)**

Klebsiella pneumoniae subsp. *ozaenae,* was one of the most dominant bacteria isolated from feeding surfaces, mouthparts and regurgitant droplets of *B. tryoni* (Drew and Lloyd 1987). The bacterium was isolated from the gut of *B. tryoni,* however, it was not a dominant species (Thaochan et al. 2009). These would be the only mentions of this bacterium in *B. tryoni*. It is not commonly known in other tephritids, with the only other record of this bacterium being from *B. cucurbitae* (Thaochan et al. 2010). The role of this bacterium in tephritids is unknown.

***Klebsiella pneumoniae* (Schroeter 1886) Trevisan 1887 (NCBI 2011)**

The only record of *Klebsiella pneumoniae* naturally occurring in the guts of tephritids in Australia where isolations were made from wild populations of *B. tryoni*, *B. jarvisi* and *B. cacuminata* and from laboratory populations of *B. tryoni* (Fitt and O'Brien 1985). The potential for using *K. pneumoniae* as a bacterial food source was screened along with *K. oxytoca*, which produced unconvincing results (Meats et al. 2009). The source of the isolates used was not clarified and thus there is a possibility that non-effective strains were used.

The potential of *K. pneumoniae* as a symbiont in other tephritid flies has been well investigated. In most research, *K. pneumoniae* was used with another bacterium, usually *P. agglomerans*. Research conducted to understand the role of *K. pneumoniae* and *P. agglomerans* in adult *C. capitata,* found that the bacteria are easily acquired from natural food sources and ingested (horizontally transmitted) and strains were also passed through all life stages and can be vertically transmitted through successive generations (Lauzon et al. 2009). This behaviour of being horizontally and vertically transmittable suggests a symbiotic relationship. An increase in male mating efficiency was observed when *K. pneumoniae* and *P. agglomerans* were added to the diet of mass-reared *C. capitata* (Niyazi et al. 2004). There were observed improvements in damaged gut of irradiated *C. capitata* when irradiated adults consumed a diet that contained *K. pneumoniae* and *P. agglomerans* (Lauzon et al. 2009). It was then concluded that *K. pneumoniae* and *P. agglomerans* jointly participate in the catabolism of nitrogen in the gut of *R. pomonella.* (Lauzon et al. 2009). Literature supports *K. pneumoniae* as a worthy candidate for use in *B. tryoni* probiotic diet screening.

***Kluyvera intermedia* (Izard et al. 1980) Pavan et al. 2005 (NCBI 2011)**

In the only record of *Kluyvera intermedia* in *B. tryoni*, the bacterium (written as *Enterobacter intermedius*) was identified using a culture dependent method (API 20E) from a bacterial culture of the midgut of an adult male *B. tryoni* (Thaochan et al. 2009). In research using molecular technique 16s rRNA, *K. intermedia* was detected as the predominant species of bacteria in *B. cucurbitae* (Thaochan et al. 2010). The role or significance of *K. intermedia* in tephritids is still unknown.

***Lelliottia amnigena* (Izard et al. 1981) Brady et al. 2013 (NCBI 2011)**

The only record of *Lelliottia amnigena* (recorded as the synonym *Enterobacter amnigenus*) on *B. tryoni* was from the crop and gut of adult specimens (Thaochan et al. 2009). The role or association of *L. amnigena* to tephritids is still unclear.

***Pantoea agglomerans* (Ewing and Fife 1972) Gavini et al. 1989 (NCBI 2011)**

Pantoea agglomerans was one of the most isolated species of bacteria from the gut of *B. tryoni* and related Australian species, *B. neohumeralis*, *B. cacuminata* and *B. musae* (Lloyd et al. 1986). Follow up research conducted to understand the role of the gut bacteria in *B. tryoni* and *B. cacuminata*, identified *P. agglomerans* as one of the bacteria that is ingested and regurgitated by adult flies when feeding and ovipositing (for females) (Drew and Lloyd 1987). *Pantoea agglomerans* was one of the "attractive-bacteria" which included *E. cloacae*, and *K. oxytoca* that was concluded to attract flies to a host plant (Drew and Lloyd 1987). To date, these works by Lloyd et al. (1986) and Drew and Lloyd (1987) would be the only records of *P. agglomerans* on *B. tryoni*. There has not been any discovery of *P. agglomerans* in *B. tryoni* in the last decade despite more research being done on gut bacteria of tephritid flies in the last decade then when the when the initial discovery was made.

Pantoea agglomerans has been commonly associated with other tephritids species outside of Australia; *C. capitata* (Lauzon et al. 2009; Niyazi et al. 2004), *R. pomonella* (Lauzon et al. 1998; MacCollom et al. 1992) and *B. cucurbitae* (Thaochan et al. 2010). *Pantoea agglomerans* and *K. pneumoniae* were observed to be easily acquired by adult *C. capitata* from natural food sources that have high nitrogen content i.e. bird faeces and insect frass, and ingested strains were passed through all life stages and can be vertically transmitted through successive generations (Lauzon et al. 2009). The fact that these bacteria are vertically transmittable suggests a symbiotic relationship. One proven association of *P. agglomerans* and a tephritid was observed when the bacteria degraded and detoxified phloridzin, a plant derived compound toxic to *R. pomonella* (Lauzon et al. 2003). When *P. agglomerans* and *K. pneumoniae* were added to the diet of mass-reared *C. capitata*, the male mating efficiency was enhanced (Niyazi et al. 2004). The research on other tephritids suggests *P. agglomerans*

is a worthy candidate for evaluation in a probiotic diet for *B. tryoni*, provided a stain of *P. agglomerans* can be isolated again from *B. tryoni*.

***Proteus mirabilis* Hauser 1885 (NCBI 2011)**

Proteus mirabilis is not a common bacterial species in tephritids and to date there is only one record of this bacterium in *B. tryoni* (Lloyd et al. 1986) and one record in *B. oleae* (Tsiropoulos 1983). Both of these isolations were from wild populations. Since no new records of tephritid bacteria have included this species especially after molecular tools were used for bacterial identification, this raises the concern of misidentification of this species.

Proteus vulgaris Hauser 1885 (Approved Lists 1980) emend. Judicial Commission 1999 (NCBI 2011)

Proteus vulgaris was isolated from wild and laboratory-reared populations of *B. tryoni* (Fitt and O'Brien 1985; Lloyd et al. 1986). It was also isolated from other tephritid flies including *B. jarvisi* (Fitt and O'Brien 1985; Lloyd et al. 1986), *B. neohumeralis* (Fitt and O'Brien 1985; Lloyd et al. 1986) and *A. ludens* (Martinez et al. 1994). The role of *P. vulgaris* in tephritids is not yet understood.

***Providencia rettgeri* (Hadley 1918) Brenner et al. 1978 (NCBI 2011)**

Providencia rettgeri has been identified from the gut, mouthparts and regurgitated fluids of *B. tryoni* and surfaces of host fruits (Drew and Lloyd 1987; Fitt and O'Brien 1985; Lloyd et al. 1986; Thaochan et al. 2009). It has been isolated from the gut of other tephritids including *B. cucurbitae* (Thaochan et al. 2010) and *B. tau* (Khan et al. 2014). The only investigation into the potential use of *P. rettgeri* as a symbiont was where *P. rettgeri* (recorded as *Proteus rettgeri*) and *K. oxytoca* were added to the diet of *B. tau* and the fecundity was monitored but no significant effects were observed (Khan et al. 2014). At this stage, the role of *P. rettgeri* in tephritids is unknown.

***Pseudomonas fluorescens* Migula 1895 (NCBI 2011)**

Pseudomonas fluorescens was isolated mainly from the pupae and head and crop of *B. tryoni* (Fitt and O'Brien 1985). There has not been any new record of isolation of this bacterium from *B. tryoni* since then. A strain of the bacterium was used as a natural food for *B. tryoni* (Drew et al. 1983). Adult insects were observed to be more attracted to the food source that contained the bacteria, however insects fed the *P. fluorescens*-enriched diet died quicker than the control insects (Drew et al. 1983). The bacterium was observed to be easily taken up by the fruit fly and establishes well in the alimentary canal of adult insects.

Records of this bacterium on other species include isolations from eggs of *B. neohumeralis* and larvae of *B. cacuminata* (Fitt and O'Brien 1985) and guts of adult *B. oleae* (Tsiropoulos 1983). A strain of this bacterium was also used in attractiveness tests of *R. pomonella* but it was not as effective as *P. agglomerans* (MacCollom et al. 1992). It is likely that *P. fluorescens* is an attractive bacterium like *P. agglomerans.* However, more work is required to fully understand the role this bacterium plays in tephritids.

***Stenotrophomonas maltophilia* (Hugh 1981) Palleroni and Bradbury 1993 (NCBI 2011)**

The only record of *Stenotrophomonas maltophilia* in *B. tryoni* was from culture of the adult heads (Fitt and O'Brien 1985). Apart from the isolation of the bacterium from almost-ripe host fruits (Drew and Lloyd 1987), the only other record of this bacterium in another tephritid was from wild and laboratory reared *B. zonata* (Reddy et al. 2014). When used in attractiveness test, *B. zonata* that were fed proteins were more attractive to *S. maltophilia* (Reddy et al. 2014).

The limited literature available on *S. maltophilia* in tephritids suggests that it might be an attractive bacterium. However, more work will be needed to confirm this suggestion. It is possible that *S. maltophilia* plays a symbiotic role in tephritids and should be a candidate to consider for probiotic diet screening.

Pseudomonas oryzihabitans **Kodama et al. 1985 (NCBI 2011)**

Pseudomonas oryzihabitans was isolated from the crop of *B. tryoni* (Thaochan et al. 2009). There are no records of this bacterium in other tephritids except for a mentioned in *B. oleae* in a review paper (Estes et al. 2012). The role of *P. oryzihabitans* in tephritids is still unclear.

Raoultella ornithinolytica **(Sakazaki et al. 1989) Drancourt et al. 2001 (NCBI 2011)**

The only record of this bacterium in *B. tryoni* was from the isolation of the midgut (Thaochan et al. 2009). At this time, the role of this bacterium in tephritids is unclear.

Raoultella terrigena **(Izard et al. 1981) Drancourt et al. 2001 (NCBI 2011)**

The presence of *Raoultella terrigena* in tephritid flies has been a recent discovery where molecular identification was used. The first record was isolation from the crop and midgut of *B. tryoni* and *B. cacuminata* (Thaochan et al. 2009). Since then, there have been two more records of this species in tephritids, *viz Bactrocera cacurbitae* (Thaochan et al. 2010), and *Bactrocera minax* where it was one of the dominant species (Wang et al. 2014a). The role of this bacterium in tephritid flies is still unknown.

Serratia liquefaciens **(Grimes and Hennerty 1931) Bascomb et al. 1971 (NCBI 2011)**

Serratia liquefaciens was the dominant larval bacterial species isolated from *B. tryoni* larvae (Fitt and O'Brien 1985). It was also isolated from adult *B. tryoni* and other Australian *Bactrocera* species including *B. jarvisi*, *B. neohumeralis* and *B. cacuminata* (Fitt and O'Brien 1985; Lloyd et al. 1986) and the Mexican fruit fly *A. ludens* (Martinez et al. 1994). *Serratia liquefaciens* was observed to secrete protease, which suggests the role of this bacterium in protein metabolism for the tephritids (Fitt and O'Brien 1985).

However, a concern for this species is the lack of records of this bacterium in the last decade when more improved techniques of bacterial isolation and identification were employed, thus

raising concerns of misidentification. Despite this, should *S. liquefaciens* be isolated from *B. tryoni* again, it is a candidate that warrants further investigation for probiotic formulation.

Serratia marcescens Bizio 1823 (NCBI 2011)

Serratia marcescens was isolated from the gut of *B. tryoni* (Fitt and O'Brien 1985; Thaochan et al. 2009). Isolations of this bacterial species have been made from other tephritids including *B. oleae* (Estes et al. 2014), *B. jarvisi* (Fitt and O'Brien 1985; Tsiropoulos 1983), *B. minax* (Wang et al. 2014a) and *C. capitata* (Campos et al. 2007). The studied effects of *S. marcescens* in tephritid flies have been of two extremes. A strain of this bacteria isolated from *C. capitata* was entomopathogenic and was trailed as a potential biological control agent of citrus pest *Phyllocnistis citrella* (Campos et al. 2007). Another strain of *S. marcescens* was studied for use as a potential biological attractant of *B. tryoni* (Howie 2007). The role of *S. marcescens* in tephritid flies varies between strains. Before further screening for use in a probiotic diet formulation, it must be established if the strain is not entomopathogenic.

***Serratia odorifera* Grimont et al. 1978 (NCBI 2011)**

The identification of *Serratia odorifera* using molecular techniques from isolates from the midgut of female *B.* tryoni by Thaochan et al. (2009), was the only record of this bacterial species in *B. tryoni* and possibly in tephritids as well. The role of this bacterium in tephritid flies is still unknown.

Appendix 1.1 References

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Appendix 2.1: Alpha diversity metrics of 58 gut samples from *Bactrocera tryoni* reared on artificial diets, calculated at 97% identity level, after rarefaction of tenerals to 10 sequence reads and matures to 5,500 sequence reads.

Sample ID	Raw sequence reads	Reads after OTU picking and singleton removal	Reads after chimera removal	OTUs before rarefaction	OTUs after rarefaction	Chao1	Simpson	Shannon	Good
ELNIF01	36,136	19,658	19,606	25	2	2	0.18	0.469	0.9
ELNIF02	17,530	7,943	7,911	17	1	1	0	0	1
ELNIF03	1,223	118	88	19	3	4	0.34	0.922	0.8
ELNIM01	1,461	173	169	12	1	1	0	0	1
ELNIM02	858	166	156	14	1	1	0	0	1
ELNIM03	2,794	1,239	1,228	14	2	2	0.42	0.881	1
ELNUF01	1,662	704	695	10	1	1	0	0	1
ELNUF02	2,264	1,001	990	5	1	1	0	0	1
ELNUF03	657	17	15	8	8	29	0.84	2.846	0.3
ELNUM01	1,315	457	455	12	1	1	0	0	1
ELNUM02	8,089	3,789	3,776	15	1	1	0	0	1
ELNUM03	2,374	1,084	1,071	16	1	1	0	0	1
HCNUF01	1,369	42	28	13	7	8.5	0.84	2.722	0.6
HCNUF02	511	37	24	12	9	23	0.88	3.122	0.2

HCNUF03	365	37	24	15	10	55	0.9	3.322	0
HCNUM01	511	17	11	8	8	13	0.86	2.922	0.4
HCNUM02	383	38	15	9	7	8.5	0.84	2.722	0.6
HCNUM03	2,575	46	41	16	6	9	0.76	2.322	0.6
HCNUM04	822	20	12	7	7	12	0.82	2.646	0.5
ELSIF01	79,340	36,085	32,718	56	31	40	0.814	2.742	1
ELSIF02	58,176	26,221	25,563	52	35	57.75	0.76	2.403	1
ELSIF03	119,749	61,442	57,800	69	32	51.5	0.634	1.91	1
ELYIF01	35,187	16,531	15,873	34	23	29	0.758	2.352	1
ELYIF02	66,777	27,562	26,322	49	34	52.2	0.733	2.35	1
ELYIF03	46,307	20,385	18,918	35	23	28.25	0.635	1.858	1
ELSIM01	54,123	23,648	20,820	58	43	78	0.605	2.149	1
ELSIM02	135,635	53,193	46,204	63	25	30	0.508	1.558	1
ELSIM03	116,225	50,035	41,522	70	34	43.333	0.734	2.445	1
ELSIM04	32,740	14,293	12,569	50	39	66.5	0.743	2.389	1
ELYIM01	98,640	44,663	42,832	89	43	100	0.778	2.551	1
ELYIM02	72,273	31,285	29,982	63	35	44	0.734	2.361	1
ELYIM03	92,122	39,619	37,117	71	37	64.5	0.73	2.404	1
ELSUM01	55,218	31,779	31,328	38	19	20	0.111	0.474	1
ELSUM02	53,557	25,441	22,502	57	33	57	0.536	1.38	1
ELSUM03	47,330	23,637	22,594	43	27	34.2	0.603	1.674	1
ELSUM04	36,958	16,781	13,822	45	35	65.6	0.546	1.681	1
ELYUF01	38,547	15,332	14,565	50	40	67.2	0.789	2.548	1
ELYUF02	46,673	21,248	20,529	157	102	159.652	0.695	2.215	0.99

ELYUF03	68,238	32,431	30,788	52	27	29.5	0.702	2.118	1
ELSUM01	58,925	23,937	22,739	40	27	40.75	0.39	1.154	1
ELSUM02	43,897	20,943	19,648	49	29	42.75	0.652	1.944	1
ELSUM03	45,760	19,573	18,803	40	19	20.5	0.465	1.439	1
ELYUM01	34,237	14,272	14,084	55	41	48.091	0.792	2.587	1
ELYUM02	76,272	34,002	31,888	72	44	54.909	0.745	2.471	1
ELYUM03	62,358	28,154	27,490	77	43	120	0.756	2.499	1
HCSUF01	34,712	15,292	14,755	30	20	29.333	0.126	0.462	1
HCSUF02	39,241	16,652	16,564	24	17	20.333	0.082	0.354	1
HCSUF03	65,188	27,074	26,996	44	16	17	0.365	1.144	1
HCYUF01	76,875	34,783	32,586	36	21	24.75	0.236	0.76	1
HCYUF02	60,806	26,958	25,065	25	12	17	0.009	0.056	1
HCYUF03	72,492	32,319	31,318	40	21	23	0.385	1.195	1
HCYUF04	31,498	14,232	7,850	26	22	88	0.365	1.099	1
HCSUM01	32,941	14,502	14,176	38	26	44.333	0.615	1.766	1
HCSUM02	60,550	26,857	25,465	34	16	19.75	0.058	0.268	1
HCSUM03	44,810	20,220	19,856	34	27	40.2	0.469	1.428	1
HCYUM01	62,267	25,528	24,839	32	20	29.333	0.374	1.122	1
HCYUM02	76,692	32,602	31,923	35	19	34	0.113	0.406	1
HCYUM03	37,451	12,386	12,011	22	15	24.333	0.019	0.107	1

Sample IDs are as per Table 1. OTUs = number of operational taxonomic units calculated at 97 % similarity; Chao1= estimate of species richness; Shannon and Simpson indices estimate diversity; Good's equation measures coverage.

Appendix 2.2: ANOVA of 16S rRNA gene sequence reads of teneral and mature adult *Bactrocera tryoni.*

Treatment factors		All					Tenerals					Mature				
		n	$\bar{x}$	SE	*F*	*p*	n	$\bar{x}$	SE	*F*	*p*	n	$\bar{x}$	SE	*F*	*p*
Life stage	Tenerals	19	1911.316	1076.351	85.149	<0.001***										
	Mature	39	25190.359	1674.838												
Irradiation (FFPF)	Irradiated	19	23020.950	3939.679	2.412	0.129	6	4859.667	3198.175	1.296	0.282	13	31403.08	3676.836	4.8921	0.03675 *
	Unirradiated	19	15672.740	2620.421			6	1167.000	544.801			13	22367.69	1780.026		
Sex	Male	29	17272.480	2758.424	0.024	0.877	9	693.400	370.694	1.459	0.244	19	25998.32	2400.5	0.2165	0.6444
	Female	29	17856.450	2553.224			10	3264.556	2212.767			20	24422.8	2385.374		
Colony origin (Unirradiated)	FFPF	19	15672.740	2620.421	0.152	0.699	6	1167.000	544.801	5.231	0.043*	13	22367.69	1780.026	0.0388	0.8454
	HIE	20	14177.950	2789.607			7	22.143	4.008			13	21800.31	2263.295		
Adult diet (Mature)	Full											19	25051.58	2132.357	0.0064	0.9369
	Sugar											20	25322.2	2616.427		

Significance codes: ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1
n = sample size, $\bar{x}$ = mean, SE = standard error, *F* = F value, *p* = P value

Appendix 2.3: ANOVA of number of OTUs in *Bactrocera tryoni*.

Treatment factors		All					Tenerals					Mature				
		n	$\bar{x}$	SE	*F*	*p*	n	$\bar{x}$	SE	*F*	*p*	n	$\bar{x}$	SE	*F*	*p*
Life stage	Tenerals	19	4.053	0.774	55.979	<0.001***										
	Mature	39	30.051	2.386												
Irradiation (FFPF)	Irradiated	19	23.368	3.692	0.189	0.666	6	1.667	0.333	0.170	0.689	13	33.385	1.842	0.424	0.521
	Unirradiated	19	26.263	5.540			6	2.167	1.167			13	37.385	5.864		
Sex	Male	29	21.276	2.876	0.013	0.911	10	3.500	0.969	0.552	0.468	19	30.632	2.301	0.055	0.816
	Female	29	21.793	3.605			9	4.667	1.258			20	29.500	4.171		
Colony origin (Unirradiated)	FFPF	19	26.263	5.540	3.819	0.058.	6	2.167	1.167	20.928	<0.001***	13	37.385	5.864	9.055	0.006**
	HIE	20	15.300	1.494			7	7.714	0.522			13	19.385	1.180		
Adult diet (Mature)	Full											19	32.737	4.521	1.211	0.278
	Sugar											20	27.500	1.760		

Significance codes: ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘.’ 0.1 ‘ ’ 1

n = sample size, $\bar{x}$ = mean, SE = standard error, *F* = F value, *p* = P value

The four MS Excel files below can be viewed and downloaded by using the password protected CloudStor link:

https://cloudstor.aarnet.edu.au/plus/s/g5VrCDHYJL9CzjI

Password: DeaneWoruba2018!

Appendix 2.4: Chapter 2 OTU table

Appendix 3.1: Chapter 3 OTU table

Appendix 4.1: Chapter 4 OTU table

Appendix 5.1: Chapter 5 OTU table