Composition and physiological roles of gut microbiota in the False Coding Moth (*Thaumatotibia leucotreta*)

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

MASTER OF SCIENCE

Of

RHODES UNIVERSITY

By

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June 2023

Declaration

I, Perryn Heather Richardson (g16r3453), hereby declare that this thesis submitted is my own work. It is being submitted for the degree of Master of Science at Rhodes University. It has not been previously submitted for assessment of any other degree at any other university or other body, organisation outside of the university.

Authors signature

26/06/2023

Date

Abstract

Gut microbiota can have a profound influence on host performance, behaviour and fitness. For False Codling Moth (FCM), *Thaumatotibia leucotreta* (Lepidoptera: Tortricidae), a major pest of citrus in South Africa, little work has been undertaken to date on gut microbe diversity or its influence on the host. This thesis aimed to i) characterise the gut microbiome of FCM under laboratory conditions and in FCM from the field, ii) and produce moths with reduced gut microbiota through egg dechorionation, which was followed by iii) the measurement of a suite of physiological traits, namely mass, survival and thermal stress in FCM from normal laboratory, dechorionated laboratory and field collected larvae that may be indicative of overall field performance. We aimed to directly test the hypothesis that gut microbial diversity partly determines insect performance and fitness by measuring its effects on growth, development, and tolerance to cold temperatures in FCM.

FCM eggs that underwent dechorionation with sodium hypochlorite had an overall effect on larval survival, egg morphology and both larval and adult moth physiological measures. Increasing concentrations of sodium hypochlorite significantly decreased insect survival, ($\chi^2(1, n = 10\ 850) = 21.724$, p-value < 0.0001), with a concentration of ≈3.69% as the concentration limit (p-value < 0.001). Successful dechorionation of FCM was achieved with a wash of sodium hypochlorite at around 3.69% concentration and was visually confirmed by reduction of FCM egg surface area, ($\chi^2(25, n = 260)$ p-value < 0.0001) and Scanning Electron Micrographs of the egg morphology. The gut microbiome of FCM from the different focus treatments was successfully characterized. Identification of the dominant bacterial families in these microbiomes revealed *Xanthobacteraceae*, *Beijerinckiaceae* and *Burkholderiaceae* in both the laboratory reared and field collected larvae, which suggests their systematic association with *T. leucotreta*. The most abundant genera were revealed as *Bradyrhizobium*, *Methylobacterium* and *Burkholderia-Caballeronia-Paraburkholderia.*

Comparison of larval mass showed that treatment (dechorionated or not) had a significant effect on larval mass ($\chi^2(2, n = 230) = 22.703$, p-value < 0.001), field larvae were heavier than both control larvae and larvae with a disrupted gut microbiome. However, adult insects with a disrupted gut microbiome had more mass than individuals from the control and field-collected larvae with intact gut microbiomes ($\chi^2(2, n = 230) = 39.074$, p-value < 0.001). Despite the difference in mass between larval treatments, there was no significant

difference in relative protein (χ^2(2, n = 24) = 5.680, *p*-value = 0.06), carbohydrate (χ^2(2, n = 24) = 3.940, *p*-value = 0.14) or lipid (χ^2(2, n = 24) = 6.032, *p*-value = 0.05) content between individuals from the control and dechorionated treatments and field-collected individuals. Turning to thermal physiology, insects collected from the field took significantly longer to recover from chill coma than both laboratory treatments with intact and disrupted gut microbiomes (χ^2(2, n = 129 = 39.659, *p*-value < 0.001). In addition, exposure to cold stress showed that treatment had a significant effect on insect mortality (χ^2(2, n = 272) = 9.176, *p*-value = 0.01), with individuals from the control and dechorionated treatment being less likely to die after experiencing cold stress compared to field-collected individuals. Differences in the mass and thermal tolerance of insects with intact and disrupted gut microbiota suggest that gut microbiota may play an important role in the cold performance of *T. leucotreta,* and these findings constitute the basis for future molecular work on the functions of these bacterial taxa. This research highlights the need for consideration of the effects of *T. leucotreta* microbiome in current pest control programs.

Acknowledgments

- Firstly, I would like to express my gratitude and appreciation to my supervisors Dr. Candice Ann Owen and Prof. John S. Terblanche for their help and advice throughout this degree. Thank you for helping me keep on track when I became overly excited about all sorts of ideas and for providing me with support and guidance to overcome the challenges that we encountered.
- To Mathew Goddard from River Bioscience, thank you for supplying me with FCM eggs and rearing advice throughout the project and for supporting this research.
- Thank you to Dr. Candice Coombes for the many discussions about FCM handling and rearing and for all the lab support that helped to make these experiments run more smoothly.
- A big thank you to Henrieka Bosua from Stellenbosch University, Applied Physiology and Ecology lab for all her help and support both in the lab and outside of it.
- Thank you to Dr. Anandi Bierman from Stellenbosch University, Applied Physiology and Ecology lab for training me in Next Generation Sequencing techniques and bioinformatics that have been central to this project.
- To Clarissa Mouton from XSIT for help in sourcing FCM eggs and pupae when the project was in its early stages.
- To Darrelle Lee from Spellbound labs for handling my many inquiries about lab equipment and supplies.
- Thank you to Marvin Randall from the Electron Beam Lab from Rhodes University for his much-appreciated help and for training me in the use of light microscopy and Scanning Electron Microscopy which helped me to nurture these skills.
- To Gwynneth Matcher from SAIAB for the NGS advice and NGS workshop training that has proved to be invaluable throughout this research.
- To Sibylle von Boetticher from Separations Science for always being willing to provide me with NGS advice and support.
- Thank you to Rhodes University, the Centre for Biological Control, and Citrus Research International (CRI) for the financial support for this research as it would not have been possible without the support.

Table of Contents

Chapter 1

General Introduction

Mass rearing of insects

Mass rearing programs have been designed for various insects with the goal of production for biological pest control, for human and animal food or for insect products (Morales-Ramos *et al*., 2014). The foundation of inundative biological control (biocontrol) is the massive release of arthropods and entomopathogens reared for that purpose (Morales-Ramos *et al*., 2014; Somerville *et al*., 2019). The False Codling Moth (FCM), *Thaumatotibia leucotreta* (Meyrick 1913) (Lepidoptera: Tortricidae), previously known as *Cryptophlebia leucotreta*, is endemic to South Africa and has adapted to become one of the five major pests of cultivated crops of stone fruits, pome fruits and certain vegetables, but it is primarily a citrus pest. Furthermore, *T. leucotreta* is a phytosanitary risk for the international trade of citrus that negatively affects export opportunities (Moore, 2002; Adom *et al.,* 2021).

The adult FCM has a wingspan of around 12-16 mm. The forewings are predominantly gray with distinctive markings, including dark spots and a whitish band (Daiber, 1980). The hindwings are light gray (Daiber, 1980). The larvae have a yellow to pink body colour (depending on larval maturity) with distinct brown markings (Daiber, 1980). The life cycle of *T. leucotreta* typically involves four main stages: egg, larva, pupa, and adult. The larvae hatch from eggs laid on the surface of fruits and then tunnel into the fruit for feeding and development (Daiber, 1980; Moore, 2002). After reaching maturity, the larvae exit the fruit and pupate in protected places such as soil or plant debris (Daiber, 1980). The adults emerge from pupae, and the cycle repeats (Daiber, 1980; Moore, 2002). Due to adult female moths laying their eggs on the rind of fruits where the neonate larvae hatch and then bore through to the fruits center causing increased fruit drop and reducing crop yield (Moore, 2002). *Thaumatotibia leucotreta* does not go through winter diapause and can have five to six overlapping generations per year (Daiber, 1980; Moore, 2002).

There are variety of control measures that are in use against FCM. Methods include pheromone traps and monitoring programs whereby pheromone traps are deployed to monitor adult moth populations and detect their presence in agricultural fields (Malan *et al*., 2018). These traps use synthetic pheromones that attract male moths, allowing farmers to assess population levels and monitor the effectiveness of control measures (Malan *et al*., 2018). Regular monitoring helps determine the timing and intensity of subsequent control

interventions (De Villiers & Pringle, 2007; Malan *et al*., 2018). Insecticides are used when necessary to control FCM populations. The choice of insecticide depends on factors such as the crop, timing, and severity of infestation. Selective insecticides that target the moth while minimizing harm to beneficial insects are preferred (De Villiers & Pringle, 2007; Malan *et al*., 2018). To reduce the risk of resistance development, it is important to rotate insecticide classes and use them judiciously following recommended application rates and timing (Hofmeyr & Pringle, 1998; Malan *et al*., 2018). Certain cultural practices play a crucial role in managing FCM. Sanitation measures, such as removing and destroying infested fruits, can help reduce the population's overall size and minimize the spread of the pest (Malan *et al*., 2018). Proper disposal of fruit waste and crop residues also prevents larvae from pupating and emerging as adults (Kirkman & Moore, 2008; Malan *et al*., 2018). Good orchard hygiene practices, including pruning, weed control, and removal of alternative host plants, can create a less favorable environment for the moth (Kirkman & Moore, 2008). Biological control agents are employed as part of an integrated pest management approach. Several parasitoid wasps, such as *Trichogramma* spp., have been identified as natural enemies of FCM (Carpenter *et al*., 2007). These wasps lay their eggs inside the eggs of the moth, preventing the development of the pest (Carpenter *et al.*, 2007). Augmentative releases of these beneficial insects can help suppress FCM populations (Moore, 2002; Malan *et al*., 2018).

Effective control of the pest has been achieved through an Sterile Insect Technique (SIT) program operated by XSIT (Pty) Ltd, Citrusdal (Barnes *et al*., 2015; Hofmeyr *et al*., 2019; Boersma, 2021). SIT, which is an important technique in the context of this thesis, consists in the production and release of a large quantity of sterile insects into a wild population for the purpose of population suppression (Parker, 2005). The SIT program involves the production of reproductively sterile *T. leucotreta* for SIT cropland application. This strategy involves mass-rearing T. leucotreta moths that are irradiated with gamma rays to reduce the fertility of both male and female insects. As a result, when partially sterilized moths mate with their wild counterparts, inherited sterility is achieved (Klaasen, 2005; Somerville *et al*., 2019; Boersma, 2021). In SIT, as well as all the above-mentioned applications involving mass rearing of insects in biological control programs, it is crucial that artificially reared and manipulated individuals are successfully able to compete with wild insects for mates (Dyck *et al*., 2005; Boersma, 2021). This thesis explores this topic by assessing the physiological implications of mass rearing insects for downstream applications.

Physiological implications of mass rearing insects

The mass rearing of insects for SIT programs regularly employ the use of cold temperatures to immobilize the insects for handling, transport and the preparation of insects for release (Bloem *et al*., 2005; Dowell *et al*., 2005; Barnes *et al*., 2015). In the *T. leucotreta* SIT program, prior to irradiation and aerial area-wide release of the moths, the *T. leucotreta* adult moths are put into a state of inactivity for easy and relatively stress-free transportation, which is done by exposing the moths to cold temperatures between 0℃ and 5℃ for up to 24 hours prior to release (Dowell *et al.,* 2005; Nepgen *et al.,* 2015). Once released, the sterile males then incorporate with the natural population of moths to suppress the pest population by breeding with wild females, resulting in inherited sterility of their offspring (Dowell *et al*., 2005; Barnes *et al*., 2015, Boersma, 2021). However, this tactic relies on the competitive fitness of the sterile moths compared to their wild counterparts for successful mating with wild females. The cold treatment of mass reared insects prior to release may have long term effects on the moths' fitness and may impact their potential to mate with wild females (Boardman *et al*., 2013; Teets & Denlinger, 2013). Boardman *et al.* (2013) noted that the possible effects of disruption of hormone homeostasis and metabolic resource uptake by cold stress may have long-term effects on *T. leucotreta* fitness, as the insect's ability to effectively induce biochemical repair mechanisms are dependent on these physiological processes. This thesis explores this topic by assessing the impact of the gut microbiome on the thermal tolerance of *T. leucotreta* and possible influences on its physiology, an often-overlooked factor of mass reared insects (Qadri *et al*., 2020; Vogel *et al*., 2022).

Host-symbiont interactions

Research into the gut microbiota of insect pests and mass reared insects has provided valuable insight into insect physiology and host-microbe interactions. For example, Cheng *et al.* (2017) found that the gut symbiont *Citrobacter* provides its host, the oriental fruit fly, *Bactrocera dorsalis* (Hendel 1912) (Diptera: Tephritidae), with the ability to degrade the pesticide trichlorphon. Another example is the ability of *Bacillus cereus*, *Enterobacter asburiae* and *Pantoae agglomerans* isolated from the gut of diamondback moth, *Plutella xylostella* (Linnaeus 1758) (Lepidoptera: Plutellidae), to degrade acephate and confer pesticide resistance to its host (Ramya *et al*., 2016). Conversely, the symbiont *Wolbachia* has been found to increase the susceptibility of African army worm, *Spodoptera exempta* (Walker 1856) (Lepidoptera: Noctuoidea), to viral infections, effectively reducing host competence (Graham *et al.,* 2012). Because of these impacts on host physiology, some studies have shown

that symbionts can be added as a probiotic to insect feed that enhances larval weight and speeds up larval development, as seen in Black soldier fly, *Hermetia illucens* (Linnaeus 1758) (Diptera: Stratiomyidae) (Gorrens *et al.,* 2021; Franks *et al.,* 2021). Lepidopteran pests are no exception. Research by Somerville *et al.* (2019) on the competitive fitness of transgenic diamondback moth, *P. xylostella*, showed that inoculation with *Enterobacter cloacae* during rearing improved larval growth and male competitive fitness compared to moths that did not receive the symbiont. This improvement of fitness was achieved by providing the benefit of priming the host immune system against pathogen infection while also normalizing the insect's physiology across rearing facilities to offset potential side effects when rearing insects in sterile conditions (Somerville *et al.,* 2019).

Benefits to the insect host provided by gut microbiota create regulatory feedbacks in the insect's metabolism that contribute to its overall physiology and has the potential to influence the host's ability to tolerate environmental stress (Soen, 2014). For example, in *Rhagoletis pomonella* (Walsh 1867) (Diptera: Tephritidae) a range of bacteria identified in the insects' gut, *Pantoea*, *Klebsiella* and *Enterobacter,* are deposited during oviposition onto apples and have been shown to provide certain nutrients and proteins essential for larval development (Lauzon, 2003; Behar *et al*., 2008, 2009). Furthermore, research on the interaction between gut microbiota and thermotolerance of *Drosophila melanogaster* (Meigen 1830) (Diptera: Drosophilidae) by Henry & Colinet (2018) found that fly recovery and survival from acute cold stress were hindered in flies that had a disrupted gut microbiome. Tolerating thermal stress is of vital importance to insect survival, and alteration of an insect's energy reserves or metabolism by symbiotic microbes can impact its thermal tolerance and have long term effects on its fitness (Teets & Denlinger, 2013). The implication of the effect of gut microbes on its host's fitness is significant in the case of insects that are mass reared and regularly exposed to cold stress, as in the case of *T. leucotreta* that is used in SIT programs that rely on the sterile insect's ability to be reproductively competitive for pest population suppression. Although recent work conducted by van der Merwe *et al.* (2022) on the yeasts present within the gut of *T. leucotreta* has shown that the yeasts *Meyerozyma guilliermondii*, *Pichia kudriavzevii*, *Pichia kluyveri* and *Hanseniaspora uvarum* improve larval development and mortality, little work has been done to identify the bacteria present within the gut microbiome of *T. leucotreta* and infer any influence that they may have on host physiology.

Microbiome of mass reared insects

The mass rearing of insects is an essential component in the production of insects for various industries, and an understated feature of mass rearing is its reliance on the microbiome of insects (Jordan & Tomberlin, 2021). The insect gut is home to a diverse community of symbiotic microbes, bacteria, archaea and various eukaryotes that play essential roles in biological processes such as nutrient absorption and immune system health (Figure 1.1) (Douglas, 2015; Gurung *et al*., 2019; Muñoz-Benavent *et al*., 2021). These microbes are crucial for host development and survival, with microbes accounting for around 10% of an insect's biomass (Shin *et al.*, 2011; Sommer & Bäckhed, 2013; Douglas, 2015; Matos *et al.*, 2017; Gurung *et al.*, 2019; Qadri *et al.,* 2020). Recent research into the insect microbiome has highlighted that microbial symbionts, mainly bacteria, influence insect traits such as growth; development; pesticide and pathogen resistance; protection against parasitism; reproduction; and behaviour (Douglas, 2015; Sugio *et al*., 2015; Almeida *et al*., 2017, van den Bosch & Welte, 2017; Gressel, 2018; Paniagua-Voirol *et al*., 2018; Qadri *et al*., 2020; Muñoz-Benavent *et al*., 2021). Comparatively, dysbiosis (the imbalance or change in gut microflora associated with ill health) in insect gut microbiota has the potential to negatively affect host physiology by depletion or absence of essential metabolites provided by symbiotic microbes (Ridley *et al*., 2012; Russell *et al*., 2013; Guo *et al*., 2014). This thesis will focus on both the characterisation and role of the gut microbiota in *T. leucotreta* physiological performance.

Motivation and aims

Little to no research has been conducted on the microbial profile or influence that the gut microbiome has on *Thaumatotibia leucotreta* physiology from laboratory reared insects or field populations and how their thermal tolerance compares. To identify and investigate the effect of gut microbes on *T. leucotreta* physiology, the transmission of maternal microbes in *T. leucotreta* eggs was disrupted by removal of the chorion layer of the egg, a process known as dechorionation. Vertical transmission of microbes between the mother and her offspring likely occurs during the egg stage (Mereghetti *et al.,* 2017; Henry & Colinet, 2018; Paniagua Voirol *et al.,* 2018). The aim of chapter 2 was to characterize the microbiome and develop a bacterial community profile for *T. leucotreta*, which was performed using 16S RNA sequencing for identification and community assessment of the bacteria present within the gut of larvae with intact and disrupted gut microbiota with further comparison to larvae collected from the field (Broderick *et al*. 2004; Douglas, 2018; Wilches *et al*., 2021). In this chapter, it was hypothesized that the predominant bacterial genera found in other Lepidoptera, such as

Enterobacter, *Flavobacterium*, *Enterococcus*, *Gamma-proteobacteria*, *Enterobacter, Pantoea, Bacillus*, *Pseudomonas* and *Acinetobacter* would be identified (Walsh & Webster, 2003, van der Hoeven *et al*., 2008, Robinson *et al.*, 2010). Furthermore, the microbiome of *T. leucotreta* field populations should have higher bacterial diversity than laboratory reared FCM (Robinson *et al*., 2010).

Once a procedure for effective dechorionation of *T. leucotreta* eggs was determined, larvae were reared on sterile artificial diet for the purpose of measuring certain physiological traits. Chapter 3 aimed to investigate the physiological effects of a disrupted gut microbiome focused on the cold tolerance of *T. leucotreta* adults. It was hypothesized that *T. leucotreta* with altered (e.g., restricted diversity or decreased abundance) gut microbiota would have differences in their larval and adult mass and macronutrient content when compared to normally laboratory reared and field collected larvae (Douglas, 1998; Ridley *et al*., 2012). Additionally, *T. leucotreta* with altered gut microbiota would be more susceptible to cold stress compared to moths with their microbiome intact, as seen in *D. melanogaster* (Henry & Colinet, 2018). Measuring the thermal tolerance of *T. leucotreta* with normal and altered gut microbiomes was done by inducing chill coma and recording moth recovery time and exposing adult moths to cold stress at a temperature nearing their lethal limit and recording moth survival (Boardman *et al*., 2012; Henry & Colinet, 2018). Overall, this research seeks to present the first characterized bacterial profile for the microbiome of *T. leucotreta*. Also, the expected differences in the measurements of the above outlined physiological traits between insects with intact and altered gut microbiota would suggest that gut microbiota play an important role in both the mass rearing and environmental fitness of *T. leucotreta*, highlighting the need for consideration of *T. leucotreta* microbiome in pest control programs and SIT (Jordan & Tomberlin, 2021).

Figures

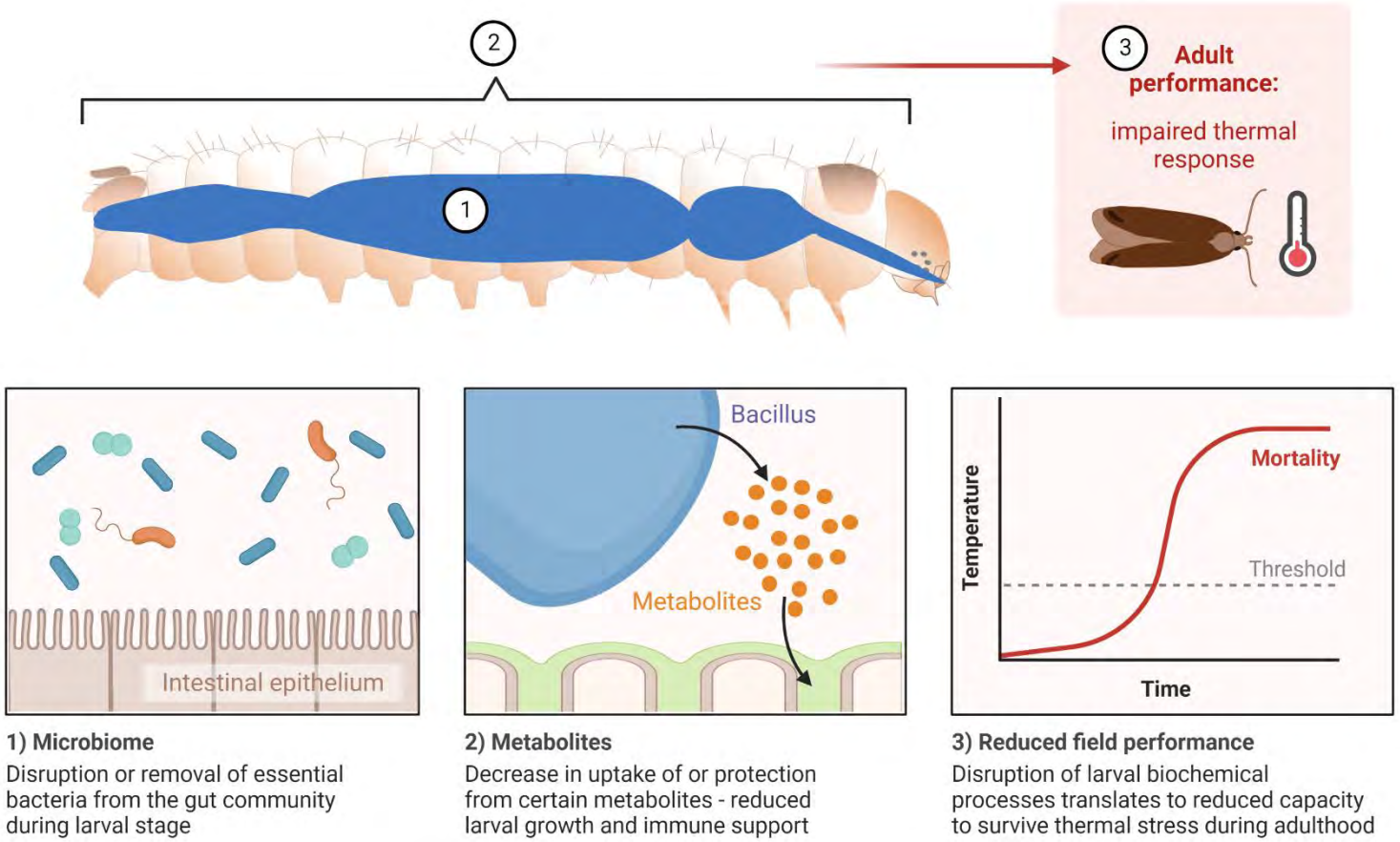

Figure 1.1 The effects of mass rearing conditions on host-microbe interactions showing the consequences on insect physiology and thermotolerance (Image created in Biorender.com).

Chapter 2

False Codling Moth gut microbiome sequencing and bacterial community composition

Introduction

Symbiosis is the intimate association between organisms of different species (Saffo, 1992; Britannica, 2022). All associations between a host and their endosymbionts are inherently intimate, as the symbionts can either be obligate or facultative in nature (Sugio *et al.,* 2015; He *et al.,* 2017; Duplouy & Hornett, 2018). The microbes that inhabit the insect gut have evolved many symbiotic associations that are involved in metabolism regulation, nutrient absorption, detoxification of toxic compounds and protection from pathogens, among other functions (Yun *et al.,* 2014; Douglas, 2015; Paniagua-Voirol *et al.*, 2018). Gurung *et al.* (2019) believe that pest insects should be seen as "mini ecosystems" due to the complex interactions between the host and its endosymbionts. Yun *et al.* (2014) argue that to better understand the ecological roles of insect symbionts, and the role that host-microbe interactions play in physiology and microbial ecology, a comprehensive analysis of the bacterial diversity within the host is essential.

The gut of many Lepidopteran species present a challenge to endosymbiotic microbes due to their diet range and neutral to acidic midgut (Berenbaum, 1980; Broderick *et al*., 2004; Dillon & Dillon, 2004). However, due to the extreme chemical environment of the insect midgut, Broderick *et al.* (2004) theorise that unusual microbial phylotypes result from these environments and likely provide benefits to their insect host. For example, in the gypsy moth, *Lymantria dispar dispar* (Linnaeus 1758) (Lepidoptera: Erebidae), the gut community is dominated by the bacterium *Enterococcus faecalis*, which actively acidifies its surrounding environment and is thought to suppress the pathogen *Bacillus thuringiensis*, which is activated in alkaline conditions (Wilson & Benoit, 1993). To put it simply, the bacteria alter midgut conditions to benefit their own survival to outcompete other microbes, which is a phenomenon likely occurring in other gut microbiomes too (Broderick *et al.,* 2004). Another example where gut microbes may contribute to insect health by pathogen suppression, again seen in the gypsy moth, is the presence of a phylotype of *Serratia* in gut samples (Grimont & Grimont, 2006). *Serratia* is an opportunistic pathogen in insect mass production facilities that has a negative impact on its host when the gut microbial community structure of the mass reared population becomes unbalanced (Grimont & Grimont, 2006).

Despite the presence of unusual phylotypes in insect gut microbiomes, taxonomic classification of microbes is still possible through Next Generation Sequencing (NGS) (Broderick *et al*., 2004; Yun *et al*., 2014; Douglas, 2018; Wilches *et al*., 2021). Taxonomic classification across 21 insect orders by Yun *et al.* (2014) showed that the predominant bacterial phyla that inhabit the insect gut are *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Actinobacteria* and *Tenericutes.* At the family level, *Anaplasmataceae* and *Enterobacteriaceae* were dominant and in Lepidoptera, the predominant genus was *Wolbachia* (Yun *et al*., 2014). A later study by Weinert *et al*. (2015) estimated that 52% of terrestrial arthropods are infected with *Wolbachia* as a heritable symbiont, albeit as an intracellular symbiont. Furthermore, various studies have found that the predominant bacterial species in Lepidoptera such as the cabbage white butterfly (*Pieris rapae*) (Linnaeus 1758) (Lepidoptera: Pieridae) and tobacco hornworm (*Manduca sexta*) (Linnaeus 1763) (Lepidoptera: Sphingidae), are species of *Enterobacter*, *Flavobacterium*, *Enterococcus*, *Gamma-proteobacteria*, *Enterobacter, Pantoea, Bacillus*, *Pseudomonas* and *Acinetobacter* (Steinhaus, 1941; Walsh & Webster, 2003; van der Hoeven *et al*., 2008, Robinson *et al*., 2010, Hammer *et al*., 2017).

Regardless of the rapid increase in insect microbiome research over the last decade and the valuable insights garnered by this research in insect biology, biochemistry, and pest management, only a handful of studies have been conducted on insect microbiomes in South Africa. One of the earliest studies assessed bacterial diversity in two *Apis mellifera* species (Linnaeus 1758) (Hymenoptera: Apidae) and found that the gut microbiome comprised mostly of *Lactobacillus*, *Bifidobacterium*, *Bartonella*, *Gluconacetobacter* and *Serratia* (Jeyaprakash *et al*., 2003); with the bacteria being likely contributors in insect nutrition, digestion pathogen defence and most importantly, sugar utilization and carbohydrate breakdown (Moran, 2015). Most microbiome research in South Africa has been focused on insects that are important for the food and feed sector such as the edible termites *Macrotermes falciger* and *Macrotermes natalensis* (Holmgren 1910) (Blattodea: Termitidae) as well as *Hermetia illucens* (Linnaeus 1758) (Diptera: Stratiomyidae). In the termites *M. falciger* and *M. natalensis*, gut bacteria contribute to host metabolism by degrading oligosaccharides and providing essential nutrients (Schnorr *et al*., 2019). The most abundant taxa were identified as *Ruminococcaceae*, *Christensenellaceae*, *Alistipes, Treponema* and *Desulfovibrio*, with notable unique species of *Lactovum* and *Citrobacter* (Schnorr *et al*., 2019). Research on the *Hermetia illucens* gut microbiome has revealed that insect diet and bacterial community composition in the gut

influences the metabolism of the insect and directly effects larval fat accumulation (Greenwood *et al*., 2021). The dominant genera across wild type and mass reared *H. illucens* were identified as *Firmicutes*, *Actinobacteria*, *Bacteroidetes* with the most abundant taxa being *Morganella*, *Providencia*, *Lactobacillus*, *Enterococcus* and *Proteus* (Greenwood *et al*., 2021). *Citrobacter* was noted as a biomarker for distinguishing between the wild type and mass reared *H. illucens*. Javal *et al*. (2022) attributed the ability of *Cacosceles newmannii* (Thomson 1877) (Coleoptera: Cerambycidae) to shift from its ancestral host plants to sugar cane facilitated by changes in bacterial diversity in the gut microbiome. From this research, bacteria from the family Enterobacteriaceae were dominant, with the most ubiquitous genera identified as *Enterobacter* and *Klebsiella* (Javal *et al*., 2022). The top genera were identified as *Serratia*, *Enterobacter/Rosenbergellia*, *Lactococcus*, *Klebsiella/Enterobacter*, *Gryllotalpicola*, *Enterococcus*, *Burkholderia/Caballeronia/ Paraburkholderia*, *Bradyrhizobium* and *Allorhizobium/Neorhizobium* (Javal *et al*., 2022). Additionally, the bacterial taxa *Bacillus* and *Burkholderia* were found to provide cellulose, pectin, xylan and starch degradation as well as plant compound detoxification important to the insect host.

Concerning Lepidoptera, research conducted by Whitaker *et al*. (2016) on the microbiome of various Lycaenid species originating from South Africa (but included many species that originated elsewhere) was the only point of reference at the time of writing. Whitaker *et al*. (2016) investigated the bacteria present in the Lycaenid gut microbiome and found no significant difference in the species diversity, richness or composition between carnivorous or predatory caterpillars. The most abundant bacterial genera detected in the Lycaenids were identified as *Alicyclobacillus*, *Methylobacterium*, *Acinetobacter* and *Agrobacterium*. The notable bacteria detected were *Pantoea, Buchnera, Serratia* and *Wolbachia* (Whitaker *et al*. 2016). Overall, the authors attributed the source of the bacterial gut communities in the Lycaenids to their food source and environment (Whitaker *et al*., 2016).

With the ever improving methods for rapid and cost effective NGS approaches for microbiome research, microbiota profile data has allowed for increased understanding of the emergence of metabolism related diseases and host phenotype development in animal models (Ross *et al*., 2013). From animal model research it has been shown that microbiota profiles can be used to predict certain mass-related traits and final carcass weight, which has led to researchers such as Camarinha-Silva *et al*. (2017) and Khanal *et al*. (2020) to reason that it is possible to accelerate and improve desirable livestock traits when production is coupled with methods for selective optimisation of host genotype and microbiota. Following this line of thought,

Greenwood *et al*. (2021) argue that microbiome profiles can be applied to *H. illucens* mass production as their research has shown that larval fat accumulation is influenced by microbiome composition in a similar fashion to that seen in animal models. Whether microbiome profiles can be applied to mass produced lepidoptera has not been thoroughly researched but the concept is becoming more attractive due to the pressures of changing agricultural practices (Qadri *et al*., 2020).

The goal of this chapter was to characterise the bacterial species richness and composition of the larval gut bacterial community of *Thaumatotibia leucotreta.* This chapter employed culture-independent methods and included the presence of larval gut samples from *T. leucotreta* from the field for effective comparison with laboratory reared larvae. It was reasoned that *T. leucotreta* from the field was likely to have bacterial phylotypes that differ to laboratory reared larvae as the species has adapted to tolerating an acidic environment (inside the citrus fruit) due to is status as a pest of citrus (Bloem *et al.,* 2003; Hofmeyr *et al.,* 2015). This research seeks to address the knowledge gap on the bacteria present in the gut microbiome of *T. leucotreta* in the hope that these findings constitute the basis for further research on the role of these microbe taxa on moth physiology and stress tolerance phenotyping.

Methods

Insect colonies and rearing

Thaumatotibia leucotreta colonies were maintained in the Department of Zoology and Entomology (DZE), Rhodes University (RU), in a controlled environment room at a temperature of 25℃±2℃, 50-60% relative humidity and a 12-hour photoperiod (L12:D12). For the Control and Dechorionated treatments, the *T. leucotreta* eggs that were used to establish the colonies were sourced from the River Bioscience mass rearing facility in Addo (Eastern Cape Province, South Africa). Eggs were supplied on A4 size sheets of wax paper as *T. leucotreta* prefer to oviposit on a waxy surface if given the option; this is the standard egg collection method used in the mass rearing facility. Field-collected *T. leucotreta* last instar larvae were sourced by Citrus Research International (CRI) technicians from infested fruit collected in citrus orchards in Sundays River Valley (33°25′S, 25°27′E) (Eastern Cape Province, South Africa). These were collected at various times throughout the year in 2021/2. Once collected, larvae were stored in individual glass tubes containing a plug of artificial diet and closed off with cotton wool. The tubes were then transported to RU where some were used for gut dissection and others were allowed to pupate at 25℃ in a climate-controlled room.

The *T. leucotreta* (mass-reared and field-collected) were reared and maintained on an artificial diet developed by Moore *et al*. (2014) and were kept in separate sterile rearing tanks.

Development of dechorionation procedure

Dechorionation of *T. leucotreta* eggs was based on the procedure used by Henry and Colinet (2018) and originally developed by Koyle *et al*. (2016). Modifications were made to the dechorionation procedure to accommodate *T. leucotreta* egg biology (Figure 2.1) for the purpose of disrupting initial microbiome establishment in juvenile larvae in the production of an insect culture with reduced gut microbiota (Figure 2.2) (Mereghetti *et al.,* 2017; Henry & Colinet, 2018; Paniagua-Voirol *et al.,* 2018). In this section of the study, both a control treatment group and a dechorionated treatment group were employed. The purpose was to determine an appropriate concentration of sodium hypochlorite for efficient dechorionation of T. leucotreta eggs, as well as to prepare larvae with reduced gut microbiota while preserving the viability of the eggs. This process was repeated for three replicates. A series of sodium hypochlorite solutions of increasing concentrations starting at 0.51% sodium hypochlorite to 7.45% sodium hypochlorite in deionized water in increasing increments of 0.15% was used to test for a suitable dechorionation concentration (Figure 2.2). Eggs were supplied on A4 size sheets of wax paper. All egg sheets used throughout these experiments were first cut into smaller 2 cm × 2 cm egg sheet pieces. For the dechorionation treatment, egg sheets were first rinsed in 70% ethanol to remove any debris from the egg sheet surface such as loose eggs and wing scales. Egg sheets were then immersed in a solution of sodium hypochlorite for 2.5 minutes to allow for chorion layer to be stripped from the egg. Egg sheets were then rinsed in a solution of dilute bleach and deionised water to remove possible bacterial contamination after dechorionation. Lastly egg sheets were rinsed in sterile deionised water to remove any remaining solvents from the eggs. This procedure was repeated for each concentration of sodium hypochlorite that was tested and compared with the control treatment (Table 2.1). After treatment, the egg sheets were placed individually onto sterile artificial diet, kept in a controlled environment chamber as described below and observed daily for two weeks to assess egg hatching. Controlled environment chambers. Artificial diet was prepared for the Control and Dechorionated treatments by mixing 60 g dry diet with 60 ml deionised water in a 300g glass jar (diet media was sourced from River Bioscience). The glass jars were then stoppered with cotton wool and placed in an autoclave for 20 minutes at 121℃. After the autoclave cycle, the glass jars were placed in tanks that have been cleaned with 3.5% bleach

and sprayed with 70% ethanol (to ensure their sterility) and allowed to cool overnight before treated *T. leucotreta* eggs (egg treatments detailed above) were placed inside.

Egg hatching was visually observed by the disappearance or reduction of pink and black eggs from the egg sheet surface. Once eggs hatch, they turn clear. Unhatched eggs remain pink (presence of early-stage embryo) or black in colour (presence of late-stage embryo as the head capsule becomes darker in colour). Eggs that did not hatch after two weeks were considered non-viable. Counts of this hatching success were performed after the two-week period by digitally counting hatched and unhatched eggs using ImageJ from photographs captured with an OLYMPUS DP72 camera of the egg sheets under an OLYMPUS SZX stereo microscope. The surviving insects were counted at each life stage to measure survival of the insects treated at various sodium hypochlorite concentrations.

Imaging and visual analysis of egg surface

In a separate experiment, new egg sheets were washed with increasing concentrations of sodium hypochlorite and were immediately viewed under an OLYMPUS SZX stereo microscope to assess if there was a reduction in surface area in egg size from the dechorionation treatment as this would indicate removal of the chorion layer. Images of the egg sheets were captured with an OLYMPUS DP72 camera. Surface area of the eggs was measured to the square micrometer (μm^2) from these images using Stream Motion (OLYMPUS Stream, 2006).

Scanning Electron Microscopy (SEM) was further used to visually analyse eggs from both the control and dechorionation treatments to confirm removal of the chorion layer from the eggs. Approximately 60 *T. leucotreta* eggs from each treatment were used for SEM. In preparation for SEM, the wax paper sheets housing the egg samples that had undergone the various treatments were transferred to glass vials and dehydrated in ethanol. After dehydration, the eggs were critical point dried in CO_2 (Polaron E3000, Quorum) for 3 hours and then mounted on self-adhesive double-sided carbon tape on aluminum stubs. After mounting, the specimens were sputter coated with gold using a Q150RS Sputter Coater by Quorum. The eggs were then examined with a VEGA Tescan SEM at 20.00kV at the Electron Beam Laboratory at Rhodes University (RU; Eastern Cape Province, South Africa). Egg morphology between the control and dechorionated eggs was visually compared by looking at the general appearance of the eggs and close-up imaging of surface texture. Eggs with their chorion layer intact would have a spumaline fringe present around the eggs and the eggs would appear relatively smooth with

slight pitting and almost round (Klowden, 2007; Gautam *et al*., 2015). In contrast, dechorionated eggs would not have a spumaline fringe and the surface of the egg would appear heavily pitted, revealing the subchorionic ultrastructure.

Gastrointestinal tract extraction procedure

To obtain gut samples from *T. leucotreta* larva without disrupting the gastrointestinal (GI) tract of the larvae and contaminating the sample, a 5-step method was used to remove the entire GI tract sample from each specimen (Figure 2.3). Larvae were first rinsed in 70% ethanol to surface sterilise the body. Placing the larva in a sterile Petri dish, the head capsule and anal segment of the larval body were removed with a sterile scalpel. Size 6 forceps were used to grasp the hindgut section of the GI tract and by gently pulling, the GI tract was released from the body cavity. The GI tract was then placed in a 2mL microcentrifuge tube with 100% ethanol. Three whole GI tracts from three different specimens in the same treatment group constituted for 1 gut sample. Pooling of GI tracts was required to ensure sufficient Bacterial DNA quantity for downstream purposes. Gut samples were collected from last instar larvae from the Control (n=18), Dechorionated (n=15) and Field (n=18) collected larvae. Dissections were performed under sterile conditions. Samples were stored in 100% ethanol at -80°C until DNA extraction.

Bacterial DNA extraction from larval gut samples

The whole gut samples from the Control, Dechorionated and the Field-collected larvae were then used to identify bacteria by high-throughput sequencing of the DNA amplicon coding for the 16S rRNA gene regions V3 and V4 (Integrated DNA Technologies, Whitehead Sci-South Africa) (Table 2.3). The whole gut of each larva was first removed from the storage tube and then dried at 40℃ for 2 hours in 2mL microcentrifuge tubes using a Thermomixer (Eppendorf, Germany). Once dry, the samples were bead-beaten for 2 × 30s at 30Hz on a Mixer Mill (Retsch, MM400) using sterile 3-mm glass beads. The dry sample powder was then suspended in 400µl 100% ethanol and homogenised via vortexing. Following this, approximately 50µl of each sample was placed in a Collection Microtube 96 well plate, one sample per well, using a pipette with a filter tip and dried at 40℃ for 2 hours using a ThermoMixer (Eppendorf, Germany). Samples were tracked and their placement in the plate was recorded.

The Qiagen DNeasy 96 Blood & Tissue Kit (Qiagen, Germany) was used to extract the bacterial DNA from 19 samples (larval gut samples and 2 technical control samples) following the manufacturers guidelines along with adaptations made by Javal *et al*. (2022), whereby a

mechanistic cell lysis procedure was added to the protocol. Bacterial contaminants associated with the laboratory environment were controlled for by including a positive control (composing of 96 bacterial variants) and negative controls (ZymoBIOMICS Microbial Community DNA Standard, ZymoBIOMICS Microbial Community DNA Standard II [Log Distribution] and ATCC® 20 Strain Staggered Mix Genomic Material). Briefly, after lysis by incubation overnight at 56℃, the lysate was bead-beaten for 5 minutes with sterile 3mm glass beads in a Mixer Mill (Retsch, MM400) at the maximum speed setting. The aqueous phase was separated by centrifugation at 6,000g for 5 minutes. All pre-PCR steps were done with filter tips on the pipettes and laboratory procedures were designed to prevent DNA contamination of the PCR products.

Microbial DNA analysis of gut bacterial assemblage

PCR was performed using 16S rRNA primers V3 and V4 (Table 2.3). 16S primer sequences were designed according to recommended sequences provided by the National Institute for Communicable Diseases (NICD) of South Africa. The resulting genomic DNA was purified using the AMPure XP reagent (Beckman Coulter Life Sciences, USA) and analysed for sample quality using the Agilent D1000 ScreenTape System prior to library prep at the NICD. Library preparation was performed on the Illumina MiSeq system for 16S Ribosomal RNA gene amplicons at the NICD (Illumina, USA). 16S metagenomic sequencing was conducted on the NextSeq 500 (Illumina, USA).

Sequence data processing for bacterial community analyses

The resultant 38 FASTQ sequences were quality-checked using FASTQC v0.11.5 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). Bases with quality scores < Q20 were trimmed using Trimmomatic v0.36 (Bolger *et al.,* 2014) and reads with <200 base pairs (bps) were removed with BioPython v3.6.1. Cleaned FASTQ sequences were converted to FASTA using Samtools v1.10, *fastq_to* function (Li *et al.,* 2009). Unique read sequences and abundances were identified with Usearch (Edgar, 2010). The chimeras were filtered and an OTU Table with 99% sequence identity was constructed. QIIME2 vs 2022.8 (Bolyen *et al.,* 2019) was used to convert OTU tables to BIOM format. Operational taxonomic units (OTUs) were assigned taxonomy using the SILVA v132 database (Quast *et al.,* 2012). Metadata was added to taxonomic assignments and the resulting biom files converted to text files containing percentage of the respective taxa present in each sample (van den Berg *et al*., 2020). Overall, this procedure resulted in the creation of 38 text files containing OTU

identification from order to genus level and correlating relative abundance which could be used for data collation per sample per treatment.

Statistical analysis – Insect survival and egg surface area

All statistical analyses was performed using R version 3.6.3 (RStudio Team 2019). Insect survival through life stages at different concentrations and a control was analysed using a logistic General Linear Mixed Model with binomial family and logit link. This model was run with a random intercept included to account for multiple measures per repeat that were not independent of each other (Bolker *et al*., 2009); the package *lme4* was used to run this model (Bates, 2011). Model fit was assessed using *DHARMa* v0.4.6 (Hartig & Lohse, 2022) by simulating the models residuals. *car* v3.1.1 (Fox *et al.,* 2022) was used to perform an ANOVA on the models to assess the difference in survival between concentration categories and life stages. Post-hoc analyses was performed using pairwise comparisons between life stages and concentrations with the *emmeans* package v1.8.2 (Lenth *et al.,* 2022).

Statistical analyses – Bacterial community analysis

R packages include *phyloseq* v1.38.0 (McMurdie & Holmes, 2013) and *vegan* v2.6.4 (Oksanen *et al*., 2020). The whole resultant dataset of 193 taxa was first curated by filtering otu reads identified as ambiguous taxa, uncultured bacteria, plant mitochondria, taxa from the positive control and taxa unidentified at the genus level to produce a final dataset of 155 taxa. Analyses on bacterial data were conducted on the curated dataset at the family and genus level. Bacterial community complexity was measured using two indices of α-diversity that consider both specific richness and evenness of relative abundances: the Shannon and Simpson indexes (Kim *et al.,* 2017). This was combined with *UpsetR* analyses, which were performed to characterize shared bacterial genera in the larval gut microbiome between the treatments (Conway *et al.,* 2017).

Difference between treatments (Control, Dechorionated and Field) was tested using Kruskal-Wallis tests and combined with a post hoc pairwise comparison using Wilcoxon rank tests with false discovery rate (FDR) correction (Anderson, 2001). Bacterial community differences between all samples were then measured using Bray-Curtis dissimilarity on log transformed data, together with an Non-metric multidimensional scaling (NMDS) plot on two dimensions. Using this dissimilarity matrix, community composition differences between treatments were tested by applying Permutational multivariate analysis of variance (PERMANOVA) with 1000 permutations using *adonis2* (Javal *et al.*, 2022). Shared bacteria

genera in relation to treatments were determined by filtering the dataset to indicate genera that were present between all three treatments and was combined with Similarity Percentage Analyses (SIMPER) assessing the average contribution of variables to the differences observed in the Bray-Curtis dissimilarity matrix (Javal *et al.,* 2022). Stacked bar plots of bacterial genera shared between the three treatments was created by filtering the overall dataset to the genera that were present in all three treatments, combined with *UpSetR* analysis. A final stacked bar plot of the most common bacterial genera shared by all three treatments was created by filtering the dataset for the top 10 genera (relative abundance > 15 000).

Results

Dechorionation of FCM eggs

Overall, treating *T. leucotreta* eggs with increasing concentrations of sodium hypochlorite significantly influenced insect survival from the egg stage to adult life stage, (χ^2(1, n = 10 850) = 21.724, *p*-value < 0.0001) (Figure 2.4). Pairwise comparisons between concentrations revealed that sodium hypochlorite concentration from 3.69% and above significantly decreased survival from egg through to the adult life stage (*p*-value < 0.001); with mortality increasing with eggs exposed to higher concentrations of sodium hypochlorite. Therefore, a concentration of 3.69% was chosen as the limit for sodium hypochlorite exposure during dechorionation.

Increasing sodium hypochlorite concentration had a strong effect on the surface area of laboratory *T. leucotreta* eggs, with higher sodium hypochlorite concentrations producing a greater decrease in surface area (χ^2(25, n = 260) *p*-value < 0.0001) (Figure 2.5). This finding together with the above life stage analysis resulted in the determination that a sodium hypochlorite concentration of 3.69% was suitable for dechorionation. For observational proof that effective dechorionation at 3.69% concentration of sodium hypochlorite, SEM was used to compare eggs prepared under the control and dechorionation procedures. SEM imaging of laboratory reared eggs from the control procedure and the dechorionation procedure show a marked change in appearance (Figure 2.6). Eggs that underwent dechorionation had an exposed vitelline membrane as observed by the pitted and wrinkled surface of the egg (Figure 2.6A). Furthermore, SEM imaging showed that the spumaline and chorion fringe that are present on control treatment eggs had been removed from the dechorionated eggs, further supporting that the eggs treated with 3.69% sodium hypochlorite had been completely dechorionated (Figure 2.6).

Larval gut bacterial community

The general characteristics of the sequencing datasets are shown in Table 2.3. The 193 bacterial variants found represented 61 bacterial families, including 34 taxa whose genus was unknown. From the whole dataset, there were 155 taxa whose taxonomy was resolved to a single genus (Table 2.4). Across all treatments, *Xanthobacteraceae* (29%), *Beijerinckiaceae* (14%) and *Burkholderiaceae* (10%) were the most abundant families and the genera *Bradyrhizobium* (29%), *Methylobacterium* (14%) and *Burkholderia-Caballeronia-Paraburkholderia* (9%) were the most abundant (Table 2.4, Figure 2.7 & 2.8).

Gut microbiome comparison between larval treatments

There was no significant difference in species richness ($\chi^2(2, n = 17) = 4.192$, p-value = 0.123), species abundance ($\chi^2(2, n = 17) = 0.746$, p-value = 0.689), Shannon diversity ($\chi^2(2, n = 17) = 0.044$, p-value = 0.978) and Simpsons index ($\chi^2(2, n = 17) = 0.563$, p-value = 0.755) from the three different treatments (Figure 2.9). Both the Shannon and Simpson indices indicated that there was no significant difference in bacterial diversity between the treatments (Figure 2.9). However, the *UpSet* plot showed larvae from the field had the highest number of unique genera (59 unique taxa) compared to both the control (25 unique taxa) and dechorionated (30 unique taxa) treatments (Figure 2.10). The Field-collected sample genera were dominated by *Bradyrhizobium* (20%), *Wolbachia* (18%) and *Gluconobacter* (11%) (Figure 2.8). Between the three sample types, larvae from the Control treatment shared the most genera with larvae from the Field (10 taxa), which was dominated by genera from the family *Burkholderiaceae* (10%) (Figure 2.8). The PERMANOVA on Bray-Curtis dissimilarities revealed a significant difference in terms of β-diversity between the control, dechorionated and field samples ($F=2.163$; $p=0.003$).

The NMDS axis distinguished larvae collected from the field to larvae in the control and dechorionated (Figure 2.11). There is some overlap indicating shared bacterial taxa with the control treatment neatly nestled between the dechorionated treatment and field-collected samples (Figure 2.11). SIMPER analyses did not reveal any genera responsible for the differentiation between larvae from the control and dechorionated treatments. However, SIMPER analysis did reveal 12 genera responsible for the differentiation between larvae from the control treatment and larvae collected from the field, including three genera unique to the larvae collected from the field (Table 2.6). SIMPER analysis also revealed 33 genera responsible for the differentiation between larvae from the dechorionated treatment and larvae

collected from the field, which included 1 genus unique to larvae from the field (Table 2.7). Finally, 18 genera were found across all three treatments (Figure 2.11), with *Bradyrhizobium* accounting for a total of 49%, followed by *Burkholderia-Caballeronia-Paraburkholderia* at a total of 15% and lastly, *Staphylococcus* at a total of 11%.

Discussion

This investigation demonstrates that the dechorionation of *T. leucotreta* eggs is possible and that the process successfully reduced the bacterial load in the larval microbiome. Increasing sodium hypochlorite concentration had a significant effect on insect survival from egg to adult life stage at concentrations above 3.69%. A concentration of 3.69% Sodium hypochlorite was also shown to reduce surface area of *T. leucotreta* eggs and effectively remove the chorion layer of the egg structure without significantly impacting egg hatching or survival. This is thus a recommended concentration for use in future experiments by other researchers attempting to remove the chorion of insect eggs with similar morphology.

The consistent occurrence of bacterial taxa from the families *Xanthobacteraceae*, *Beijerinckiaceae* and *Burkholderiaceae* in both the laboratory reared and field collected larvae suggests they may have a systematic association with *T. leucotreta*. The analysis revealed high relative abundance reads to the genera *Bradyrhizobium* (29%), *Methylobacterium* (14%) and *Burkholderia-Caballeronia-Paraburkholderia* (9%). The abundance of these bacteria in the larval stage supports the notion that they are associated with the feeding stage of the larvae and may outcompete other bacteria (Robinson *et al*., 2010; Sugio *et al*., 2015). The findings of this chapter not only identify components of the *T. leucotreta* gut microbiome, which has never been done before, but also highlight compositional similarities of larval gut communities recorded in cabbage white butterfly, tobacco hornworm and other lepidoptera (Steinhaus, 1941; van der Hoeven *et al*., 2008; Robinson *et al*., 2010; Chen *et al*., 2016; Mereghetti *et al*., 2017). More importantly, the findings of this study corroborate with bacteria identified in the gut microbiome of other South African insects (Jeyaprakash *et al*., 2003; Schnorr *et al*., 2019; Greenwood *et al*., 2021; Javal *et al*., 2022). Notably, the similarities between the gut bacteria of *T. leucotreta* and various Lycaenids from South Africa suggest that the bacteria *Pantoea*, *Methylobacterium*, *Acinetobacter* and *Serratia* may have important functional roles in or are common to Lepidoptera (Whitaker *et al*., 2016).

However, it cannot be distinguished if these bacteria are obligate, facultative or transitive (as seen in Lycaenidae) endosymbionts in *T. leucotreta* without further analysis of the presence

of the bacteria in all feeding and developmental life stages of the insect (Sugio *et al*., 2015; Schnorr *et al*., 2019). In this study, *T. leucotreta* larvae collected from infested fruit in the Sundays River valley indicate the presence and dominance of *Wolbachia* species in the wild population that was not seen in the laboratory reared larvae, which suggests establishment of this bacteria in field populations. *Wolbachia* was also found in wild Lycaenids collected in South Africa although its function has yet to be elucidated without further research and it is unknown if this bacterium is derived from the insects food source (Whitaker *et al*., 2016). The identification of *Wolbachia* in only the gut samples of field collected *T. leucotreta* and has interesting implications for biological control of this pest, as seen in the case of *Wolbachia* infection in *Spodoptera exempta* (Walker 1856) (Lepidoptera: Noctuidae) where the pathogen increased the insect hosts susceptibility to the baculovirus *Spodoptera exempta* nucleopolyhedrovirus (Graham *et al*., 2012). Research into the specific strain(s) of *Wolbachia* in *T. leucotreta* could prove beneficial in terms of whether or not it influences the insects' susceptibility to the *Cryptophlebia leucotreta* ganuloviruse (CrleGV) and *Cryptophlebia peltastica* nucleopolyhedrovirus used to in pest management programs (Moore, 2021). Overall, this finding suggests that the population of T. leucotreta utilized in mass rearing programs may possess a competitive advantage over the wild population. Consequently, it is crucial to closely monitor any supplementation of the mass-reared population with wild insects to prevent the inadvertent introduction of Wolbachia. The reason behind this caution lies in the uncertainty surrounding whether the Wolbachia strains present here express antimicrobial properties to suppress competing bacteria or serves any other functional role within the physiology of these insects.

The diversity metrics between the field collected and laboratory reared larvae did not differ. Yet laboratory reared larvae had higher relative abundance of bacterial species irrespective of whether they were from the control or dechorionated treatment. This finding implies that rearing environment plays a significant role in enriching certain bacterial species of the larval microbiome as seen in gut microbiome of *Lymantria dispar* and several other Lepidopteran species (Broderick *et al*., 2004, 2009). The laboratory reared larvae used in this study were derived from a mass rearing colony which has been maintained under strict conditions and reared on sterile artificial diet to avoid outside contamination. This could explain the differences in bacterial abundance in their microbial communities. Bacterial abundance in the laboratory reared larvae was further decreased by the dechorionation process as seen in dechorionated larvae, which suggests that certain microbial species are maternally transmitted

during the egg stage or that bacteria with the ability to supress other bacteria was removed (Mereghetti *et al*., 2017; Paniagua-Voirol *et al*., 2018). However, the eventuality that such a complex community of bacteria may not be a maternal transmission but due to an imperfect technique of producing germ-free individuals due to the difficulty of producing truly sterile individuals. This study provides evidence of possible maternal transmission of microbiota in *T. leucotreta* and is the first finding of its kind recorded in this insect species. To verify this finding, it is recommended that bacterial DNA extraction and taxonomic analysis be performed on *T. leucotreta* egg masses in a manner similar to that performed by Chen *et al*. (2016) on the eggs of *Spodoptera littoralis*. Although insects collected from the field had higher bacterial species richness than laboratory reared insects as discussed above, the laboratory insects had a higher bacterial species abundance, which may be due to the presence of *Serratia* which was only detected in the control treatment and is a known opportunistic pathogen in insect mass rearing facilities and has the capability to suppress other bacterial species (Grimont & Grimont, 2006). This study was successful in dechorionating *T. leucotreta* eggs, which has never before been attempted with this species. Ultimately, dechorionation of *T. leucotreta* eggs effectively reduced the diversity of microbes in the gut microbiome community of last instar larvae, suggesting that establishment of the gut microbiome begins at the egg stage for this species.

On analysing the bacterial species present in the gut microbiome of the control and dechorionated treatment larvae and field-collected larvae, 59 unique genera were detected only in the field-collected larvae. These genera were dominated by *Bradyrhizobium*, *Wolbachia* and *Gluconobacter*. Species of *Bradyrhizobium* are known environmental contaminants present in microbiome studies due to its common presence in the environment (Kumar *et al.,* 2022), which indicates potential contamination during the study process. It should be noted that *Wolbachia* is widely distributed amongst insects and is thought to infect over 60% of all insects (Hilgenboecker *et al*., 2008). However, certain bacterial species of *Wolbachia* and *Gluconobacter* are known insect endosymbionts that affect host biochemistry (Graham *et al*., 2012; He *et al*., 2017; Malacrinò *et al*., 2018). *Wolbachia* strains influence their insect host by manipulating the hosts reproductive system or by making the host more susceptible to viral infection as seen in *S. exempta* (Walker 1856) (Lepidoptera: Noctuidae) (Werren *et al*., 2008; Graham *et al*., 2012). The presence of *Gluconobacter* in insect midguts has been shown to metabolize and detoxify phenolic glycosides and increase the insects hosts adaptability as seen in *Ceratitis capitata* (He *et al*., 2017; Malacrinò *et al*., 2018). It is likely

that *Gluconobacter* may play this role in the gut of *T. leucotreta* and allow it to detoxify phenolic compounds in citrus fruit as it was only detected in the field collected insects (Lou & Ho, 2017).

In this study, the bacteria present in the *T. leucotreta* gut microbiome were characterised using 16S sequencing and aided by disrupting microbiome establishment during the egg stage. Further investigation into *T. leucotreta* gut microbiome community robustness after disturbance and community composition at each life stage would allow for further insight into microbial community dynamics and possible effects that they may have on host metabolism, pathogen suppression and pesticide resistance. The results of this study have important implications for the mass rearing of *T. leucotreta* for pest control programs. The first being that *T. leucotreta* is mass reared for the production of virus for CrleGV-based biopesticide (Moore *et al*., 2015). Due to the ability of certain bacteria to suppress competing pathogens, viral load build-up in *T. leucotreta* could be hindered by the presence of certain bacteria. Alternatively, understanding the infection of the biopesticide to wild *T. leucotreta* could be better understood through insights into pathogen suppression conveyed by the microbiome. Secondly, implementing microbial controls or microbiome analysis into the rearing practices of *T. leucotreta* for Sterile Insect Technique could aid in detection of pathogens and contaminants prior to reaching lethal levels in the mass rearing facility as a preventative measure against loss of insect product, as experienced in the rearing facilities of *H. illucens* (Qadri *et al*., 2020; Vandeweyer *et al*., 2021). Moreover, further investigation of the gut microbiome of *T. leucotreta* is key to preventing the spread of microbial and pesticide resistance in the species, with the added benefit of enhancing our understanding plant-pest interactions and opening new routes for economic exploitation (Leftwich *et al.*, 2016; Mereghetti *et al.*, 2017; Qadri *et al*., 2020).

Figures and Tables

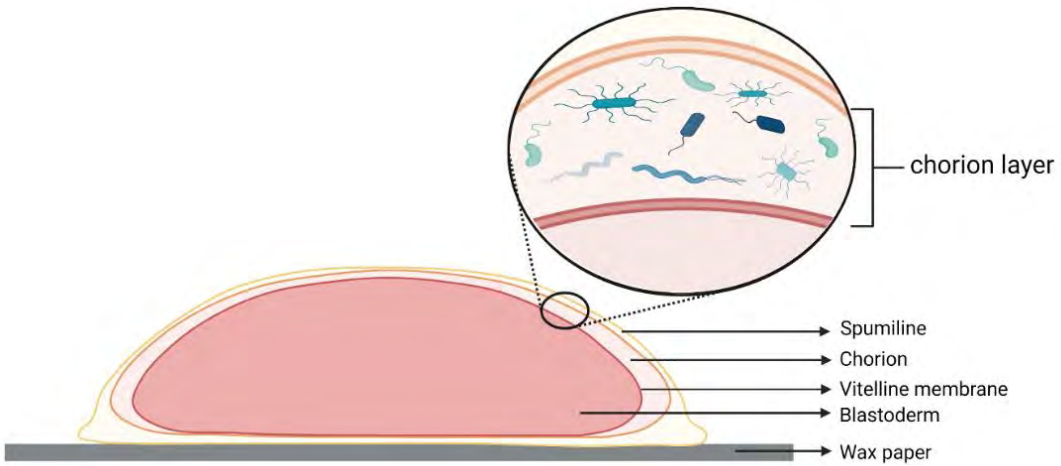

Figure 2.1. The structure of a *Thaumatotibia leucotreta* egg indicating the presence of maternal microbes in the chorion. (Figure based on insect egg anatomy described by Klowden (2007) and Gautam *et al.* (2015). [Image created in Biorender.com]

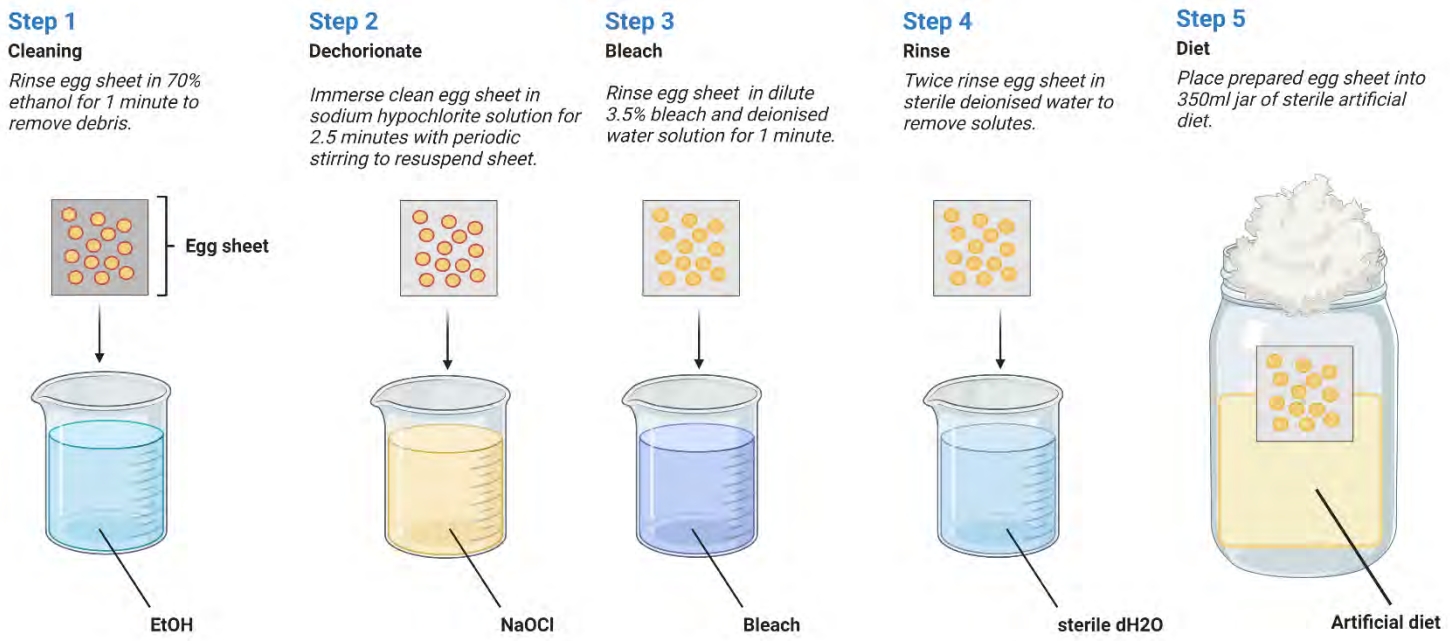

Figure 2.2. Preparation of dechorionated *Thaumatotibia leucotreta* eggs in 5 steps [Image created in Biorender.com].

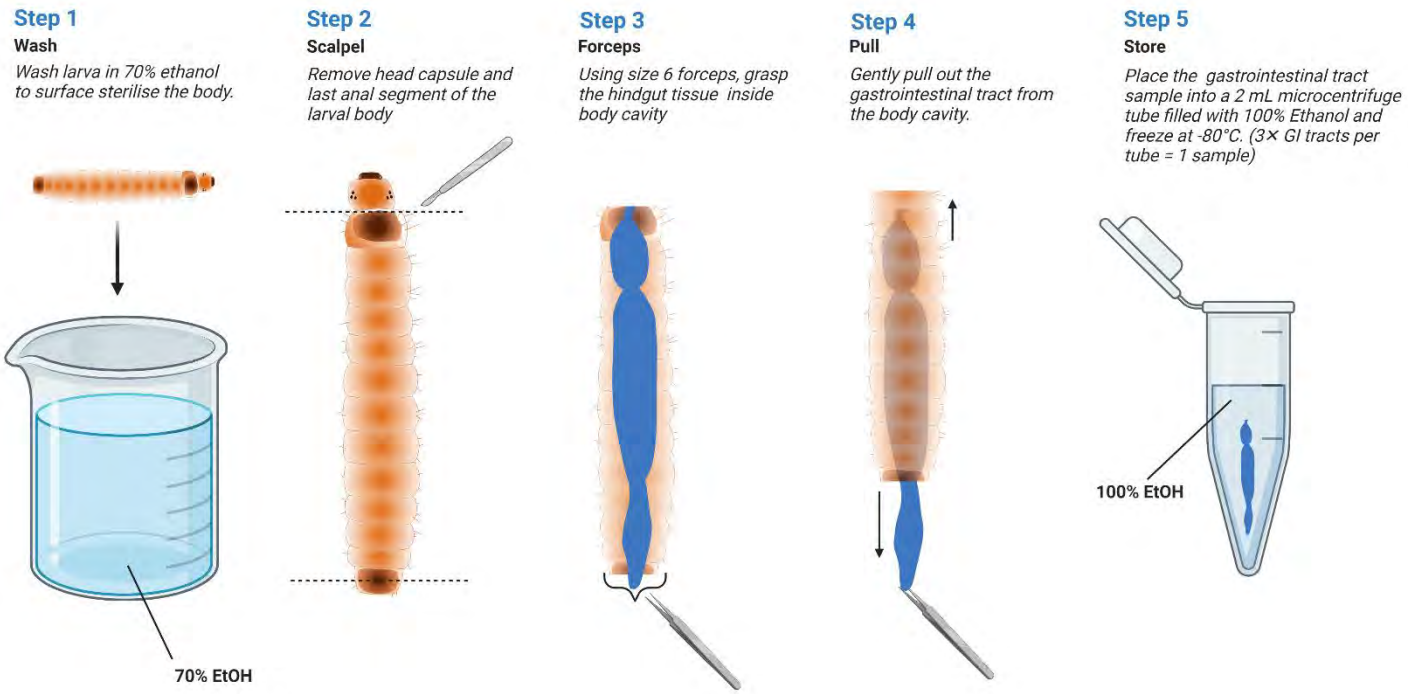

Figure 2.3. The 5-step method used to remove the whole gastrointestinal tract from *Thaumatotibia leucotreta* larva for the purpose of storing gut samples for gut microbiome analysis (Image created in Biorender.com).

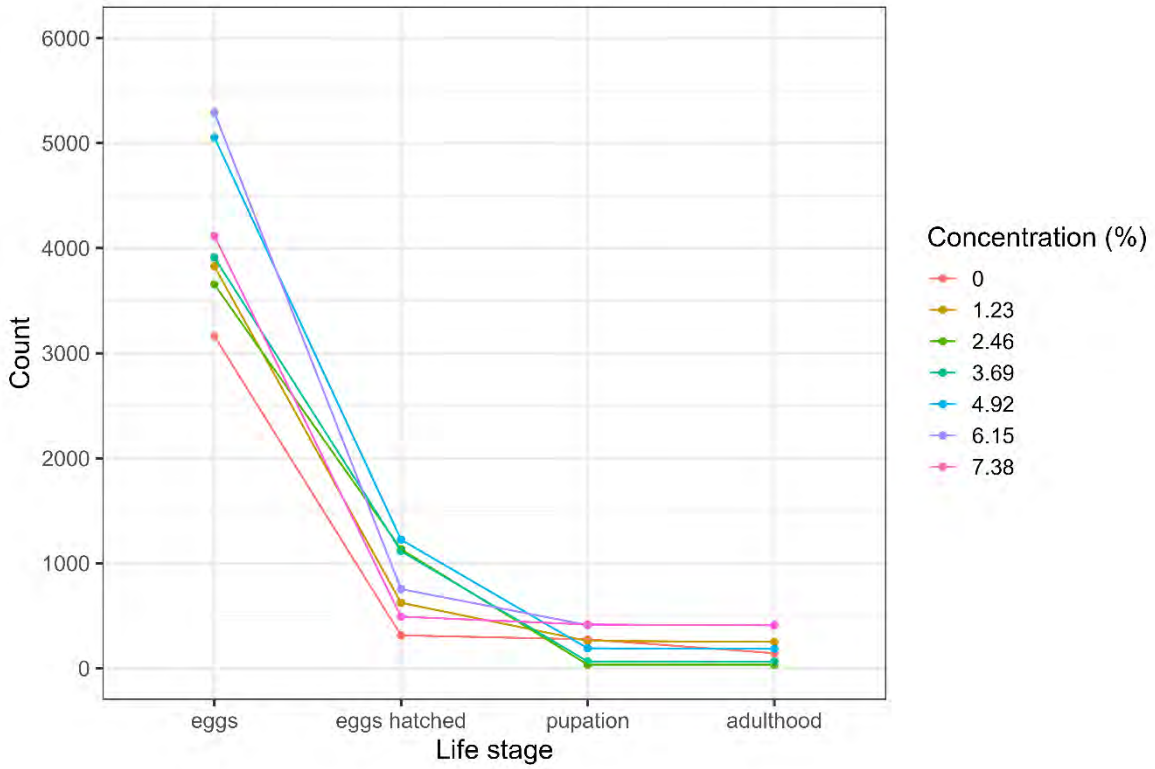

Figure 2.4. Number of *Thaumatotibia leucotreta* at each life stage beginning at the egg stage treated with various concentrations of sodium hypochlorite from 0% up to 7.38%. The egg stage indicates the number of eggs used at each concentration category. The eggs hatched stage indicates the number of eggs that hatched from the corresponding concentration category in the egg stage. The pupation stage indicates the number of larvae that reached pupation from the corresponding concentration category in the eggs hatched staged. The adult stage indicates the number of insects that successfully reached the adult stage from the corresponding concentration category from the pupation stage. Each sodium hypochlorite concentration category is represented by different colours.

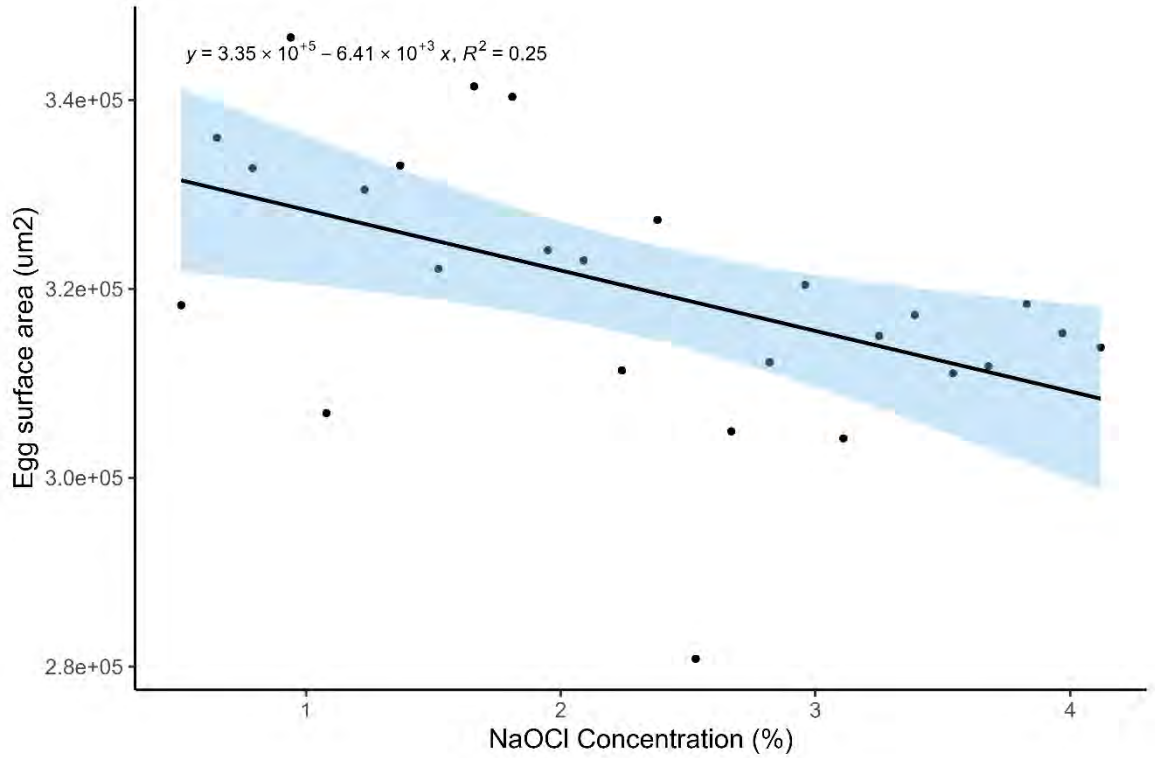

Figure 2.5. Change in surface area of *Thaumatotibia leucotreta* eggs after dechorionation treatment as measured at different concentrations of sodium hypochlorite in dH_2O starting at 0.51% increasing to 4.12%.

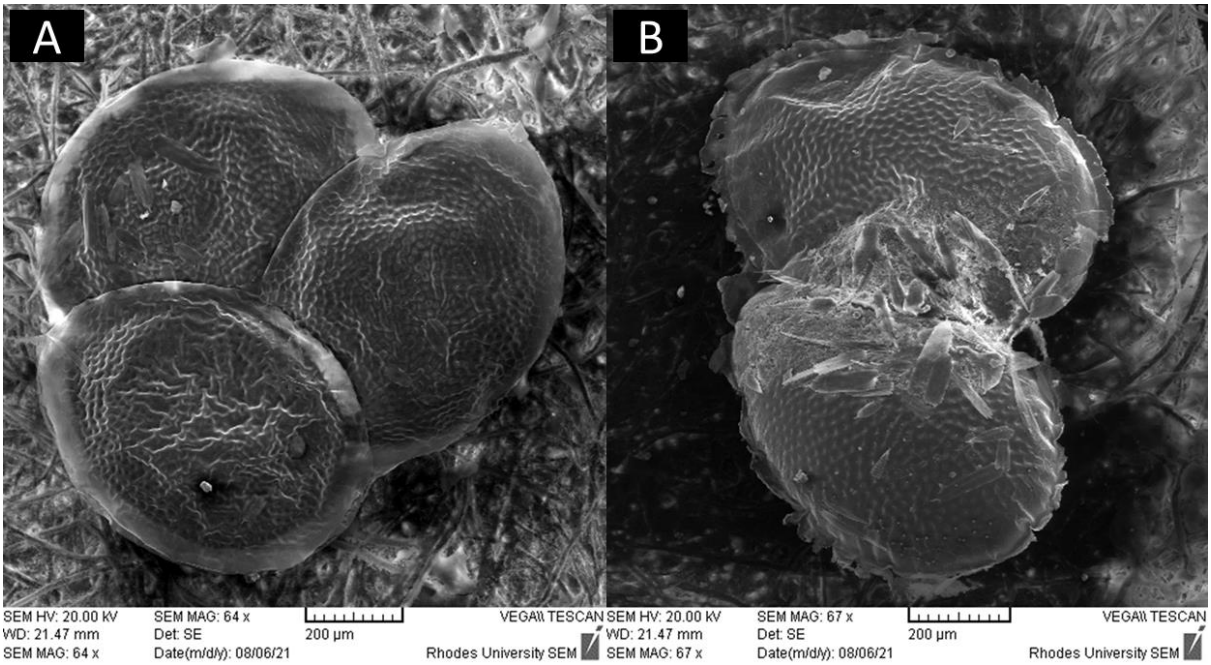

Figure 2.6. Scanning Electron Micrographs of *Thaumatotibia leucotreta* eggs. (A) Dechorionated treatment (3.69% sodium hypochlorite) group with the spumaline fringe and chorion removed and the subchorionic ultrastructure of the egg visible; (B) Control treatment (0% sodium hypochlorite) group with the spumaline fringe and chorion visibly intact.

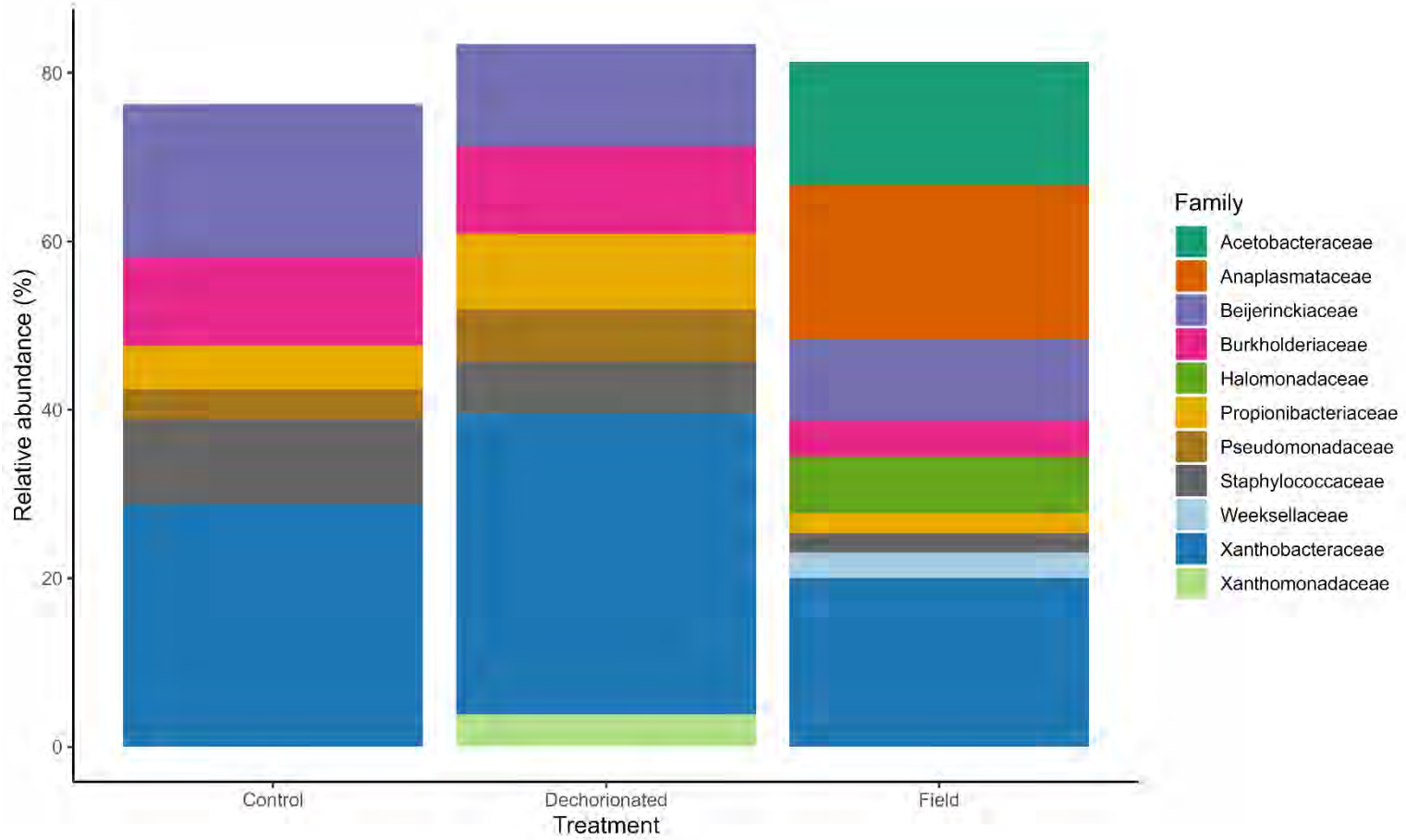

Figure 2.7. Composition of bacterial taxa at the family level that account for over 2% of taxa in the gut microbiome of *Thaumatotibia leucotreta* larvae from the following treatments: Control - larvae from standard lab rearing procedure; Dechorionated - larvae reared from eggs that underwent dechorionation; and Field – field-collected larvae found in infested fruit. Each taxon is represented by different colours.

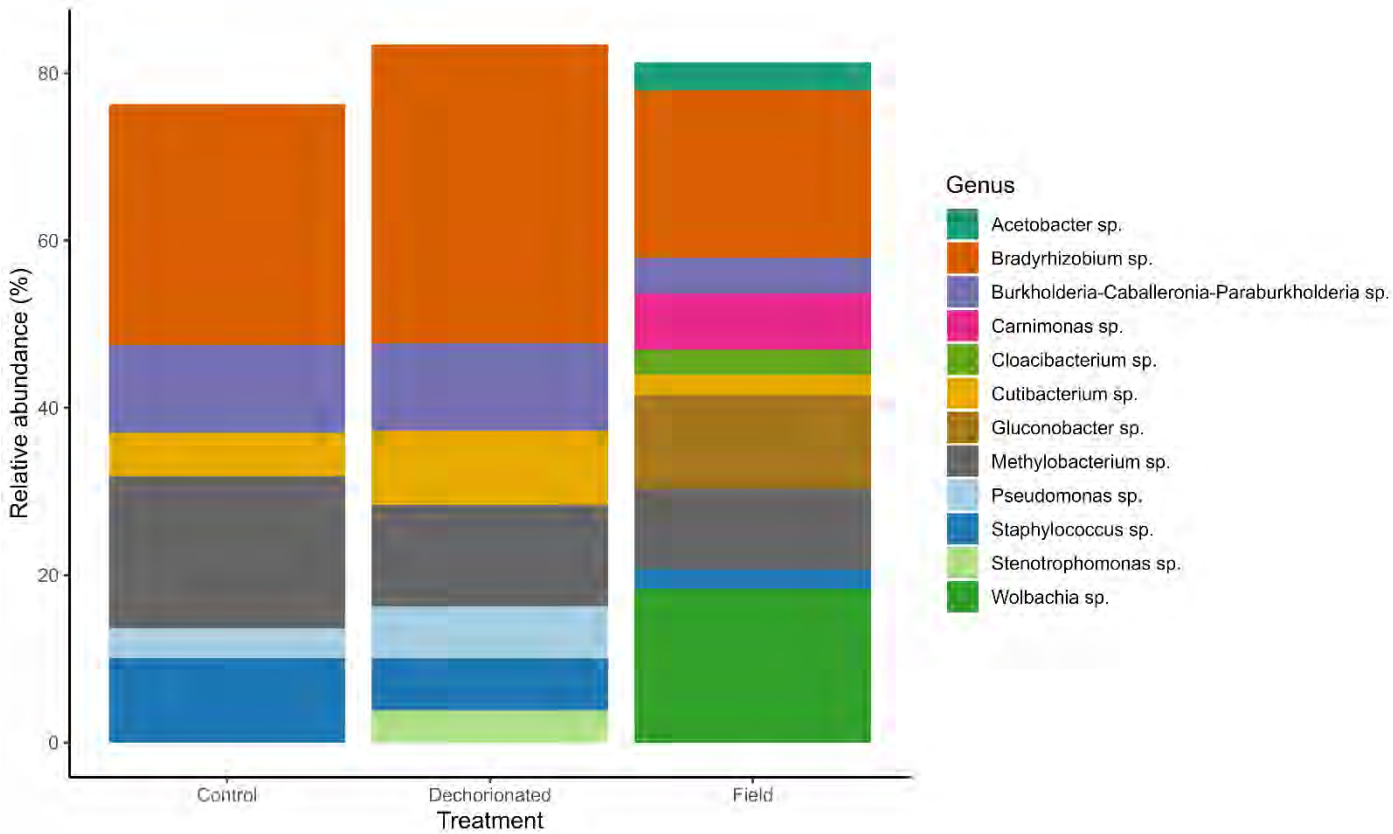

Figure 2.8. Composition of bacterial taxa at the genus level that account for over 2% of taxa in the gut microbiome of *Thaumatotibia leucotreta* larvae from a laboratory reared colony or collected from infested citrus fruit. Control - larvae from standard lab rearing procedure. Dechorionated - larvae reared from eggs that underwent dechorionation and Field – field-collected larvae found in infested fruit. The relative abundance count of taxa are represented by different colours.

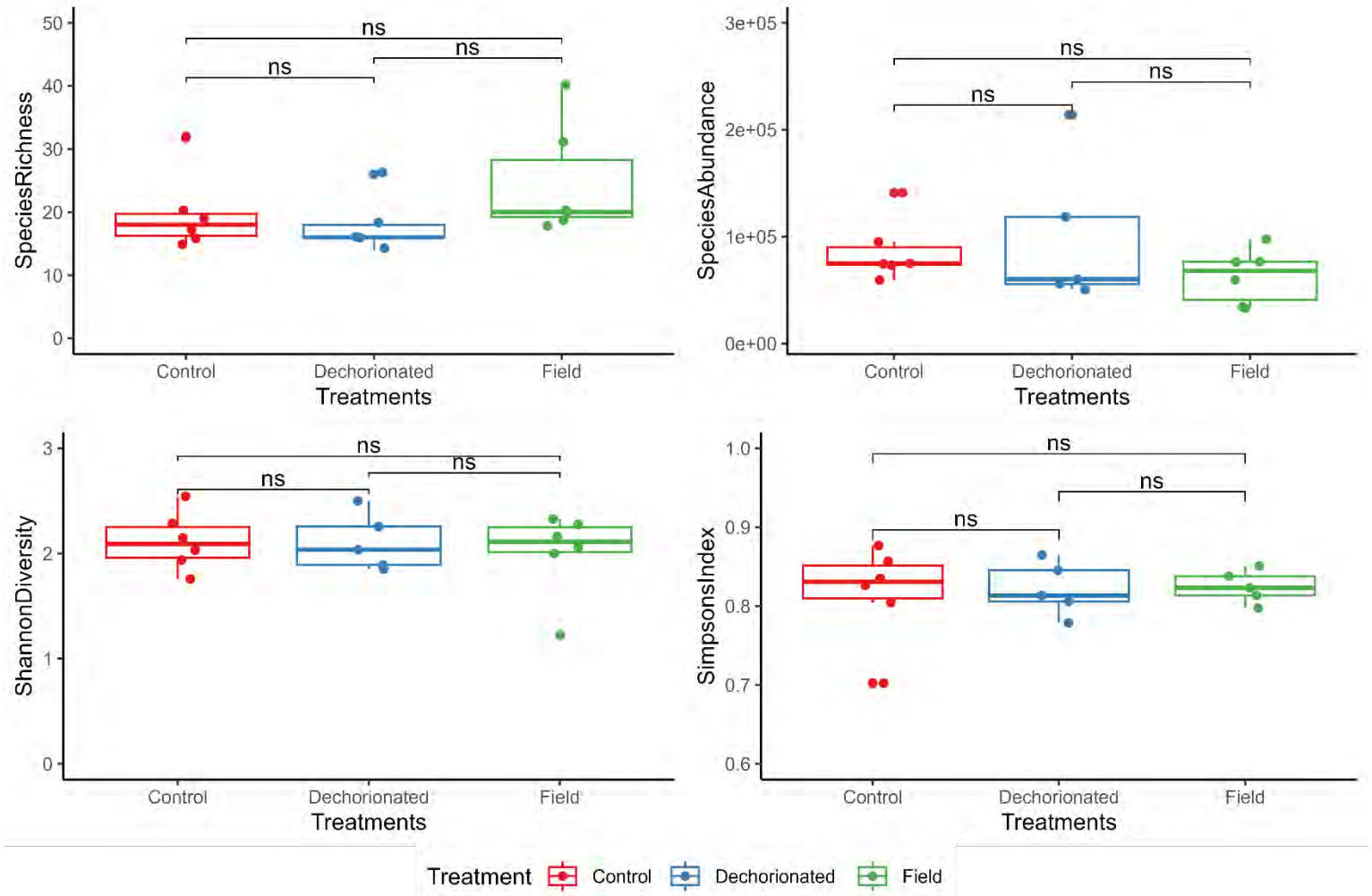

Figure 2.9. Species richness, species abundance, Shannon, and Simpson α-diversity indices of bacterial community observed in the gut microbiome of *Thaumatotibia leucotreta* larvae under three treatments. Boxes and whiskers = interquartile range and mean.

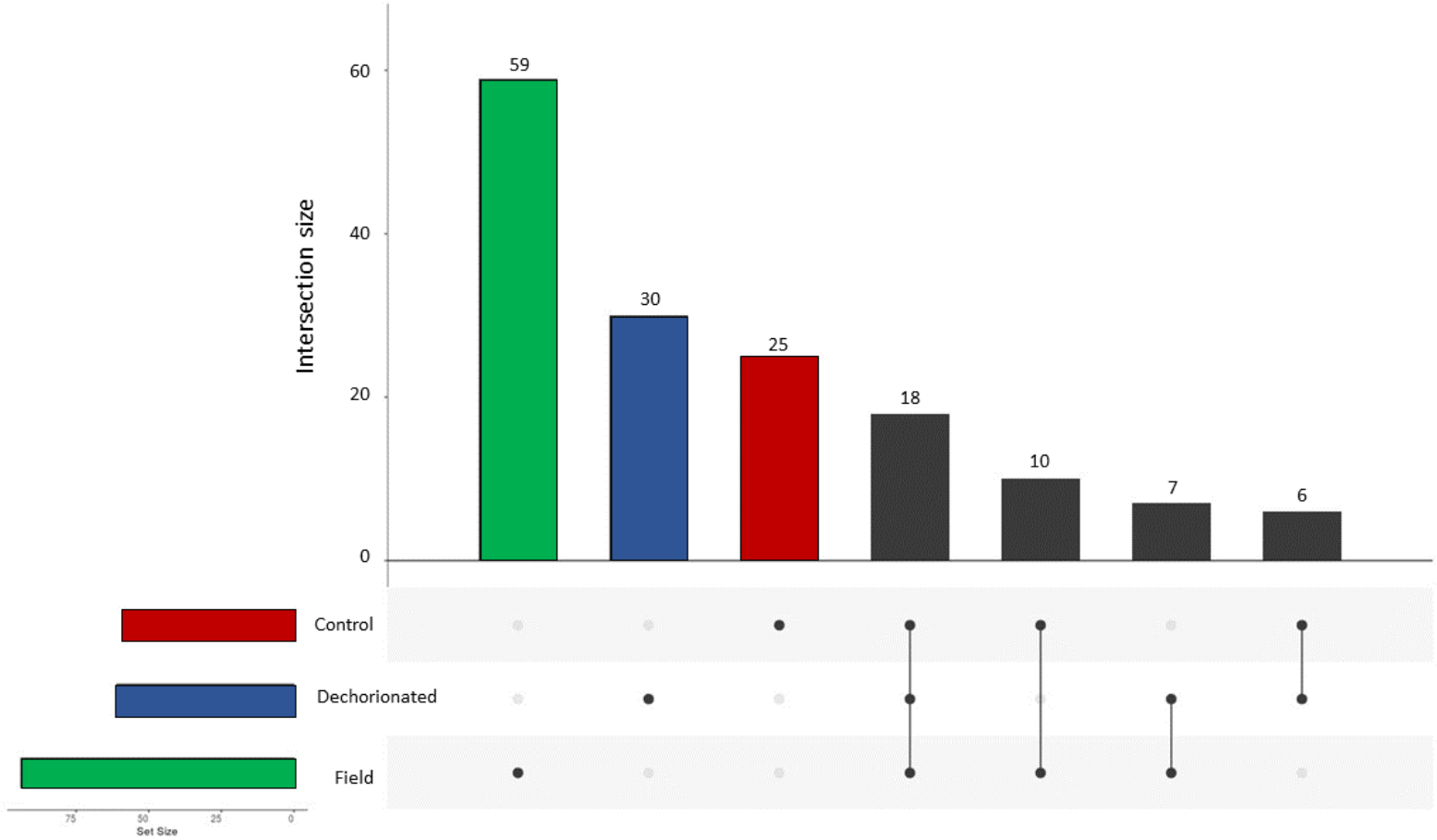

Figure 2.10. UpSetR plot showing intersections (number of bacterial genera) observed in the gut microbiome of *Thaumatotibia leucotreta* larvae between treatments (Plot created with UpSetR Shiny app (https://gehlenborglab.shinyapps.io/upsetr/)). The first three bars from the left indicate the number of bacterial genera unique to the control, dechorionated treatment and field-collected larvae. The fourth bar indicates the number of genera shared between the control, dechorionated treatments and field-collected larvae. The remaining three bars show the number of bacterial genera shared between the specified treatments.

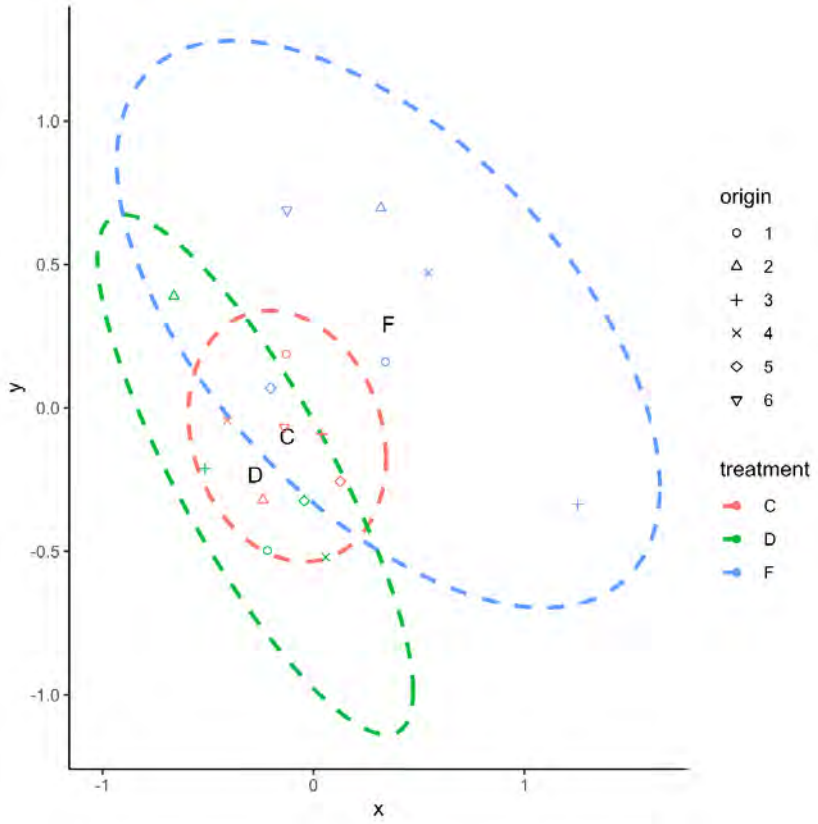

Figure 2.11. β-diversity of the bacterial communities in the larval gut microbiome of *Thaumatotibia leucotreta* from three treatments. C – Control treatment, D – Dechorionation treatment and F – Field-collected larvae. Non-metric Multidimensional Scaling (NMDS) plot shows microbiome communities based on Bray-Curtis dissimilarities. Origin refers to the sample from which the data originates.

Table 2.1. *Thaumatotibia leucotreta* egg treatment procedures.

Treatment	**Step 1**		**Step 2**		**Step 3**		**Step 4**
	Procedure	Time	Procedure	Time	Procedure	Time	
Control	Surface sterilize egg sheets in 70% Ethanol	2 minutes	Rinse egg sheets in 90 ml dilute bleach solution	1 minute	Rinse eggs sheets in sterile deionized water twice		
Dechorionated	Surface sterilize egg sheets in 70% Ethanol	2 minutes	Wash egg sheets in *X concentration of Sodium hypochlorite with sterile deionized water	2.5 minutes	Rinse eggs in 90 ml dilute bleach solution	1 minute	Rinse eggs sheets in sterile deionized water twice

Table 2.2: 16SrRNA primer sets for amplification prior to sequencing.

Primer name	Primer name	Primer sequence 5’ - 3’	Gene
16S Amplicon PCR Forward Primer	16SrRNA_FCMm_F	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG	16S rRNA
16S Amplicon PCR Reverse Primer	16SrRNA_FCMm_R	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC	

Table 2.3: General characteristics of sequencing data for each *Thaumatotibia leucotreta* larval gut sample according to treatment.

Treatment	Sample	Number of reads	Average length of reads	Number of OTUs
Control	C1	334805	301	179/161
	C2	168746	301	82/59
	C3	187560	301	141/125
	C4	145589	301	118/71
	C5	175545	301	84/68
	C6	157297	301	97/74
Dechorionated	D1	584158	301	269/233
	D2	133572	301	96/69
	D3	207867	301	62/45
	D4	140246	301	101/58
	D5	123509	301	46/33
Field-collected	F1	126356	301	56/49
	F2	150283	301	47/46
	F3	164200	301	135/104
	F4	151794	301	108/74
	F5	174067	301	58/54
	F6	115812	301	68/44

Table 2.4. Bacterial genera representing resolved taxa found in the gastro-intestinal tract of *Thaumatotibia leucotreta* from each larval treatment.

Taxa		Presence in Treatments (Yes = *)		
Family	**Genus**	**Control**	**Dechorionated**	**Field**
Acetobacteraceae	*Acetobacter*			*
	Ameyamaea			*
	Gluconacetobacter			*
	Gluconobacter			*
	Komagataeibacter			*
Actinomycetaceae	*F0332*		*	
Aerococcaceae	*Aerococcus*			*
Alteromonadaceae	*Alishewanella*		*	
	Salinimonas		*	
	Rheinheimera	*		
Anaplasmataceae	*Wolbachia*			*
Anemone flaccida	*Anemone flaccida*	*		
Archangiaceae	*Cystobacter*		*	
Bacillaceae	*Aeribacillus*	*		
	Bacillus	*	*	*
	Geobacillus		*	
	Ornithinibacillus		*	
Bacteroidaceae	*Bacteroides*			*
Bdellovibrionaceae	*OM27 clade*		*	
Beijerinckiaceae	*1174-901-12*		*	*
	Methylobacterium	*	*	*
Brevibacteriaceae	*Brevibacterium*	*		
Burkholderiaceae	*Acidovorax*	*		

	Alcaligenes			*
	Aquabacterium	*	*	
	Burkholderia-Caballeronia-Paraburkholderia	*	*	*
	Comamonas	*		*
	Delftia	*		
	Massilia	*		*
	Noviherbaspirillum			*
	Oxalicibacterium			*
	Pelomonas	*		*
	Schlegelella	*		*
	Tepidicella	*		
	Tepidimonas	*	*	*
	Variovorax			*
Caulobacteraceae	*Brevundimonas*	*	*	*
	Phenylobacterium			*
Cellulomonadaceae	*Actinotalea*	*		
Cellvibrionaceae	*Cellvibrio*	*		
Clostridiaceae 1	*Clostridium sensu stricto 1*			*
Corynebacteriaceae	*Corynebacterium 1*	*	*	*
	Lawsonella	*	*	
Coxiellaceae	*Coxiella*			*
Cupressus chengiana	*Cupressus chengiana*	*		
Cyanobiaceae	*Cyanobium PCC-6307*			*
Defluviitaleaceae	*Defluviitaleaceae UCG-011*			*
Deinococcaceae	*Deinococcus*		*	*
Demequinaceae	*Demequina*		*	
Dermabacteraceae	*Brachybacterium*	*		*
Devosiaceae	*Pelagibacterium*			*
Dietziaceae	*Dietzia*	*	*	*

Enterobacteriaceae	*Enterobacter*	*		*
	Erwinia			*
	Escherichia-Shigella	*		
	Hafnia-Obesumbacterium			*
	Klebsiella	*	*	*
	Pantoea	*	*	*
	Photorhabdus			*
	Pragia	*		
	Proteus			*
	Providencia	*		*
	Rosenbergiella			*
	Serratia	*		
	Tatumella			*
	Xenorhabdus	*		
Family X	*Thermicanus*			*
Family XII	*Exiguobacterium*		*	
Flavobacteriaceae	*Flavobacterium*		*	
	Myroides		*	
Gaiellaceae	*Gaiella*		*	
Geodermatophilaceae	*Blastococcus*			*
Halomonadaceae	*Carnimonas*			*
	Halomonas		*	
	Kushneria			*
	Oceanospirillum			*
Hydrogenophilaceae	*Hydrogenophilus*	*		
Intrasporangiaceae	*Ornithinicoccus*			*
	Janibacter		*	
	Knoellia		*	
	Phycicoccus		*	
	Serinicoccus			*

Kineosporiaceae	*Pseudokineococcus*			*
	Quadrisphaera			*
Lachnospiraceae	*CHKCI001*			*
	Lachnospira			*
	Lachnospiraceae NK3A20 group		*	
Microbacteriaceae	*Curtobacterium*	*		*
	Herbiconiux		*	
	Leifsonia		*	
	Leucobacter			*
	Microbacterium			*
	Pseudoclavibacter			*
Micrococcaceae	*Arthrobacter*	*		
	Glutamicibacter	*		
	Pseudarthrobacter	*		
Micromonosporaceae	*Actinoplanes*		*	
Moraxellaceae	*Acinetobacter*	*		*
	Enhydrobacter		*	*
Nakamurellaceae	*Nakamurella*			*
Neisseriaceae	*Kingella*	*		
	Simonsiella		*	
Nocardioidaceae	*Aeromicrobium*		*	
	Marmoricola	*		
	Nocardioides	*	*	*
Parachlamydiaceae	*Candidatus Protochlamydia*			*
Pasteurellaceae	*Haemophilus*			*
Planococcaceae	*Planococcus*			*
Porphyromonadaceae	*Porphyromonas*			*
Propionibacteriaceae	*Acidipropionibacterium*	*	*	
	Cutibacterium	*	*	*
	Friedmanniella	*		

	Microlunatus			*
	Propionicimonas			*
Pseudomonadaceae	*Pseudomonas*	*	*	*
Rhizobiaceae	*Mesorhizobium*	*	*	
	Phyllobacterium			*
Rhizobiales Incertae Sedis	*Phreatobacter*		*	
Rhodanobacteraceae	*Dyella*			*
	Frateuria			*
	Paenirhodobacter			*
Rhodobacteraceae	*Paracoccus*		*	*
Rhodocyclaceae	*Dechloromonas*	*		
Rubrobacteriaceae	*Rubrobacter*		*	
Ruminococcaceae	*Ethanoligenens*			*
	Ruminococcaceae UCG-014			*
	Ruminococcus 1			*
Solirubrobacteraceae	*Solirubrobacter*	*		
Sphingobacteriaceae	*Pedobacter*			*
	Sphingobacterium	*	*	
	Altererythrobacter			*
	Novosphingobium	*		
	Qipengyuania			*
	Sphingomonas	*	*	*
	Sphingopyxis		*	
Spirochaetaceae	*Sphaerochaeta*		*	
Staphylococcaceae	*Staphylococcus*	*	*	*
Streptococcaceae	*Streptococcus*	*	*	*
Streptomycetaceae	*Streptomyces*		*	
Thermaceae	*Thermus*	*		*
Thermoanaerobacteraceae	*Thermoanaerobacter*			*
Veillonellaceae	*Veillonella*		*	

Weeksellaceae	*Bergeyella*		*	*
	Chryseobacterium		*	*
	Cloacibacterium	*	*	*
	Riemerella			*
Xanthobacteraceae	*Bradyrhizobium*	*	*	*
	Pseudolabrys	*		
	Rhodoplanes	*	*	
	Rhodopseudomonas		*	*
Xanthomonadaceae	*Arenimonas*		*	
	Lysobacter		*	
	Stenotrophomonas	*	*	*
	Thermomonas			*

Table 2.5. Bacterial genera explaining the difference between field collected *Thaumatotibia leucotreta* larvae and larvae from the control treatment according to SIMPER analysis (RA = Relative Abundance).

Family	**Genus**	**Control RA**	**Field RA**	***p*-value**
Beijerinckiaceae	*Methylobacterium*	93567	37288	0.02
Acetobacteraceae	*Gluconobacter*	0	43154	0.02
Burkholderiaceae	*Tepidimonas*	8481	2048	0.02
Acetobacteraceae	*Acetobacter*	0	12850	0.04
Brevibacteriaceae	*Brevibacterium*	5072	0	0.04
Enterobacteriaceae	*Proteus*	0	344	0.04
Enterobacteriaceae	*Serratia*	190	0	0.04
Cupressus chengiana	*Cupressus chengiana*	5	0	0.04
Enterobacteriaceae	*Xenorhabdus*	6801	0	0.05
Moraxellaceae	*Acinetobacter*	5237	1189	0.05
Cellvibrionaceae	*Cellvibrio*	681	0	0.05
Bacillaceae	*Aeribacillus*	313	0	0.05

Table 2.6. Bacterial genera explaining the difference between field collected *Thaumatotibia leucotreta* larvae and larvae from the dechorionated treatment according to SIMPER analysis (RA = Relative Abundance).

Family	**Genus**	**Dechorionated RA**	**Field RA**	***p*-value**
Xanthomonadaceae	*Stenotrophomonas*	19151	127	0.01
Acetobacteraceae	*Acetobacter*	0	12850	0.02
Actinomycetaceae	*F0332*	2100	0	0.02
Halomonadaceae	*Halomonas*	1688	0	0.02
Micromonosporaceae	*Actinoplanes*	1584	0	0.02
Rhizobiales Incertae Sedis	*Phreatobacter*	925	0	0.02
Gaiellaceae	*Gaiella*	524	0	0.02
Nocardioidaceae	*Aeromicrobium*	457	0	0.02
Xanthomonadaceae	*Arenimonas*	2	0	0.02
Family XII	*Exiguobacterium*	2708	0	0.03
Bdellovibrionaceae	*OM27 clade*	2309	0	0.03
Flavobacteriaceae	*Flavobacterium*	1736	0	0.03
Bacillaceae	*Geobacillus*	1611	0	0.03
Veillonellaceae	*Veillonella*	1673	0	0.03
Intrasporangiaceae	*Knoellia*	2593	0	0.03
Bacillaceae	*Ornithinibacillus*	1171	0	0.03
Rhizobiaceae	*Mesorhizobium*	1100	0	0.03
Archangiaceae	*Cystobacter*	892	0	0.03
Lachnospiraceae	*Lachnospiraceae NK3A20 group*	786	0	0.03
Spirochaetaceae	*Sphaerochaeta*	551	0	0.03
Intrasporangiaceae	*Janibacter*	507	0	0.03
Beijerinckiaceae	*1174-901-12*	280	2	0.03
Xanthomonadaceae	*Lysobacter*	5	0	0.03
Sphingomonadaceae	*Sphingopyxis*	3	0	0.03
Neisseriaceae	*Simonsiella*	2	0	0.03
Microbacteriaceae	*Herbiconiux*	2	0	0.03

Microbacteriaceae	*Leifsonia*	2	0	0.03
Alteromonadaceae	*Salinimonas*	2	0	0.03
Streptomycetaceae	*Streptomyces*	2	0	0.03
Propionibacteriaceae	*Cutibacterium*	44284	9049	0.04
Moraxellaceae	*Enhydrobacter*	4716	681	0.04
Rubrobacteriaceae	*Rubrobacter*	4183	0	0.05
Alteromonadaceae	*Alishewanella*	1393	0	0.05

Chapter 3

The effects of gut microbial community structure on insect host physiology

Introduction

As illustrated in chapter one, microbial symbionts convey benefits to their hosts by providing metabolic services such as breaking down and metabolizing food particles, and detoxifying toxic compounds (Douglas, 2018). These microbe-host benefits in insects can directly impact host physiology and can be seen in host growth and development, and environmental fitness (Muñoz-Benavent *et al.*, 2021). Insects require their endosymbionts to provide them with essential nutrients such as amino acids, vitamins and lipids due to the nutritionally limited environment that many exist in (Douglas, 1998). Furthermore, symbiotic microbes may also have a protective role in terms of their host's physiology by aiding in their ability to tolerate thermal stress (Gurung *et al.,* 2019). For the most part, bacteria are the most studied microbes in microbiome research due to their ability to provide their hosts with benefits relating to nutrition, temperature stress tolerance and protection against natural enemies (Gurung *et al.,* 2019).

Nutritional mutualisms likely emerged from insect-bacteria co-evolution in nutrient-poor environments (Skidmore & Hansen, 2017). For example, larvae of *Bactrocera oleae,* the Olive fruit fly (Rossi, 1790) (Diptera: Tephritidae) rely on the bacterial endosymbiont *Candidatus* to develop in unripe olives as the bacteria are able to counteract and metabolize oleuropein, which is a phenolic compound produced by the plant to inhibit pest damage (Ben-Yosef *et al.,* 2015). As another example, some aphids rely on heritable bacteria to assist them nutritionally (Corbin *et al.*, 2017). These bacteria have also been shown to have an effect on the aphid's thermal tolerance (Corbin *et al.,* 2017). Mereghetti *et al.* (2017) argue that it is the symbiotic associations between insects and microbes that allow insects to colonize new ecological niches, as these associations represent an evolutionary driving force that provides the insect host with new or altered phenotypes. For example, the gut of the lepidopteran pest *Spodoptera littoralis* (Boisduval, 1833) (Lepidoptera: Noctuidae) has been found to always be colonized by *Enterococcus mundtii* despite varied diets and across all life stages (Mereghetti *et al.,* 2017). It is hypothesized that the bacteria play an active role in the physiology of the host as *E. mundtii* has been found to produce an antimicrobial peptide (AMP) in the gut lumen of the

host (Chen *et al*., 2016). This creates a chemical barrier against pathogens and increases host fitness (Shao *et al.*, 2017).

The effect that symbiotic microbes have on host physiology is not well understood and current research is only beginning to reveal the role that these microbes have in the biological processes of the host. In a study conducted by Henry and Colinet (2018), it was revealed that symbiotic gut microbiota in *Drosophila melanogaster* (Meigen 1830) (Diptera: Drosophilidae) directly affected the insect's ability to tolerate thermal stress. The study concluded by revealing that disruption of the *D. melanogaster* microbiome through egg dechorionation, which is the removal of the chorion layer of the egg by chemical means, decreased the insect's ability to tolerate and survive cold stress (Henry & Colinet, 2018). The theory behind this phenomenon is that gut microbiota establish roles within the insect hosts metabolic system that creates regulatory feedbacks, which benefit the hosts overall physiology, thus influencing the hosts ability to tolerate environmental stress (Soen, 2014).

The use of cold temperatures to induce immobilization and chill coma in insects for easier handling and transport is a common practice in Sterile Insect Technique (SIT) programs (Dowell *et al*., 2005). Chill coma is a term that refers to neuromuscular impairment in the insect body induced at mild to low temperatures and is a reversible paralytic state (Andersen *et al.*, 2015). Understanding the role of the insect microbiome and its effect on ecologically relevant traits has the potential to improve Integrated Pest Management (IPM) strategies that target economically important pests, such as *Thaumatotibia leucotreta* (Meyrick, 1913) (Lepidoptera: Tortricidae) (False Codling Moth – FCM), and pave the way to pest control strategies that manipulate the host-microbe association (Douglas, 2015; Mereghetti *et al.,* 2017). Over the past two decades, much research on *T. leucotreta* has been on how to control the pest in the field, understanding its thermal physiology and to mass rear the insect for SIT programs or biopesticide production (Moore, 2002; Bloem *et al*., 2005; Carpenter *et al*., 2007; Stotter & Terblanche, 2009; Boardman *et al*., 2013; Moore *et al*., 2014; Boersma & Carpenter, 2016; Malan *et al*., 2018; Nepgen *et al*., 2018; Boersma *et al*., 2019; Adom *et al.*, 2021). However, little to no research has been conducted on the microbial profile or effects that the gut microbiome has on *T. leucotreta* physiology from field populations or laboratory reared insects and how their performance in the field compares.

In microbiome research, disruption of gut microbiota can be achieved by supplementing the insects diet with antibiotics (Robinson *et al*., 2010), or by dechorionation of the insect egg as done by Henry and Colinet (2018). Although it must be noted that although antibiotics are an

inexpensive, rapid and efficient means of eliminating microbes in the microbiome; the use of antibiotics has the disadvantage of negatively impacting the study organisms' physiology (Kennedy *et al*., 2018). For example, antibiotic treatment with streptomycin in *Bactrocera dorsalis* was associated with reduced egg hatching rate and growth (He *et al*., 2017). The addition of rifampin to the diets of the termites *Zootermopsis angusticollis* (Hagen, 1858) (Blattodea: Archotermopsidae) and *Reticulitermes flavipes* (Kollar, 1837) (Blattodea: Rhinotermitidae) resulted in severe long term fitness effects of the colonies by disrupting nutrient acquisition by reproductive members and reducing fecundity (Rosengaus *et al*., 2011). Coupled with the physiological effect of antibiotic treatment, Rosengaus *et al*. (2011) found that the treatment shifted the bacterial community towards Gram-negative microorganisms. More specifically, antibiotic treatment using chloramphenicol and vancomycin in *Bombyx mori* (Linnaeus, 1758) (Lepidoptera: Bombycidae), resulted in the disruption of lipid, carbohydrate and amino acid metabolic processes as well as caused oxidative damage to the gastrointestinal tract (Li *et al*., 2020). Due to the possible negative side effects of antibiotic treatment on insect physiology, this study focused on the use of egg dechorionation as a means of disrupting gut microbiota for effective comparison between treatments as outlined in the above chapter.

The purpose of this chapter was to investigate a suit of physiological traits of *T. leucotreta* that are indicative of field performance to test whether gut microbial diversity interacts with and partly determines insect performance and fitness, including insect growth and tolerance to cold temperatures. These traits of *T. leucotreta* with undisrupted (control), disrupted gut microbiota (Dechorionated) and field collected larvae (Field) were compared.

Methods

Insect colonies and rearing

Thaumatotibia leucotreta were reared as per the methods described in Chapter 2 for the preparation of the control, dechorionated treatments and field-collected individuals.

Insect mass and macronutrient concentration

Final instar *T. leucotreta* larvae, which was observed when the larvae turn pink in colour approximately 14 days after hatching, (n=100 per treatment except for the field-collected samples where n=30) and adults (n=100 per treatment except for the field-collected samples where n=30) from the control, dechorionated treatments and field-collected insects were first weighed. The juvenile insects were carefully removed from the diet substrate using fine soft

forceps and placed in a 2mL microcentrifuge tube, their fresh masses were immediately recorded in milligrams using a fine balance scale housed at the Department of Zoology and Entomology (DZE), Rhodes University (RU). Adult moths were weighed using a fine balance scale 24 hours after eclosion, and weights recorded as fresh mass. Last instar *T. leucotreta* larvae from each treatment (control treatment n=8, dechorionated treatment n=8 and field-collected individuals n=8) were chosen at random and used for protein, lipid and carbohydrate concentration analysis. Larvae were prepared by individually flash freezing in liquid nitrogen and then placed in individual 2mL microcentrifuge tubes before being stored at -80℃ until the next steps could be completed at Stellenbosch University (SU; Western Cape Province, South Africa).

Once at SU, the specimens were individually freeze-dried in liquid nitrogen using a VirTis Benchtop Pro Freeze dryer and then reweighed to obtain their dry mass to the nearest 0.1mg. From the freeze-dried specimens, nine individuals were randomly selected from each treatment group for a total of 24 specimens that were used for body composition analysis.

Whole freeze-dried *T. leucotreta* larvae were individually placed in 2mL microcentrifuge tubes with a sterile glass bead and were homogenized in 180µl lysis buffer consisting of 100mM KH_2PO_4, 1Mm EDTA and 1mM DTT at a pH of 7.4 using a Retsch™ (MM 400) automatic multitube vortexed for five minutes at the maximum setting (Foray *et al.,* 2012). The protein, lipid and carbohydrate content were then determined for each individual insects homogenate from each treatment using colourimetric methods designed by Van Handel (1965) and modified into a 96-well microplate format by Foray *et al.* (2012) with modifications by Cuff *et al*. (2021) (see specific details below). All chemical reagents and constituents were supplied by Sigma Aldrich (Cape Town, Western Cape Province, South Africa).

Protein determination

Following the methods of Foray *et al.* (2012), eight prepared samples from the control, dechorionated treatments and field-collected individuals were centrifuged in a Prism™ Microcentrifuge for 15 minutes, 80 g at 4℃, to allow for sedimentation of cell debris. Using an adjustable volume pipette with a filtered tip, 1.5µl of supernatant was transferred from each sample into a borosilicate 96-well microplate. Each sample was replicated three times within the plate. In each sample well, 250µl of Bradford reagent was introduced and left to incubate at room temperature for 20 minutes. Prior to microplate reading, the microplates were shaken

at 10Hz for 3 seconds in the microplate reader. Protein concentration was measured spectrophotometrically using a Tecan™ (Spark 10M) microplate reader at 595nm using a dilution-series of Bovine Serum Albumin in the buffer solution as the standard.

Lipid determination

In accordance with the methods by Foray *et al.* (2012), the lipids were dissolved from the previously prepared homogenate by adding 20µl of sodium sulphate to the solution with 4.5µl lysis buffer, and 1500µl chloro-methanol (1:2 v/v) to solubilize the lipid. After vortexing, the samples were then centrifuged in the Prism™ Microcentrifuge at 800g for 15 minutes at 4℃. Using an adjustable volume pipette, 100µl of sample supernatant was moved into 2mL microcentrifuge tubes to undergo overnight evaporation in a fume hood. After total evaporation of sample solution, 10µl of 98% Sulphuric acid was added into each sample tube using an adjustable volume pipette with a filtered tip. The sample tubes were then incubated at 90℃ for 2 minutes in a Labomiz™ water bath. The tube racks with the sample tubes were then wrapped in aluminum foil and cooled on ice for 5 minutes. Vanillin reagent was prepared by mixing Vanillin with orthophosphoric acid of 68% for a solution concentration of 1.2g/l. 210µl of Vanillin reagent. The prepared Vanillin reagent solution was added to each sample tube and then thoroughly vortexed. Each sample was transferred via adjustable volume pipette in duplicates of 200µl of the solution to 96-well borosilicate microplates. Prior to microplate reading, the microplates were shaken at 10Hz for 15 minutes in the microplate reader. Lipid absorbance was measured spectrophotometrically using a Tecan™ (Spark 10M) microplate reader at 525nm using a dilution-series of glycerol trioleate in the buffer solution as the standard.

Carbohydrate determination

Carbohydrates were dissolved from the homogenate by adding 20µl of sodium sulphate to the solution with 4.5µl lysis buffer, and 1500µl chloro-methanol (1:2 v/v) to solubilize the water-soluble carbohydrates, in accordance with methods by Foray *et al.* (2012). After vortexing the tubes, the samples were then centrifuged at 800g for 15 minutes at 4℃. Sample supernatant was transferred using an adjustable volume pipette in duplicates of 100µl into 2mL microcentrifuge tubes for evaporation for over 48 hours in a fume hood. After total evaporation of the sample solution, 250µl of chloro-methanol (1:2 v/v) was added to each sample tube and thoroughly vortexed. Next, individual 15mL Falcon tubes were each filled with 200µl of the sample solution. Anthrone reagent was prepared by mixing anthrone powder and 70% Sulphuric acid for a solution concentration of 1.42g/l. 4.8ml of anthrone reagent was

added to the falcon tubes containing the sample solution and incubated at 90℃ for 15 minutes in a water bath. After incubation, the falcon tubes were placed in ice for 5 minutes to cool. After cooling, each sample was transferred in duplicates of 250µl into 96-well borosilicate microplates. Carbohydrate absorbance was measured spectrophotometrically using a Tecan™ (Spark 10M) microplate reader at 625nm using a dilution series of glucose in triple distilled sterile water with 4.8ml of anthrone reagent.

Chill coma recovery time

To determine the chill coma recovery times (CCRTs), *T. leucotreta* adults that were 24 hours old after eclosion from the control, dechorionated treatments and field-collected larvae were sorted into individual 2mL microcentrifuge tubes and placed in a Styrofoam float in a 50/50 water/glycol-filled water bath (Grant R52 with GP200 programmable controllers, United Kingdom), then exposed to a temperature of 0±1°C for two hours following Boardman *et al.,* (2012).

After the cold exposure period, the specimens were placed into individual labelled petri dishes, where they were laid on their backs at 25°C while being continually monitored for two hours. Recovery time was recorded for each insect as the time taken to regain mobility and stand unaided.

Cold stress survival

Acute cold stress tests were performed on adult FCM moths 24 hours after eclosion from the control, dechorionated treatments and field-collected larvae with four sample groups used per treatment (Figure 3.1). Moths were stored individually in 2mL microcentrifuge tubes plugged with cotton wool and the tubes were then placed in a Styrofoam float. For sample group one, randomly selected individuals (n=25 from each treatment) were exposed to 10°C for four hours in a water bath (Grant R52 with GP200 programmable controllers, United Kingdom) for a cold hardening pre-treatment step (Figure 3.1). Simultaneously, for sample group two, randomly selected individuals (n=25 from each treatment) were kept at a constant 25°C for four hours (Figure 3.1). After the four-hour pre-treatment, all sample groups were plunged straight into a 50/50 water/glycol-filled water bath at -7°C±1°C for two hours. Temperatures and timing of this experiment were sourced from Terblanche *et al*. (2017) to push the thermal limit of the moths to effectively test the sensitivity of their thermal stress responses despite this temperature not being used in SIT.

After the cold stress exposure, moths were kept in their individual 2mL microcentrifuge tubes, given a strip of filter paper moistened with dilute sugar water and allowed to recover at 25°C in a controlled environment chamber for 24 hours before survival counts were recorded. This experiment was repeated four times for laboratory treatment groups and once for field collected insects for a total sample size of 100 moths for each treatment category.

Statistical analysis - Mass

All statistical analyses was performed using R version 3.6.3 (RStudio Team 2019). Separate general linear models (GLMs), one for the last instar larvae and one for the adults, with gaussian distributions and an identity link function were used to investigate the effect of treatment on insect mass from the control, dechorionated treatments and field-collected specimens. Model fits were assessed using *DHARMa* v0.4.6 (Hartig & Lohse, 2022) by simulating the models residuals and viewing AIC scores. *car* v3.1.1 (Fox *et al.,* 2022) was used to perform an ANOVA on the model to analyze the difference between the means of the treatments. All post-hoc analyses were performed using pairwise comparisons between treatments with the *emmeans* package v1.8.2 (Lenth *et al.,* 2022).

Statistical analysis – Macronutrient content

Absorbance values for protein, lipid and carbohydrates were converted to content (mg) using standard curves generated using the standard dilution series in each assay. The amount of each macronutrient present in each total *T. leucotreta* specimen was estimated by using the linear equation derived from the calibration curves of the standards. The water content of larvae was calculated by subtracting larval dry mass from larval fresh mass.

General Linear Models (GLMs) were used to assess the effect of treatment on the fresh mass, dry mass and water content of larvae from the control, dechorionated treatments and field-collected larvae. As a result of non-normal data distributions, determined by a Shapiro-Wilks test, models were generated with square root transformation of the fresh mass, dry mass and mass of water variable. Square root transformation of these variables were used as the response variables to account for heteroscedasticity of the data. Model fits were assessed by viewing AIC scores and plotting residuals using base R version 3.6.3 (RStudio Team, 2019). *car* v3.1.1 (Fox *et al.,* 2022) was used to perform ANOVAs on the models to assess the difference in fresh mass, dry mass and mass of water between the treatments. Additionally, post-hoc analyses were performed on the models using pairwise comparisons between treatments with the *emmeans* package v1.8.2 (Lenth *et al.,* 2022).

General linear mixed models (GLMM), using the package *glmmTMB* v1.1.4 (Brooks *et al*., 2022), were used to investigate the effect of treatment on macronutrient content from the protein, lipid, carbohydrate assays. Initial linear models assessed the interactions between protein, lipid, and carbohydrate content with treatments after controlling for insect sample dry mass. Dry mass was used to establish how composition of macronutrients varied relative to the final condition of each *T. leucotreta* specimen after each treatment to correct for the interactive effect of body mass on macronutrient percentage in body composition. Model fits were assessed using *DHARMa* v0.4.6 (Hartig & Lohse, 2022) by simulating the models residuals and viewing AIC scores. *car* v3.1.1 (Fox *et al.,* 2022) was used to perform an ANOVA on the model to assess the difference in macronutrient content between the treatmants. All post-hoc analyses were performed using pairwise comparisons between treatments with the *emmeans* package v1.8.2 (Lenth *et al.,* 2022).

Statistical analysis - CCRT

To examine the effects of treatment on CCRT, a GLM was fitted with log transformed CCRT as the response variable (log transformation was necessary to ensure homogeneity of variance) and treatment as the predictor variable using the log1p function using base R version 3.6.3. Model fits were assessed using *DHARMa* v0.4.6 (Hartig & Lohse, 2022) by simulating the models residuals and viewing AIC scores. *car* v3.1.1 (Fox *et al.,* 2022) was used to perform a Levene's test on homogeneity of variance across groups on the model and an ANOVA on the model to analyse the difference between the means of the treatments. All post-hoc analyses were performed using pairwise comparisons between treatments with the *emmeans* package v1.8.2 (Lenth *et al.,* 2022).

Statistical analysis – Cold stress survival

To examine the effects of treatment and cold hardening or direct exposure temperature steps on FCM cold stress survival, a GLM was fit with binomial distribution and a probit link function. Insect mortality was the response variable with treatment, temperature and sex as the predictor variables. The first model was run to test for additive effects of treatment and temperature. The second model was run to test for interaction effects between the predictor variables. Model fits were assessed using *DHARMa* v0.4.6 (Hartig & Lohse, 2022) by simulating the models residuals. Collinearity was checked using the *performance* package v0.10.1 (Lüdecke *et al.,* 2022) . The package *car* v3.1.1 (Fox *et al.,* 2022) was used to run an ANOVA on the best fit model. All post-hoc analyses were performed using pairwise

comparisons between treatments with the *emmeans* package v1.8.2 (Lenth *et al.*, 2022). Effect plots of the models were made using *ggeffects* v1.1.4 (Lüdecke, 2018).

Results

Insect mass and macronutrient concentration There were significant differences in the masses of larvae from the three different treatments, (χ^2(2, n = 230) = 22.703, *p*-value < 0.001) (Figure 3.2A). Larvae from the dechorionated treatment weighed significantly more than larvae from the control treatment (*p*-value = 0.030) (Figure 3.2A), while field-collected samples were significantly heavier than both laboratory reared treatments (*p*-value < 0.001; *p*-value = 0.011) (Figure 3.2A).

Similarly, there were significant differences in the masses of adults from the three different treatments (χ^2(2, n = 230) = 39.074, *p*-value < 0.001) (Figure 3.2B). Results for adult mass showed that moths from the dechorionated treatment weighed significantly more than moths from the control treatment (*p*-value < 0.0001) and from moths from the field-collected samples (*p*-value < 0.001; Figure 3.2B). Additionally, moths from the control treatment weighed more than those from the field-collected samples (*p*-value = 0.022; Figure 3.2B). There was a significant difference in larval fresh mass between the between control, dechorionated treatments and field-collected samples, (χ^2(2, n = 24) = 36.668, *p*-value < 0.0001). Pairwise comparison of fresh mass between the treatments showed that all three treatments differed significantly from one another (*p*-value < 0.02) (Figure 3.3), with samples from the field having the highest fresh mass. There was also a significant difference in dry mass between the treatment samples (χ^2(2, n = 24) = 34.971, *p*-value < 0.0001) (Figure 3.3). Pairwise comparisons of larval dry mass indicated that the dechorionated individuals had a higher dry mass than the control individuals (*p*-value < 0.0001) and the field-collected individuals (*p*-value = 0.0001) (Figure 3.3). In support of these patterns, there was a significant difference in water content between the treatments (χ^2(2, n = 24) = 8.969, *p*-value = 0.011) (Figure 3.3). Pairwise comparisons showed that the dechorionated individuals had a higher water content than the control individuals (*p*-value = 0.033). The field-collected samples had a higher water content than the control treatment (*p*-value = 0.028) (Figure 3.3).

The investigation of the macronutrient contents revealed there were no significant differences in protein (χ^2(2, n = 24) = 5.680, *p*-value = 0.06) , carbohydrate (χ^2(2, n = 24) = 3.940, *p*-value = 0.14) or lipid (χ^2(2, n = 24) = 6.032, *p*-value = 0.05) content between the three different treatments whilst controlling for dry mass (Figure 3.4).

Chill coma recovery time

There was a significant difference overall in Chill Coma Recovery Time (CCRT) between the treatments (χ^2(2, n = 129 = 39.659, *p*-value < 0.001) (Figure 3.5). Pairwise comparisons indicated that the CCRTs were not significantly different between the control and dechorionated treatments (*p*-value = 0.846) (Figure 3.5). However, CCRT was significantly different between the dechorionated treatment and field-collected samples (*p*-value < 0.0001), with insects from the dechorionated treatment recovering from chill coma faster (Figure 3.5). Similarly, CCRT was significantly different between the control treatment and field-collected samples (*p*-value < 0.0001), with insects from the control treatment recovering faster (Figure 3.5).

Cold stress survival

An additive probit model without interaction terms between treatments, sex and temperature proved the best fit due to low collinearity. There was a significant difference in treatment in cold stress survival between the control, dechorionated treatments and field-collected samples (χ^2(2, n = 272) = 9.176, *p*-value = 0.01). Neither temperature (*p*-value = 0.138) nor sex (*p*-value = 0.153) affected cold stress survival of insects in the control, dechorionated treatments and field-collected samples.

Pairwise comparison of the treatments showed that insects from the dechorionated treatment showed a significant difference in mortality compared to insects from the control treatments (*p*-value = 0.024), with insects from the control experiencing higher mortality (Figure 3.6). Similarly, there was a significant difference in mortality between dechorionated treatment and field-collected samples (*p*-value = 0.04), with insects from the dechorionated treatment suffering less mortality than those from the field-collected samples (Figure 3.6). There was no significant difference in mortality between the control treatment and field-collected samples (*p*-value = 0.998; Figure 3.6).

Discussion

The findings of this study suggest that there is an interaction between gut microbiota and the thermotolerance and physiology of *T. leucotreta*, which results in fitness differences between treatments. As gut microbes have been shown to play important roles in insect development and nutrient absorption, analysing insect mass is an indirect way of measuring their effect on insect physiology (Douglas, 1998; Paniagua-Voirol *et al.*, 2018). The results of this study showed that *T. leucotreta* with reduced gut microbiota through egg dechorionation had

increased body mass in both their larval and adult life stages compared to insects with intact gut microbiomes from the control treatment and field-collected individuals. Larvae collected in the field had a significantly higher body mass than larvae with disrupted gut microbiomes and control larvae. However, in the adult life stage, moths with disrupted gut microbiome are significantly larger than both control and field moths. Comparatively, larvae from the dechorionated treatment had a higher dry mass than larvae from the control treatment and field-collected samples.

This difference in dry mass during the larval stage is likely linked to the increased mass of *T. leucotreta* in the adult life stage. Larval mass is a determining factor in pupation and adult mass in insects that undergo metamorphosis (Davidowitz *et al*., 2003; Boggs & Freeman, 2005). In an early study by Kaufman *et al*. (1989), the influence of the microbiome on host food utilization and development was highlighted when conventionally reared insects outperformed germ-free insects. This pattern has since been repeated in studies focusing on mass produced insects such as *Hermetia illucens* whereby removal of certain microbes from the microbiome negatively impacts larval development and adult fitness (Jordan & Tomberlin, 2021). However, it has also been revealed that supplementation of specific microbes to the microbiome can improve larval development (Somerville *et al*., 2019). Based on the aforementioned findings, larval development, which is linked to adult fitness, appears to depend largely on two factors: diet source and microbiome composition (Davidowitz *et al.*, 2003; Boggs & Freeman, 2005). Javal *et al*. (2022) revealed that the differing larval development and adult weight in *Cacosceles newmannii* on various diets may be due to hindered nutrient extraction. In this study, laboratory reared *T. leucotreta* were raised on a sterile artificial diet developed to meet their nutritional needs whereas insects collected from the field were retrieved from infested fruit. While differences in larval mass between dechorionated and field collected larvae could be explained by the removal or bacteria that suppress other microbes, the indistinguishable effects of food source may obscure the true effect of microbiota in driving the development of this phenotype. It is recommended that future research focused on the relationship between host traits and the microbiome in *T. leucotreta,* use a common garden experiment by rearing individuals derived from mass-reared colony and field collected individuals on sterilized fruit or artificial diet seeded with gut homogenate from different origins.

As there is a difference seen in the fresh and dry mass larvae and adults of *T. leucotreta*, it was expected that there would be a difference in macronutrient composition between the larval

treatments. Interestingly, on analysis of water content and the protein, lipid and carbohydrate content between the control, field and dechorionated larvae, there was no significant differences in macronutrient content but there was a difference in water content. Although there was no significant difference in lipid, protein and carbohydrate content, it does not rule out difference in other body components such as chitin and other nutrient subcategories, for example, differing ratios between triglycerides and phospholipids. With our experimental setup, we could not evidence changes in composition of protein, lipid and carbohydrate concentration associated with the differences in bacterial composition of our three treatments. This is not consistent with results from studies conducted on *H. illucens* and *D. melanogaster* (Leitão-Gonçalves *et al*., 2017; Greenwood *et al*., 2021). However, these results do not rule out whether the gut microbiome of *T. leucotreta* has no role in other biochemical processes such as hormone homeostasis which is linked to water content in the insect body (Waterson *et al*., 2014). The mutualistic relationship between gut microbes and their host is a factor that influences host energy metabolism and the composition of the microbiome has been associated to certain metabolic diseases in animal models (Bäckhed *et al*., 2005; Cani & Delzenne, 2009; Gáliková *et al*., 2015). For instance, in *D. melanogaster*, the commensal bacterium *Lactobacillus plantarum* has been identified as playing a critical role in systemic growth of its host through metabolite production coupled with upregulation of genes in the host (Layalle *et al*., 2008; Storelli *et al*., 2011). To uncover the mechanistic roles of components of the *T. leucotreta* gut microbiome, we recommend that future investigations focus on metagenomic approaches involving gut transcriptome analysis to this area of study (Douglas, 2018).

Investigation into the ability of *T. leucotreta* to tolerate environmental stress after disruption of its gut microbiota revealed that gut microbes have the potential to influence the hosts overall physiology, as theorised by Soen (2014). Results of the CCRT tests showed that insects collected from the field took longer to recover from chill coma than larvae from both the control and dechorionated treatment. Coupled with the CCRT analysis, results of cold stress survival testing showed that insects with a disrupted gut microbiome are less susceptible to cold stress than insects with an unaltered gut microbiome. These findings are inconsistent to what was seen in physiological tests conducted on *D. melanogaster* by Henry and Colinet (2018) with regards to cold stress. These results suggest there is a possible relationship between the gut microbiome of *T. leucotreta* and host thermotolerance. However, this relationship cannot be appropriately contextualised in this study in that only cold tolerance

was tested. Although the cold stress testing of *T. leucotreta* is suitable for application to industry practices seen in the cold immobilisation of the insect used in SIT programs (Dowell *et al*., 2005), the testing did not provide the whole view of *T. leucotreta* thermotolerance capabilities since it only focused on cold stress tolerance and did not include heat tolerance. Complete assessment of on whether microbiota disruption influences the mechanisms underlying thermal stress tolerance needs to include analysis of heat stress due to bacteria themselves being thermally sensitive (Wernegreen, 2012; Moran, 2016).

Alternatively, the result that insects from the dechorionated treatment were more likely to survive cold stress than insects with intact microbiomes may indicate that the removal of certain pathogens present at subclinical levels resulted in a phenomenon known as pathobiont elimination (Lee *et al*., 2013). This elimination could have led to a reduction in physiological stress and subsequently allowed the host to allocate its energy reserves more effectively (Grimont & Grimont, 2006; Ardia *et al*., 2012).

By removing the pathogens, the dechorionated insects potentially freed up energy that was once dedicated to immune system functioning (Ardia *et al*., 2012). The immune system plays a crucial role in defending the host against infections and maintaining overall health (Chambers & Schneider, 2012). However, it requires significant energy resources to operate efficiently. When the immune system is constantly engaged in combatting pathogens, the energy that could have been allocated to other physiological processes, such as stress response, may be limited (Ayres, 2013; 2013).

In the context of cold stress, where the ability to withstand and adapt to low temperatures is vital, the redirection of energy resources away from immune system functioning towards other mechanisms becomes advantageous(Sinclair *et al*., 2013; Henry & Colinet, 2018). The dechorionated insects, with their reduced pathogen load and potentially enhanced energy reserves, were better equipped to respond to the environmental stress of cold temperatures.

This observation highlights the intricate balance between immune system functioning and the allocation of energy reserves in insects. It suggests that the removal of certain pathogens at subclinical levels can positively influence the host's ability to cope with environmental stressors, as energy resources are no longer monopolized by immune responses. Further research in this area could provide valuable insights into the interplay between pathogen presence, immune system dynamics, and the overall fitness of insect populations.

Figures and Tables

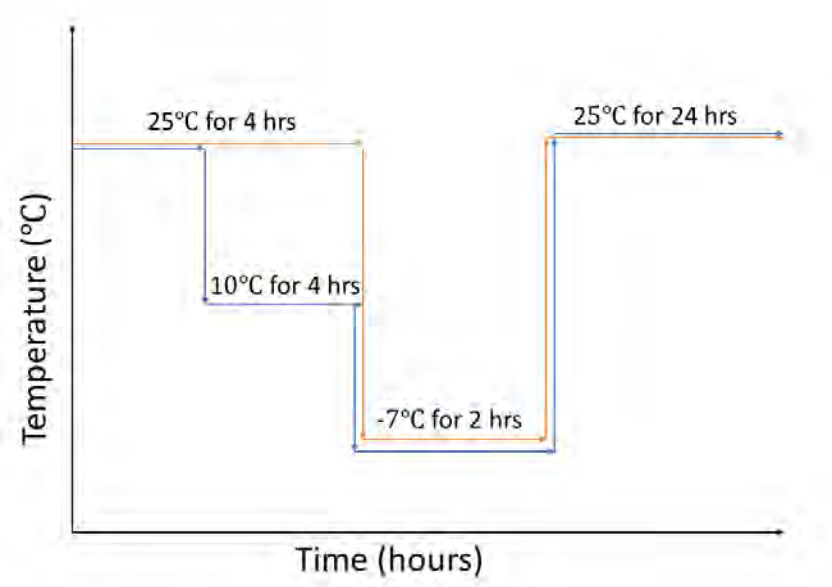

Figure 3.1. Schematic of temperature stress treatments and times allotted for each step during cold stress survival experiments. Orange arrows indicate moths that experienced a temperature regime whereby the 1) moths have been reared from eggs with chorion intact and 2) moths that have been reared from eggs that have been dechorionated. Blue arrows indicate moths that have experienced a stepped temperature regime to induce cold hardening whereby 3) moths have been reared from eggs with chorion layer intact and with 4) moths that have been reared from eggs that have been dechorionated.

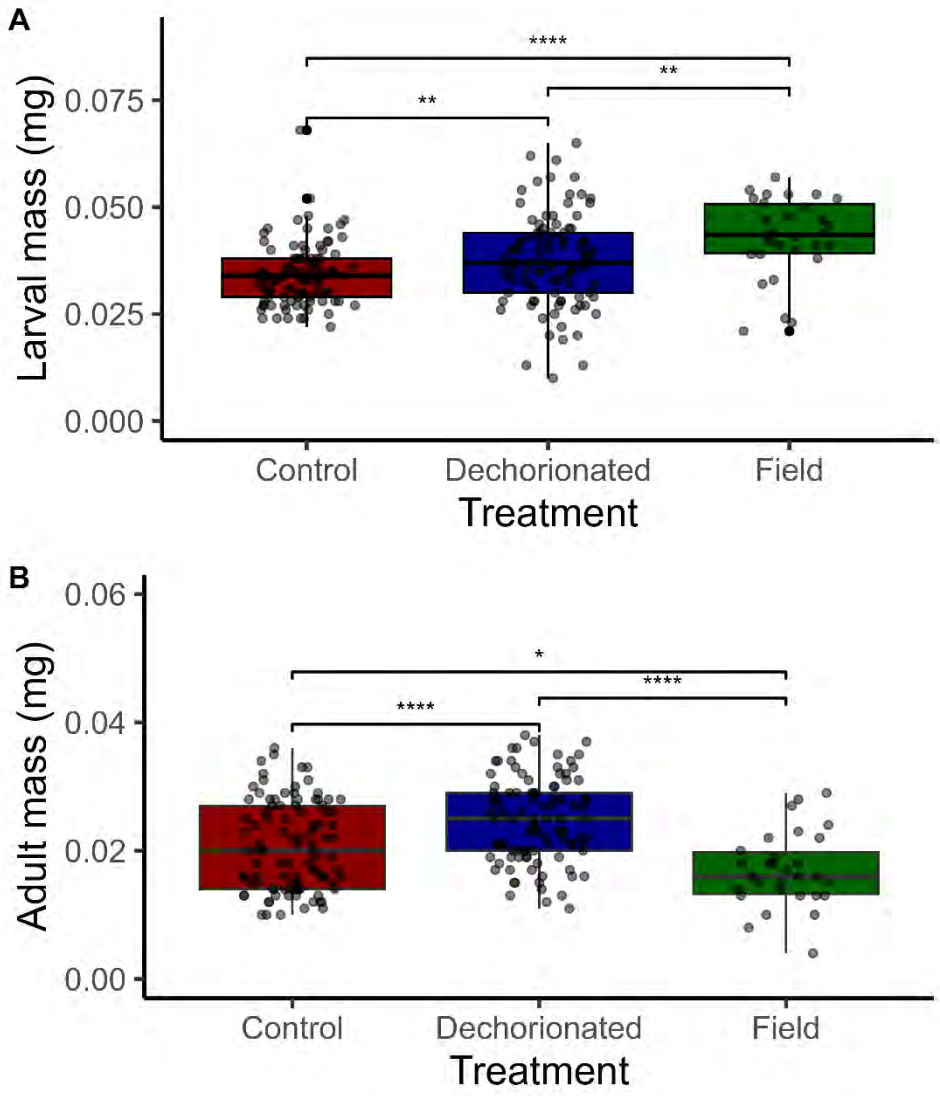

Figure 3.2. The mass of *Thaumatotibia leucotreta* from the control, dechorionated treatments and field-collected samples for A) last instar larvae and B) adults. Boxes and whiskers = interquartile range and mean, and dots outside of boxes = outliers. Level of significance (*p*-value) indicated by stars: 'NS' = 0.05, '*' = 0.01, '**' = 0.001, '***' = 0.001, '****' = 0.

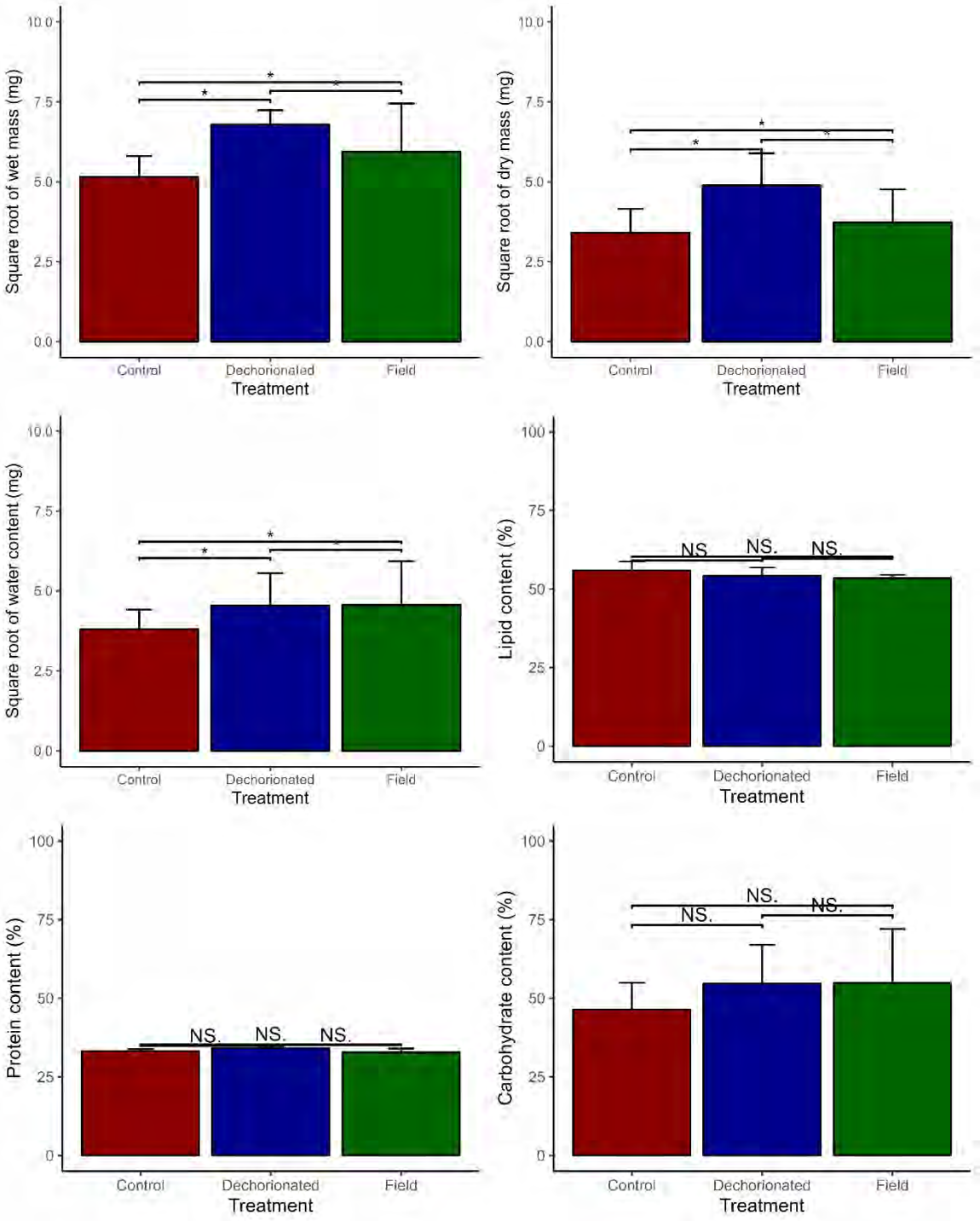

Figure 3.3. Overall composition of *Thaumatotibia leucotreta* last instar larvae indicating fresh mass, dry mass and water content; and with macronutrient composition as a percentage of dry mass. Significant differences between variables indicated by star '*' or "NS" for no significance.

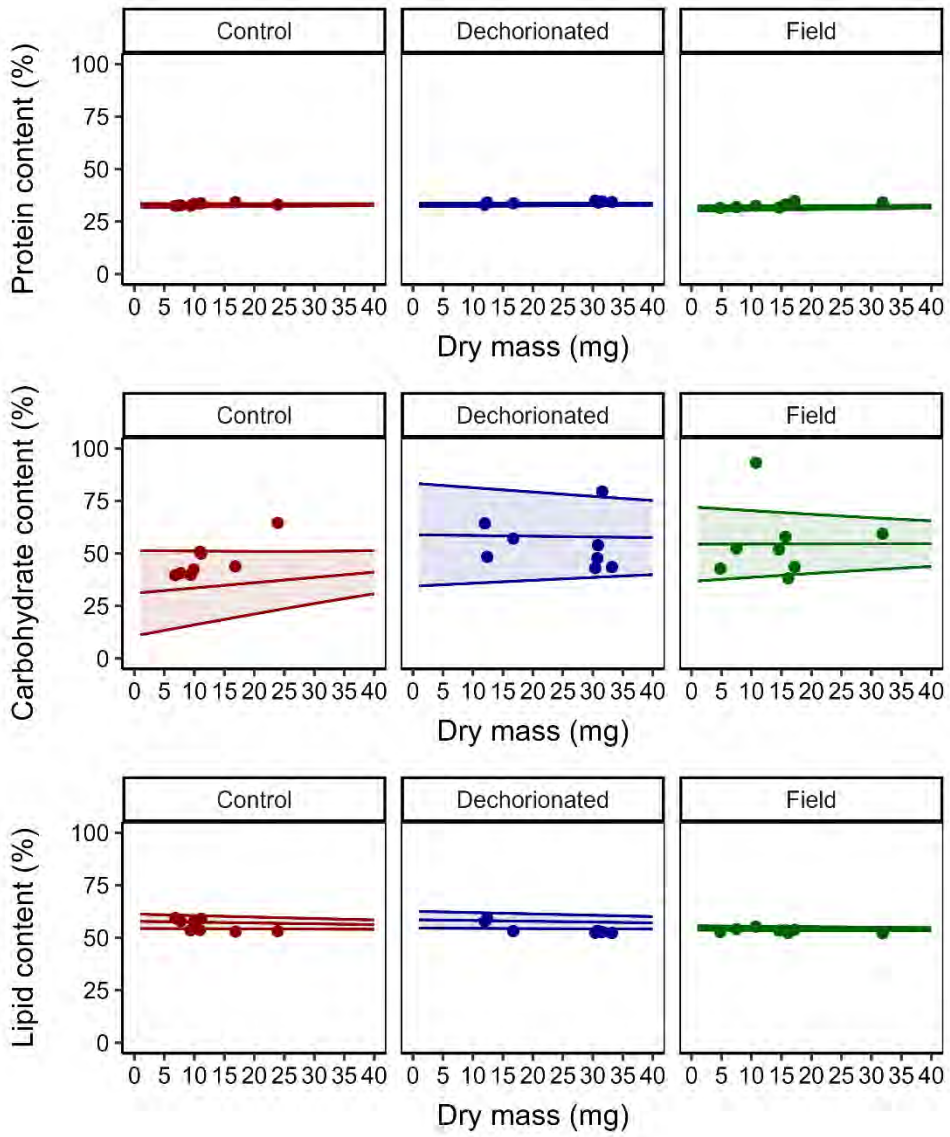

Figure 3.4. The macronutrient composition of *Thaumatotibia leucotreta* last instar larvae across three treatments correcting for dry mass of larvae. Ribbons indicate confidence intervals.

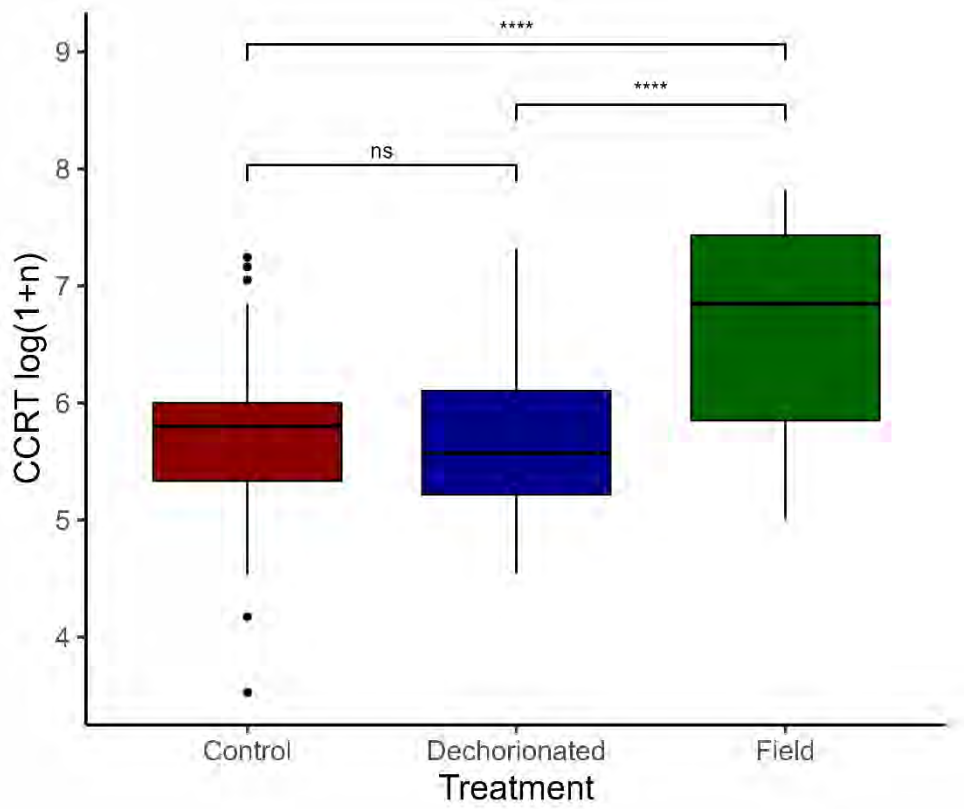

Figure 3.5. Log transformed chill coma recovery time (CCRT) of adult *Thaumatotibia leucotreta* from the control, dechorionated treatments and field-collected samples. Boxes and whiskers = interquartile range and mean, and dots outside of boxes = outliers. Level of significance (*p*-value) indicated by stars: 'ns' = 0.05, '*' = 0.01, '**' = 0.001, '***' = 0.001, '****' = 0.

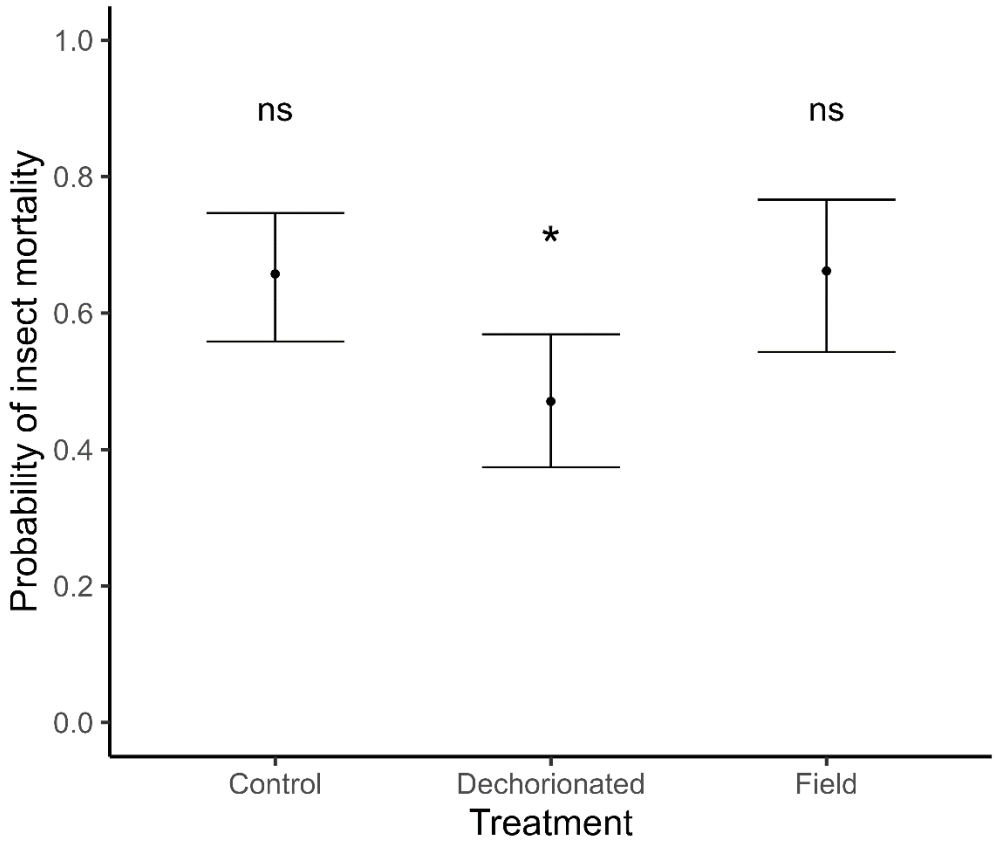

Figure 3.6. Effects plot showing the predicted probabilities of mortality from cold stress for adult *Thaumatotibia leucotreta* from the control, dechorionated treatments and field-collected samples. Error bars indicate Standard Error. Level of significance (*p*-value) indicated by, 'ns' > 0.05, '*' = 0.02.

Chapter 4

Conclusion

Thesis Overview

Overall, this study explored the host-microbe physiological interaction of *Thaumatotibia leucotreta* (Meyrick 1913) (Lepidoptera: Tortricidae) and bacteria present in its gut microbiome. The predominant bacterial families found to inhabit the gut microbiome were identified as *Xanthobacteraceae*, *Beijerinckiaceae* and *Burkholderiaceae* with genera *Bradyrhizobium* (29%), *Methylobacterium* (14%) and *Burkholderia-Caballeronia-Paraburkholderia* (9%) as having the highest relative abundances. It was shown that bacterial diversity was highest in specimens collected from the field compared to laboratory reared larvae, which is expected based on the heterogeneity of the environments that the insects are reared in. Larvae that were reared from eggs that underwent dechorionation were also shown to have the lowest bacterial diversity, which indicated that the process of egg dechorionation effectively disrupted gut microbiome establishment. In total there are 18 bacterial genera that were found across all three treatments with *Bradyrhizobium* accounting for a total of 49%, followed by *Burkholderia-Caballeronia-Paraburkholderia* at a total of 15% and lastly, *Staphylococcus* at a total of 11% which suggests that these genera make up the core bacterial microbiota for *T. leucotreta* and has similarities with other studies that sought to identify components of the lepidopteran gut microbiome (Walsh & Webster, 2003; van der Hoeven *et al*., 2008; Robinson *et al*., 2010). This is the first study to characterise the bacterial microbiome of *T. leucotreta* and will provide a useful foundation from which other researchers can study other facets of the *T. leucotreta* or African Lepidopteran host-microbe interactions. More specifically, the findings of this thesis are relevant to the mass rearing practices of *T. leucotreta* for SIT programs and biopesticide production in that researchers incorporate microbiome applications into further investigations on host competitive fitness and pest-pathogen interactions.

This study supports the notion that disruption of the insect microbiome influences host physiology that may result in fitness differences between treated and untreated insects (Douglas, 1998, 2015; Gurung *et al*., 2019; Muñoz-Benavent *et al*., 2021). Regarding thermal physiology, this study suggests that there may be an interaction between the gut microbiome of *T. leucotreta* and its ability to survive thermal stress. The reduction of bacterial diversity and increase in bacterial load in dechorionated larvae was associated with

increased larval fresh mass and larval dry mass which appears to increase the likelihood of surviving cold stress. This result is surprising given that insects that have had their microbiome disrupted generally do not perform well when compared to their counterparts that have their gut microbiome intact (Henry & Colinet, 2018). This finding suggests that the bacterial composition in larvae raised from dechorionated eggs positively influenced larval development that in turn improved physiological traits of the adults. Moreover, it was shown that insects collected from the field that had high bacterial diversity in their gut microbiome took longer to recover from chill coma than their laboratory reared counterparts. These findings provide a basis for future research focused on the analysis of microbiome disruption and its influence on thermotolerance and long term effects on insect fitness due to the alteration of the insects energy reserves (Teets & Denlinger, 2013). Owing to the fact that insects used in SIT programs need to be able to compete with the wild population, enhancing the understanding of pest biology and metabolic influences have the potential to improve pest management practices (Qadri *et al.*, 2020).

The biochemical mechanisms underlying the ability of *T. leucotreta* to tolerate cold stress and suppress pathogens are complex, yet microbiome research has the potential to provide insight into these mechanisms that may prove to be economically useful. Overgaard and Macmillan (2017) state that the loss of ion homeostasis across gut epithelia is directly responsible for chill injuries. Findings by Overend *et al*. (2016) suggest that complex interactions among pH, ion transporters and bacteria take place in the midgut. Nutrition not only plays an essential role in an insect's ability to tolerate cold temperatures, it is also vital in microbe-host interactions (Ridley *et al.,* 2012, Colinet *et al.*, 2013). The work of Wong *et al*. (2014) showed that disruption and elimination of microbiota from the gut microbiome of *Drosophila melanogaster* (Meigen 1830) (Diptera: Drosophilidae) induced changes in nutrient acquisition in the insect body, which had negative consequences on storage of fatty acids and carbohydrates. As these compounds are linked to insect response to stress, thermal tolerance is likely to be affected. This study found no significant differences in the content of protein, lipids and carbohydrates between field collected and laboratory reared larvae with intact and disrupted microbiomes that would suggest the role of these macronutrients in the cold stress tolerance of *T. leucotreta*. However, further investigation into chitin concentration, fatty acid storage or hormone homeostasis and possible metabolic roles of the gut microbiota in *T. leucotreta* would provide further insight into these biochemical mechanisms (Layalle *et al*., 2008; Waterson *et al*., 2014).

Implications of the study

The implications of this study not only include insights into this pest's biology but also on the pest management of *T. leucotreta* and mass rearing practices utilised in Sterile Insect Technique programs (SIT). This research provides evidence that microbiome establishment begins at the egg stage for this species and that bacteria within the gut of *T. leucotreta* influences larval growth and host physiology.

Firstly, it must be mentioned that there is a real possibility that bacteria in the gut of *T. leucotreta* may contribute to the development of pesticide resistance by detoxifying pesticidal compounds (Hofmeyr & Pringle, 1998), a process that has been seen in other insect pests such as *Bactrocera dorsalis, Bactrocera olea* and *Hypothenemus hampei* for example (Gressel, 2018). Additionally, research conducted by Ramya *et al*. (2016) isolated the bacteria *Bacillus cereus*, *Enterobacter asburiae* and *Pantoae agglomerans* from diamondback moth in India that could degrade insecticidal compounds. This study also identified bacteria from the genus *Bacillus*, *Enterobacter* and *Pantoea* in the gut microbiome of *T. leucotreta* and taxonomic analysis at the species level would provide a more accurate representation of these bacteria and their possible roles in the microbiome (Ramya *et al*., 2016).

Secondly, the findings of this study provide evidence for the potential improvement of mass reared *T. leucotreta* by harnessing the microbiome, which could be done in the form of a supplement to the sterile artificial diet or improved methods for egg sterilisation (Gorrens *et al*., 2021). This study showed a marked increase in larval mass of larvae whose gut microbiome was altered at the egg stage. Producing larvae of increased size is relevant to mass rearing *T. leucotreta* for the purpose of biopesticide production, whereby higher yields of virus could be produced with fewer insects (Morales-Ramos *et al*., 2014; Moore, 2021). On the topic of mass rearing *T. leucotreta* for virus production, further research into the role of the microbiome on immune function and pathogen suppression in this pest species has the potential to enhance our understanding of the susceptibility of *T. leucotreta* to *Cryptophlebia leucotreta* granulovirus (CrleGV) and *Cryptophlebia peltastica* nucleopolyhedrovirus. To date there is no research on the microbe-microbe interactions in *T. leucotreta* that may influence the hosts susceptibility to pathogen infection as seen in other insects (Grimont & Grimont, 2006; Graham *et al*., 2012). Larvae of increased mass or priming of the immune system of insects for release could be achieved through the supplementation of commensal bacteria or probiotic to the artificial diet of mass reared *T.*

leucotreta (Somerville *et al*., 2019; Gorrens *et al*., 2021). Likely bacterial genus candidates for *T. leucotreta* artificial diet supplementation are *Phycicococcus*, *Rubrobacter*, *Exiguobacterium*, *Knoellia*, *OM27 clade*, bacteria *F0332* and *flavobacterium* as they had the highest relative abundances of the bacteria that were unique to the dechorionated treatment larvae. However, species level identification of these bacteria would need to be performed for a more refined application.

Lastly, applying microbiome analysis to mass rearing programs of *T. leucotreta* would provide various benefits that may enhance the thermotolerance of sterile *T. leucotreta* for release in SIT programs. Overall, the results of the study suggest there is a relationship between the gut microbiome of *T. leucotreta* and thermotolerance, and that *T. leucotreta* with altered gut microbiomes were more likely to survive cold stress than those with intact microbiomes from the field. Thus, it appears that mass produced insects reared on artificial diet share a comparable fitness level with the insects in the wild population. However, a complete assessment on whether gut microbiome disruption influences the mechanisms underlying thermal stress tolerance needs to include analysis of heat stress as well to determine if mass reared insects are truly able to better tolerate temperature stress when released into the environment during SIT programs (Wernegreen, 2012; Moran, 2016). The ability to tolerate heat stress in the field is just as important to mass reared insects used in SIT programs as the ability to tolerate cold stress during irradiation and transport due to their effects on hormone homeostasis and energy metabolism (Lorenz & Gade, 2009; Boardman *et al*., 2013). Understanding these mechanisms may provide valuable insight that helps refine insect rearing techniques and release conditions of *T. leucotreta* in pest control programs as well as provide a better understanding of this pests' biology.

Recommendations

The data provided in this study provide the foundation for future research into the gut microbiome of *T. leucotreta.* Further investigation into species-level identification of the bacteria and their functional roles in the host metabolism would provide improved insight into this pests' biology and may provide alternative solutions to pest management practices. Further research into the possibility that certain species of bacteria such as *Bacillus*, *Enterobacter, Wolbachia, Serratia* and *Pantoea* may contribute pathogen suppression or the development of pesticide resistance in the species is needed. Moreover, the mechanism of how reduced bacterial diversity in mass reared *T. leucotreta* and possible probiotic supplementation into artificial diet improves overall insect physiology highlights the need

for further research in this area. The application of microbial checks to the mass-rearing practices of *T. leucotreta* holds the most economic potential in the short term. This approach offers several advantages, including the potential increase in larval yield, improved pathogen detection in mass rearing facilities, and the ability to standardize insect production throughout the rearing facilities (Somerville *et al*., 2019).

Overall, a hologenomic approach of investigation into the host-microbe influence on *T. leucotreta* physiology and thermotolerance has untapped potential in the control and understanding of this agricultural pest (Alberdi *et al*., 2022). The reason being that the microbiome provides protective effects to its host through metabolic processes such as colonization-mediated stimulation of gene upregulation of oxidative stress response pathways during temperature stress (Nakagawa *et al*., 2016). Combining assays on host gene expression and microbial metagenomics has provided researchers such as Fontaine and Kohl (2023) with the necessary tools to determine that the microbiome of *Lithobates clamitan*, green frogs, provides a buffering effect that protects its host from heat stress. Although these approaches are still relatively new in their application on insect host-microbe interactions , they provide a more complete understanding on the development of emergent phenotypes in host physiology as influenced by the host microbiome (Lynch & Hsiao, 2019; Qadri *et al*., 2020).

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Appendices

Appendix 1. BioPython script

```
#!/usr/bin/env python
"""
%prog to clean sequences - remove short sequences, remove excess N's and duplicate sequences
"""
import sys
from Bio import SeqIO
def sequence_cleaner(fasta_file, min_length=0, por_n=100):
    # Create our hash table to add the sequences
    sequences = {}
# Using the Biopython fasta parse we can read our fasta input
    for seq_record in SeqIO.parse(fasta_file, "fasta"):
        # Take the current sequence
        sequence = str(seq_record.seq).upper()
        # Check if the current sequence is according to the user parameters
        if (
            len(sequence) >= min_length
            and (float(sequence.count("N")) / float(len(sequence))) * 100 <= por_n
        ):
            # If the sequence passed in the test "is it clean?" and it isn't in the
            # hash table, the sequence and its id are going to be in the hash
            if sequence not in sequences:
                sequences[sequence] = seq_record.id
            # If it is already in the hash table, we're just gonna concatenate the ID
```

```
                    # of the current sequence to another one that is already in the hash table
                    else:
                        sequences[sequence] += "_" + seq_record.id
# Write the clean sequences
# Create a file in the same directory where you ran this script
            with open("clear_" + fasta_file, "w+") as output_file:
                # Just read the hash table and write on the file as a fasta format
                for sequence in sequences:
                    output_file.write(">" + sequences[sequence] + "\n" + sequence + "\n")
    print("CLEAN!!!\nPlease check clear_" + fasta_file)
        userParameters = sys.argv[1:]
        try:
            if len(userParameters) == 1:
                sequence_cleaner(userParameters[0])
            elif len(userParameters) == 2:
                sequence_cleaner(userParameters[0], float(userParameters[1]))
            elif len(userParameters) == 3:
                sequence_cleaner(
                    userParameters[0], float(userParameters[1]), float(userParameters[2])
                )
            else:
                print("There is a problem!")
          except:
            print("There is a problem!")
```