SYMBIOTIC MICROBE RESPONSE TO BLACK SOLDIER FLY (DIPTERA: STRATIOMYIDAE) LARVAL HOST STARVATION AND LARVAL DEVELOPMENT IN LARGE-SCALE PRODUCTION

A Dissertation

by

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ABSTRACT

In recent years, the black soldier fly, *Hermetia illucens*, (Diptera: Stratiomyidae) has been ranked high in sustainable agriculture due to its ability to transform organic waste into insect protein and compost. Numerous companies globally are implementing this concept, even though our understanding of this insect is still limited. This dissertation investigated how the insect larval host interacts with its associated bacteria when the host is starved as well, the actual production system for this insect at a large scale. By investigating the relationship of the bacteria community and the larval host during starvation, I determined a metabolically active microbial community shift in response to the larval host starvation. I also determined a decrease (P=0.0025) in microbial community diversity at the phylum level, and I identified six standout genera (*Actinomyces*, *Microbacterium*, *Enterococcus*, *Sphingobacterium*, *Weissella*, and *Leucobacter*) that had significantly different relative abundances between treatments. When investigating the microbial activities during the above-mentioned condition, I determined there were 103 microbial genes significantly different across treatments. These findings can lead to protein production optimization by manipulating the associated microbe in the feed substrate for the black soldier fly larvae to induce higher food intake.

Scale of study impacted life-history traits of the black soldier fly and its production. I determined results from the previous studies done on bench-top scale might not apply to the real world. Because of changing the rearing conditions, larval life-history traits exhibited significant differences in survivorship, biomass conversion rate, and larval sizes.

The *H. illucens* larvae in the small container had 28.2% lower survivorship than in the large rearing tray ($P<0.05$), and they are 24.7% larger in size than in the large tray ($P<0.05$). However, the bioconversion rate in the large tray is 2.7% bigger than in the small containers ($P<0.05$).

Research in this dissertation identified few issues that might cause management troubles in the production system, such as density of larval inoculation in the rearing system, and the microbe associated with the food substrate that might cause variability in the production system, though more works are needed to be done in order to understand the mechanisms as well as to find solutions for production optimization.

DEDICATION

This dissertation is dedicated to my daughter and to my wife who have been very understanding on the toughness of this work and have encouraged me for the entire time. I dedicate this dissertation to my advisor Dr. Jeffery Tomberlin for his acceptance of me to this wonderful laboratory; without him seeing the potential in me at the beginning, I would not have the chance to pursue a higher degree after receiving my undergraduate degree from Penn State. I appreciate his continuous encouragement and support at every step of my work towards completing this dissertation. I also dedicate this dissertation to my co-advisor Dr. Heather Jordan for her continuous encouragement and hand by hand training during the whole process. Thank you to my academic committee who have guided me through the process and kept me on track. Thank you to my mother and father for providing all necessary support for me to complete this work.

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NOMENCLATURE

ATP	Adenosine triphosphate
BSF/BSFL	Black Soldier Fly/Black Soldier Fly Larvae
OTU	Operational Taxonomic Unit
T	Time
TAMU	Texas A&M University

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CHAPTER I

INTRODUCTION

Overview

The black soldier fly, *Hermetia illucens* (Diptera: Stratiomyidae), is found in tropic and temperate regions around the world (Tomberlin and Sheppard 2002). The black soldier fly has been recognized as a beneficial species for its biological and ecological impact on the environment (Furman et al. 1959, Kilpatrick and Schoof 1959, Tomberlin et al. 2002). In addition, adult black soldier flies do not feed, as they rely on nutrients accumulated during larval development, and rarely enter residential areas (Tomberlin and Sheppard 2002). Thus, they are not considered a pest.

Black soldier fly larvae actively feed on a wide range of decaying materials, ranging from plant residuals (Simmons and Dove 1942) to carrion, such as human remains (Lord et al. 1994). By feeding on the waste, black soldier fly larvae also impact the associated bacterial profiles. For example, black soldier fly larvae have been shown to inactivate *Escherichia coli* (Liu et al. 2008) and *Salmonella* spp. populations in different types of manure (Erickson et al. 2004, Liu et al. 2008, Lalander et al. 2013, Lalander et al. 2015).

Black Soldier Fly as Feedstuff

Black soldier fly prepupae can be used as feed for livestock. The last instar has a nutrient profile of 35% lipid and 42% crude protein (Craig Sheppard et al. 1994); they also have been tested successfully as supplemental feedstuffs for many livestock and is especially interested in the aquaculture industry. Bondari and Sheppard (Bondari and Sheppard 1981) found that both channel catfish (*Ictalurus punctatus*) and blue tilapia (*Tilapia aurea*) fingerlings fed 100% larvae meal did not have differential growth development than the control group. The sensory test in the same study also indicated there was no taste difference between fish fed commercial diet or black soldier fly larval meal. A follow up study confirmed this finding and further indicated that the chopped dry larval meal could improve the feed conversion rate by 28% for blue tilapia (Bondari and Sheppard 1987). Similar growth test done on rainbow trout (*Oncorhynchus mykiss*) revealed that with a 50% black soldier fly larval meal in the diet, the feed to fish body weight conversion rate is significantly higher than the current commercial diet (St-Hilaire et al. 2007). In addition, in a study of supplementing commercial diet with dried black soldier fly larvae for turbot (*Psetta maxima*) researchers found that the black soldier fly larvae meal could be used to replaces 33% of the diet without negatively impacting the turbot growth rate (Kroeckel et al. 2012).

Although many insect species, such as mealworm, *Tenebrio molitor* (Bovera et al. 2015), earthworm, *Eisenia fetida* (Chaves et al. 2015), and locust, *Acrida cinerea* (Liu and Lian 2003), could be used as animal feed, the black soldier fly has greater potential for

industrialization due to its robust ability to feed on a variety of different waste materials as previously mentioned. For example, black soldier fly larvae can successfully feed and develop on pig manure (Newton et al. 2005), poultry manure (Sheppard et al. 1994), and food wastes (Barry 2004, Nguyen et al. 2013).

Potential

Now is the time to industrialize insects as a means to reduce food waste entering landfills while producing protein for livestock, poultry, and aquaculture feed. According to Food and Agriculture Organization (FAO) of the United Nations, currently 40% of global food production becomes waste that is transported to landfills (Gustavsson et al. 2011). This portion of the waste is responsible for adding 3.3 billion tonnes of greenhouse gas to the planet's atmosphere. In addition to its environmental impacts, it also causes a direct economic loss of $750 billion annually (FAO 2013). Scientists have invested tremendous amount of effort seeking a way to address this global issue. Because black soldier fly larvae can consume these wastes and convert them into usable protein and lipid, they have been proposed as a potential solution for this issue.

Challenges

Although many insect species, such as mealworm, *Tenebrio molitor* (Bovera et al. 2015), earthworm, *Eisenia fetida* (Chaves et al. 2015), and locust, *Acrida cinerea* (Liu and Lian 2003), could be used as animal feed, the black soldier fly has greater potential for

industrialization due to its robust ability to feed on a variety of different waste materials as previously mentioned. For example, black soldier fly larvae can successfully feed and develop on pig manure (Newton et al. 2005), poultry manure (Sheppard et al. 1994), and food wastes (Barry 2004, Nguyen et al. 2013).

However, there are current limitations to the use of the black soldier fly as a means to mass manage food waste. Although controlled lab studies indicate black soldier fly larvae can reduce targeted pathogens, no information is available on how these results translate to industrial scale. Secondly, there is expected variation in development due to nutrient and texture variation in the food waste being fed to the larvae. Third, the quality of the end product larvae could vary depends on the inflow waste quality, for example, food waste with high cellulose content has less accessible nutrients, and thus the larvae take longer time to develop and are smaller in size. The development variation due to the diversity and quality of food wastes could be huge as (Nguyen et al. 2013) reported that black soldier fly larvae fed on pig liver develop into prepupae stage (22.5 day) is 33.8% faster than those fed on pig manure (34 day); and the larvae fed on chicken feed has a average prepupal weight of 184.3 mg versus 113.3 mg of those fed on pig manure, accounting for 62.7% weight difference than pig manure fed larvae. In real-world application such variation is problematic, as the inflow of waste type is not going to be consistent from day to day, and the resulting inconsistency of development will cause difficulties in the factorial flow line operation.

In order to develop the black soldier fly larvae into a waste management tool and method for producing feedstuff, there are three main aspects to be investigated: 1) how to increase the efficiency of the waste-biomass conversion rate by manipulating the symbiont structure, or employing probiotic pre-treatments, 2) determine large scale production parameters, and 3) reduce the risk of problematic microbial contamination by applying probiotic *Lactobacillus* fermentation as pre-treatment.

In order to increase the efficiency of industrialized black soldier fly larval conversion rates, I propose that the symbiotic microbes play an important role in regulating the development of black soldier fly larvae. Evidences of such relationship have been found on termites (Isoptera) (Taprab et al. 2005), roaches (Blattaria) (Todaka et al. 2010), and beetle larvae (Coleoptera) (Egert et al. 2005) that endosymbionts play an critical role degrading cellulose materials for the host and facilitating nutrient digestion. In addition, the probiotic product Rid-X (Reckitt Benckiser Inc. Parsippany, NJ) when provided in the diet can aid in the breakdown of complex and indigestible compounds such as hemicellulose and cellulose resulting in more nutrients being available to black soldier fly larvae, and thus increase the bio-conversion rate (Zheng et al. 2012). Yet, the effect of how the probiotic treatment on the food impacts the invertebrate's growth is not well-known. By understanding how symbionts interact with the host, black soldier fly larval development rate or biomass production can be optimized by manipulating the microbes in its feeding environment. Specifically, I am interested in investigating symbiotic microbe response to host stress under conditions of larval starvation. My objective is to

characterize the microbial function associated with black soldier fly larvae fed and starved and aiming to identify the genes responsible for host feeding behavior.

Although previous studies produced appealing results of rearing black soldier fly larvae with numerous types of food, all research to date has been on laboratory-scale as listed above. Thus, the results of applying these data to an industry-scale facility are not known, and such a data set is in urgent need by industry to have more accurate understanding of the process and how to optimize production.

Previous studies have found that the black soldier fly larvae digestion has antimicrobial effect under certain conditions (Erickson et al. 2004, Liu et al. 2008, Lalander et al. 2013, Lalander et al. 2015); however, there is still risk that the end product is contaminated by pathogen due to poor management, such as storing the food waste in open space, or being contaminated when transporting. Specifically, I am interested in the most common food-borne bacteria genera such as coliform, enterococci, staphylococci, and heterotrophic bacteria. Since *Lactobacillus* has been used for food preserving for thousands of years because it produces lactic acid and bacteriocin that can suppress pathogens (O'sullivan et al. 2002), here I propose using Lactobacillus culture as probiotic treatments to the waste before feeding them to the black soldier fly larvae. The pretreatment will serve for pathogen inactivation as well as nutrient pre-digestion, and therefore the larvae as end product is free of contamination. I will determine if fermented food is suitable for black soldier fly larvae development.

The objectives in this dissertation are:

Objective I

Microbial Community Structure in Response to Host Starvation of Black Soldier Fly Larvae (Diptera: Stratiomyidae)

Hypothesis

Ho: There will be no differences in microbial structure of black soldier fly larvae fed and starved.

Ha: There will be differences in microbial structure of black soldier fly larvae fed and starved.

Objective II

Microbial Function in Response to Host Starvation of Black Soldier Fly Larvae (Diptera: Stratiomyidae)

Hypothesis

Ho: There will be no differences in microbial function of black soldier fly larvae fed and starved.

Ha: There will be differences in microbial function of black soldier fly larvae fed and starved.

Objective III

Determine the differences of black soldier fly larval life-history between large-scale production systems and bench-top scale system

Hypothesis

Ho: Black soldier fly larval life-history in benchtop scale is not different than in large scale

Ha: Black soldier fly larval life-history in benchtop scale is different than in large scale

The above objectives were designed to improve the efficiency and security of the current black soldier fly production industry.

References

Barry, T. 2004. Evaluation of the economic, social, and biological feasibility of bioconverting food wastes with the black soldier fly (*Hermetia illucens*). Ph.D. dissertation, University of North Texas, Denton.

Bondari, K., and D. Sheppard. 1981. Soldier fly larvae as feed in commercial fish production. Aquaculture 24: 103-109.

Bondari, K., and D. C. Sheppard. 1987. Soldier fly, *Hermetia illucens* L., larvae as feed for channel catfish, *Ictalurus punctatus* (Rafinesque), and blue tilapia, *Oreochromis aureus* (Steindachner). Aquaculture and Fisheries Management 18: 209-220.

Bovera, F., G. Piccolo, L. Gasco, S. Marono, R. Loponte, G. Vassalotti, V. Mastellone, P. Lombardi, Y. A. Attia, and A. Nizza. 2015. Yellow mealworm larvae (*Tenebrio molitor* , L.) as a possible alternative to soybean meal in broiler diets. British Poultry Science 56: 569-575.

Chaves, R. C., R. Queiroga de Paula, B. Gücker, I. Gonçalves Boëchat, I. Evódio Marriel, and A. Oliveira Teixeira. 2015. An alternative fish feed based on earthworm and fruit meals for tilapia and carp postlarvae. Uma ração alternativa baseada em farinha de minhoca e frutas para alevinos de tilápia e carpa. 13: 15-24.

Egert, M., U. Stingl, L. D. Bruun, B. Pommerenke, A. Brune, and M. W. Friedrich. 2005. Structure and topology of microbial communities in the major gut

compartments of Melolontha melolontha larvae (Coleoptera: Scarabaeidae). Applied and Environmental Microbiology 71: 4556-4566.

Erickson, M. C., M. Islam, C. Sheppard, J. Liao, and M. P. Doyle. 2004. Reduction of *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Enteritidis in chicken manure by larvae of the black soldier fly. Journal of Food Protection 67: 685-690.

FAO. 2013. Food wastage footprint-Impacts on natural resources: summary report. Food and Agriculture Organization of the United Nations.

Furman, D. P., R. D. Young, and P. E. Catts. 1959. *Hermetia illucens* (Linnaeus) as a Factor in the Natural Control of Musca domestica Linnaeus1. Journal of Economic Entomology 52: 917-921.

Gustavsson, J., C. Cederberg, U. Sonesson, R. Van Otterdijk, and A. Meybeck. 2011. Global food losses and food waste. Food and Agriculture Organization of the United Nations, Rome.

Kilpatrick, J. W., and H. Schoof. 1959. Interrelationship of water and *Hermetia illucens* breeding to *Musca domestica* production in human excrement. The American Journal of Tropical Medicine and Hygiene 8: 597-602.

Kroeckel, S., A. G. E. Harjes, I. Roth, H. Katz, S. Wuertz, A. Susenbeth, and C. Schulz. 2012. When a turbot catches a fly: evaluation of a pre-pupae meal of the Black Soldier Fly (*Hermetia illucens*) as fish meal substitute - growth performance and chitin degradation in juvenile turbot (*Psetta maxima*). Aquaculture 364: 345-352.

Lalander, C., S. Diener, M. E. Magri, C. Zurbrugg, A. Lindstrom, and B. Vinneras. 2013. Faecal sludge management with the larvae of the black soldier fly (*Hermetia illucens*) - from a hygiene aspect. Science of the Total Environment 458: 312-318.

Lalander, C. H., J. Fidjeland, S. Diener, S. Eriksson, and B. Vinnerås. 2015. High waste-to-biomass conversion and efficient *Salmonella* spp. reduction using black soldier fly for waste recycling. Agronomy for Sustainable Development 35: 261-271.

Liu, C.-m., and Z.-m. Lian. 2003. Influence of *Acrida cinerea* replacing Peru fish meal on growth performance of broiler chickens. Journal of Economic Animal 7: 48-51.

Liu, Q., J. K. Tomberlin, J. A. Brady, M. R. Sanford, and Z. Yu. 2008. Black soldier fly (Diptera: Stratiomyidae) larvae reduce *Escherichia coli* in dairy manure. Environmental Entomology 37: 1525-1530.

Lord, W. D., M. L. Goff, T. R. Adkins, and N. H. Haskell. 1994. The black soldier fly Hermetia illucens (Diptera: Stratiomyidae) as a potential measure of human postmortem interval: observations and case histories. Journal of Forensic Sciences 39: 215-222.

Newton, G., D. Sheppard, D. Watson, G. Burtle, C. Dove, J. Tomberlin, and E. Thelen. Year. Published. The black soldier fly, *Hermetia illucens*, as a manure management/resource recovery tool, pp. 5-7. *In*, Symposium on the State of the Science of Animal Manure and Waste Management, 2005.

Nguyen, T. T. X., J. K. Tomberlin, and S. Vanlaerhoven. 2013. Influence of resources on *Hermetia illucens* (Diptera: Stratiomyidae) larval development. Journal of Medical Entomology 50: 898-906.

O'sullivan, L., R. Ross, and C. Hill. 2002. Potential of bacteriocin-producing lactic acid bacteria for improvements in food safety and quality. Biochimie 84: 593-604.

Sheppard, D. C., G. L. Newton, S. A. Thompson, and S. Savage. 1994. A value added manure management system using the black soldier fly. Bioresource Technology 50: 275-279.

Simmons, S. W., and W. E. Dove. 1942. Waste Celery as a Breeding Medium for the Stablefly or " Dog Fly," with Suggestions for Control. Journal of Economic Entomology 35: 709-715.

St-Hilaire, S., C. Sheppard, J. K. Tomberlin, S. Irving, L. Newton, M. A. McGuire, E. E. Mosley, R. W. Hardy, and W. Sealey. 2007. Fly prepupae as a feedstuff for rainbow trout, *Oncorhynchus mykiss*. Journal of the World Aquaculture Society 38: 59-67.

Taprab, Y., T. Johjima, Y. Maeda, S. Moriya, S. Trakulnaleamsai, N. Noparatnaraporn, M. Ohkuma, and T. Kudo. 2005. Symbiotic fungi produce laccases potentially involved in phenol degradation in fungus combs of fungus-growing termites in Thailand. Applied and Environmental Microbiology 71: 7696-7704.

Todaka, N., T. Inoue, K. Saita, M. Ohkuma, C. A. Nalepa, M. Lenz, T. Kudo, and S. Moriya. 2010. Phylogenetic analysis of cellulolytic enzyme genes from representative lineages of termites and a related cockroach. PloS One 5: e8636.

Tomberlin, J. K., and D. C. Sheppard. 2002. Factors influencing mating and oviposition of black soldier flies (Diptera: Stratiomyidae) in a colony. Journal of Entomological Science 37: 345-352.

Tomberlin, J. K., D. C. Sheppard, and J. A. Joyce. 2002. Selected life-history traits of black soldier flies (Diptera: Stratiomyidae) reared on three artificial diets. Annals of the Entomological Society of America 95: 379-386.

Zheng, L., Y. Hou, W. Li, S. Yang, Q. Li, and Z. Yu. 2012. Biodiesel production from rice straw and restaurant waste employing black soldier fly assisted by microbes. Energy 47: 225-229.

CHAPTER II

STARVATION SHIFTS ASSOCIATED BACTERIA COMMUNITY IN BLACK SOLDIER FLY (DIPTERA: STRATIOMYIDAE) LARVAE

Summary

The impact of starvation on associated metabolically active gut bacterial communities in a host is poorly understood. Deciphering potential shifts in metabolically active associated bacterial communities could potentially lead to discoveries of mechanisms regulating the sensation of hunger. We used the black soldier fly larvae (BSF) as a model to explore this relationship as foundational information of bacteria-BSF interactions have been established, as well as the potential use of BSF larvae (BSFL) as a potential agent for consuming and converting organic waste to valuable protein for use as animal feed. Bacteria associated with food substrates of BSFL impact associated life-history (i.e., larval development), but whether the larval host state could have impact on the associated metabolically active microbe community is not known. In this study, we discovered a metabolically active microbial community shift due to larval host starvation, and identified several key bacteria associated with BSFL hunger state changes. At the phylum level, the community diversity decreased significantly while the host was starved (P=0.0025); while at the genus level, DESeq2 analysis identified six genera being significantly different (q<0.05) both at 24 and 48 post initiation of starvation: *Actinomyces*, *Microbacterium*, *Enterococcus*, *Sphingobacterium*, *Weissella*, and *Leucobacter*. Our study demonstrated

the associated metabolically active microbial community could be influenced by the larval host feeding status. Future studies in bacterial communities should explore these shifts as they could serve as a mechanism regulating feeding habits of the BSF in response to starvation.

Introduction

Sensations of hunger are caused by an imbalance between energy intake and expenditure (Friedman et al. 1999). One immediate response to hunger is assumed to be the drive to increase food consumption. Malnutrition, on the other hand, is not as simple as hunger, and is defined as either inadequate or excessive consumption of dietary substances ultimately leading to the development of undernutrition or obesity, respectively, and their corresponding health sequelae (Elia 2017). Though malnutrition is not the same as hunger, they both may often be connected (Friedman et al. 1999, Elia 2017).

Recent studies have shown evidence of gut microorganisms playing significant roles in influencing nutritional decisions, digestion, and metabolism (Read and Holmes 2017). Many of these organisms have coevolved within their host to perform a number of functions the host would otherwise be unable to accomplish on its own. For instance, gut-associated bacteria rapidly adapt to changes in host diet through changes in population and induction of signaling compounds and degradation enzymes that facilitate digestion through absorption and metabolism of complex molecules (Read and Holmes 2017). Characterization of bacteria is therefore important for understanding the comprehensive

physiology of the gastrointestinal tract microbiota, and its relationship to both hunger and malnutrition.

Given their ease in rearing and manipulation, insects offer great benefits as model hosts for studying these processes. They represent the most diverse group of known organisms, and account for approximately 80% of Earth's animal species (IISE 2011). Insects also occupy both terrestrial and aquatic habitats, and provide natural ecosystem services often taken for granted, such as herbivory, food for animals, and pollination. Additionally, many insects drive ecosystem processes such as nutrient recycling and decomposition.

Studies have investigated the impact of symbionts, as well as gut microbiomes on nutrient absorption, brain function, behavior, overall health, and the consequences of diet on insect associated microbiomes (Feldhaar 2011). However very few have analyzed data from nutrient deprivation or starvation, and a literature review revealed only one study investigating the effects of prolonged nutrient deprivation on insect gut microbiota (Tinker and Ottesen 2016). These studies will be important for our comprehensive understanding of how change in nutrient status impacts insect, and other hosts', gut microbiomes, and subsequent changes in insect-microbe functional processes.

The black soldier fly (BSF), *Hermetia illucens* (L.) (Diptera: Stratiomyidae) is a well-known non-pest fly species that, along with associated microbes, converts decomposing matter into proteins and lipids (Diener et al. 2009, Yu et al. 2011). Black soldier fly larvae (BSFL) undergo a fast-growing period and have a huge appetite. Individual BSF

eggs weigh 0.025 mg (Cammack 2016), and can develop into 170 mg prepupae in about three weeks (Tomberlin et al. 2002). BSFL consume a wide range of organic matter, such as animal manure (Sheppard et al. 1994, Newton et al. 2005, Zhou et al. 2013) and restaurant waste (Nguyen et al. 2015), and transform them into high quality protein approved for use in the aquaculture industry as feed (Bondari and Sheppard 1987).

Therefore, we took advantage of the known BSF biology and feeding behavior, as well as taking into account its importance in nutrient recycling, to investigate the total associated metabolically active prokaryotic shift due to host starvation and subsequent nutrient deprivation. More specifically, we analyzed changes in BSFL gut metabolically active prokaryotic community diversity during nutrient deprivation by sequencing the total prokaryotic RNA, as a first step for understanding microbe-host interactive metabolism, and in contribution to the long-term goal of optimizing BSF sustainable agricultural systems.

Materials and Methods

Sample Collection

Sixty-six clutches (approximately 40K eggs) (Tomberlin et al. 2002) of BSF eggs were collected from a colony maintained in the Forensic Laboratory for Investigative Entomological Sciences (FLIES) Facility at Texas A&M University. Larvae were fed standard Gainesville diet (Hogsette 1992) for 16 days, before being partitioned into Control and Treatment group, with a setup of four replicates. From day 16, larvae from

the Control group were being fed daily, whereas larvae in the Treatment group were Starved until the end of the experiment (Figure II-1). Five larval sub-samples (80 to 100 mg each) were taken from each replicate of Control and Treatment groups at three time points (T0 H, T24 H, and T48 H after starvation), and subjected to RNA isolation. To have a base understanding of the initial microbiota structure in the starting feed, one sample of Gainesville diet was collected (100 mg) at the beginning of the experiment. Due to the single sample collection, this feed sample data was only included in results within the heat map. All data analyses were carried out without inclusion of the feed data, unless otherwise specified.

Microbial Total RNA Isolation and Purification

Sample RNA was isolated using Trizol® reagent and chloroform. Isolated RNA samples were purified using the Direct-zol™ RNA MiniPrep kit, followed by a prokaryotic RNA enriching step with the MICROBEnrich™ Kit. The NEB Ultra RNA Library Kit (New England Biolabs) was used to convert the RNA to cDNA, avoiding steps to remove rRNA, while preserving total RNA concentrations. Established cDNA libraries were multiplexed using NEBNext Oligos for Illumina to create five libraries. Resulting multiplexed libraries were submitted for shotgun whole metatranscriptome sequencing using an Illumina® HiSeq 2000 instrument.

Raw paired-end reads from whole metatranscriptome sequences were demultiplexed and trimmed, and adapters removed using the FasQC tool kit. Pair-end sequences were

generated using Pandaseq (version 2.10) (Masella et al. 2012). Taxonomic classification and KO function prediction were assessed using the most up-to-date Parallel-Meta software at the time (version 3.3.1) (Su et al. 2012). Sequences from replicate samples were concatenated into a single fastq file, representing one treatment, prior to software analysis, when generating a distribution bar chart and rarefaction curve.

Statistical Analysis

Additional statistical analyses were performed using RStudio (Version 0.99.903), which was built on R software (Version 3.3.1) (Team 2013). The Heatmap2 package was employed to analyze genera count data from Parallel Meta output results, and to provide a heat map distribution of the genera present over 3% of relative abundance across all treatments. To determine microbial input of the feed and to illustrate the change from feed to larvae, we included an extra set of data of the Gainesville diet, which was collected and sequenced as a pilot study. Because we only sequenced one feed sample, and it cannot provide any statistical data, we only used feed data in discussion to provide inferences on microbes that were detected originally in the feed.

For the structure analysis, DESeq2 package was used to detect genera with differential abundance between treatments (Love et al. 2014). Tests were conducted treating T24 H and T48 H as separate events, where raw count data from Control T24 H were tested against Starved T24 H, and Control T48 H were tested against Starve T48 H. Significance was determined with FDR adjusted *p* value (q-value) less than 0.05.

For the functional prediction comparison analysis, the Parallel Meta software was able to generate KEGG annotated table of each sample, and DESeq2 package was used analyze the KO table output from Parallel Meta software to detect differences of functional prediction between treatments. Tests were done by treating all controlled samples at T0, T24, and T48 as Control treatment, and all starved samples at T24 and T48 as Starved treatment, Significance was determined with FDR adjusted *p* value (q-value) less than 0.05.

Results

Sequences using Illumina® HiSeq, followed by quality filtering, yielded 10.35 GB of sequence data (862,168 sequences) from the total 20 samples, with 101 base pair sequence length for each sample. All reads passed FastQC quality check with a mean quality score over 30. The forward and reverse sequence files were then merged into paired end sequence files using Pandaseq (version 2.10) with default settings resulting in an average of 500 MB of sequencing data per sample. Parallel Meta 3.3.1 was able to classify BSF assembled rRNA sequences into 10 Phyla, 24 Classes, 40 Orders, 66 Families, 117 Genera, 26 Species, and 10691 Operational Taxonomic Units (OTUs) (Table II-1)**.** Limited by the database inclusion, the genera level provided the most informative classification. Adequacy of sequencing depth was based on OTU rarefaction curves. For example, as the lowest amount of sequence was generated in the Starved T24 H treatment, no more OTUs were identified with sequence numbers above 93967 (Figure II-2b).

Diversity Comparison

Overall, the Shannon Diversity Index revealed a higher diversity and even community in the Control than in the Starved group (P = 0.0025), at the phylum level (Figure II-3). Based on the OUT calculated Shannon index, Control at T0 H was the most diverse and even treatment, and started to decrease as either feeding or starvation progressed, with diversity further decreasing in Control T48 H, Starved T24 H, Control T24 H, and Starved T48H (Figure II-2a). At the experiment end, Control larvae continually fed had a higher Shannon Diversity Index of 6.886 at the at T48 H timepoint, which is higher than that of the Starved cohort of 6.532 at T48 H (Figure II-2a).

Phylum

Among the 11 identified Phyla, six of those Phyla were identified with a significantly different relative abundance between the Control and Starved cohort ($q < 0.05$). Actinobacteria was the most dominant Phylum shared by samples from both Control and Treatment, followed by Proteobacteria, Firmicutes, Euryarchaeota, and Bacteroidetes (Figure II-4). While Actinobacteria was the most abundant Phylum among all treatments, this phylum was found in higher abundance in the Starved cohort ($q < 0.001$). Besides Actinobacteria, Bacteroidetes and Verrucomicrobia were also found with higher abundance in the Starved cohort ($q<0.01$ and $q<0.001$, respectively. Table II-2). On the other hand, Firmicutes, Euryarchaeota, Crenarchaeota, and Planctomycetes were found

decreased within the Starved cohort. Notably all of them had decreased by 50% or more (Table II- 2).

Genera

Actinomyces was identified as the most abundant genus with an average abundance of 15.34% across all samples. *Actinomyces* was found with increasing abundance over time in larval samples, no matter the treatment, but were found negligibly present (0.02%) in the original feed sample (Figure II-5). The genera were more present at a higher density in the Starved samples (19.0% at T24 H and 27.2% at T48 H) than in the respective Control samples (9.2% at T24 H and 16.6% at T48 H). *Weissella* was found 10 times more abundant in the Control, than in the Starved cohort. A total of 82 genera had significantly ($q < 0.05$) different abundance between treatments at T24 H, and 64 genera at T48 H. The heat map illustrates additional, remarkable microbiota genera diversity across treatments and feed (Figure II-5). For example, *Pyrobaculum* and *Cerasicoccus* were not found in the original feed, but were present in larval samples, though in low abundance (less than 4%).

When assessments were based on the FDR adjusted *P* value (q-value), six genera with a minimum abundance of 3% at any treatment were identified as significantly different between T24 H and T48 H: *Actinomyces*, *Microbacterium*, *Enterococcus*, *Sphingobacterium*, *Weissella*, *Leucobacter* (Figure II-6). *Enterococcus* and *Weissella* were found in significantly lower abundance in the Starved cohort than in the Control at

respective time points ($q < 0.05$), while others, including *Actinomyces*, *Microbacterium*, *Sphingobacterium*, and *Leucobacter* were found higher in the Starved cohort than in the Control at the respective time point ($q < 0.05$) (Figure II-6). It is worth noting that the patterns of changes varied depending on the taxa. For example, *Enterococcus* and *Weissella* showed a trend of decreasing abundance over time in the Starved cohort, while their abundance remained at a higher level in the Control. On the other hand, the abundance of *Actinomyce*s and *Leucobacter* continued to increase over time, moreso in the Starved cohort. Nonetheless, *Microbacterium* and *Sphingobacterium* divergenced with respect to abundance, where they showed increasing density in the Starved cohort and lower density in the Control (Figure II-6, Table II- 3).

Functional Prediction

The Parallel Meta pipeline software was able to link 6709 predicted gene functions to the KEGG database. The DESeq2 package identified 4401 genes that were statistically different ($q < 0.05$) between Control and Treatment. Based on the log2 fold changes, we identified 10 prediction genes that's being down regulated in the started treatment group, as well as 10 prediction genes that's up regulated. The genes being down regulated were mostly in the metabolism and genetic information processing family; and the up regulated genes were mostly in genetic information process and environmental information processing family.

Discussion

The goal of this study was to investigate the total associated metabolically active microbiota shift due to host starvation and subsequent nutrient deprivation, where samples included ectosymbiomes and endosymbiomes. Overall, phylum level diversity associated with Starved larvae was lower than that of the microbial communities from the fed BSFL cohorts ($P = 0.0025$). Among all the genera with at least 3% abundance at any time point, *Actinomyces*, *Microbacterium*, *Enterococcus*, *Sphingobacterium*, *Weissella*, and *Leucobacter* showed significantly different abundance at the respective time points ($q < 0.05$). Results showed a shift in the microbiota associated with feeding and fasting. Taxa were identified that were found in both Starved and Control fed groups, as well as those significantly associated individually with either group. Taxa identified in both groups, but not found in the feed, could likely constitute members of a core microbiome, though their specific role in host biology needs further investigation.

Decreasing community diversity as well as the evenness implied host starvation resulted in some members within the metabolically active microbial community becoming dominant and outcompeting those not able to tolerate starvation stress. These dominating bacteria may also aid in host adaptation to starvation. For instance, *Actinomyces* is known to produce enzymes for degradation of chitin and plant material, and is also known to produce antibiotics against pathogenic fungus (Sharma 2014). These microbial characteristics could help the host survive during prolonged nutrient deprivation.

At the Phylum level, Actinobacteria was the most dominant metabolically active phylum shared by samples from both Control and Treatment but was found in higher abundance in the Starved cohort. Additionally, Bacteroidetes and Verrucomicrobia were also found with higher abundance in the Starved cohort. On the other hand, Firmicutes, Euryarchaeota, Crenarchaeota, and Planctomycetes were found more abundant among the fed cohort. These results, though with some differences, are similar to other studies investigating microbiome shifts and limited nutrient availability. For instance, using a Burmese python model, Costello, et al (Costello et al. 2010) demonstrated an abundance of Bacteroidetes during fasting that shifted towards a post-prandial abundance of Firmicutes. Crawford et al also showed that after a 24 H fast, mice showed a significant increase in the abundance of Bacteroidetes species and a corresponding decrease in Firmicutes (Crawford et al. 2009). However, our study differed from a study of the microbiome of the American Cockroach, *Periplaneta americana*, who showed a relatively stable microbiome during dietary shifts (Tinker and Ottesen 2016). Study design and model organism may account for data result differences.

Bacteria-host interactions are at the forefront of scientific research, especially in the area of host manipulation and life-history interference (Oliver et al. 2003), with great implications toward human and animal health and behavior. For instance, manipulating host feeding behavior has been hypothesized to be beneficial to gut microbiota, since many microbes sequester nutrients through host-acquired food sources (Alcock et al. 2014). Furthermore, recent data from human studies suggest decreased gut microbiota diversity

is linked to an increased food intake in colic infants. In that study, infants with colic had lower microbial diversity and cried more often, which the authors hypothesized would result in parental attention and possibly increased feeding (de Weerth et al. 2013). The increased energy intake was hypothesized to likely benefit the Phylum Proteobacteria, who was found in higher density than those without colic. These data also suggested the possibility that Proteobacteria were manipulating their hosts through dysphoria induction (de Weerth et al. 2013).

In contrast, gut microbiota can also change host-feeding behavior through modulation of host receptor gene expression. One study showed that germ free mice possessed different taste receptors for fat detection on the tongue and also in the intestines when compared to wild-type mice (Duca et al. 2012). Another mouse study showed inclusion of *Lactobacillus* mixtures in food promoted hormones inducing less food intake and concurrently reducing other hunger-inducing hormones (Yadav et al. 2013).

While many studies have focused on the addition of nutrients into the system and impact on microbial structure, a literature review revealed only limited investigations of bacterial community shifts during host starvation. In circumstances of deficient nutrients in a starving host environment, commensal gut microbes likely also experience prolonged starvation events, where sustaining life is a vital task. Many bacteria use quorum-sensing to communicate and obtain information on bacterial density in their environment, and to achieve group cooperation for phenotypic changes to adapt to environmental changes, such as forming biofilms or changing physiological processes (Li and Tian 2012). Under

starvation conditions, bacteria are likely to show a group stress response. A well-described example is *Mycococcus xanthus,* who forms a fruiting body with hardy spores in response to starvation by producing cell-cell signaling and social cooperation (Velicer et al. 2000, West et al. 2006, Li and Tian 2012). Furthermore, a growing body of knowledge has shown that quorum-sensing molecules are also responsible for inter-kingdom communication (Hughes and Sperandio 2008). As such, microbes possibly, through mechanisms of modulated host or microbe gene, hijacking host signals, or production of microbial chemical signals, induce host dysphoria to induce host hunger and consequently, nutrient intake.

Therefore, besides the information of what bacteria was in there, the information of what were they doing at different host stage were also important. The functional prediction analysis of this study indicated that the bacteria in the starved host had less activity of DNA replication as well as lower level of metabolism, suggesting these bacteria might have entered dormant stage in response to the lack of nutrient source. On the other hand, there were more genetic information processing activities in the control group, indicating an active growing bacteria community in the feeding host. A more depth discussion of the functional analysis will be carried out in a separate paper.

Basic understanding of complex insect-microbe interactions and their associated metabolic capabilities will be a key factor in utilizing these systems for application in solving practical environmental and societal issues. Insects, particularly BSFL, are a tenable answer for sustainable agriculture and waste management (Sheppard et al. 1994,

St-Hilaire et al. 2007a). BSF occur throughout temperate and tropical regions globally, are not a pest species, consume organic waste such as manure, and can be used as an ingredient for livestock, poultry, and aquaculture feed. Furthermore, data demonstrate BSFL digestion reduces pathogens from within the waste (Erickson et al. 2004, Liu et al. 2008, Lalander et al. 2013, Lalander et al. 2015). To date, researchers have shown that BSF larvae can reduce *Escherichia coli* O157:H7 and *Salmonella* Enteritidis in chicken manure (Erickson et al. 2004, Liu et al. 2008, Lalander et al. 2013, Lalander et al. 2015), *E. coli* in dairy manure (Liu et al. 2008), and *Salmonella* spp. in human feces as well as in mixed organic wastes (Lalander et al. 2013, Lalander et al. 2015). Consequently, industrialization of BSF has been proposed as a means to sustainably convert organic waste (i.e., animal manure or food waste) to protein. As of 2017, BSFL are the only insects approved for use in or as fish feed in the United States (e.g., salmonid species) and Europe (IPIFF 2020). Asia also has a long history of consuming insects or using insects as animal feed ingredients, but no regulations to date have specified BSF as animal feed.

Although the industry is evolving and growing rapidly, many aspects of the basic biology of this species are still unclear. Some studies determined BSF larval gut microbial community structure was significantly influenced by the type of the food consumed (Jeon et al. 2011, Zheng et al. 2013). Other studies demonstrated the inoculation of both associated gut bacteria (Yu et al. 2011) or probiotics (Zheng et al. 2012) into waste could improve larval growth. However, the mechanism of how the microbe influence the

nutrient uptake ability of the larval host is unclear. Also, the system has not been optimized for converting waste to protein.

One possible approach is to keep the larvae hungry constantly, by maintaining a microbiota that is associated with host hunger. In this system, "hunger-related" microbiota might serve as signaling agents for hunger induction or satiation in the larval host. This hunger trigger would presumably promote increased feeding, and ultimately waste to protein conversion, meeting farmers' goals for higher biomass per capita; especially in the BSFL production industry. Furthermore, microbial community members may be identified that would provide added nutritional value and nutritional access for larval population and waste conversion.

The community structures revealed in this study are quite different from gut microbial structures in other studies of BSFL. Zheng et al. (Zheng et al. 2013) surveyed standard grain diet fed 7-d-old BSFL associated symbionts, and found, that Bacteroidetes (54.4%) was the most abundant phylum, followed by the Firmicutes and Proteobacteria. Jeon et al. (Jeon et al. 2011) surveyed three groups of 8-d-old BSFL gut microflora fed with restaurant waste, cooked rice, and calf forage, and found that the microbial community structures were directly influenced by the intake feed. For example, those fed restaurant waste showed Bacteroidetes (67.36%) and Proteobacteria (18.85%) as predominant phyla, whereas Proteobacteria (54.01%) and Firmicutes (42.27%) were dominant when BSFL fed on cooked rice. Larvae who fed on calf forage had a more evenly distributed community with Proteobacteria (31.08%), Actinobacteria (24.58%), Firmicutes (23.46%),

and Bacteroidetes (20.51%) (Jeon et al. 2011). In both of these previous studies, Bacteroidetes was found to be the most abundant phylum. In our studies, BSFL fed Gainesville diet at T0 H (11-d-old), which showed to have associated microbial communities predominated by Proteobacteria (31.42%), followed by Euryarchaeota (16.86%) and Actinobacteria (16.56%). These differences underscore the importance of diet considerations in selective pressure for influence on resident gut microbiomes. Other contributors, such as larval age, rearing environment, host genetics, and early microbial exposure may also account for some differences. It is also worth mentioning that these two previous studies used 16S rRNA targeted pyrosequencing techniques, while our study used a total RNA metagenomic shotgun sequencing technology and profiled the metabolically active community and could be the major contributor for the community profile differences.

Our taxonomic resolution was able to identify specific genera, however, only 17.7% sequences were identified on the species level, indicating these sequences belonged to poorly characterized, BSFL specific lineages. Ultimately, species identification within our samples will be necessary for targeted manipulation as well as to determine probiotic versus pathogenic potential. For instance, though many *Weissella* strains have both pre- and probiotic properties, some are opportunistic pathogens to humans, and *W. ceti* is pathogenic to farmed rainbow trout (Oncorhynchus myk*iss*) (Figueiredo et al. 2012). Further investigations are required to determine if such a community structure containing possible pathogenic bacteria in BSFL would cause animal infections. In addition,

Campylobacter was found in both feeding and starved larvae. Some *Campylobacter* ssp. are human pathogens causing diarrhea, cramping, abdominal pain, and fever in those infected, and is the most common cause of diarrheal illness in the United States (CDC 2014). *Campylobacter* organisms are wildly present in livestock such as chicken flocks and can be transmitted through common water sources and contact with infected feces (CDC 2014). However, most *Campylobacter* can be easily killed through proper food handling. Furthermore, a literature review revealed no history of *Campylobacter* as commensals of insect guts; thus, our finding merits more in-depth study.

In conclusion, our metagenomic shotgun sequencing data revealed that starvation of the larval host led to a dramatic shift of the associated metabolically active microbial community. Although the biological meaning of such a shift remains undetermined, investigating the functions of those specific taxa associated with nutrient rich or replete states will be valuable for elucidating inter-kingdom communication and contribution of microbes in waste conversion and nutrient uptake. This increased knowledge would allow utility for designing methods and strategies for improved bioconversion efficiency through microbial manipulation to evolve into a solution of increasing protein production and waste reduction.

References

Alcock, J., C. C. Maley, and C. Aktipis. 2014. Is eating behavior manipulated by the gastrointestinal microbiota? Evolutionary pressures and potential mechanisms. Bioessays 36: 940-949.

Barry, T. 2004. Evaluation of the economic, social, and biological feasibility of bioconverting food wastes with the black soldier fly (*Hermetia illucens*). University of North Texas.

Bondari, K., and D. Sheppard. 1981. Soldier fly larvae as feed in commercial fish production. Aquaculture 24: 103-109.

Bondari, K., and D. C. Sheppard. 1987. Soldier fly, *Hermetia illucens* L., larvae as feed for channel catfish, *Ictalurus punctatus* (Rafinesque), and blue tilapia, *Oreochromis aureus* (Steindachner). Aquaculture and Fisheries Management 18: 209-220.

Bovera, F., G. Piccolo, L. Gasco, S. Marono, R. Loponte, G. Vassalotti, V. Mastellone, P. Lombardi, Y. A. Attia, and A. Nizza. 2015. Yellow mealworm larvae (*Tenebrio molitor*, L.) as a possible alternative to soybean meal in broiler diets. British Poultry Science 56: 569-575.

CDC, C. f. D. C. a. P. 2014. Campylobacter. Centers for Disease Control and Prevention, United States.

Chaves, R. C., R. Queiroga de Paula, B. Gücker, I. Gonçalves Boëchat, I. Evódio Marriel, and A. Oliveira Teixeira. 2015. An alternative fish feed based on

earthworm and fruit meals for tilapia and carp postlarvae. Uma ração alternativa baseada em farinha de minhoca e frutas para alevinos de tilápia e carpa. 13: 15-24.

Costello, E. K., J. I. Gordon, S. M. Secor, and R. Knight. 2010. Postprandial remodeling of the gut microbiota in Burmese pythons. The ISME Journal 4: 1375-1385.

Crawford, P. A., J. R. Crowley, N. Sambandam, B. D. Muegge, E. K. Costello, M. Hamady, R. Knight, and J. I. Gordon. 2009. Regulation of myocardial ketone body metabolism by the gut microbiota during nutrient deprivation. Proceedings of the National Academy of Sciences 106: 11276-11281.

de Weerth, C., S. Fuentes, and W. M. de Vos. 2013. Crying in infants: On the possible role of intestinal microbiota in the development of colic. Gut Microbes 4: 416-421.

Diener, S., C. Zurbrugg, and K. Tockner. 2009. Conversion of organic material by black soldier fly larvae: establishing optimal feeding rates. Waste Management & Research 27: 603-610.

Duca, F. A., T. D. Swartz, Y. Sakar, and M. Covasa. 2012. Increased oral detection, but decreased intestinal signaling for fats in mice lacking gut microbiota. PLoS One 7: e39748.

Egert, M., U. Stingl, L. D. Bruun, B. Pommerenke, A. Brune, and M. W. Friedrich. 2005. Structure and topology of microbial communities in the major gut compartments of *Melolontha melolontha* larvae (Coleoptera: Scarabaeidae). Applied and Environmental Microbiology 71: 4556-4566.

Elia, M. 2017. Defining, Recognizing, and Reporting Malnutrition. The International Journal of Lower Extremity Wounds doi: 10.1177/1534734617733902.

Erickson, M. C., M. Islam, C. Sheppard, J. Liao, and M. P. Doyle. 2004. Reduction of *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Enteritidis in chicken manure by larvae of the black soldier fly. Journal of Food Protection 67: 685-690.

FAO. 2013. Food wastage footprint-Impacts on natural resources: summary report. http://www.fao.org/3/i3347e/i3347e.pdf. Food and Agriculture Organization of the United Nations.

Feldhaar, H. 2011. Bacterial symbionts as mediators of ecologically important traits of insect hosts. Ecological Entomology 36: 533-543.

Figueiredo, H., F. Costa, C. Leal, G. Carvalho-Castro, and R. Leite. 2012. *Weissella* sp. outbreaks in commercial rainbow trout (*Oncorhynchus mykiss*) farms in Brazil. Veterinary Microbiology 156: 359-366.

Friedman, M. I., P. Ulrich, and R. Mattes. 1999. A figurative measure of subjective hunger sensations. Appetite 32: 395-404.

Furman, D. P., R. D. Young, and P. E. Catts. 1959. *Hermetia illucens* (Linnaeus) as a Factor in the Natural Control of *Musca domestica* Linnaeus. Journal of Economic Entomology 52: 917-921.

Gustavsson, J., C. Cederberg, U. Sonesson, R. Van Otterdijk, and A. Meybeck. 2011. Global food losses and food waste. Food and Agriculture Organization of the United Nations, Rome.

Hogsette, J. A. 1992. New diets for production of house flies and stable flies (Diptera: Muscidae) in the laboratory. Journal of Economic Entomology 85: 2291-2294.

Hughes, D. T., and V. Sperandio. 2008. Inter-kingdom signalling: communication between bacteria and their hosts. Nature Reviews Microbiology 6: 111-120.

IISE. 2011. State of observed Species. International Institute for Species Exploration.

IPIFF. 2020. EU Legislation. https://ipiff.org/insects-eu-legislation/.

Jeon, H., S. Park, J. Choi, G. Jeong, S. Lee, Y. Choi, and S. Lee. 2011. The intestinal bacterial community in the food waste-reducing larvae of *Hermetia illucens*. Current Microbiology 62: 1390-1399.

Kilpatrick, J. W., and H. Schoof. 1959. Interrelationship of water and *Hermetia illucens* breeding to *Musca domestica* production in human excrement. The American Journal of Tropical Medicine and Hygiene 8: 597-602.

Kroeckel, S., A. G. E. Harjes, I. Roth, H. Katz, S. Wuertz, A. Susenbeth, and C. Schulz. 2012. When a turbot catches a fly: evaluation of a pre-pupae meal of the Black Soldier Fly (*Hermetia illucens*) as fish meal substitute - growth performance and chitin degradation in juvenile turbot (*Psetta maxima*). Aquaculture 364: 345-352.

Lalander, C., S. Diener, M. E. Magri, C. Zurbrugg, A. Lindstrom, and B. Vinneras. 2013. Faecal sludge management with the larvae of the black soldier fly (*Hermetia illucens*) - from a hygiene aspect. Science of the Total Environment 458: 312-318.

Lalander, C. H., J. Fidjeland, S. Diener, S. Eriksson, and B. Vinnerås. 2015. High waste-to-biomass conversion and efficient *Salmonella* spp. reduction using black soldier fly for waste recycling. Agronomy for Sustainable Development 35: 261-271.

Li, Y.-H., and X. Tian. 2012. Quorum sensing and bacterial social interactions in biofilms. Sensors 12: 2519-2538.

Liu, C.-m., and Z.-m. Lian. 2003. Influence of *Acrida cinerea* replacing Peru fish meal on growth performance of broiler chickens. Journal of Economic Animal 7: 48-51.

Liu, Q., J. K. Tomberlin, J. A. Brady, M. R. Sanford, and Z. Yu. 2008. Black soldier fly (Diptera: Stratiomyidae) larvae reduce *Escherichia coli* in dairy manure. Environmental Entomology 37: 1525-1530.

Lord, W. D., M. L. Goff, T. R. Adkins, and N. H. Haskell. 1994. The black soldier fly *Hermetia illucens* (Diptera: Stratiomyidae) as a potential measure of human postmortem interval: observations and case histories. Journal of Forensic Sciences 39: 215-222.

Love, M. I., W. Huber, and S. Anders. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biology 15: 550.

Masella, A. P., A. K. Bartram, J. M. Truszkowski, D. G. Brown, and J. D. Neufeld. 2012. PANDAseq: paired-end assembler for illumina sequences. BMC Bioinformatics 13: 31.

Newton, G., D. Sheppard, D. Watson, G. Burtle, C. Dove, J. Tomberlin, and E. Thelen. Year. Published. The black soldier fly, *Hermetia illucens*, as a manure management/resource recovery tool, pp. 5-7. *In*, Symposium on the State of the Science of Animal Manure and Waste Management, 2005.

Nguyen, T. T., J. K. Tomberlin, and S. Vanlaerhoven. 2015. Ability of Black Soldier Fly (Diptera: Stratiomyidae) Larvae to Recycle Food Waste. Environmental Entomology 44: 406-410.

Nguyen, T. T. X., J. K. Tomberlin, and S. Vanlaerhoven. 2013. Influence of resources on *Hermetia illucens* (Diptera: Stratiomyidae) larval development. Journal of Medical Entomology 50: 898-906.

O'sullivan, L., R. Ross, and C. Hill. 2002. Potential of bacteriocin-producing lactic acid bacteria for improvements in food safety and quality. Biochimie 84: 593-604.

Oliver, K. M., J. A. Russell, N. A. Moran, and M. S. Hunter. 2003. Facultative bacterial symbionts in aphids confer resistance to parasitic wasps. Proceedings of the National Academy of Sciences 100: 1803-1807.

Read, M. N., and A. J. Holmes. 2017. Towards an integrative understanding of Diet–Host–Gut microbiome interactions. Frontiers in Immunology 8: 538.

Sharma, M. 2014. Actinomycetes: source, identification, and their applications. Int. J. Curr. Microbiol. App. Sci 3: 801-832.

Sheppard, D. C., G. L. Newton, S. A. Thompson, and S. Savage. 1994. A value added manure management system using the black soldier fly. Bioresource Technology 50: 275-279.

Simmons, S. W., and W. E. Dove. 1942. Waste Celery as a Breeding Medium for the Stablefly or " Dog Fly," with Suggestions for Control. Journal of Economic Entomology 35: 709-715.

St-Hilaire, S., K. Cranfill, M. A. McGuire, E. E. Mosley, J. K. Tomberlin, L. Newton, W. Sealey, C. Sheppard, and S. Irving. 2007a. Fish offal recycling by the black soldier fly produces a foodstuff high in omega-3 fatty acids. Journal of the World Aquaculture Society 38: 309-313.

St-Hilaire, S., C. Sheppard, J. K. Tomberlin, S. Irving, L. Newton, M. A. McGuire, E. E. Mosley, R. W. Hardy, and W. Sealey. 2007b. Fly prepupae as a feedstuff for rainbow trout, *Oncorhynchus mykiss*. Journal of the World Aquaculture Society 38: 59-67.

Su, X., J. Xu, and K. Ning. 2012. Parallel-META: efficient metagenomic data analysis based on high-performance computation. BMC Syst Biol 6 Suppl 1: S16.

Taprab, Y., T. Johjima, Y. Maeda, S. Moriya, S. Trakulnaleamsai, N. Noparatnaraporn, M. Ohkuma, and T. Kudo. 2005. Symbiotic fungi produce laccases potentially involved in phenol degradation in fungus combs of fungus-growing termites in Thailand. Applied and Environmental Microbiology 71: 7696-7704.

Team, R. C. 2013. R foundation for statistical computing. Vienna, Austria 3.

Tinker, K. A., and E. A. Ottesen. 2016. The core gut microbiome of the American cockroach, *Periplaneta americana*, is stable and resilient to dietary shifts. Applied and Environmental Microbiology 82: 6603-6610.

Todaka, N., T. Inoue, K. Saita, M. Ohkuma, C. A. Nalepa, M. Lenz, T. Kudo, and S. Moriya. 2010. Phylogenetic analysis of cellulolytic enzyme genes from representative lineages of termites and a related cockroach. PloS One 5: e8636.

Tomberlin, J. K., and D. C. Sheppard. 2002. Factors influencing mating and oviposition of black soldier flies (Diptera: Stratiomyidae) in a colony. Journal of Entomological Science 37: 345-352.

Tomberlin, J. K., D. C. Sheppard, and J. A. Joyce. 2002. Selected life-history traits of black soldier flies (Diptera: Stratiomyidae) reared on three artificial diets. Annals of the Entomological Society of America 95: 379-386.

Velicer, G. J., L. Kroos, and R. E. Lenski. 2000. Developmental cheating in the social bacterium *Myxococcus xanthus*. Nature 404: 598-601.

West, S. A., A. S. Griffin, A. Gardner, and S. P. Diggle. 2006. Social evolution theory for microorganisms. Nature Reviews Microbiology 4: 597-607.

Yadav, H., J.-H. Lee, J. Lloyd, P. Walter, and S. G. Rane. 2013. Beneficial metabolic effects of a probiotic via butyrate-induced GLP-1 hormone secretion. Journal of Biological Chemistry 288: 25088-25097.

Yu, G., P. Cheng, Y. Chen, Y. Li, Z. Yang, Y. Chen, and J. K. Tomberlin. 2011. Inoculating poultry manure with companion bacteria influences growth and development of black soldier fly (Diptera: Stratiomyidae) larvae. Environmental Entomology 40: 30-35.

Zheng, L., Y. Hou, W. Li, S. Yang, Q. Li, and Z. Yu. 2012. Biodiesel production from rice straw and restaurant waste employing black soldier fly assisted by microbes. Energy 47: 225-229.

Zheng, L., T. L. Crippen, B. Singh, A. M. Tarone, S. Dowd, Z. Yu, T. K. Wood, and J. K. Tomberlin. 2013. A survey of bacterial diversity from successive life stages of black soldier fly (Diptera: Stratiomyidae) by using 16S rDNA pyrosequencing. Journal of Medical Entomology 50: 647-658.

Zhou, F., J. K. Tomberlin, L. Zheng, Z. Yu, and J. Zhang. 2013. Developmental and waste reduction plasticity of three black soldier fly strains (Diptera: Stratiomyidae) raised on different livestock manures. Journal of Medical Entomology 50: 1224-1230.

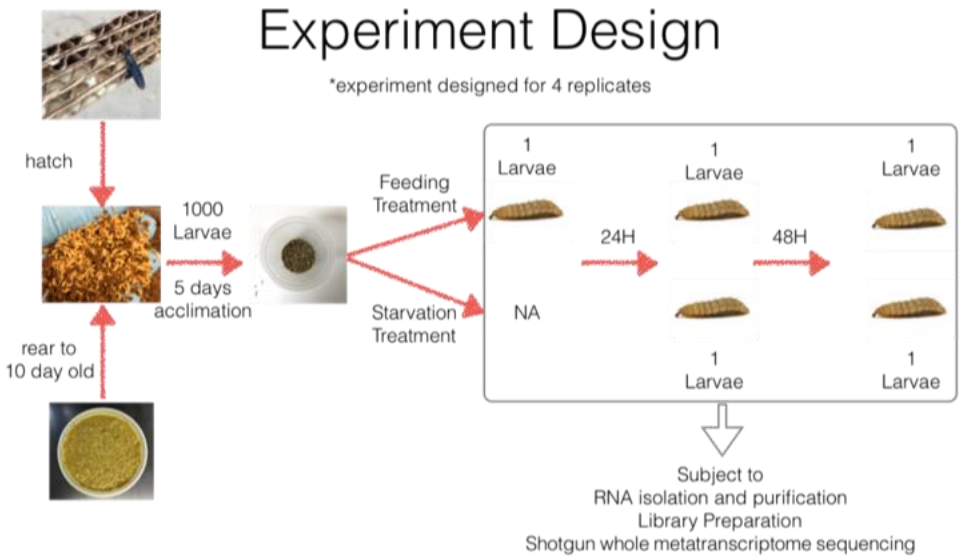

Figure II-1 The workflow of the experiment design. Neonates hatched from the eggs were fed 70% Gainesville diet till 10 days old, and then partitioned into 1000 larvae per lot with additional 5 day feeding for acclimation. On the 15th day, the larvae, with the frass, were further split into two cups, 500 larvae with equal amount of the frass each. One cup represented the control group, in which the larvae were being fed daily (20 g,2 hour prior to the sampling, and 30 g after the sampling), until the end of the experiment. The other cup represented the starvation treatment, in which the larvae were no longer receiving any food for the rest of the experiment. Five subsamples were taken from each replicate and subjected to RNA isolation and library preparation for shotgun sequencing

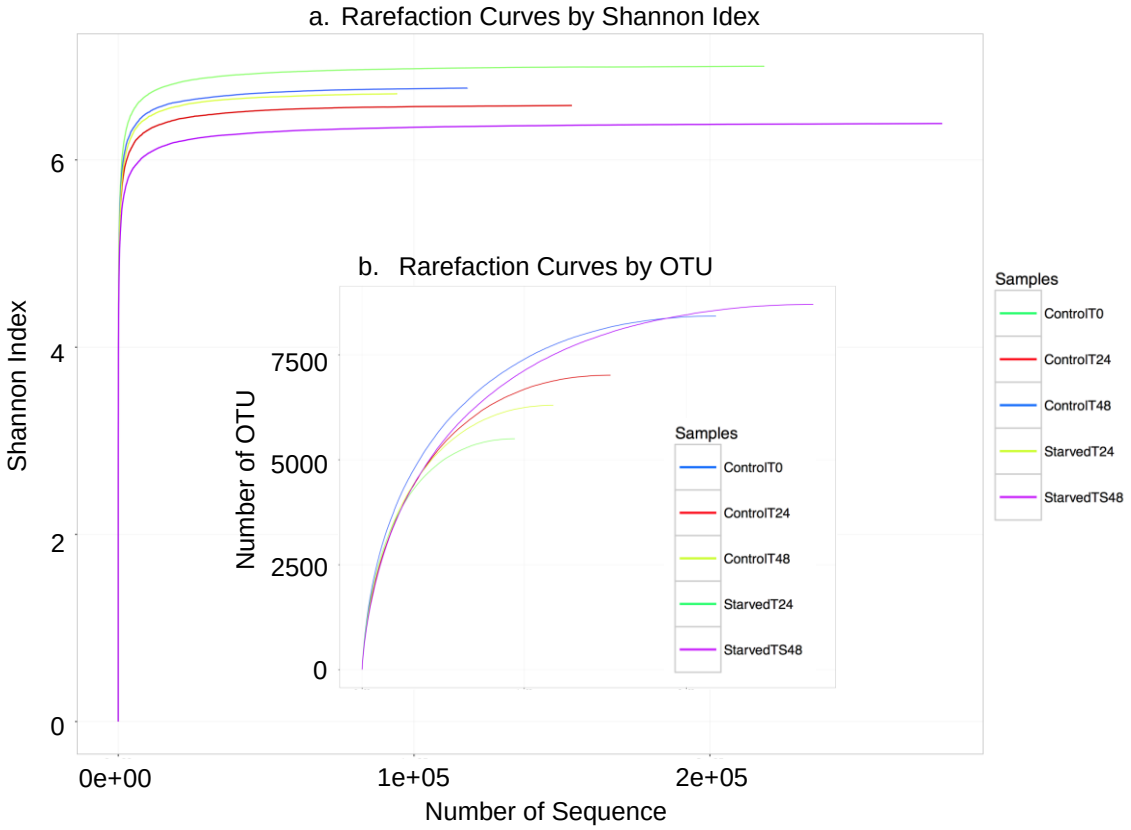

Figure II-2 a) Rarefaction curve with Shannon diversity index. Generally, the Shannon index value was higher in the control groups than in the starvation groups, indicating there were loss of community diversity and evenness as a function of time and treatment. b) Rarefaction Curve on OTUs.

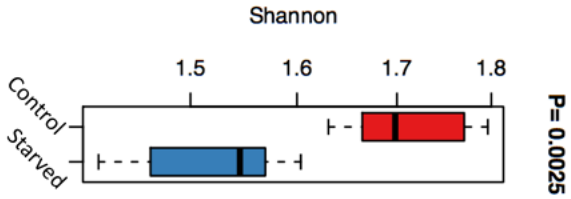

Figure II-3 Alpha diversity at phylum level. The control group revealed a higher community diversity

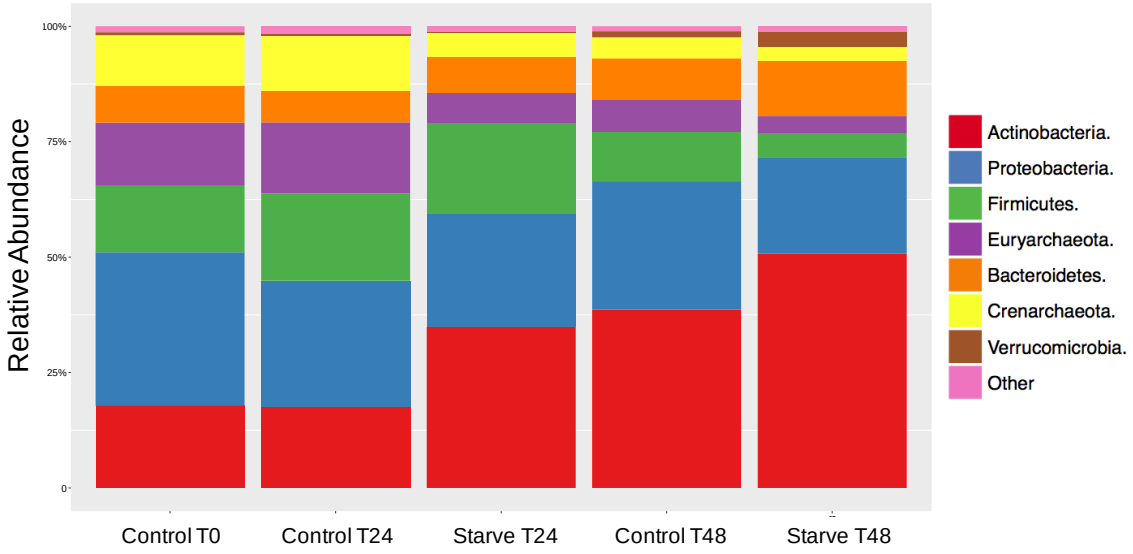

Figure II-4 Distribution chart on relative abundance at phylum level. The distribution chart revealed that the community structures were different across treatment.

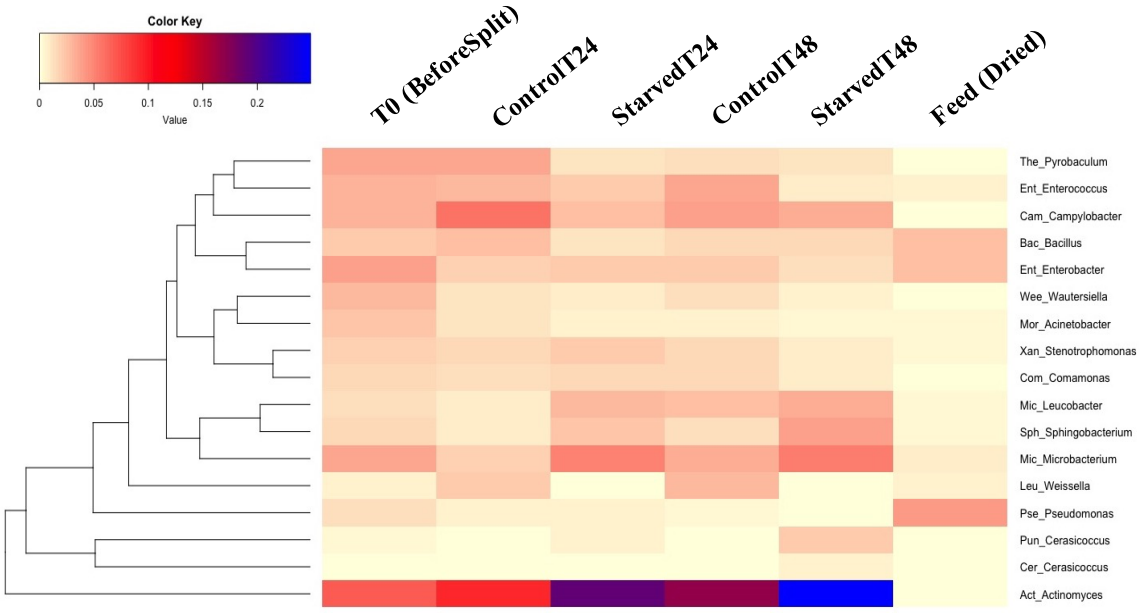

Figure II-5 Heatmap showing differentiated genera occurrence among treatments and in the feed

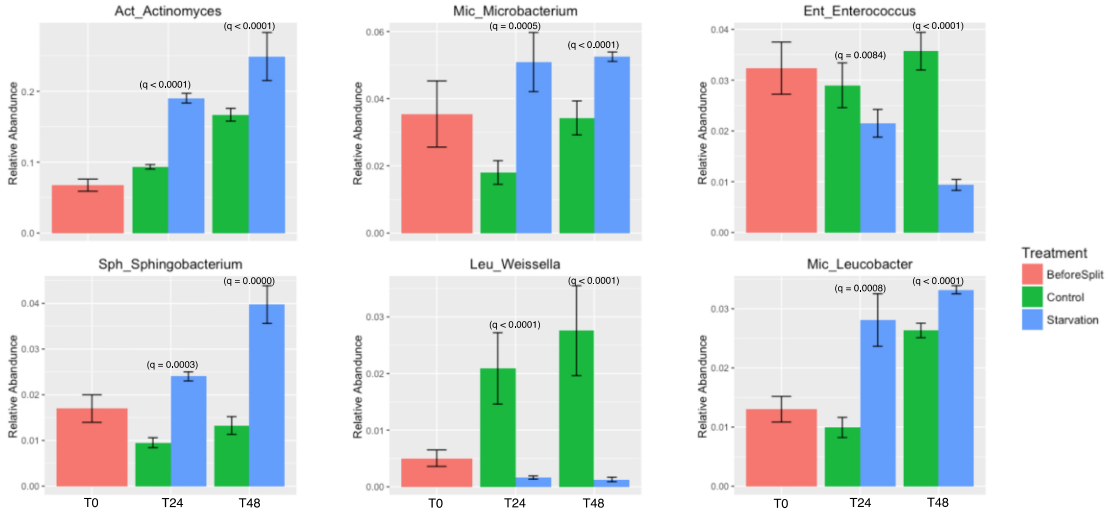

Figure II-6 The top 6 genera with relative abundance that were significantly different at both T24 and T48, with a minimum abundance of 3% in any treatments.

Table II-1. Parallel-Meta classification result

Taxa Level	Identified taxa member
Phyla	10
Class	24
Order	40
Family	66
Genus	117
Species	26
OTU	10691

Table II-2 Phylum members that were present in significant different abundance between control and starved groups. Significance were determined by DESeq2 package with a cutoff q-value of 0.05.

Phyla	**Mean Control[a]**	**Mean Starved[b]**	**q-value**
Actinobacteria	0.230	0.433	<0.001
Firmicutes	0.178	0.082	<0.001
Euryarchaeota	0.135	0.061	<0.01
Bacteroidetes	0.067	0.107	<0.01
Crenarchaeota	0.096	0.040	<0.001
Verrucomicrobia	0.005	0.027	<0.001
Planctomycetes	0.002	0.001	<0.05

[a]Mean abundance among all control samples at T0, T24, and T48

[b]Mean abundance among all starved samples at T24 and T48

Table II-3 Anova test result of the slopes of abundance change comparison between fed and starved cohort of the highlighted genera.

Genera	DF	Residuals	F - value	p
Act_Actinomyces	1	20	6.31	0.02
Mic_Microbacterium	1	20	1.44	0.24
Ent_Enterococcus	1	20	11.53	0
Sph_Sphingobacterium	1	20	22.269	0
Leu_Weissella	1	20	10.24	0
Mic_Leucobacter	1	20	1.31	0.27

CHAPTER III

SHIFT OF MICROBIAL GENE EXPRESSION LEVEL IN RESPONSE TO HOST STARVATION OF BLACK SOLDIER FLY LARVAE

Summary

`Insect and their associated microbes have long coevolved, but little is still known on how they impact each other during variable conditions, including metabolic stress. Black soldier fly larvae represent a great model for investigating this relationship due to their lifestyle associated with complex microbial activities in extreme environments. Furthermore, this information will be greatly appreciated by the industry of mass-producing black soldier fly larvae as a potential alternative to increase protein production efficiency. In this study, we employed metatranscriptomic shotgun sequencing to investigate larval host starvation-induced functional shifts of the associated microbes. Black soldier fly larvae were fed or were under fasting conditions for up to 48 hrs where their microbes were then processed for analysis. We discovered 104 prokaryotic genes that were expressed significantly ($q < 0.05$) between the starved and feeding cohort. Among these, 72 were downregulated in the Starved treatment, most being associated with metabolism and information processing. The other 32 were upregulated with approximately 20% of those being characterized as enzyme related or associated with transport systems. Our study demonstrated that metabolic stress induced by black soldier fly larval fasting had a great impact on the associated functional bacterial response.

Introduction

In the host environment, access to carbohydrates, amino acids, and minerals varies widely. A host's gut can be relatively rich in certain nutrients, but are highly restricted during fasting or starvation, and nutrient availability is further modulated by the activities of the host microbiota (Xia et al. 2014, Nesvorna et al. 2019). Relying on their host for survival, gut bacteria have developed strategies to compensate for periods of host starvation.

Next-generation sequencing has significantly increased our understanding of the composition, diversity, and the role of the gut microbiota in health and disease has greatly expanded (Bashiardes et al. 2016). However, gut microbial composition provides little insight into the interactions between the microbiota and its host. Research is now beginning to expand beyond compositional analysis toward a more functional understanding of host-microbe interactions in an effort to elucidate mechanisms of these host-gut microbe interactions.

The role of the gut microbiota in host metabolism and appetite has been primarily investigated in regard to obesity and metabolic syndromes in humans (Ley 2010, Tilg and Kaser 2011). Studies have shown that microbes can induce dysphoria that changes feeding behavior, and there is also evidence for a selective influence of diet on microbes (Alcock et al. 2014, Bradlow 2014).

However, host-microbe interactions with regards to functioning during feeding and fasting is also of interest in the insect-based bioconversion industry, especially in the field

of black soldier fly larva (BSFL) mass-production. The black soldier fly (BSF), *Hermetia illucens*, (L.) (Diptera: Stratiomyidae) is a beneficial insect widely spread across the globe in temporal regions, and their ability to digest a wide range of organic waste as larvae into valuable insect protein has won themselves a crown jewel in recent years (Tomberlin 2020). Furthermore, the BSF is a good model system for studying insect-microbe interactions due to its occurrence in habitats where complex microbial activities are naturally present (Jeon et al. 2011, Zheng et al. 2013), and it has very likely evolved multiple strategies to coexist with these microbes. For instance, BSFL have been found to inhibit a wide range of pathogenic bacteria, such as *Escherichia coli* (Erickson et al. 2004, Liu et al. 2008) and *Salmonella* spp. (Erickson et al. 2004, Lalander et al. 2013, Lalander et al. 2015). On the other hand, BSFL associated bacteria contribute to host nutrition by aiding to degrade organic material. For instance, BSFL can better transform restaurant waste after the application of Rid-X®, a product containing a proprietary blend of microbes, and can better digest chicken manure with the presence of gut isolated *Bacillus subtilis* (Yu et al. 2011, Xiao et al. 2018).

An incentive to study starvation related microbial activity in the BSFL is to determine whether microbial mediated mechanisms could be shaped to increase appetite for more efficient BSFL waste-to-protein conversion. A study of the feeding behavior of BSFL determined individual larvae eat sporadically, with only half of its time in feeding and the other half wandering (5 minutes rotation) (Shishkov et al. 2019). If we could find a means

to increase their feeding times by inducing hunger, then the efficiency of waste transformation could be increased.

Investigating larval starvation-induced microbial activity changes could also lead to new possible avenues to increase protein production efficiency by understanding more about their interkingdom interactions. In this study, we used BSFL as a model organism to investigate how larval host fasting impacts functional activities of the associated microbes. We sought to measure gene expression from a microbial community under selective pressure and with higher functional resilience to metabolic stress. From this, our goal was to identify microbial genes associated with feeding and those leading to functional stability during starvation.

Materials and Methods

Colony Maintenance

BSF eggs were collected from a colony maintained in the Forensic Laboratory for Investigative Entomological Sciences (FLIES) Facility at Texas A&M University. The eggs were collected in three layers of 2 cm (w) by 2 cm (h) x 3 cm (l) cm corrugated cardboard blocks taped to the inside a 2 L plastic bucket 3 cm above approximately 500 g of the Gainesville diet (30% alfalfa meal, 20% corn meal, and 50% wheat bran) saturated with water (Hogsette 1992). Cardboard was replaced daily. Cardboard containing eggs was placed in a ~1 L deli cup and maintained in an incubator at approximately 70% RH, 27°C, and 12:12 L:D until hatch (Sheppard et al. 2002).

BSF Feeding Conditions

Sixty-six clutches (approximately 40K eggs) (Tomberlin et al. 2002) of BSF eggs were collected within an eight-hour window using cardboard egg traps. Upon hatching, larvae were maintained using methods described in Sheppard et al. Larvae were fed 50 g 70% saturated Gainesville diet on the day of hatching. An additional 50 g of feed was given daily from day four to ten. On the eleventh day, 4,000 larvae were collected from the pool and partitioned equally into four containers (~1L) (ChoiceHD brand from webstaurantstore.com), representing four technical replicates denoted as A, B, C, and D. For each replicate, larvae were fed 50 g Gainesville diet at 70% moisture daily for an additional five days in order to allow the larvae to acclimate. On day 16, each replicate was split into two containers. The first group representing the control group was fed 50 g Gainesville diet daily until the end of the experiment. The second group, representing the starvation treatment, was not fed for the remainder of the experiment. Each of the 4 cups of control was given 20 g feed two hours prior to larval sampling, and an additional 30 g feed after samples were taken (Figure III-11). Five sub-samples (80 to 100 mg each) of larvae were taken from each replicate of the control and treatment at three time points (T0, T24 and T4 after starvation) and placed in 2 ml sterile centrifuge tubes with 0.5 g glass beads and 1 mL Trizol®, immediately followed by manual tissue homogenization.

Microbial Total RNA Isolation and Purification

Manually homogenized samples were further processed with a bead beater (BioSpec) for two minutes. After the first minute of homogenization, samples were placed on ice for a two-minute incubation period to avoid overheating and subsequent RNA degradation, and then processed to the second minute of bead beating. After homogenization, samples were incubated at room temperature for 5 min, followed by centrifugation at 12,000xg for 10 min at 4°C to pellet excess particulate. Following this, the supernatant in each sample was transferred to a new tube containing 0.2 mL chloroform, followed by vortexing and incubation at room temperature for 3 min. Samples were centrifuged at 12,000xg for 15 min at 4°C, and resulting product was distinguished into three phases: upper, aqueous RNA phase, the middle, DNA interphase, and lower, protein and lipid organic phase.

The upper, aqueous RNA phases were transferred to a clean tube containing 0.5 mL isopropanol and incubated at room temperature for 10 min followed by a centrifugation at 12,000xg for 10 min at 4°C. Supernatants were removed, and RNA pellets were washed twice using 1 mL 75% ethanol and subsequent centrifugation at 7,500xg for 5 min at 4°C. The RNA pellets were air-dried, then resuspended in RNase free water. Resuspended RNA samples were pooled for each replicate, resulting in 20 RNA samples from larval samples and one RNA sample isolated from feed samples, that were stored at -20°C.

RNA was purified using the Direct-zol™ RNA MiniPrep kit, following the manufacturer's protocol. Resulting prokaryotic RNA was enriched using the MICROBEnrich™ Kit, following the MICROBEnrich™ Kit Protocol that captures and

removes up to 90% of eukaryotic RNA, resulting in prokaryotic RNA enrichment. Enrichment results were confirmed using a Gel electrophoresis method (Figure III-4). If severe eukaryotic RNA contamination was detected, the respective samples were purified with the MICROBEnrich™ Kit for a second time. The enriched RNA products were then quantified with a Qubit® 2.0 Fluorometer, and then ran on a gel to determine RNA quality.

The NEB Ultra RNA Library Kit (New England Biolabs) was used to convert the RNA to cDNA, avoiding steps to remove rRNA, and preserving total RNA concentrations. Established cDNA libraries were multiplexed using NEBNext Oligos for Illumina to create five libraries. Resulting multiplexed libraries were submitted for shotgun whole metatranscriptome sequencing using an Illumina® HiSeq 2000 instrument.

Raw paired-end reads from whole metatranscriptome sequences were demultiplexed, and trimmed, and adapters removed using the FasQC tool kit. Quality checks were performed to remove low quality reads (3% in average). *De novo* assembling of the clean reads was performed with Trinity software (Grabherr et al. 2011), and each assembled transcript was given a unique gene name (e.g. g0_i0, g100290_i0), following with software Coding Potential Calculator 2 (CPC2) (Kang et al. 2017) to select out a pool of transcript that has coding potential. The unigenes with coding potential were then input into BLAST (Altschul et al. 1997) against 8 databases with respect to multiple functional categories, including Nr (Deng et al. 2006), Swiss-Prot (Apweiler et al. 2004), GO (Ashburner et al. 2000), eggnog/COG (Tatusov et al. 2000), KOG (Koonin et al. 2004),

CAZy (Levasseur et al. 2013, Lombard et al. 2014), KEGG (Kanehisa et al. 2004), and Pfam (Finn et al. 2014), to gain annotation information.

R packages DESeq2 (Love et al. 2014) were used to identify differential expression among treatments (treating T24 and T48 versus S24 and S48) with cut off value of $q<0.05$; R packages ggplot2 was used to plot the figures, including a heatma of statistically significantly differentially expressed genes, and a PCoA plot with 95% ellipses to demonstrate distances between expressed gene profiles across samples and treatments.

Results

There were 110.9 million reads generated from the sequencing, with 96.92% clean reads. *De novo* assembling resulted in 100,291 unique genes, and within which we gained a dataset of 5,581 genes with coding potential. DESeq2 identified 104 coding genes that had significantly different expressed levels between Fed and Starved cohorts. Among these, 32 were upregulated and 72 were downregulated in the Starved cohort.

A PCoA plot showed gene expression was separated among each treatment (Figure III-2). Genes associated with samples associated with the T0 timepoint were clustered together. Two samples within the T24 timepoint clustered close to the T0 timepoint, while the other two clustered at a distance. The T48 samples also clustered close to the T0 and T24 timepoint samples. Samples from the Starved cohort at T24 were more dispersed, but were closest to the T48 samples. Two of the samples from the S48 timepoint were

clustered together, but at a distance from the other samples, while the other two were clustering together near the T0 samples.

Additionally, a heatmap was created and demonstrated a clear shift of gene expression among the 104 differentially expressed genes between treatments (Figure III-3). Among those downregulated were 2 genes encoding proteins involved in general metabolism, 2 involved in amino acid metabolism, 3 in lipid metabolism, 14 involved in carbohydrate metabolism, and 2 genes encoding proteins for energy metabolism. Additionally, six genes were downregulated that are involved in environmental information processing including genes for flagellar assembly (*mot*A), response to oxidative stress (*sod*A), and a type IX secretion system (Table III- 1, Table III-2).

Twenty genes involved in genetic information processing were also downregulated, including 14 involved in transcription or translation, five encoding for heat shock proteins and chaperones for responding to heat or osmotic stress, and one, *sec*A, that has a central role in coupling the hydrolysis of ATP to the transfer of proteins into and across the cell membrane. There were 7 genes involved in signaling and cellular processes, including genes encoding transporters, and an ATPase. Finally, 16 genes did not correspond to any known function, and were listed as hypothetical (Table III-1).

Functional annotation of the 14 of the 32 upregulated genes in the Starved cohort were classified as hypothetical genes (Table III-1). However, two genes were found to be involved in carbohydrate metabolism, including a chitinase (*chi*A) and glutamate decarboxylase (*gad*A). Two upregulated genes also encoded for a Type IX Secretion

System (T9SS) type A sorting domain-containing protein involved in environmental information processing and protein secretion. Seven genes encoding enzymes were also upregulated. These enzymes included cell wall hydrolases, lysozyme-like enzymes, and a thioredoxin-like enzyme.

Three upregulated genes were involved in signaling and cellular processing and encoded transporters. Also, three upregulated genes were involved in general metabolism and included genes for an α-mannosidase, one for an oxygen-dependent coproporphyrinogen oxidase, and one for a cytochrome P450. Finally, one gene was upregulated that was identified as RNA-dependent RNA polymerase, involved in genetic information processing (Table III- 1).

Discussion

In this study, we have used shotgun metatranscriptomics to describe changes to gut microbial function during host metabolic stress. Our study demonstrated that host starvation of BSFL had a significant impact on the functional gene expression of the associated microbes. Data showed 104 genes were expressed at significantly different levels between Starved and Fed treatments (Table III-2).

Starvation of the host also means nutrient and space deficiency for the associated microbes. With this shift, we expected decreased levels of genes for metabolism and a shift to genes involved in housekeeping and stress response. Among those shown downregulated genes in the Starved cohort, 23 were involved in carbohydrate metabolism,

fermentation and other metabolic pathways and 33 were involved in information processing. The results were not surprising because microbes were also suffering stress and likely shutting down gene expressions related to proliferation and shifting to more survival related gene expression under the starved condition. But interestingly, several genes involved in stress response were downregulated, including *sod*A, encoding superoxide dismutase, and genes encoding for proteins for response to heat and osmotic shock. Active feeding by BSFL increases temperatures within their local environment (Xiao et al. 2018). This temperature increase potentially activates microbial stress response. Furthermore, when nutrients and food sources are plentiful, as in the Fed cohort, microbial richness and diversity is also increased (Chapter II), thus increasing competition for space and resources.

Alternatively, in the Starved cohort, decreased microbial diversity, which tends to be associated with larger populations of the same species, *Actinomyces* for instance (Chapter II), may result in individual species having more energy and resources and a higher capacity for host manipulation, because fewer resources are spent on competition. In this instance, bacterial crossfeeding, wherein one species of bacteria provides nutrients for another species, may also be occurring (Smith et al. 2019). This concept is in agreement with other studies that showed bacterial metabolites and crossfeeding can influence host satiety pathways and have extensive effects on appetite and feeding behavior (Fetissov 2017, Yang et al. 2018).

Many fewer genes were upregulated within the Starved cohort compared to the Fed cohort. An oxygen-dependent coproporphyrinogen oxidase was upregulated that is involved in heme biosynthesis. The functions served by hemes are diverse and dependent upon the nature of the heme moiety and the protein to which it is bound. Many bacteria contain a diversity of environmentally regulated heme-containing respiratory cytochromes in which the heme has a redox function for aerobic and anaerobic respiration (Mayfield et al. 2011). There are other non-respiratory-chain cytochromes, such as cytochrome P450s, one of which upregulated also within the Starved cohort, that uses heme as a cofactor to metabolize a broad range of substrates, most commonly serving as a monooxygenase (Kelly and Kelly 2013). Cytochrome P450s are associated with secondary metabolism and have also been shown to contribute to microbial pathogenicity against insects, being associated with cuticle lipid metabolism. The upregulation of these genes suggests their importance in specialized roles in helping the BSFL gut microbes to compete in their niche under metabolic stress.

We also observed higher abundance and transcription of genes encoding for a type A sorting domain-containing protein, that may function in protein secretion. The T9SS is a terminal branch of the general secretory pathway (GSP) and a means to secrete substrates across the cytoplasmic membrane in a Sec-dependent manner into the periplasm (Chen et al. 2011, Chatzi et al. 2013). The conserved C-terminal domain functions as an outer membrane translocation signal for export of virulence factors or other proteins to the cell

surface, potentially including hydrolases such as chitinase and lysozymes, as indicated by upregulation of these genes in the Starved cohort, as well (Veith et al. 2017).

Bacteria produce chitinases in order to supply nitrogen and carbon as a source of nutrients, or for parasitism (Rathore and Gupta 2015). Many invasive bacteria produce chitinases to allow them to invade into and to survive in host cells, or to utilize chitin as a source of energy and nutrition. Chitinases play an important role in bacterial pathogenesis wherever host contains chitin (Busby et al. 2012). As a result, bacteria can live on or invade into the chitin-containing host promptly and efficiently (Rathore and Gupta 2015). Genes encoding microbial chitinases have also been identified that were not useful for utilizing chitin as a sole source of carbon, suggesting that they might have biological or physiological functions other than binding to chitinous material or breaking down chitin. These genes could also be playing important roles not only in the physiology of the gut microbes but also in the interaction with host cells, although this point requires more research.

But, overall, bacterial lysozymes are poorly characterized, though sequences homologous to eukaryotic lysozymes were recently identified (Yamamoto et al. 2014, D'Angelo et al. 2016, Palud et al. 2018). The possibility exists that BSFL associated gut bacteria in the Starved cohort produced these lytic agents and delivered them, potentially through the upregulated T9SS into the periplasm of adjacent bacterial cells causing their lysis (Chou et al. 2012). The production of bacteriolytic exoenzymes is a property of many bacterial species and is one means to sequester released nutrients (Vollmer et al. 2008).

Additionally, we identified an upregulated α-mannosidase, predicted to be important in shaping the nutrient availability of the intestinal ecosystem. In the event that dietary components are lacking, gut bacteria can grow on eukaryotic glycans, secreted by the host's epithelium (Martens et al. 2008). Bacteria have developed many complex catabolic pathways, including the utilization of α-mannosidases to catalyze glycan assimilation and ensure metabolic regulation. Indeed, recent research has revealed that certain symbiotic gut-resident bacteria maintain a selective advantage over microbial competitors through the ability to use α-mannosidases to catabolize α-linked mannopolysaccharides as a sole carbon source (Cuskin et al. 2015). In addition, the Starved cohort showed upregulation of an outer membrane lipocalin family protein and *sus*C, a TonB-dependent oligosaccharide transporter. The lipocalins are a family of proteins which transport small hydrophobic molecules such as steroids and lipids, while the TonB-dependent transporter mediates transport of oligosaccharides from the surface of the outer membrane to the periplasm for subsequent degradation (Lakshmi et al. 2015, Bolam and van den Berg 2018). TonB dependent transport of BSFL associated glycans into the microbial periplasm would allow for subsequent deconstruction by α-mannosidase and internalization for use as a carbon source.

Several cell wall hydrolases (CWH) were also upregulated in the Starved cohort. Often, CWH may also be related to biogenesis of the cell wall and membrane (Sanchez et al. 2008). But, CWH activity can also have effects on a wide range of physiological functions from bacterial adhesion, biofilm formation, protein secretion, conjugation,

virulence and immune response. Additionally, many of these enzymes participate in cell-wall recycling as a mechanism for conserving energy and for bacterial growth (Vermassen et al. 2019). When the bacterial cells are faced with a sudden loss of carbon, recycling of cell wall material would allow bacterial survival by completing a last round of cell division before growth arrest (Park and Uehara 2008).

Another interesting finding was the upregulation of *gad*A, encoding a glutamate decarboxylase in the Starved cohort. This gene converts glutamate to gamma-aminobutyrate (GABA). The production of GABA has been shown to confer resistance against acidic pH conditions in many bacterial species. Also, many bacteria decarboxylate glutamate to GABA to enhance metabolic energy (Mazzoli and Pessione 2016).

Further, GABA is an inhibitory neurotransmitter, produced by both multicellular organisms and prokaryotes, and are considered as a common means of interkingdom communication, even between phylogenetically distant organisms (Lyte 2011). It has been hypothesized that such a common language contributes to the homeostatic regulation of gut microbiota and possibly to the functioning of the brain and behavior (Lyte 2011).

GABA is used as a food supplement and has broad human and animal health benefits, including reducing stress and anxiety (Inagawa et al. 2005, Nakamura et al. 2009, Yoto et al. 2012, Boonstra et al. 2015). Notably, GABA is becoming increasingly recognized in host energy metabolism. GABA regulates insulin secretion and inhibits proinflammatory cells and the secretion of a large number of inflammatory molecules (Bhandage et al. 2018). Though more work needs to be undertaken, recent data suggests that manipulating

gut microbes based on dietary changes or fecal transplants can also impact GABA levels. For instance, *Lactobacillus rhamnosus* is able to regulate central GABA receptor expression in a vagus nerve–dependent manner, and it was recently shown to increase central GABA concentrations and subsequently improve insulin sensitivity. Furthermore, GABA supplements have also been produced by fermenting *Lactobacillus* and other lactic acid bacteria (David et al. 2014, Mazzoli and Pessione 2016, Kootte et al. 2017).

The impact on GABA has also been shown in insects where GABA signaling to insulin-producing cells in *Drisophila* is more prevalent during metabolic stress. A study by Enel et al. showed that metabotropic GABA receptors expressed on insulin producing neurosecretory cells in the *Drosophila* brain are involved in inhibitory regulation of insulin signaling (Enell et al. 2010). Data from that study showed reduction of lifespan was more prominent in GABA receptor knockdown flies exposed to starvation or desiccation than in normally fed flies. GABA signaling also led to *Drosophila* being able to store carbohydrates and lipids longer during 12-24 hour periods of starvation and desiccation (Enell et al. 2010).

GABA signaling may be one mechanism leading to insulin regulation, and thus, increased GABA supplementation may be one way to increase BSFL carbon and fat storage during periods of starvation. Another option might be engineering GABA (or utilizing GABA receptor agonists) deficient BSFL as a means to increase feeding and organic waste conversion. BSFL development is regulated by the larval fat body in response to nutrition, where the fat body secretes signals that lead to the release of insulin-

like hormones from the brain that ultimately lead to larval growth (Gold et al. 2018). These signals are suppressed during starvation or on low-protein diets and display reduced growth rate and increase development time. Therefore, microbial GABA may be part of a stress response, also aiding in mediating effects on metabolism and lifespan during metabolic stress. Bacterial strains producing or altering these GABA could most likely play a central role in influencing appetite and energy metabolism via modulation of the BSFL central nervous system, though further work should be done to elucidate this.

Our data suggest the existence of mechanisms regulating bacterial physiology in correspondence with the nutritional status in their host BSFL associated environment. But, although informative, our findings cannot adequately reflect the physiologic state of the BSF during either fed or starved conditions. And, more work should be done to measure global connections between microbe-host feedback loops, including how BSFL immune response (Vallet-Gely et al. 2008), and insulin-like signaling (Shin et al. 2011) shapes microbial structure and function. For example, we would expect BSFL to have a role in determining selection pressure on members of the gut microbiota. How the immune system has coevolved with the microbiota remains unclear, but it is important to accept that members of the microbiota are not necessarily evolving in a constant environment, but rather one that also responds and adapts. Indeed, starvation would conceivably increase BSFL immune response, that would also alter the gut environment and microbial populations, that would then lead to further immune response alterations. Consistent with the Red Queen Hypothesis (Van and Van Valen 1973), microbes would likely adapt gene

expression under metabolic stress conditions for continual improvement of scavenging and functional stability as adaptation to physiological changes within the BSFL host.

These results taken together, suggest a system that gut microbes use to adjust to limited nutrients and adjust their metabolisms accordingly, thus benefiting individual species, other bacteria through crossfeeding, and conceivably, the host, who also obtains caloric intake through microbial metabolism (Xu et al. 2004). Furthermore, microbially mediated GABA production may be a means by which BSFL can survive periods of starvation and desiccation. In conclusion our study demonstrated the host starvation of black soldier fly larvae has a significant impact on the functional gene expression of the associated bacteria community. Further work is needed to investigate whether these responses were just for self-regulation for the bacteria, or the responses were also influencing BSFL.

References

Alcock, J., C. C. Maley, and C. Aktipis. 2014. Is eating behavior manipulated by the gastrointestinal microbiota? Evolutionary pressures and potential mechanisms. Bioessays 36: 940-949.

Altschul, S. F., T. L. Madden, A. A. Schäffer, J. Zhang, Z. Zhang, W. Miller, and D. J. Lipman. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Research 25: 3389-3402.

Apweiler, R., A. Bairoch, C. H. Wu, W. C. Barker, B. Boeckmann, S. Ferro, E. Gasteiger, H. Huang, R. Lopez, and M. Magrane. 2004. UniProt: the universal protein knowledgebase. Nucleic Acids Research 32: D115-D119.

Ashburner, M., C. A. Ball, J. A. Blake, D. Botstein, H. Butler, J. M. Cherry, A. P. Davis, K. Dolinski, S. S. Dwight, and J. T. Eppig. 2000. Gene ontology: tool for the unification of biology. Nature Genetics 25: 25-29.

Bashiardes, S., G. Zilberman-Schapira, and E. Elinav. 2016. Use of metatranscriptomics in microbiome research. Bioinformatics and Biology Insights 10: BBI. S34610.

Bhandage, A. K., Z. Jin, S. V. Korol, Q. Shen, Y. Pei, Q. Deng, D. Espes, P. O. Carlsson, M. Kamali-Moghaddam, and B. Birnir. 2018. GABA Regulates Release of Inflammatory Cytokines From Peripheral Blood Mononuclear Cells

and CD4(+) T Cells and Is Immunosuppressive in Type 1 Diabetes. EBioMedicine 30: 283-294.

Bolam, D. N., and B. van den Berg. 2018. TonB-dependent transport by the gut microbiota: novel aspects of an old problem. Curr Opin Struct Biol 51: 35-43.

Boonstra, E., R. de Kleijn, L. S. Colzato, A. Alkemade, B. U. Forstmann, and S. Nieuwenhuis. 2015. Neurotransmitters as food supplements: the effects of GABA on brain and behavior. Front Psychol 6: 1520.

Bradlow, H. L. 2014. Obesity and the gut microbiome: pathophysiological aspects. Hormone Molecular Biology and Clinical Investigation 17: 53-61.

Busby, J. N., M. J. Landsberg, R. M. Simpson, S. A. Jones, B. Hankamer, M. R. Hurst, and J. S. Lott. 2012. Structural analysis of Chi1 Chitinase from Yen-Tc: the multisubunit insecticidal ABC toxin complex of Yersinia entomophaga. J Mol Biol 415: 359-371.

Chatzi, K. E., M. F. Sardis, S. Karamanou, and A. Economou. 2013. Breaking on through to the other side: protein export through the bacterial Sec system. Biochem J 449: 25-37.

Chen, Y. Y., B. Peng, Q. Yang, M. D. Glew, P. D. Veith, K. J. Cross, K. N. Goldie, D. Chen, N. O'Brien-Simpson, S. G. Dashper, and E. C. Reynolds. 2011. The outer membrane protein LptO is essential for the O-deacylation of LPS and the co-ordinated secretion and attachment of A-LPS and CTD proteins in Porphyromonas gingivalis. Mol Microbiol 79: 1380-1401.

Chou, S., N. K. Bui, A. B. Russell, K. W. Lexa, T. E. Gardiner, M. LeRoux, W. Vollmer, and J. D. Mougous. 2012. Structure of a peptidoglycan amidase effector targeted to Gram-negative bacteria by the type VI secretion system. Cell Rep 1: 656-664.

Cuskin, F., E. C. Lowe, M. J. Temple, Y. Zhu, E. Cameron, N. A. Pudlo, N. T. Porter, K. Urs, A. J. Thompson, A. Cartmell, A. Rogowski, B. S. Hamilton, R. Chen, T. J. Tolbert, K. Piens, D. Bracke, W. Vervecken, Z. Hakki, G. Speciale, J. L. Munoz-Munoz, A. Day, M. J. Pena, R. McLean, M. D. Suits, A. B. Boraston, T. Atherly, C. J. Ziemer, S. J. Williams, G. J. Davies, D. W. Abbott, E. C. Martens, and H. J. Gilbert. 2015. Human gut Bacteroidetes can utilize yeast mannan through a selfish mechanism. Nature 517: 165-169.

D'Angelo, T., R. Oshone, F. Abebe-Akele, S. Simpson, K. Morris, W. K. Thomas, and L. S. Tisa. 2016. Permanent Draft Genome Sequence for Frankia sp. Strain EI5c, a Single-Spore Isolate of a Nitrogen-Fixing Actinobacterium, Isolated from the Root Nodules of Elaeagnus angustifolia. Genome Announc 4: 4.

David, L. A., C. F. Maurice, R. N. Carmody, D. B. Gootenberg, J. E. Button, B. E. Wolfe, A. V. Ling, A. S. Devlin, Y. Varma, M. A. Fischbach, S. B. Biddinger, R. J. Dutton, and P. J. Turnbaugh. 2014. Diet rapidly and reproducibly alters the human gut microbiome. Nature 505: 559-563.

Deng, Y., J. Li, S. Wu, Y. Zhu, Y. Chen, F. He, Y. Chen, L. Y. Deng, J. LI, and S. WU. 2006. Integrated nr database in protein annotation system and its localization. Computer Engineering. 32. 71-72.

Enell, L. E., N. Kapan, J. A. Soderberg, L. Kahsai, and D. R. Nassel. 2010. Insulin signaling, lifespan and stress resistance are modulated by metabotropic GABA receptors on insulin producing cells in the brain of *Drosophila*. PLoS One 5: e15780.

Erickson, M. C., M. Islam, C. Sheppard, J. Liao, and M. P. Doyle. 2004. Reduction of *Escherichia coli* O157:H7 and *Salmonella enterica* serovar Enteritidis in chicken manure by larvae of the black soldier fly. Journal of Food Protection 67: 685-690.

Fetissov, S. O. 2017. Role of the gut microbiota in host appetite control: bacterial growth to animal feeding behaviour. Nat Rev Endocrinol 13: 11-25.

Finn, R. D., A. Bateman, J. Clements, P. Coggill, R. Y. Eberhardt, S. R. Eddy, A. Heger, K. Hetherington, L. Holm, and J. Mistry. 2014. Pfam: the protein families database. Nucleic Acids Research 42: D222-D230.

Gold, M., J. K. Tomberlin, S. Diener, C. Zurbrugg, and A. Mathys. 2018. Decomposition of biowaste macronutrients, microbes, and chemicals in black soldier fly larval treatment: A review. Waste Manag 82: 302-318.

Grabherr, M. G., B. J. Haas, M. Yassour, J. Z. Levin, D. A. Thompson, I. Amit, X. Adiconis, L. Fan, R. Raychowdhury, and Q. Zeng. 2011. Trinity: reconstructing

a full-length transcriptome without a genome from RNA-Seq data. Nature Biotechnology 29: 644.

Hogsette, J. A. 1992. New diets for production of house flies and stable flies (Diptera: Muscidae) in the laboratory. Journal of Economic Entomology 85: 2291-2294.

Inagawa, K., S. Seki, M. Bannai, Y. Takeuchi, Y. Mori, and M. Takahashi. 2005. Alleviative effects of gamma-aminobutyric acid (GABA) on behavioral abnormalities in aged dogs. J Vet Med Sci 67: 1063-1066.

Jeon, H., S. Park, J. Choi, G. Jeong, S. Lee, Y. Choi, and S. Lee. 2011. The intestinal bacterial community in the food waste-reducing larvae of *Hermetia illucens*. Current Microbiology 62: 1390-1399.

Kanehisa, M., S. Goto, S. Kawashima, Y. Okuno, and M. Hattori. 2004. The KEGG resource for deciphering the genome. Nucleic Acids Research 32: D277-D280.

Kang, Y.-J., D.-C. Yang, L. Kong, M. Hou, Y.-Q. Meng, L. Wei, and G. Gao. 2017. CPC2: a fast and accurate coding potential calculator based on sequence intrinsic features. Nucleic Acids Research 45: W12-W16.

Kelly, S. L., and D. E. Kelly. 2013. Microbial cytochromes P450: biodiversity and biotechnology. Where do cytochromes P450 come from, what do they do and what can they do for us? Philos Trans R Soc Lond B Biol Sci 368: 20120476.

Koonin, E. V., N. D. Fedorova, J. D. Jackson, A. R. Jacobs, D. M. Krylov, K. S. Makarova, R. Mazumder, S. L. Mekhedov, A. N. Nikolskaya, and B. S. Rao.

2004. A comprehensive evolutionary classification of proteins encoded in complete eukaryotic genomes. Genome Biology 5: R7.

Kootte, R. S., E. Levin, J. Salojarvi, L. P. Smits, A. V. Hartstra, S. D. Udayappan, G. Hermes, K. E. Bouter, A. M. Koopen, J. J. Holst, F. K. Knop, E. E. Blaak, J. Zhao, H. Smidt, A. C. Harms, T. Hankemeijer, J. Bergman, H. A. Romijn, F. G. Schaap, S. W. M. Olde Damink, M. T. Ackermans, G. M. Dallinga-Thie, E. Zoetendal, W. M. de Vos, M. J. Serlie, E. S. G. Stroes, A. K. Groen, and M. Nieuwdorp. 2017. Improvement of Insulin Sensitivity after Lean Donor Feces in Metabolic Syndrome Is Driven by Baseline Intestinal Microbiota Composition. Cell Metab 26: 611-619 e616.

Lakshmi, B., M. Mishra, N. Srinivasan, and G. Archunan. 2015. Structure-Based Phylogenetic Analysis of the Lipocalin Superfamily. PLoS One 10: e0135507.

Lalander, C., S. Diener, M. E. Magri, C. Zurbrugg, A. Lindstrom, and B. Vinneras. 2013. Faecal sludge management with the larvae of the black soldier fly (*Hermetia illucens*) - from a hygiene aspect. Science of the Total Environment 458: 312-318.

Lalander, C. H., J. Fidjeland, S. Diener, S. Eriksson, and B. Vinnerås. 2015. High waste-to-biomass conversion and efficient *Salmonella* spp. reduction using black soldier fly for waste recycling. Agronomy for Sustainable Development 35: 261-271.

Levasseur, A., E. Drula, V. Lombard, P. M. Coutinho, and B. Henrissat. 2013. Expansion of the enzymatic repertoire of the CAZy database to integrate auxiliary redox enzymes. Biotechnol Biofuels 6: 41.

Ley, R. E. 2010. Obesity and the human microbiome. Current Opinion in Gastroenterology 26: 5-11.

Liu, Q., J. K. Tomberlin, J. A. Brady, M. R. Sanford, and Z. Yu. 2008. Black soldier fly (Diptera: Stratiomyidae) larvae reduce *Escherichia coli* in dairy manure. Environmental Entomology 37: 1525-1530.

Lombard, V., H. Golaconda Ramulu, E. Drula, P. M. Coutinho, and B. Henrissat. 2014. The carbohydrate-active enzymes database (CAZy) in 2013. Nucleic Acids Res 42: D490-495.

Love, M. I., W. Huber, and S. Anders. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biology 15: 550.

Lyte, M. 2011. Probiotics function mechanistically as delivery vehicles for neuroactive compounds: Microbial endocrinology in the design and use of probiotics. Bioessays 33: 574-581.

Martens, E. C., H. C. Chiang, and J. I. Gordon. 2008. Mucosal glycan foraging enhances fitness and transmission of a saccharolytic human gut bacterial symbiont. Cell Host Microbe 4: 447-457.

Mayfield, J. A., C. A. Dehner, and J. L. DuBois. 2011. Recent advances in bacterial heme protein biochemistry. Curr Opin Chem Biol 15: 260-266.

Mazzoli, R., and E. Pessione. 2016. The Neuro-endocrinological Role of Microbial Glutamate and GABA Signaling. Front Microbiol 7: 1934.

Nakamura, H., T. Takishima, T. Kometani, and H. Yokogoshi. 2009. Psychological stress-reducing effect of chocolate enriched with gamma-aminobutyric acid (GABA) in humans: assessment of stress using heart rate variability and salivary chromogranin A. Int J Food Sci Nutr 60 Suppl 5: 106-113.

Nesvorna, M., V. Bittner, and J. Hubert. 2019. The mite *Tyrophagus putrescentiae* hosts population-specific microbiomes that respond weakly to starvation. Microbial Ecology 77: 488-501.

Palud, A., H. Scornec, J. F. Cavin, and H. Licandro. 2018. New Genes Involved in Mild Stress Response Identified by Transposon Mutagenesis in *Lactobacillus paracasei*. Front Microbiol 9: 535.

Park, J. T., and T. Uehara. 2008. How bacteria consume their own exoskeletons (turnover and recycling of cell wall peptidoglycan). Microbiol Mol Biol Rev 72: 211-227.

Rathore, A. S., and R. D. Gupta. 2015. Chitinases from Bacteria to Human: Properties, Applications, and Future Perspectives. Enzyme Res 2015: 791907.

Sanchez, B., M. C. Champomier-Verges, P. Anglade, F. Baraige, C. G. de Los Reyes-Gavilan, A. Margolles, and M. Zagorec. 2008. A preliminary analysis of *Bifidobacterium longum* exported proteins by two-dimensional electrophoresis. J Mol Microbiol Biotechnol 14: 74-79.

Sheppard, D. C., J. K. Tomberlin, J. A. Joyce, B. C. Kiser, and S. M. Sumner. 2002. Rearing methods for the black soldier fly (Diptera: Stratiomyidae). Journal of Medical Entomology 39: 695-698.

Shin, S. C., S.-H. Kim, H. You, B. Kim, A. C. Kim, K.-A. Lee, J.-H. Yoon, J.-H. Ryu, and W.-J. Lee. 2011. *Drosophila* microbiome modulates host developmental and metabolic homeostasis via insulin signaling. Science 334: 670-674.

Shishkov, O., M. Hu, C. Johnson, and D. L. Hu. 2019. Black soldier fly larvae feed by forming a fountain around food. Journal of the Royal Society Interface 16: 20180735.

Smith, N. W., P. R. Shorten, E. Altermann, N. C. Roy, and W. C. McNabb. 2019. The Classification and Evolution of Bacterial Cross-Feeding. Frontiers in Ecology and Evolution 7: 153.

Tatusov, R. L., M. Y. Galperin, D. A. Natale, and E. V. Koonin. 2000. The COG database: a tool for genome-scale analysis of protein functions and evolution. Nucleic Acids Research 28: 33-36.

Tilg, H., and A. Kaser. 2011. Gut microbiome, obesity, and metabolic dysfunction. The Journal of Clinical Investigation 121: 2126-2132.

Tomberlin, J. K., D. C. Sheppard, and J. A. Joyce. 2002. Selected life-history traits of black soldier flies (Diptera: Stratiomyidae) reared on three artificial diets. Annals of the Entomological Society of America 95: 379-386.

Tomberlin, J. K. H., A. van. 2020. Black soldier fly from pest to 'crown jewel' of the insects as feed industry: an historical perspective. Journal of Insects as Food and Feed 6 (1): 1-4.

Vallet-Gely, I., B. Lemaitre, and F. Boccard. 2008. Bacterial strategies to overcome insect defences. Nature Reviews Microbiology 6: 302-313.

Van, V., and L. Van Valen. 1973. A new evolutionary law. Evol. Theory. 1:1–30

Veith, P. D., M. D. Glew, D. G. Gorasia, and E. C. Reynolds. 2017. Type IX secretion: the generation of bacterial cell surface coatings involved in virulence, gliding motility and the degradation of complex biopolymers. Mol Microbiol 106: 35-53.

Vermassen, A., S. Leroy, R. Talon, C. Provot, M. Popowska, and M. Desvaux. 2019. Cell Wall Hydrolases in Bacteria: Insight on the Diversity of Cell Wall Amidases, Glycosidases and Peptidases Toward Peptidoglycan. Front Microbiol 10: 331.

Vollmer, W., B. Joris, P. Charlier, and S. Foster. 2008. Bacterial peptidoglycan (murein) hydrolases. FEMS Microbiol Rev 32: 259-286.

Xia, J. H., G. Lin, G. H. Fu, Z. Y. Wan, M. Lee, L. Wang, X. J. Liu, and G. H. Yue. 2014. The intestinal microbiome of fish under starvation. BMC Genomics 15: 266.

Xiao, X., L. Mazza, Y. Yu, M. Cai, L. Zheng, J. K. Tomberlin, J. Yu, A. van Huis, Z. Yu, and S. Fasulo. 2018. Efficient co-conversion process of chicken manure into protein feed and organic fertilizer by *Hermetia illucens* L. (Diptera: Stratiomyidae) larvae and functional bacteria. Journal of Environmental Management 217: 668-676.

Xu, J., H. C. Chiang, M. K. Bjursell, and J. I. Gordon. 2004. Message from a human gut symbiont: sensitivity is a prerequisite for sharing. Trends Microbiol 12: 21-28.

Yamamoto, E., H. Muramatsu, and K. Nagai. 2014. *Vulgatibacter incomptus* gen. nov., sp. nov. and *Labilithrix luteola* gen. nov., sp. nov., two myxobacteria isolated from soil in Yakushima Island, and the description of *Vulgatibacteraceae* fam. nov., *Labilitrichaceae* fam. nov. and *Anaeromyxobacteraceae* fam. nov. Int J Syst Evol Microbiol 64: 3360-3368.

Yang, H., M. Yang, S. Fang, X. Huang, M. He, S. Ke, J. Gao, J. Wu, Y. Zhou, H. Fu, C. Chen, and L. Huang. 2018. Evaluating the profound effect of gut microbiome on host appetite in pigs. BMC Microbiol 18: 215.

Yoto, A., S. Murao, M. Motoki, Y. Yokoyama, N. Horie, K. Takeshima, K. Masuda, M. Kim, and H. Yokogoshi. 2012. Oral intake of gamma-aminobutyric acid affects mood and activities of central nervous system during stressed condition induced by mental tasks. Amino Acids 43: 1331-1337.

Yu, G., P. Cheng, Y. Chen, Y. Li, Z. Yang, Y. Chen, and J. K. Tomberlin. 2011. Inoculating poultry manure with companion bacteria influences growth and development of black soldier fly (Diptera: Stratiomyidae) larvae. Environmental Entomology 40: 30-35.

Zheng, L., T. L. Crippen, B. Singh, A. M. Tarone, S. Dowd, Z. Yu, T. K. Wood, and J. K. Tomberlin. 2013. A survey of bacterial diversity from successive life stages

of black soldier fly (Diptera: Stratiomyidae) by using 16S rDNA pyrosequencing. Journal of Medical Entomology 50: 647-658.

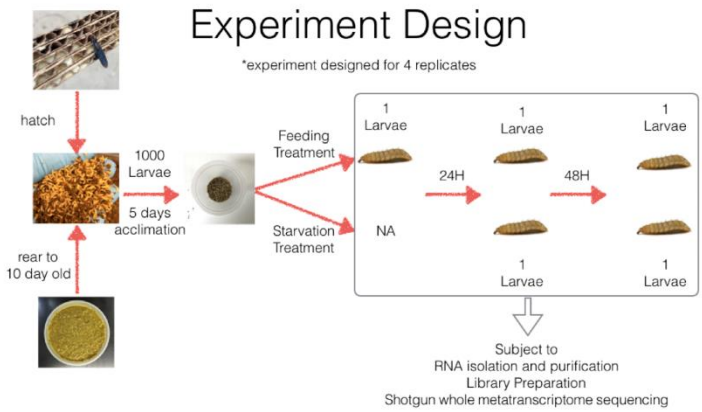

Figure III-1 The workflow of the experiment design. Neonates hatched from the eggs were fed 70% Gainesville diet till 10 days old, and then partitioned into 1000 larvae per lot with additional 5 day feeding for acclimation. On the 15th day, the larvae, with the frass, were further split into two cups, 500 larvae with equal amount of the frass each. One cup represented the control group, in which the larvae were being fed daily (20 g, 2 hour prior to the sampling, and 30 g after the sampling), until the end of the experiment. The other cup represented the starvation treatment, in which the larvae were no longer receiving any food for the rest of the experiment. Five subsamples were taken from each replicate and subjected to RNA isolation and library preparation for shotgun sequencing

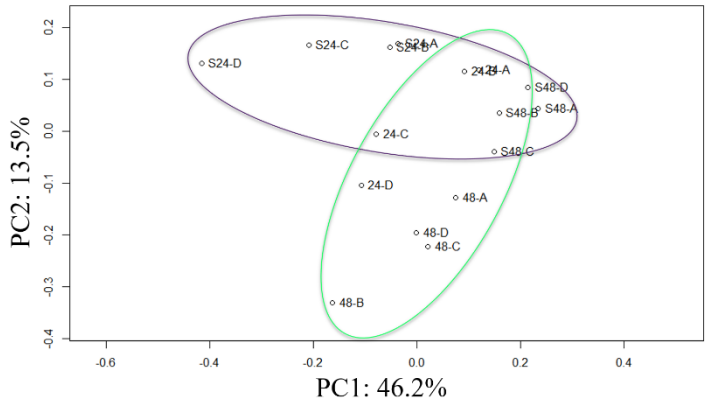

Figure III-2 PCoA analysis of the two treatments showed distinct difference.

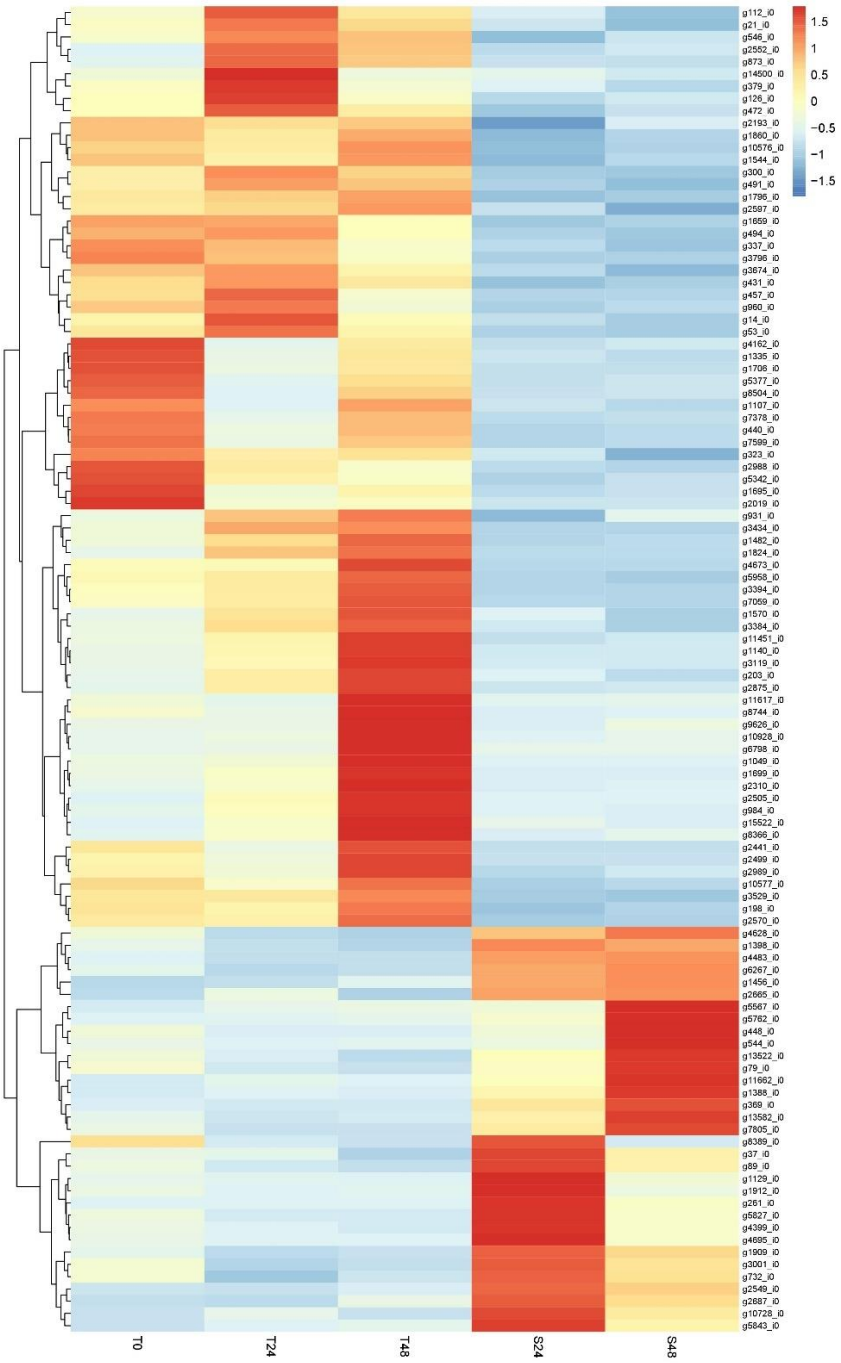

Figure III-3 Heatmap of the 108 genes detected differentially expressed among treatments. 4 were suspected from eukaryotic and being excluding for following discussion. Among which 72 were found downregulated in the starved cohort and 32 were found upregulated.

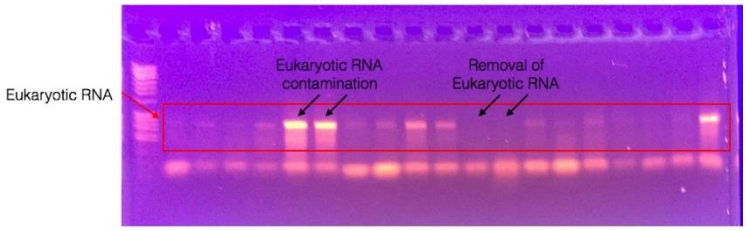

Figure III-4 Eukaryotic RNA contamination shown on an electrophoresis gel. We used a gel electrophoresis method to check the quality of the RNA product after processing through the MICROBEnrich™ Kit. If severe eukaryotic RNA contamination was detected, we will process the respective sample with MICROBEnrich™ Kit following by a gel check again, until satisfied.

Table III-1 KEGG Pathway annotation counts on the 104 significantly expressed genes between starved and fed. Most downregulated genes in the starved cohort were those involved in metabolism and most of those found upregulated in the starved cohort were poorly categorized hypothetical genes.

KEGG Pathway	**Genes**
Downregulated	
Amino acid metabolism	2
Carbohydrate metabolism	14
Energy Metabolism	2
Environmental Information Processing	6
Genetic Information Processing	20
Lipid metabolism	3
Metabolism	2
Signaling and Cellular processes	7
Hypothetical	16
Upregulated	
Carbohydrate metabolism	2
Glycan biosynthesis and metabolism	1
Environmental Information Processing	2
Enzyme	7
Genetic Information Processing	1
Hypothetical	28

Table III-2 Annotation information on the 104 genes found differently expressed between starved and fed cohorts.

Unigene ID	Protein	BlastX Description	KEGG Category	Gene	Log2Fold
g2193_i0	WP_123856702.1	3-hydroxyisobutyrate dehydrogenase [Chryseobacterium nakagawai]	Amino acid metabolism	mmsB	-1.4081
g3674_i0	RKO09898.1	glutathione S-transferase [Macrococcus caseolyticus]	Amino acid metabolism	gst	-2.8973
g1699_i0	WP_002828899.1	phosphopyruvate hydratase (enolase) [Weissella paramesenteroides]	Carbohydrate metabolism		-5.9665
g2875_i0	WP_140837237.1	zinc-binding dehydrogenase [Weissella paramesenteroides]	Carbohydrate metabolism	butB	-4.6969
g2310_i0	WP_150191497.1	bifunctional acetaldehyde-CoA/alcohol dehydrogenase [Weissella paramesenteroides]	Carbohydrate metabolism	adhC	-4.578
g1482_i0	WP_002828557.1	pyruvate kinase [Weissella paramesenteroides]	Carbohydrate metabolism	pyk	-4.1121
g5958_i0	WP_110446869.1	RpiB/LacA/LacB family sugar-phosphate isomerase [Gilliamella apis]	Carbohydrate metabolism	RpiB/LacA/LacB	-3.2743
g9626_i0	WP_044165379.1	AraC family transcriptional regulator [Bacteroides reticulotermitis]	Carbohydrate metabolism	araC	-2.8291
g112_i0	EDM77474.1	pyruvate carboxylase [Plesiocystis pacifica SIR-1]	Carbohydrate metabolism		-2.1711
g873_i0	WP_140836520.1	L-arabinose isomerase [Weissella paramesenteroides]	Carbohydrate metabolism	araA	-4.9742
g1544_i0	WP_039103767.1	phosphopyruvate hydratase [Frischella perrara]	carbohydrate metabolism	eno	-2.5483
g198_i0	WP_091120723.1	formate C-acetyltransferase [Gilliamella intestini]	Carbohydrate metabolism	pflB	-1.6478
g14_i0	WP_159397361.1	SDR family NAD(P)-dependent oxidoreductase [Sorangium cellulosum]	carbohydrate metabolism		-2.6802
g1860_i0	WP_093309558.1	S-(hydroxymethyl)glutathione dehydrogenase/class III alcohol dehydrogenase [Pseudospirillum japonicum]	carbohydrate metabolism	frmA	-2.0717
g2570_i0	WP_079040427.1	chitin binding domain-containing protein [Streptomyces prasinus]	carbohydrate metabolism		-2.863

Table III-2 Continued

Unigene ID	Protein	BlastX Description	KEGG Category	Gene	Log2Fold
g472_i0	WP_118902028.1	chitin binding domain-containing protein [Streptomyces globisporus]	carbohydrate metabolism		-2.273
g931_i0	WP_114128284.1	NADH dehydrogenase subunit 5 [Desertihabitans brevis]	Energy Metabolism		-1.3145
g2441_i0	WP_042854949.1	MULTISPECIES: photosystem II q(b) protein [Bacilli]	Energy metabolism	psbA	-5.2118
g4162_i0	WP_135836350.1	T9SS type A sorting domain-containing protein [Empedobacter sp. MRS2]	Environmental Informatio Processing		-3.1475
g3796_i0	WP_134230839.1	serine protease, partial [Oenococcus oeni]	Environmental Informatio Processing		-1.5342
g7599_i0	WP_038334988.1	MULTISPECIES: superoxide dismutase [Empedobacter]	Environmental Information Processing	sodA_1	-3.5002
g1049_i0	WP_002827845.1	PTS sugar transporter subunit IIC [Weissella paramesenteroides]	Environmental Information Processing		-5.1382
g2505_i0	WP_044164618.1	MotA/TolQ/ExbB proton channel family protein [Bacteroides reticulotermitis]	Environmental Information Processing	motA	-4.3859
g10576_i0	EFG03676.1	venom protein R-like protein [Streptomyces clavuligerus]	Environmental Information signalling		-2.7657
g21_i0	OGQ06634.1	Acetyl-CoA carboxylase activity	Lipid metabolism		-1.9211
g379_i0	RPH47422.1	long-chain fatty acid--CoA ligase [Desulfobacteraceae bacterium]	Lipid metabolism		-1.7335
g440_i0	WP_135834456.1	preprotein translocase subunit SecA [Empedobacter sp. MRS2]	Genetic Informatiom Processing	secA	-3.6871

Table III-2 Continued

Unigene ID	Protein	BlastX Description	KEGG Category	Gene	Log2Fold
g1796_i0	AJA44592.1	bacterial translation initiation factor 3 (bIF-3) [Frischella perrara]	Genetic Information Processing	infC	-2.223
g1140_i0	WP_034915554.1	elongation factor G [Gilliamella apicola]	Genetic Information Processing	fusA	-2.3971
g984_i0	WP_082031226.1	translation initiation factor IF-3 [Actinomyces polynesiensis]	Genetic Information Processing	infC	-2.4736
g1107_i0	WP_135835360.1	elongation factor Tu [Empedobacter sp. MRS2]	Genetic Information Processing	tuf	-2.8713
g4673_i0	WP_154542940.1	50S ribosomal protein L10 [Actinomycetaceae bacterium WB03_NA08]	Genetic Information Processing	rplJ	-3.0103
g1695_i0	WP_135834376.1	50S ribosomal protein L9 [Empedobacter sp. MRS2]	Genetic Information Processing	rplI	-3.5967
g431_i0	WP_132862506.1	50S ribosomal protein L5 [Stenotrophomonas sp. ATCM1_4]	Genetic Information Processing	rplE	-3.5713
g1659_i0	WP_065561374.1	50S ribosomal protein L10 [Gilliamella apicola]	Genetic Information Processing	rplJ	-3.6717
g5377_i0	WP_135834840.1	30S ribosomal protein S16 [Empedobacter sp. MRS2]	Genetic Information Processing	rpsP	-4.0865
g1824_i0	WP_002828990.1	elongation factor Tu [Weissella paramesenteroides]	Genetic Information Processing	tuf	-5.4484
g3434_i0	WP_054066302.1	30S ribosomal protein S1 [Comamonas kerstersii]	Genetic Information Processing	rpsA	-5.1256
g6798_i0	EER75693.1	ribosomal subunit interface protein [Weissella paramesenteroides ATCC 33313]	Genetic Information Processing	RaiA	-4.9827
g3394_i0	WP_140836394.1	50S ribosomal protein L1 [Weissella paramesenteroides]	Genetic Information Processing	rplA	-4.7103
g11617_i0	WP_002827298.1	Hsp20/alpha crystallin family protein [Weissella paramesenteroides]	Genetic Information Processing	hsp20	-4.7032
g2989_i0	WP_065558060.1	RNA polymerase sigma factor RpoH [Gilliamella apicola]	Genetic Information Processing	RpoH	-3.4602
g337_i0	WP_110201926.1	molecular chaperone HtpG [Kangiella spongicola]	Genetic Information Processing	htpG	-3.0425
g300_i0	WP_126755780.1	molecular chaperone DnaK [Escherichia coli]	Genetic Information Processing	dnaK	-1.9809

Table III-2 Continued

Unigene ID	Protein	BlastX Description	KEGG Category	Gene	Log2Fold
g546_i0	WP_155383606.1	DnaJ domain-containing protein [Nitriliruptoraceae bacterium ZYF776]	Genetic Information Processing	dnaJ	-1.9288
g8744_i0	WP_148298347.1	Hsp20/alpha crystallin family protein [Bacteroides reticulotermitis]	Genetic Information Processinghsp20	hsp20	-4.3742
g494_i0	OIF94626.1	hypothetical protein A7M93_19645 [Acinetobacter baumannii]	hypothetical		-5.0804
g11451_i0	WP_141146917.1	hypothetical protein [Weissella cibaria]	hypothetical		-4.2914
g10928_i0	WP_151627872.1	hypothetical protein, partial [Bacillus luti]	hypothetical		-4.0955
g3384_i0	CWB19287.1	Uncharacterised protein [Streptococcus pneumoniae]	hypothetical		-3.4053
g14500_i0	None	None	hypothetical		-3.1288
g1570_i0	CWB19287.1	Uncharacterised protein [Streptococcus pneumoniae]	hypothetical		-2.9349
g8504_i0	WP_135833917.1	hypothetical protein [Empedobacter sp. MRS2]	hypothetical		-2.829
g8366_i0	None	None	hypothetical		-2.777
g15522_i0	HAB16788.1	hypothetical protein [Verrucomicrobiales bacterium]	hypothetical		-2.5597
g126_i0	None		hypothetical		-2.7344
g10577_i0	EDY50506.1	conserved hypothetical protein [Streptomyces clavuligerus]	hypothetical		-2.6507
g5342_i0	none	none	hypothetical		-2.1986
g2665_i0	none	none	hypothetical		1.35444
g7059_i0	KKY00280.1	hypothetical protein VN21_15085, partial [Paraclostridium benzoelyticum]	hypothetical		-2.0731

Table III-2 Continued

Unigene ID	Protein	BlastX Description	KEGG Category	Gene	Log2Fold
g2597_i0	CRH04936.1	conserved protein of unknown function [include at least 3 DUF3421 domain] [magneto-ovoid bacterium MO-1]	hypothetical		-1.7136
g3529_i0	OXK53524.1	putative transmembrane protein E. coli	hypothetical		-3.0576
g3119_i0	GAE82601.1	3-oxoacyl-[acyl-carrier-protein] synthase [Bacteroides reticulotermitis JCM 10512]	Lipid metabolism		-3.5165
g2499_i0	WP_135835864.1	deoxyhypusine synthase [Empedobacter sp. MRS2]	metabolism		-4.8326
g2019_i0	WP_135834378.1	M24 family metallopeptidase [Empedobacter sp. MRS2]	metabolism		-3.9698
g457_i0	DAC72316.1	ATPase	Signalling and cellular processes		-2.3353
g53_i0	MBV35073.1	ATPase-coupled transmembrane transporter activity	Signalling and cellular processes		-5.6701
g203_i0	WP_154546478.1	heavy metal translocating P-type ATPase [Actinomycetaceae bacterium WB03_NA08]	Signalling and cellular processes		-4.3569
g960_i0	WP_132129957.1	sugar porter family MFS transporter [Anseongella ginsenosidimutans]	signalling and cellular processes	mfs	-2.9223
g1706_i0	WP_135834648.1	OmpA family protein [Empedobacter sp. MRS2]	Signalling and Cellular processes	OmpA	-3.5659
g1335_i0	WP_135834135.1	OmpA family protein [Empedobacter sp. MRS2]	Signalling and Cellular processes	OmpA	-2.9629
g7378_i0	WP_093363851.1	outer membrane beta-barrel protein [Sphingobacterium wenxiniae]	Signalling and Cellular processes		-5.0292
g1388_i0	BAO37115.1	chitinase	Carbohydrate metabolism	chiA	2.34818
g544_i0	WP_113849180.1	glutamate decarboxylase [Enterococcus gallinarum]	Carbohydrate metabolism	gadB, gadA, GAD	5.69893

Table III-2 Continued

Unigene ID	Protein	BlastX Description	KEGG Category	Gene	Log2Fold
g261_i0	RYY45361.1	T9SS type A sorting domain-containing protein [Chitinophagaceae bacterium]	Environmental Information Processing		4.60216
g4483_i0	WP_129020527.1	T9SS type A sorting domain-containing protein [Edaphocola flava]	Environmental Information Processing		4.26724
g10728_i0	EEW79284.1	cell wall-associated hydrolase [Brucella abortus NCTC 8038]	Enzyme		2.11798
g1912_i0	KNH03717.1	Cell wall-associated hydrolase [Candidatus Burkholderia brachyanthoides]	Enzyme		1.80259
g5827_i0	AOE12513.1	cell wall-associated hydrolase [uncultured bacterium]	Enzyme		1.66604
g448_i0	none	none	Enzyme		4.86661
g11662_i0		lysozyme like	Enzyme		2.23266
g3001_i0	WP_136899865.1	redoxin domain-containing protein [Sphingobacterium olei]	Enzyme		4.10799
g4399_i0	VBC09016.1	Cell wall-associated hydrolase [Burkholderia pseudomallei]	Enzyme		1.89042
g89_i0	YP_009552275.1	RNA-dependent RNA polymerase [Pythium polare RNA virus 1]	Genetic Information Processing	rdrp	2.8259
g13522_i0	WP_093363768.1	hypothetical protein [Sphingobacterium wenxiniae]	hypothetical		4.01132
g13582_i0	WP_057718750.1	hypothetical protein [Lactobacillus taiwanensis]	hypothetical		4.19934
g2665_i0	none	none	hypothetical		1.35444
g2687_i0	KWW27341.1	Uncharacterized protein AUK64_2224 [bacterium P201]	hypothetical		2.48748
g37_i0		none	hypothetical		2.31398
g4628_i0	KWW27341.1	Uncharacterized protein AUK64_2224 [bacterium P201]	hypothetical		2.46471

Table III-2 Continued

Unigene ID	Protein	BlastX Description	KEGG Category	Gene	Log2Fold
g4695_i0	WP_148918973.1	hypothetical protein [Sphingobacterium sp. SSI9]	hypothetical		5.01678
g5567_i0	none		hypothetical		2.2117
g5762_i0	none		hypothetical		2.35688
g5843_i0	EFG86088.1	hypothetical protein CLCAR_4307 [Clostridium carboxidivorans P7]	hypothetical		1.76414
g6267_i0	WP_157970338.1	hypothetical protein [Pedobacter sp. SM1810]	hypothetical		5.10748
g7805_i0	WP_093366288.1	hypothetical protein [Sphingobacterium wenxiniae]	hypothetical		4.96508
g8389_i0	VXB75181.1	hypothetical protein FRIGORI9N_420006 [Frigoribacterium sp. 9N]	hypothetical		2.93783
g2549_i0	WP_148919955.1	hypothetical protein [Sphingobacterium sp. SSI9]	hypothetical	RseC/MucC	3.61522
g1398_i0	AIA94441.1	Glyco_hydro_38C [uncultured bacterium]	Metabolism		3.05425
g1456_i0	WP_069134462.1	cytochrome P450 [Gammaproteobacteria bacterium 2W06]	Metabolism		2.7098
g1909_i0	PON14515.1	oxygen-dependent coproporphyrinogen oxidase [Candidatus Entotheonella serta]	Metabolism		2.68655
g732_i0	WP_093364513.1	TonB-dependent receptor [Sphingobacterium wenxiniae]	Signalling and Cellular Processes	susC	3.00253
g79_i0	WP_159613577.1	SusC/RagA family TonB-linked outer membrane protein [Tenacibaculum sp. KUL118]	Signalling and Cellular Processes	susC	3.99621
g1129_i0	WP_132863087.1	lipocalin family protein [Stenotrophomonas sp. ATCM1_4]	Signalling and Cellular Processes	blc	3.85746

CHAPTER IV

SELECTED LIFE-HISTORY TRAITS OF THE BLACK SOLDIER FLY (DIPTERA: STRATIOMYIDAE) PRODUCED AT AN INDUSTRIAL-SCALE IN CHINA

Summary

As the global food demand is increasing along with human population growth, there is greater need for alternative protein sources. Insect protein, especially the larvae of the black soldier fly (Diptera: Stratiomyidae), has become a key approach for solving this issue in part due to its ability to convert organic waste into insect biomass with minimal resource (e.g., land, water) requirements. However, most information utilized to develop industrial production of this species is reliant on data generated from laboratory-scaled studies. Unfortunately, scaling these data to an industrial level potentially is not linear resulting in over, or under, estimating production. In this study, we compared selected life-history traits of larval black soldier fly produced at benchtop (e.g., 1 L container with 614 larvae) and industrial scales (e.g., 29.5 L container inoculated with 10,000 larvae). Larvae were provided a single feeding (2 g/larva) in each treatment. Significant differences in the mean larval weight (24.7%), survivorship (-28.2%), and biomass conversion (2.7%) were determined between benchtop and industrial treatments. These results indicate larval number and the associated container size are important factors to consider when designing a black soldier fly factory. Furthermore, caution should be taken when applying data from

laboratory studies to industrial scale production systems as the values potentially are not linear.

Introduction

The global human population is rapidly expanding and is predicted to reach approximately 10 billion by 2050 (Nations 2017). In order to meet food demand, agricultural production will need to double (Tilman et al. 2011, Alexandratos and Bruinsma 2012). Protein, especially meat, is expected to have the greatest shortage due to inadequate resources needed for production. According to the World Wildlife Fund, beef alone requires more land than all other farmed animals and crops combined (WWF 2019).

A novel approach for meeting protein demands globally will be the mass production of insects. While this industry has a long cultural history (e.g., chupalinas (Orthoptera: Acridoidea) in Mexico, silkworms (Lepidoptera: Bombycidae) in China), mass production of insects for use as a feed is a more recent development (Hamamura 2001, Ramos-Elorduy 2002, Van Huis et al. 2015). In such cases, these insects can be mass produced and utilized as feed for livestock, poultry, and aquaculture (Van Huis et al. 2015). By using insects as the primary feed substrate, traditional materials (e.g., maize, soy) could be supplemented with insect protein or diverted directly to human consumption. Furthermore, some insects can be produced on products of little to no human value, such as food waste (Nguyen et al. 2015), livestock manure (Khan et al. 2012, Koné et al. 2017), animal waste (St‐Hilaire et al. 2007), and brewery waste (Chia et al. 2018).

Insect farming can also provide environmental benefits. Feeding these materials to insects protects air, land, and water from potential contamination (Van Huis et al. 2015). For example, the black soldier fly, *Hermetia illucens*, (L.) (Diptera: Stratiomyidae) can be fed food waste that would typically be placed in landfills *(*Khan et al. 2012). Accordingly, digestion of these materials suppresses noxious odors (Beskin et al. 2018), greenhouse gases (Perednia et al. 2017), and pathogens (Erickson et al. 2004, Liu et al. 2008, Lalander et al. 2013, Lalander et al. 2015). Furthermore, less land, water, and space, are needed to produce insects, such as the black soldier fly, than traditional animal production (Miglietta et al. 2015). Other benefits include fast development time (e.g., black soldier fly can develop to harvestable size within 14 d) (Tomberlin et al. 2002) versus beef (e.g., 12-18 months of feeding to reach the needed weight to slaughter) (WWF 2019). It is also worth noting that the full insect is edible unlike beef (48.5%) (Miglietta et al. 2015). Because of the ability of the black soldier fly to consume a variety of organic wastes, while offering benefits to the environment, it is now the 'crown jewel' of the insects as feed industry and is mass produced globally (Tomberlin and van Huis 2020), while also being approved for use as poultry feed in the USA and Canada as well as feed for select aquaculture species in the same locations as well as the European Union (AAFCO 2019, Einstein-Curtis 2019, IPIFF 2020).

Current knowledge about this species has been largely developed based on lab-scale (i.e., benchtop) studies (Diener et al. 2009, Paz et al. 2015, Beskin et al. 2018), including those major findings that have served as milestones in the black soldier fly farming

industry (Sheppard et al. 2002, Tomberlin et al. 2002, Tomberlin et al. 2009). For example, methods developed for mass production of this insect in colony were developed using 300 ml containers (Sheppard et al. 2002). While these methods serve as the cornerstone of all companies globally mass producing the black soldier fly, determining how to apply them at a larger scale is a challenge. The same can be said for other studies examining the conversion of waste into insect biomass (Lalander et al. 2019). In most instances, they were accomplished using small containers (e.g., 14 cm x 7.5 cm x 7 cm (Diener et al. 2009)), which are not necessarily the size used in mass production. For example, some industrial settings often use containers 10X larger (Yang, unpublished data).

Ultimately, scale matters when applying data from a small study to a much larger setting (McGill 2010). For example, with the black soldier fly, the temperature in an 665 mm x 435 mm x160 mm plastic bin with actively feeding larvae can be well above room temperature reaching 42℃ (Yang, unpublished data), which is not common with benchtop scale where containers are typically remain at ambient temperature (Meneguz et al. 2018b). Consequently, many "in house" studies by industry are necessary as a means to optimize black soldier fly production. Unfortunately, such data in many cases are private and have not been validated through open research channels. The objective of this study was to demonstrate the differences between benchtop and industrial scale with regards to black soldier fly production and waste conversion to insect biomass. By knowing whether the black soldier fly larvae exhibit different life-history traits at different scales, industry can better design production systems that optimize production and waste conversion.

Materials and Methods

Industrial Site Process

Experiments were conducted in a black soldier fly production facility designed and managed by JM Green Environmental Protection Ltd in Baotou, Inner Mongolia Province, China. The facility is part of a waste management project in Baotou, which is designed to recycle 100 metric tons restaurant kitchen waste per day.

Organic waste collected from local restaurants was used in the experiments. Upon delivery at the factory, the organic wastes, collected within 24 h, were first processed through an automatic sorter to remove non-food waste items, such plastic bottles and aluminum cans. Remaining waste is then ground into a slurry form with a particle size under 5 mm. The slurry was then cooked for 4 h at 80ºC before being processed through a centrifuge which produced three materials: lipids, liquids, and solids. The lipid phase was collected for sale as an ingredient for bio-diesel production, and the liquids are processed through a biogas reactor. There was usually 20 metric tons of remaining solids with 70-80% water content from a batch of 100 metric tons of raw waste, and these solid wastes were used for black soldier fly larval feedstock.

Prior to digestion with black soldier fly larvae, the solid sludge was placed in intermediate bulk containers (IBC tanks, 1000 L) and inoculated with 30 L of *Lactobacillus* culture and allowed to ferment for at least 24 h. After fermentation, which was indicated by the pH dropping from 6 to below 4, sludge, the material, now known as feedstock, was transferred into a feed mixer where ad lib amount of wheat bran was added

to reduce moisture to 70%. Once moisture content is adjusted, feedstock is pumped into growing pans, which are described below to feed the larvae.

Black Soldier Fly Population

One kilogram of black soldier fly eggs was received from a facility in Guangdong Province, China. Eggs were partitioned into 50 g allotments and placed on metal stand (20 cm in diameter) covered with a screen mesh (1.5 mm) and positioned 5cm above a neonate larval substrate in a 665 mm x 435 mm x 160 mm blue plastic bin. The neonate larval substrate was made with 50% fermented food waste prepared as beforementioned, and 50% wheat bran by volume, with water added to adjust moisture to 70%. Doing so allowed neonates to drop down to the tray and have immediate access to the feedstock. Trays were stored in a rearing room at 28ºC, 60-90% relative humidity, and 8L:16D. Neonates were allowed to feed for 4-5 d. Larvae were then separated with a 2 mm sifter and used to inoculate industrial pans containing feedstock (described below).

Experiment Design

Two trials of the following experiment were conducted. For each trial, six replicates were used for each treatment. For the industrial treatment, which is based in part on production in the Baotou facility, each replicated consisted of a 29.5 L white plastic tray (610 mm x 420 mm x 115 mm). For small-scale (benchtop), a 1 L plastic tray (155 mm x 155 mm x75 mm) was used as a replicate. Large scale containers were filled with 5 kg

feedstuff at the beginning (i.e., single feeding) of the experiment and inoculated with 10,000 larvae (2 larvae per gram of feedstuff). Each small-scale container was filled with 307 g feedstuff and inoculated with 614 larvae (2 larvae per gram of feed stuff). Accounting for amount of food provided, allowed for feed amount per larva to be excluded as a factor. The feed amounts were determined to ensure same amount of feed was placed per cm^2 so that the depths of the substrate were equal between these two containers. Both treatments were placed on the same rack for processing (Figure IV-1).

All experiments were conducted at 27-30 ºC, 60-90% RH, 8L:16D. Observations were made every 24 h. On the 7^{th} day of the experiment (harvesting point in factory) for industrial and small-scale treatments, larvae and frass from each tray/container were separated using a 5 mm mesh sieve. Total weight of harvested larvae and frass from each tray/container were recorded. Five subsamples of 10 larvae were randomly collected from each replicate and weight recorded in order to determine average larval weight.

Data Collection and Analysis

Following confirmation that data for a given variable measured met parametric requirements, an analysis of variance was performed (RStudio, version 1.1.383) followed by a Tukey's HSD (honest significant difference) to determine mean separation ($p \leq 0.05$).

Results

Final larval weight of black soldier fly larvae (Figure IV-2) resulting from the benchtop treatment was 24.7% greater than in the industrial treatment ($F_{1,22} = 177.7$, $p < 0.01$). However, survivorship (Figure IV-3) in the industrial treatment was 28.2% greater than in the benchtop treatment ($F_{1,22} = 253.1$, $p < 0.01$). And, larval mass conversion rate (Figure IV-4) was 2.7% greater in the industrial treatment ($F_{1,22} = 5.99$, $p < 0.05$).

Discussion

Black soldier fly production did not scale predictably from laboratory to industrial size in this study. Black soldier fly larvae reared at lower numbers in smaller containers (i.e., benchtop) were 24.7% bigger (Figure IV-2) than those reared at the industrial scale (i.e., each pan contained 16X more larvae are 16X bigger than the benchtop scale); however, survivorship (Figure IV-3) and conversion rates (Figure IV-4) were lower. These data have tremendous implications for the value of benchtop data being applied by industry.

At face value, these data would indicate the application of the benchtop data to an industrial production facility could grossly under-estimate production levels and conversion rates. If we were to translate benchtop data from the current study to industrial scale (i.e., system used in current study is applied in the industrial facility where the study took place), predictions would be inaccurate as far as production and thus financially. For example, using the data from the benchtop treatment to design a factory aiming to produce

100 metric tons of larvae with an average larval size of 0.15 g, they would require 5.6kg (28%) more eggs, which equals to $5,600 at current market price (Yang, unpublished data), in order to account for high mortality (Table IV-1).

In fact, larval conversion rates across the two treatments further emphasize this point. Larval mass conversion rate at the industrial scale big trays was 0.6% greater, which was statistically significant, than that at the benchtop scale. While unassuming, such a small difference translates into massive economic impact. For example, the factory used as the study site digests 20 tons of feedstuff daily with black soldier fly larvae. If the 0.6% were translated to this scale, it would equate to the additional production of 120 kg of fresh larvae, which equals to 40kg of dried product, every day. If extrapolated to one year, 0.6% increased production would yield 14,600 kg of dried product valuing over USD $250,000 in the US retail market as of 2019. Furthermore, the 0.6% greater conversion rate also means 30 cubic meter less frass a year (Yang, unpublished data), and in some cases this is preferred as it is seen as by-product of less value than the resulting larvae.

Producing larger larvae in a facility could reflect low survivorship as observed in this study. Larvae reared at the benchtop scale suffered greater mortality (Figure IV-3) than those in the industrial treatment. Consequently, those that survive experience less competition for resources. This result does not come as a surprise as it has been observed for other species. For example, size of *Lucilia cuprina*, (Wiedemann), (Diptera: Calliphoridae), which is a carrion colonizer, oscillated over generations when relegated to feed on a set carrion amount (Nicholson 1950). The initial generation over-populated the

carrion resulting in almost 100% mortality and 50% smaller flies being produced. Given the adults of the second generation were small, they under-populated the fresh carrion source (i.e., same size and quality as with the first generation) resulting in high survivorship and large adults. The same was observed in the current study. The largest larvae produced were from replicates that suffered the greatest mortality (i.e., reduced competition), while the smaller larvae were from the industrial replicates where mortality was lower (i.e., greater competition for food) Figure IV-2&3).

Defining the optimal larval density and associated feed rate within an environment (i.e., pan dimensions and food allocation) are critical for maximizing larval survival and subsequent production. As demonstrated in this study, smaller group of larvae in the smaller container resulted in less ideal survivorship, and this is not restricted to black solider fly, as described with *L. cuprina*. The suboptimal density impact insect survivorship in both ways, when higher larval density resulted in lower survivorship, opposite results were also determined with the butterfly *Euselasia chrysippe*, (Bates), (Lepidoptera: Riodinidae) where two times bigger group size resulted in 21.6 times greater survivorship (Allen 2010).

In some instances, greater larval number, to a degree, for some species can be beneficial. For example, greater larval density can suppress pathogen proliferation, such as with *Drosophila melanogaster*, Meigen, (Diptera: Drosophilidae) where fungi are known to grow on larval resources if the larval density is not above a given threshold (Wertheim et al. 2002). Optimal densities can also allow for thermoregulation which is

critical for development. Larval *L. curprina* aggregation and feeding can result in a microclimate 15 °C above ambient conditions, which allows them to develop two times faster than those at a lower density (Kotzé et al. 2016).

Such modifications of the environment (i.e., larval number, pan size, feed rate) could be critical for black soldier fly production. Under more natural conditions, black soldier fly larvae occur at high larval numbers in dung piles (Sheppard 1983, Sukarsih 1986) or carrion (Tomberlin et al. 2005), which are normally associated with unpredictable pathogen levels (Diclaro and Kaufman 2009). The ability of black soldier fly larvae to modify pH (Ma et al. 2018, Meneguz et al. 2018a) can result in suppressed pathogens (Wang et al. 2015) and improve the palatability of the food (Deshpande et al. 2015). Furthermore, black soldier fly larvae produce antimicrobial peptides (AMPs) that can inhibit a broad spectrum of bacteria (Park et al. 2014, Park et al. 2015, Vogel et al. 2018), and studies have shown that black soldier fly larvae can reduce *Escherichia coli* (Erickson et al. 2004, Liu et al. 2008), *Salmonella* spp. (Erickson et al. 2004, Lalander et al. 2013, Lalander et al. 2015), and phage (Lalander et al. 2015) in the waste. However, such processes are potentially partially regulated by larval number thus explaining the greater mortality in the low number, benchtop, treatment rather than the industrialized scale examined in the current study.

While not examined in the current study, it should be noted that the larger larvae produced at the benchtop scale may not equate quality larvae. Nutrient content of larvae can vary depending on size. Protein content is key factor regulating the use of black soldier

fly larvae as animal feed (Bondari and Sheppard 1981, Bondari and Sheppard 1987, St-Hilaire et al. 2007, Sealey et al. 2011, Widjastuti et al. 2014). However, black soldier fly larvae shift from protein to fat accumulation as they age (Liu et al. 2017). Although they only investigated nutrient content shift in relationship to larval age, other researchers have found lipid content increased exponentially with body size for other species. For example, protein content increased linearly with the growth of the yellow fever mosquito, *Aedes aegypti*, Linnaeus *in* Hasselquist, (Diptera: Culicidae); however, lipid accumulation was more exponential suggesting larger larvae would have more fat than smaller conspecifics (Timmermann and Briegel 1999). If the same occurs for black soldier fly larvae, then larger larvae potentially are not ideal due to their high fat content

This study demonstrated select life-history traits of black soldier fly larvae are impacted based on larval number and pan size (benchtop vs industrial). Standardizing the rearing containers for black soldier fly larvae production is critical for optimal production. Furthermore, while valuable, laboratory studies only scratch the surface in terms of elucidating the factors regulating larval growth and production. Future studies should consider such scale issues when evaluating industrial value of results generated and making recommendations.

References

AAFCO. 2019. Dried Black Soldier Fly Larvae. AAFCO 2019 Official Publication Association of American Feed Control Officials.

Alexandratos, N., and J. Bruinsma. 2012. World agriculture towards 2030/2050: the 2012 revision. ESA Working paper FAO, Rome.

Allen, P. 2010. Group size effects on survivorship and adult development in the gregarious larvae of *Euselasia chrysippe* (Lepidoptera, Riodinidae). Insectes Sociaux 57: 199-204.

Beskin, K. V., C. D. Holcomb, J. A. Cammack, T. L. Crippen, A. H. Knap, S. T. Sweet, and J. K. Tomberlin. 2018. Larval digestion of different manure types by the black soldier fly (Diptera: Stratiomyidae) impacts associated volatile emissions. Waste Management 74: 213-220.

Bondari, K., and D. Sheppard. 1981. Soldier fly larvae as feed in commercial fish production. Aquaculture 24: 103-109.

Bondari, K., and D. C. Sheppard. 1987. Soldier fly, *Hermetia illucens* L., larvae as feed for channel catfish, *Ictalurus punctatus* (Rafinesque), and blue tilapia, *Oreochromis aureus* (Steindachner). Aquaculture and Fisheries Management 18: 209-220.

Chia, S. Y., C. M. Tanga, I. M. Osuga, S. A. Mohamed, F. M. Khamis, D. Salifu, S. Sevgan, K. K. Fiaboe, S. Niassy, and J. J. van Loon. 2018. Effects of waste

stream combinations from brewing industry on performance of black soldier fly, *Hermetia illucens* (Diptera: Stratiomyidae). PeerJ 6: e5885.

Deshpande, S. A., R. Yamada, C. M. Mak, B. Hunter, A. S. Obando, S. Hoxha, and W. W. Ja. 2015. Acidic food pH increases palatability and consumption and extends *Drosophila* lifespan. The Journal of Nutrition 145: 2789-2796.

Diclaro, J., and P. E. Kaufman. 2009. Black soldier fly *Hermetia illucens* Linnaeus (Insecta: Diptera: Stratiomyidae). EENY 461: 1-3.

Diener, S., C. Zurbrugg, and K. Tockner. 2009. Conversion of organic material by black soldier fly larvae: establishing optimal feeding rates. Waste Management & Research 27: 603-610.

Einstein-Curtis, A. 2019. Canadian BSF producer gets CFIA approval for in-feed use. www.feednavigator.com.

Erickson, M. C., M. Islam, C. Sheppard, J. Liao, and M. P. Doyle. 2004. Reduction of *Escherichia coli* O157:H7 and *Salmonella enterica serovar Enteritidis* in chicken manure by larvae of the black soldier fly. Journal of Food Protection 67: 685-690.

Hamamura, Y. 2001. Silkworm rearing on artificial diet, Science Publishers, Inc.

IPIFF. 2020. EU Legislation. www.ipiff.org/insects-eu-legislation

Khan, H. A. A., S. A. Shad, and W. Akram. 2012. Effect of livestock manures on the fitness of house fly, *Musca domestica* L.(Diptera: Muscidae). Parasitology Research 111: 1165-1171.

Koné, N., M. Sylla, S. Nacambo, and M. Kenis. 2017. Production of house fly larvae for animal feed through natural oviposition. Journal of Insects as Food and Feed 3: 177-186.

Kotzé, Z., M. H. Villet, and C. W. Weldon. 2016. Heat accumulation and development rate of massed maggots of the sheep blowfly, *Lucilia cuprina* (Diptera: Calliphoridae). Journal of Insect Physiology 95: 98-104.

Lalander, C., S. Diener, C. Zurbrügg, and B. Vinnerås. 2019. Effects of feedstock on larval development and process efficiency in waste treatment with black soldier fly (*Hermetia illucens*). Journal of Cleaner Production 208: 211-219.

Lalander, C., S. Diener, M. E. Magri, C. Zurbrugg, A. Lindstrom, and B. Vinneras. 2013. Faecal sludge management with the larvae of the black soldier fly (*Hermetia illucens*) - from a hygiene aspect. Science of the Total Environment 458: 312-318.

Lalander, C. H., J. Fidjeland, S. Diener, S. Eriksson, and B. Vinnerås. 2015. High waste-to-biomass conversion and efficient *Salmonella* spp. reduction using black soldier fly for waste recycling. Agronomy for Sustainable Development 35: 261-271.

Liu, Q., J. K. Tomberlin, J. A. Brady, M. R. Sanford, and Z. Yu. 2008. Black soldier fly (Diptera: Stratiomyidae) larvae reduce *Escherichia coli* in dairy manure. Environmental Entomology 37: 1525-1530.

Liu, X., X. Chen, H. Wang, Q. Yang, K. ur Rehman, W. Li, M. Cai, Q. Li, L. Mazza, and J. Zhang. 2017. Dynamic changes of nutrient composition throughout the entire life cycle of black soldier fly. PLoS One 12.

Ma, J., Y. Lei, K. u. Rehman, Z. Yu, J. Zhang, W. Li, Q. Li, J. K. Tomberlin, and L. Zheng. 2018. Dynamic effects of initial pH of substrate on biological growth and metamorphosis of black soldier fly (Diptera: Stratiomyidae). Environmental Entomology 47: 159-165.

McGill, B. J. 2010. Matters of scale. Science 328: 575-576.

Meneguz, M., L. Gasco, and J. K. Tomberlin. 2018a. Impact of pH and feeding system on black soldier fly (*Hermetia illucens*, L; Diptera: Stratiomyidae) larval development. PloS One 13.

Meneguz, M., A. Schiavone, F. Gai, A. Dama, C. Lussiana, M. Renna, and L. Gasco. 2018b. Effect of rearing substrate on growth performance, waste reduction efficiency and chemical composition of black soldier fly (*Hermetia illucens*) larvae. Journal of the Science of Food and Agriculture 98: 5776-5784.

Miglietta, P., F. De Leo, M. Ruberti, and S. Massari. 2015. Mealworms for food: a water footprint perspective. Water 7: 6190-6203.

Nations, U. 2017. World Population Prospects: The 2017 Revision, World Population 2017 Wallchart. Department of Economic and Social Affairs. *ST/ESA/SER.A/398.*

Nguyen, T. T., J. K. Tomberlin, and S. Vanlaerhoven. 2015. Ability of Black Soldier Fly (Diptera: Stratiomyidae) Larvae to Recycle Food Waste. Environmental Entomology 44: 406-410.

Nicholson, A. 1950. Population oscillations caused by competition for food. Nature 165: 476-477.

Park, S.-I., J.-W. Kim, and S. M. Yoe. 2015. Purification and characterization of a novel antibacterial peptide from black soldier fly (*Hermetia illucens*) larvae. Developmental & Comparative Immunology 52: 98-106.

Park, S., B. Chang, and S. Yoe. 2014. Detection of antimicrobial substances from larvae of the black soldier fly, *Hermetia illucens* (Diptera: Stratiomyidae). Entomological Research 44: 58-64.

Paz, A. S. P., N. S. Carrejo, and C. H. G. Rodríguez. 2015. Effects of larval density and feeding rates on the bioconversion of vegetable waste using black soldier fly larvae *Hermetia illucens* (L.),(Diptera: Stratiomyidae). Waste and Biomass Valorization 6: 1059-1065.

Perednia, D., J. Anderson, and A. Rice. 2017. A comparison of the greenhouse gas production of black soldier fly larvae versus aerobic microbial decomposition of an organic feed material. Research and Reviews: Journal of Ecology and Environmental Sciences. 5: 10-16.

Ramos-Elorduy, J. 2002. Edible insects of chiapas, Mexico. Ecology of Food and Nutrition 41: 271-299.

Sealey, W. M., T. G. Gaylord, F. T. Barrows, J. K. Tomberlin, M. A. McGuire, C. Ross, and S. St-Hilaire. 2011. Sensory analysis of rainbow trout, Oncorhynchus mykiss, fed enriched black soldier fly prepupae, *Hermetia illucens*. Journal of the World Aquaculture Society 42: 34-45.

Sheppard, C. 1983. House fly and lesser fly control utilizing the black soldier fly in manure management systems for caged laying hens. Environmental Entomology 12: 1439-1442.

Sheppard, D. C., J. K. Tomberlin, J. A. Joyce, B. C. Kiser, and S. M. Sumner. 2002. Rearing methods for the black soldier fly (Diptera: Stratiomyidae). Journal of Medical Entomology 39: 695-698.

St-Hilaire, S., C. Sheppard, J. K. Tomberlin, S. Irving, L. Newton, M. A. McGuire, E. E. Mosley, R. W. Hardy, and W. Sealey. 2007. Fly prepupae as a feedstuff for rainbow trout, *Oncorhynchus mykiss*. Journal of the World Aquaculture Society 38: 59-67.

St‐Hilaire, S., K. Cranfill, M. A. McGuire, E. E. Mosley, J. K. Tomberlin, L. Newton, W. Sealey, C. Sheppard, and S. Irving. 2007. Fish offal recycling by the black soldier fly produces a foodstuff high in omega‐3 fatty acids. Journal of the World Aquaculture Society 38: 309-313.

Sukarsih. 1986. Growth and development of some filth flies in relation to moisture content of poultry manure. Penyakit Hewan 18: 134-140.

Tilman, D., C. Balzer, J. Hill, and B. L. Befort. 2011. Global food demand and the sustainable intensification of agriculture. Proceedings of the National Academy of Sciences 108: 20260-20264.

Timmermann, S., and H. Briegel. 1999. Larval growth and biosynthesis of reserves in mosquitoes. Journal of Insect Physiology 45: 461-470.

Tomberlin, J. K., D. C. Sheppard, and J. A. Joyce. 2002. Selected life-history traits of black soldier flies (Diptera: Stratiomyidae) reared on three artificial diets. Annals of the Entomological Society of America 95: 379-386.

Tomberlin, J. K., D. C. Sheppard, and J. A. Joyce. 2005. Black soldier fly (Diptera: Stratiomyidae) colonization of pig carrion in South Georgia. Journal of Forensic Science 50: JFS2003391-2003392.

Tomberlin, J. K., P. H. Adler, and H. M. Myers. 2009. Development of the black soldier fly (Diptera: Stratiomyidae) in relation to temperature. Environmental Entomology 38: 930-934.

Tomberlin, J. K., and A. van Huis. 2020. Black soldier fly from pest to 'crown jewel' of the insects as feed industry: an historical perspective. Journal of Insects as Food and Feed 6: 1-4.

Van Huis, A., M. Dicke, and J. J. van Loon. 2015. Insects to feed the world. Wageningen Academic Publishers.

Vogel, H., A. Müller, D. G. Heckel, H. Gutzeit, and A. Vilcinskas. 2018. Nutritional immunology: diversification and diet-dependent expression of antimicrobial

peptides in the black soldier fly *Hermetia illucens*. Developmental & Comparative Immunology 78: 141-148.

Wang, C., T. Chang, H. Yang, and M. Cui. 2015. Antibacterial mechanism of lactic acid on physiological and morphological properties of *Salmonella enteritidis, Escherichia coli* and *Listeria monocytogenes*. Food Control 47: 231-236.

Wertheim, B., J. Marchais, L. E. M. Vet, and M. Dicke. 2002. Allee effect in larval resource exploitation in Drosophila: an interaction among density of adults, larvae, and micro‐organisms. Ecological Entomology 27: 608-617.

Widjastuti, T., R. Wiradimadja, and D. Rusmana. 2014. The effect of substitution of fish meal by black soldier fly (*Hermetia illucens*) maggot meal in the diet on production performance of quail (*Coturnix coturnix japonica*). Scientific Papers, Series D. Animal Science 57: 125-129.

WWF. 2019. Beef, World Wild Fund for Nature. www.worldwildlife.org/industries/beef

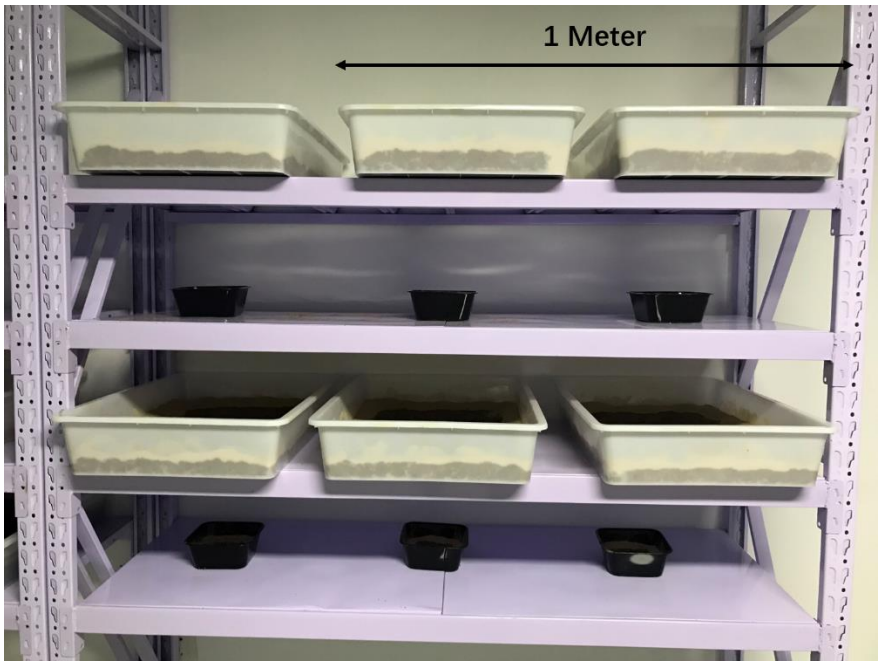

Figure IV-1. Setup for industrial and small-scale black soldier fly larval production. Pans containing feedstock and black soldier fly larvae were randomly placed on shelves in a rearing room 27-30 ºC, 60-90% RH, 8L:16D.

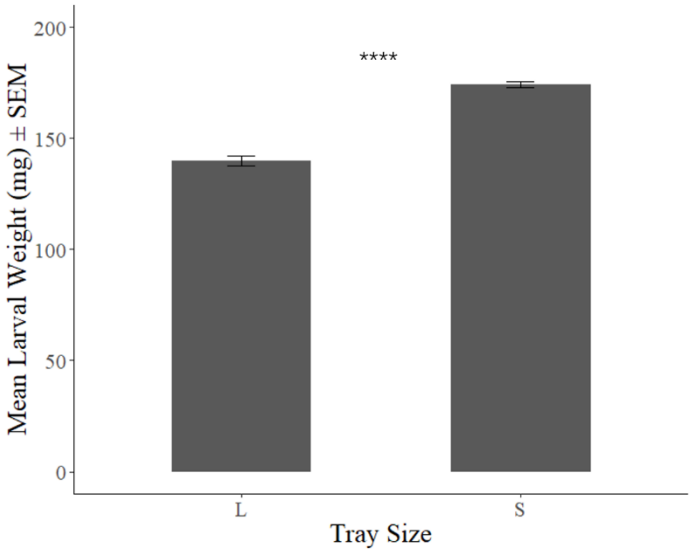

Figure IV-2. Mean larval mass weight after 7 days of rearing in different size containers. Containers were placed in 27-30 ºC, 60-90% RH environment, with 8L:16D photoperiod. **** ANOVA test result indicates mean values from the two treatments are significantly different with $p < 0.05$.

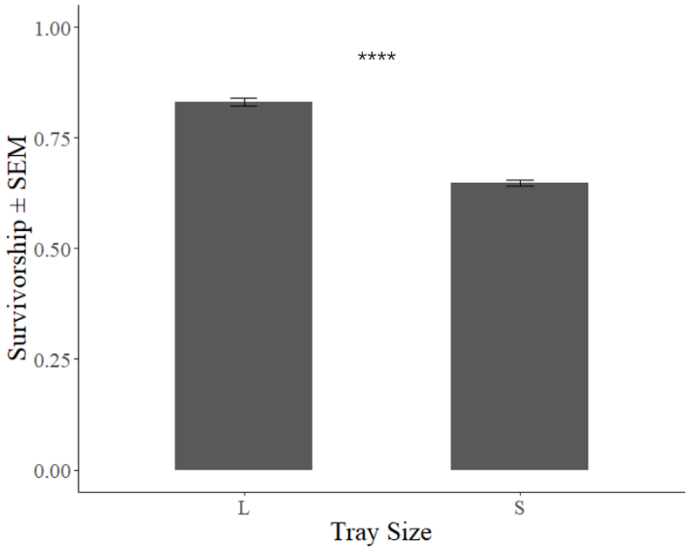

Figure IV-3. Larval survivorship after 7 days of rearing in different size containers. Containers were placed in 27-30 ºC, 60-90% RH environment, with 8L:16D photoperiod. **** ANOVA test result indicates mean values from the two treatments are significantly different with $p < 0.05$.

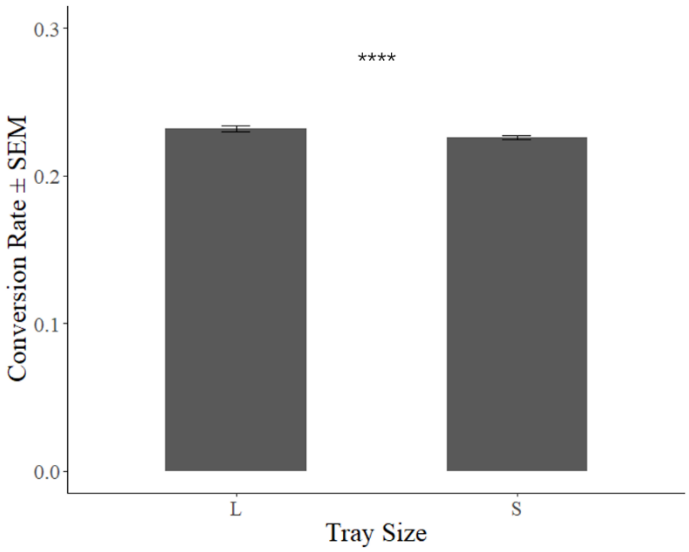

Figure IV-4. Larval mass conversion rate after 7 days of rearing from different containers. Containers were placed in 27-30 ºC, 60-90% RH environment, with 8L:16D photoperiod. The conversion rate was calculated with the total weight of larvae harvested at the end divided by the weight of the feed started in the respective containers. **** ANOVA test result indicates mean values from the two treatments are significantly different with $p < 0.05$.

Table IV-1 Projection of amount of waste and eggs needed in order to produce 100 metric tons of black soldier fly larvae using industrial large-scale data versus benchtop small-scale data.

	L[1]	**S[2]**	**% Difference**
Amount of Waste (metric ton)	431	442	2.6%
Amount of Eggs[3] (kg)	20.1	25.7	27.9%

[1]L = 83.1% survivorship, larval size = 0.15g each, conversion rate = 23.2%

[2]S = 64.8% survivorship, larval size = 0.15g each, conversion rate = 22.6%

[3]Each gram of black soldier fly egg contains 40,000 individuals (Cammack, unpublished data)

CHAPTER V

CONCLUSIONS

The black soldier fly, *Hermetia illucens*, (L.) (Diptera: Stratiomyidae) is an insect species that has been given increasing attention around the world for its potential to provide sustainable protein production for the food and feed industry (Tomberlin 2020). Its ability to convert waste into insect biomass within a short among of time is very efficient among all livestock (Diener et al. 2009). However, in order to achieve stable mass-production of black soldier fly larvae, there are many questions remaining to be answered. In this dissertation, I explored the interactions between microbes and the black soldier fly larvae, as well as scalability of its mass rearing. My researched showed starving a host, in this case black soldier fly larvae, could alter its associated microbial structure (chapter 2) and function (chapter 3). Furthermore, my research demonstrated "scale" is an important factor regulating black soldier fly production (chapter 4).

Chapters 2 and 3 demonstrate starvation impacts associated microbial structure and function in black soldier fly larvae. I determined the microbial community diversity at the phylum level waste significantly decreased in starved larvae ($q < 0.05$), and genus *Actinomyces* increased its presence through the starvation process. I also determined microbes experienced decreased gene expression, such as nutrient metabolism and environmental information processing, in the starved cohort.

My research also demonstrated data generated from laboratory studies (i.e., benchtop) do not scale up to industrial scale in a linear manner (chapter 4). I determined that black

soldier fly larvae reared in large-scale containers (10x larger) produced smaller larvae (-24.7%) than in the benchtop scale containers, though mainly due to higher mortality rate in the benchtop containers (28.2%) which resulted in a lower competition situation. The larval biomass conversion rate was found similar with only 2.7% difference, as a result of the balanced compensation between larval size and mortality rate in each treatment. My research suggested that container size had a significant impact on the life-history traits of the black soldier fly larvae, and thus selecting a container for a factory should also consider the biological factors, besides the engineering convenience, for optimal productions.

These findings (chapter 4) could be used to enhance black soldier fly production at an industrial scale. First of all, it provides guidelines at the planning phase where designers can more precisely predict output and how to achieve optimization in terms of industrial layout of the facility. For example, if the designer applied the benchtop dataset in the planning for a facility that would use larger trays for rearing, he would have overestimated the need for eggs by 28%. Secondly, my findings would encourage the industry to look for the best tray size that will carry out the optimal survival rate for the black soldier fly larvae.

There were limitations to my research. While my research (chapters 2 and 3) showed larval condition (i.e., starvation) could influence the associated microbial structure or functions, the mechanisms of how the bacteria and the larval host interact were not fully explored. Indeed, only 5253 out of 5581 genes with coding potential got a hit from the 7 gene database, so still 6% of the genes remained totally unexplored. Future studies on interkingdom communications between black soldier fly larvae and the associated bacteria

are needed to better understand the full extent to which whether this relationship exists in this system, and how significant such communication impacts the development or behavior of the larvae (Hughes and Sperandio 2008). My research cannot adequately demonstrate the physiological shift of the black soldier fly larvae during either fed or starved conditions. Simply following the rules of the Red Queen Hypothesis (Van and Van Valen 1973), one would assume associated microbes are likely under constant selective pressure from the host immune response as well as to metabolic stress conditions, and thus more works need to be done to measure the global connections between microbe-host interaction feedback loops, for instance, influence of larval host immune system (Vallet-Gely et al. 2008), and insulin-like signaling on the microbial structure and function (Shin et al. 2011).

Limitations were also identified with the industrialized experiment (chapter 4) completed. While I only tested one size container (29.5L, 610 x 420 x 115 mm) as the industrial scale, companies globally employ a variety of differently shaped and sized containers. Fundamentally, any black soldier fly farm is only doing one thing, and that is putting an identified amount of seed larvae and feedstock in a certain size container. Once this step is done, the rest of the job is just to maintain an optimal ambient condition, which has been extensively studied throughout the past decades. If these values are stable, the production output of this system should consistent and predictable. However, research is needed to determine the appropriate measures of seed larvae and diet to optimize production. Therefore, the container being used to raise the black soldier fly larvae is the basic unit of any BSF farm. For example, the L tray (29.5L, 610 x 420 x 115 mm) used in

this experiment can process 5 kg waste within 7 days. For a facility that wants to process 10 tons of feedstock per day, the facility will need to manage 14,000 trays minimum. In fact, not only the number of larvae or amount of waste per pan dependent on the container size, but all equipment in the factory, such as the hopper size and position, the conveyor belt size to move the trays, etc, is dependent on the container dimensions. Therefore, the container is the fundamental units in a facility, and all process and hardware are just to accommodate the use of this basic units.

As the industry is still searching for the ideal design of the “basic” units, the system machinery cannot be standardized, and thus the cost of the production systems is extremely high due to the customization demand and low volume manufacturing. Only once the industry has reached an agreement in terms of a container design to raise the black soldier fly larvae, can the standardization of the system machinery as well as the standard operation protocol to manage larval number and waste amount per pan be accomplished.

At the most fundamental level, my work revealed a novel aspect of insect and microbe interaction due to the state of host starvation, and discovered the size of the rearing container has a significant impact on the life-history traits of the black soldier fly larvae.

References

Diener, S., C. Zurbrugg, and K. Tockner. 2009. Conversion of organic material by black soldier fly larvae: establishing optimal feeding rates. Waste Management & Research 27: 603-610.

Hughes, D. T., and V. Sperandio. 2008. Inter-kingdom signalling: communication between bacteria and their hosts. Nature Reviews Microbiology 6: 111-120.

Shin, S. C., S.-H. Kim, H. You, B. Kim, A. C. Kim, K.-A. Lee, J.-H. Yoon, J.-H. Ryu, and W.-J. Lee. 2011. *Drosophila* microbiome modulates host developmental and metabolic homeostasis via insulin signaling. Science 334: 670-674.

Tomberlin, J. K. H., A. van. 2020. Black soldier fly from pest to 'crown jewel' of the insects as feed industry: an historical perspective. Journal of Insects as Food and Feed 6 (1): 1-4.

Vallet-Gely, I., B. Lemaitre, and F. Boccard. 2008. Bacterial strategies to overcome insect defences. Nature Reviews Microbiology 6: 302-313.

Van, V., and L. Van Valen. 1973. A new evolutionary law. Evolutionary Theory 1: 1-30.