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The effect of bacterial supplementation on black soldier fly larval growth and development

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By

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With a growing human population, food insecurity is becoming a worldwide problem. As the search for sustainable sources of protein continues, black soldier fly larvae come to the forefront as a partial solution. Full of proteins and fats, the larvae will consume most organic matter and rapidly develop into a usable form. Supplementing black soldier fly larvae with oleaginous microbes *Arthrobacter* AK19 and *Rhodococcus rhodochrous* increases their body size by 25%, potentially accelerates their development by one day, and increases their conversion efficiency. Supplementing with *Bifidobacterium breve* decreased body size, slowed development, and decreased conversion efficiency, underscoring the importance of selecting supplemental microbes and testing first on a small-scale. Promising results on the small-scale led to an industrial study, where similar results were also seen, resulting in greater optimization of this system.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

There are two definitive problems currently afflicting the world that black soldier fly farming and associated research can help mitigate. The first, global demand for food produced for human consumption is predicted to increase by 100% over the span of the next 40 years[1]. Despite efforts to keep up, agricultural production is not expected to meet the demand[2], as demand is predicted to increase by 100-110% by 2050[3] and only a 60-70% increase is predicted in animal products in the same time period[4]. Increased need for cattle and other animal proteins requires increased feed production with limited available land. The space and water needs of both livestock and production of their feed accounts for nearly 70% of all the land used in agricultural production[5]. Second, as both human and livestock populations grow there will be an increase in waste production. Manure, food scrap waste, and agricultural waste all produce greenhouse gases and noxious odors as well as serve as potential incubators for pathogenic microbes [6–8]. Therefore, safe and effective waste management solutions must be developed.

Entomophagy, the consumption of insects, has been practiced globally and throughout history, spanning nearly every culture[9]. Insects are a great source of healthy fats, proteins, and some trace elements[10]. It can be considered more environmentally responsible to grow insects as a source of protein rather than rearing animal proteins because not only do insects require twelve times less feed[5] and significantly less water[11,12]

to produce the same amount of protein as livestock, but they also produce significantly fewer greenhouse gas and ammonia emissions than any currently raised conventional livestock[13–15]. Insects do not even have to totally replace animal proteins to be able to reduce the environmental impact of livestock production systems. Portions of the animals' diets can be substituted with insect proteins[4], and the animal waste can also be managed with insects[16], which may then be used for feed. The low cost and space needs of insect rearing make it an ideal part of a solution for low-tech areas struggling with feed security but can be scaled up for higher production to address a global problem.

The black soldier fly (*Hermetia illucens* (L.)) (Diptera: Stratiomyidae) is one of about 2000 species of insect used as food[17]. The adult black soldier fly (BSF) is a non pest species, is not a known vector of disease agents, and does not bite. Additionally, the adult fly does not need to take up any food as it can survive on fat reserves accumulated during its larval stage[18]. The larvae of the black soldier fly (BSFL) are known as voracious feeders that will consume and degrade most organic materials, with a conversion rate ranging from 55% to 70%[19–21]. They are able to degrade everything from fruit and vegetables, and animal remains, and have successfully been used to manage and degrade manure[22]. These wastes can then be converted into insect biomass that is rich in both proteins and fats[23].

Substitution or partial replacement of traditional diets with BSFL has had positive results. Weaned pigs fed a diet consisting of 50% BSFL showed a 9% improvement in feed efficiency[22,24]. Similarly, a study conducted on rainbow trout showed that replacing up to 40% of the fish's diet with BSFL showed no negative effects on both the fish's physiology and the quality of meat, but unfortunately there were lower levels of healthy polyunsaturated fatty acids[25]. BSFL have also been fed to poultry, usually because they are

natural colonizers of poultry manure and have been used by farmers to help with waste management and prevent the manure from becoming a pollution issue[26,27]. In many studies, BSFL were deemed a partial substitute for soybean or corn meal feed. When used to feed broiler quails, there was no difference in yield between quails whose diet had been partially replaced with BSFL and those who ate their usual diet[28], but they did have improved amino acid levels that pushed the meat towards more nutritious, and increased the saturated and monounsaturated fatty acids found in the meat[28]. One more poultry study conducted with broiler chickens found that while feeding BSFL to the chickens did increase the levels of undesired fatty acids, defatting the BSFL decreased this effect[25].

BSFL are poised for mass production for proteins and oils as we know more about this species than any others that hold the same potential. Numerous companies both in the United States and abroad are attempting to rear them for mass production as food, as feed, and as a waste management and conversion solution. However, the system has not been optimized for maximum production of proteins and lipids or for maximum waste degradation. The first step to their optimization is performing experiments on the benchtop, in order to determine variability and efficiency in methodology. It is important, however, to recognize that differences may be found when results at a small scale are compared to those obtained at the industrial scale. Reasons for this may include the sheer number of larvae in an industrial scale, as nutrient availability and access to food for each individual will differ from the small-scale. In small scale studies, the larvae have less competition and easier access to food, as well as less surface area. On the industrial scale they must compete with thousands, not hundreds, of other larvae for resources. This in turn will influence waste conversion and feeding efficiency. Similarly, moisture content and the heat of the

entire system will not be the same as on the benchtop because of increased number of larvae seeking out food. Studies conducted on a small-scale are important for initial results, determination of important variables, and fine-tuning methodology, but must be conducted on an industrial level before these methods can be considered for application to "real world" or to a commercial setting.

BSFL have been shown to decrease the amount of pathogens such as *Salmonella enterica* and *Escherichia coli* in its substrate[29–31] and can become contaminated with the bacteria they encounter[32]. Furthermore, studies have shown beneficial effects through bacterial supplementation. For instance, inoculating poultry manure used to raise BSFL with a bacteria, *Bacillus subtilis*, increased the growth of the larvae[33]. Both of these studies show that these larvae can be influenced by probiotic additions. By definition, probiotics are "viable microorganisms that, when ingested, have a beneficial effect"[34]. In human intestinal health, probiotics are able to inhibit adherence of pathogens, compete for nutrients, and stimulate immunity[35]. In insects, probiotics have been found to have beneficial effects. One study showed that *Enterococcus kuehniella* isolated from larval moth feces and orally administered to red flour beetle larvae increased infection survival rates of the beetle larvae due to the probiotic's antimicrobial activity[36] However, in another study bees fed sugar syrup supplemented with *Lactobacillus rhamnosus* (a commercially available probiotic) were more susceptible to disease, and had a shorter lifespan[37]. The latter study underscores the importance of probiotic selection in measuring health and functional outcomes.

Bacteria also provide nutrition in the form of triglycerides and lipids that are essential for insect growth and reproduction and provides energy needed during extended

nonfeeding periods. This is particularly true during the larval stage where energy reserves are accumulated within the fat body to be utilized during metamorphosis. There is great diversity in the concentration of lipids present in bacterial species. For instance, oleaginous microbes have a high lipid content , which composes about 20% or more of their biomass[38]. Oleaginous microbes are excellent candidate organisms for the bioprocessing of chitinous waste (such as the exoskeletons of dead adult BSF), as many possess the enzymatic machinery to break down chitin and protein. Additionally, they can synthesize and accumulate triacylglycerides, similar in composition to vegetable oils, a primary material for biodiesel production[39]. In a large scale nearly closed-loop system, rearing facilities could use adult flies allowed to emerge for breeding as a portion of the media used to grow the oleaginous microbes, eliminating waste output from the system and further cementing BSFL-rearing for protein as an environmentally conscious effort.

For these reasons, I selected two oleaginous microbes for the bacterial supplement studies described within this work to supplement into the BSFL diet mixture: *Arthrobacter AK19* and *Rhodococcus rhodochrous 21198*. Both *Arthrobacter* and *Rhodococcus* species have previously been investigated as a means of bacterial hydrocarbon synthesis ultimately to be used in making biofuels[40,41].

My hypothesis was that the addition of fat-rich bacteria to the feeding substrate of BSFL would increase body mass, development rate, feed-to-body mass conversion, and nutrient density.

In the following chapters, I present methodology and results of both small-scale and industrial scale research where BSFL were supplemented with oleaginous microbes. Additionally I conducted a small-scale study where I supplemented BSFL diet with

Bifidobacterium breve, a well characterized human probiotic[42]. In all studies, I determined growth, waste conversion, and gut microbiome composition in an effort to determine the utility of bacterial supplementation in BSFL rearing and industrialization, and the role gut microbes play in BSFL diet metabolism. Results of this work demonstrate the effectiveness of bacterial supplementation to BSFL food to BSFL growth and waste conversion, and the importance of probiotic choice in feeding experiments.

CHAPTER II

MATERIALS AND METHODS

2.1 Fly colony

Black soldier fly eggs were collected from a colony at the Forensic Laboratory for Investigative Entomological Sciences (FLIES) facility at Texas A&M University. Eggs were collected in three layers of 2x3cm corrugated cardboard blocks placed above approximately 500g of spent grain diet saturated with water. The cardboard was replaced daily, and cardboard containing eggs was placed in a one-liter deli cup and held in an incubator at 70% relative humidity, 27°C and 12:12 L:D until the eggs have hatched. The larvae were shipped to Mississippi State University Department of Biological Sciences when the larvae were eleven days old for each of the experiments conducted at Mississippi State University.

2.2 Bacterial Growth and Collection

Both *Rhodococcus rhodochrous* 21198 and *Arthrobacter AK19* were grown on Luria nutrient agar and broth at pH 6.8 and 26°C for three days, then collected by either scraping the plates or centrifuging the broth and collecting the pellet. All of the collected bacteria were washed in a saline solution to remove residual nutrient media. *Bifidobacterium breve* was grown anaerobically at 37°C on plates and collected by scraping plates.

2.3 *Arthrobacter* AK19 Supplementation: Small-scale

Eleven-day old BSFL were sent to MSU from Dr. Tomberlin's lab at Texas A&M University. Larvae were divided into sets of 300 larvae, with each set placed into control or treatment containers in triplicate. A perforated plastic wrap was secured on the top of the containers to prevent escape. Larvae in control containers were fed daily with 18.0g Gainesville diet (a standard plant based diet composed of 30% alfalfa meal, 20% corn meal, and 50% wheat bran with water[43]), while treatment containers were fed 16.65g Gainesville diet supplemented with 1.35g (7.5% of diet) of *Arthrobacter* AK19. The control diets received additional water in place of a supplement to make up for the moisture difference. Initial weights of the larvae were recorded by randomly selecting 25 larvae from the containers and weighing them, as well as initial weight of the diets. Every 24 hours the larvae were separated from their feeding substrate. The feed and waste in the container were weighed, as well as the larvae in sets of 25. Containers were kept in a controlled and constant environment at room temperature. After 10 days, the experiment was stopped and the larvae and waste were immediately weighed. Waste from each replicate was collected, weighed, and dried at constant temperature (55°C) for 24H then weighed again. Remaining larvae and waste from immediate collection were frozen in -20C until further analysis.

2.4 *Bifidobacterium breve* Supplementation Experiment: Small-scale

A similar treatment plan was followed for *B. breve* as was described above, except instead of 300 larvae per cup only 100 larvae were placed into each treatment container. An identical feeding plan and percent bacteria were used. Instead of placing

the bacterial supplement into each day's diet and then feeding, *B. breve* was grown on plates anaerobically until 1% by weight of the total diet could be replaced with the supplement. The entire volume of diet required for a 10-day experiment was weighed out and prepared with the appropriate volume of water in advance. The diet was placed in an anaerobic chamber, maintained by anaerobic packs that were changed out daily. The inoculum amount was added into the diet and allowed to colonize and the diet required for the entire experiment was kept at growing temperature. The BSFL larvae in their treatment containers were left on the benchtop at room temperature with a perforated plastic wrap on top of each cup to prevent escape. In order to make sure temperature was not significantly different between the control and treatment groups, the diet and water for the control groups was also kept in the same incubator and was the same temperature during feedings.

2.5 *Arthrobacter* AK19 and *R. rhodochrous 21198* Supplementation: Industrial scale

The industrial scale experiments were conducted using *Arthrobacter* AK19 and *R. rhodochrous* 21198. Gainesville diet was used as a diet base where either 8.0g of *R. rhodochrous* 21198 or *Arthrobacter AK19* was added to 6kg of diet per pan (four pans per treatment or control, N=12 total), stirring with gloves for 30 seconds to disperse the supplement. Following this, approximately 10,000 larvae were added to each pan of either non-supplemented or supplemented. Each treatment condition (*Arthrobacte*r-supplemented, *Rhodococcus*-supplemented, and control) had 4 replicates. The larvae were allowed to feed constantly on the initially placed food source. The pans were mixed daily

to ensure that temperature spikes due to composting action did not occur. Every three days, a subset of 500 larvae was removed from the pans, weighed, and frozen for later analysis. A sample of the waste was also collected and frozen for later analysis. The experiment was carried out for 10 days. On the final day, 500 larvae from each replicate were removed, weighed, and frozen. The rest of the larvae were sifted from the waste and the total mass of the larvae, as well as the total mass of waste, was weighed. One liter of waste was saved and dried down to determine moisture content.

2.6 DNA extraction

DNA from the subsets of larvae and waste were isolated using a modified protocol of that discussed in Williamson, et al., 2014[44], quantified by a Qubit 2.0, and purified using a Qiagen DNA clean-up kit. Genomic DNA was extracted from all replicates and the extracts were subsequently pooled. The DNA was amplified with V4 primers and suggested protocols by the Earth Microbiome Project[45] and visualized by gel electrophoresis. Verified amplifying DNA was sent to Michigan State University Sequencing Facility for paired-end 16S metagenome sequencing.

2.7 DNA Sequencing

Microbial DNA samples were sequenced using Illumina MiSeq of 2 x 250 bp paired-end reads following 16S library construction, both performed by the Michigan State University Genomics Core Facility. The V4 hypervariable region of the 16S rRNA gene was amplified using dual indexed Illumina compatible primers 515f/806r as described by Kozich, JJ (2013)[46]. PCR products were normalized using Invitrogen SequalPrep DNA Normalization plates and the products recovered from the plates

pooled. This pool was cleaned up with AMPureXP magnetic SPRI beads. The pool was QC'd and quantified using a combination of Qubit dsDNA HS, Advanced Analytical Fragment Analyzer High Sensitivity NGS DNA and Kapa Illumina Library Quantification qPCR assays. Sequencing of the pooled amplicons was on an Illumina MiSeq v2 standard flow cell using a 500 cycle v2 reagent cartridge. Custom Sequencing and index primers were added to appropriate wells of the reagent cartridge as described in Kozich, et.al, 2013[46]. Base calling was done by Illumina Real Time Analysis (RTA) v1.18.54 and output of RTA was demultiplexed and converted to FastQ format with Illumina Bcl2fastq v2.19.1.

Raw fastq files barcoded Illumina 16S rRNA paired-end reads were assembled, quality-filtered, demultiplexed, and analyzed in QIIME version 1.8.0[47]. Reads were discarded if they have a quality score < Q20, contained ambiguous base calls or barcode/primer errors, and/or were reads with < 75% (of total read length) consecutive high-quality base calls. Chimeric reads were removed using the default settings in QIIME[48]. After quality control, the remaining sequences were binned into OTUs at a 97% sequence similarity cutoff using UCLUST[49]. Assembled sequence reads were classified into Operational Taxonomic Units (OTUs) on the basis of sequence similarity. The highest-quality sequences from each OTU cluster were taxonomically assigned using the RDP classifier[50] and identified using BLAST against reference sequences from the most current Greengenes 97% reference dataset (http://greengenes.secondgenome.com) [51–53]. Representative sequences of all OTUs were aligned to the Greengenes reference alignment using PyNAST[54], and low abundance OTU's (<0.0005% of reads in the total dataset) were

removed[55]. Samples were rarefied to achieve equal coverage per sample and those samples with fewer sequences were used in subsequent analyses.

2.8 Analyses of Microbial Diversity

Bacterial diversity was assessed through the Chao1 estimator and the Shannon index, calculating both indexes after subsampling with QIIME and data against the Greengenes Database, to avoid sequencing effort bias. Relative abundance was also assessed and plotted at family level using the R vegan and phyloseq statistical packages. Family level abundance less than 2% were not shown. Principal Coordinates Analysis (PCoA) and Bray-Curtis dissimilarity index were used from the R statistical package to study community composition, assessing the statistical significance of the differences in sample groupings through Bray-Curtis distance matrices and Adonis testing. Statistical tests used in the study were two-sided, and a p value of 0.05 or less was reported as statistically significant.

2.9 Determination of Functional Capacity using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt)

In order to predict genes from the metagenome, closed-reference operational taxonomic units (OTUs) were obtained from the filtered reads using QIIME version 1.8.0[47]. The biom-formatted OTUs table was then loaded to PICRUSt on the online Galaxy version in the Langille Lab (v1.1.1), alongside the Greengenes database (last updated June 2017). PICRUSt software estimates functional potential from the community metagenome using copy normalized 16S rRNA sequencing data whose gene contents are contributing to KEGG (Kyoto Encyclopedia of Genes and Genomes) identified pathways. Functionally annotated genes that were identified were compressed

into 12 general gene families. Comparisons were made between differences in annotated gene abundance from control and treatment groups to determine the percent change of treatment as compared to control groups. Only those with gene abundance at or above 25% change were considered for analysis. All raw data for the PICRUSt analyses are stored in the Jordan lab hard drive.

2.10 Random forest

The Random Forest R statistical package was used in order to determine the most important taxa for classifying samples by treatment. Random Forest is an emsemble machine learning method that fits decision trees onto subsamples of a data set. Then it combines these results to improve regression accuracy[56]. The output MeanDecreaseGini values describes which predictor variables have a greater role in splitting the data.

CHAPTER III

RESULTS

3.1 Small-scale *Arthrobacter* study

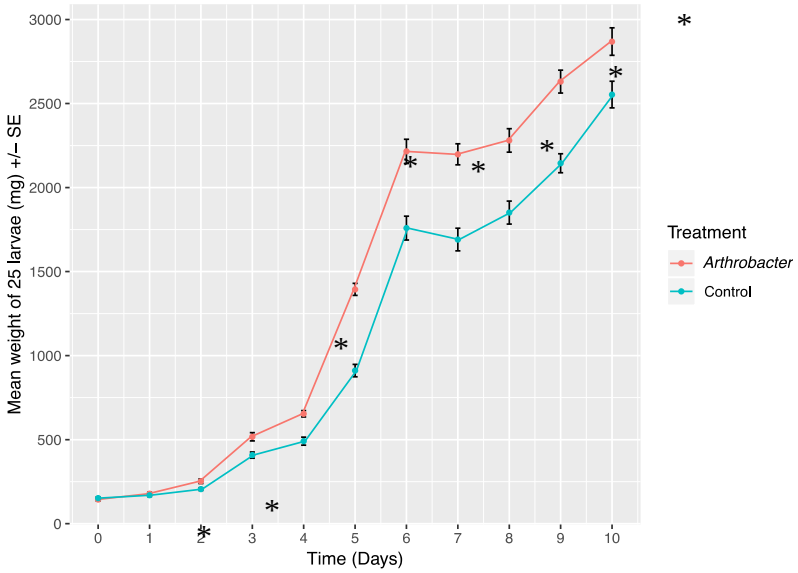

Figure 3.1 Mean daily weights for *Arthrobacter* AK19 supplemented larvae compared to control

The mean daily weights for the small-scale *Arthrobacter AK19* supplementation are presented in Figure 3.1. The mean daily mass of supplemented larvae was greater than the control-diet larvae. On the third day, larvae in treatment groups were 94% larger than in the control; interestingly, larval mass had increased 107% from day 2 to day 3, whereas control larvae only increased mass by 28% from day 2 to day 3. A similar but steadily diminishing trend was seen in later timepoints: On day 5, treatment larvae were 58% larger than control larvae and increased their mass by 113% from day 4. Despite control larvae having an 85% increase in mass compared to the day 4 timepoint, their overall mass was still less than treatment larvae. There was a significant difference in mean daily weight between treatment and control larvae at day 3 ($p = 0.007$), day 4 ($p = 0.0003$), day 5 ($p = 0.005$), day 6 ($p = 0.001$), day 7 ($p = 0.0006$), day 8 ($p = 0.007$) day 9 ($p = 0.002$) and day 10 ($p = 0.015$).

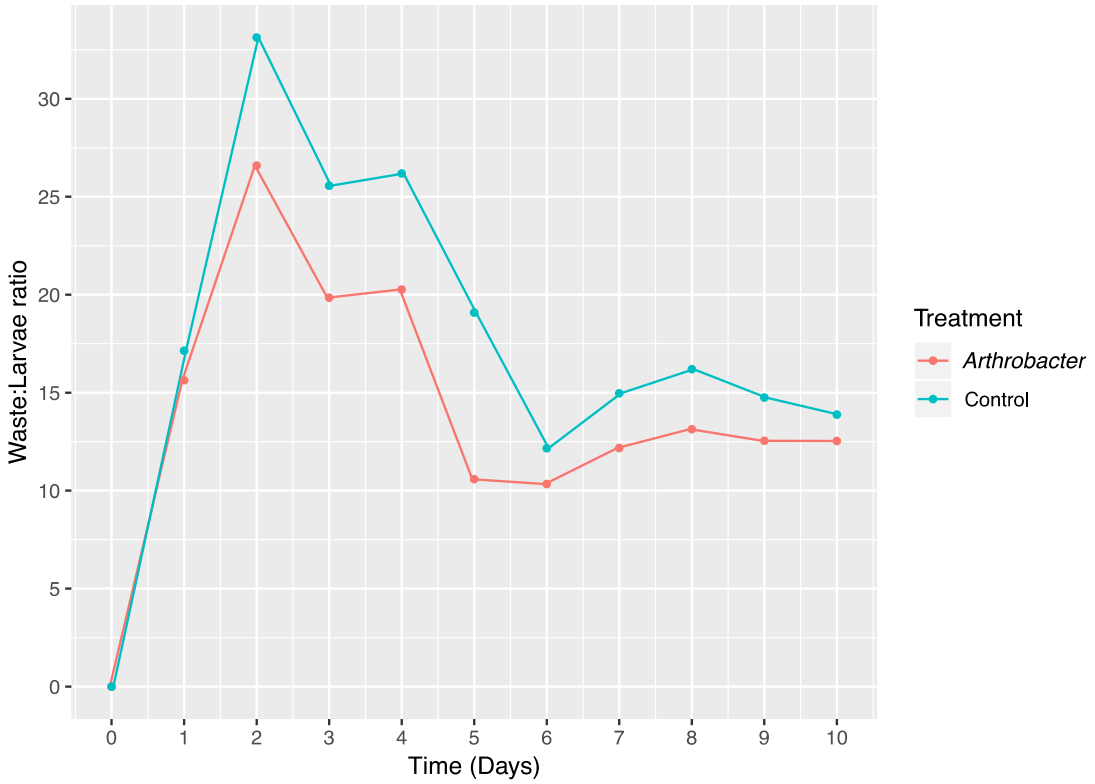

Figure 3.2 Waste:larvae ratio for *Arthrobacter* supplemented larvae as compared to control

The waste:larvae ratio was calculated for timepoints 2-10. An overall lower waste:larvae ratio was observed in *Arthrobacter* supplemented groups, revealing the bacterial supplemented larvae increased their capacity to break down and ingest the feeding substrate and convert it to biomass. The waste:larvae ratio increased during the first two days, and peaked at day 2. As the larvae continued feeding daily, the ratio decreased until the larvae neared the prepupal stage from T6-T10.

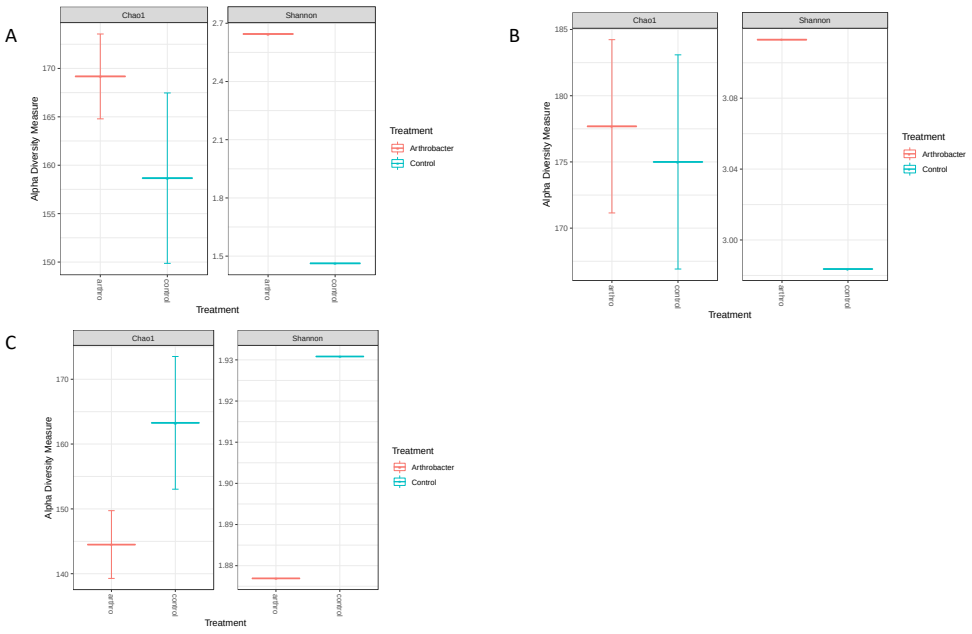

Figure 3.3 Alpha diversity measures of number of observed species (Chao1) and abundance and evenness (Shannon) in *Arthrobacter* supplemented larvae compared to control at day 7 (A), 9 (B) and 10 (C).

There was no significant difference found in BSFL microbiome species richness at day 7 or 9 (Figure 3.3A and 3.3B); however, a significant difference in richness was found for day 10 (Figure 3C). When samples were weighted on abundance, there was a statistically significant difference between treatment and control samples at all timepoints (Figure 3). At day 7 and 9, there was a higher abundance of species found in *Arthrobacter* supplemented samples, but this was reversed at day 10, at which time control samples showed an increased abundance in species.

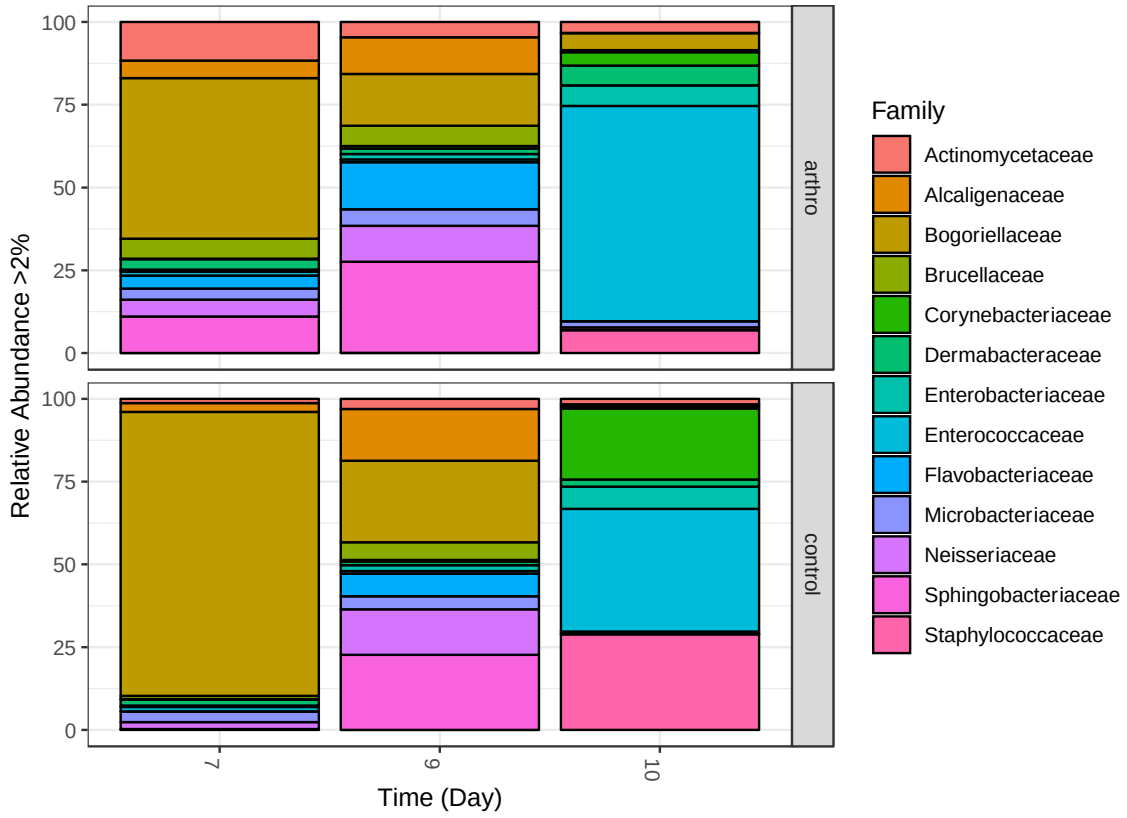

Figure 3.4 Relative abundance of Family level microbial taxa from control or *Arthrobacter* supplemented BSFL

Figure 3.4 shows the relative abundance of BSFL associated bacterial families. On day 7, the treatment group showed greater diversity and had increased abundance for every family represented, with the exception that the control group was composed of 39% more Bogoriellaceae and 9% more Enterococcaceae. Of those families that were increased in treatments at day 7, all showed over 100% increase except for a 11% increase in Microbacteriaceae and a 23% increase in Staphylococcaceae. At T9, the differences were not as apparent. Treatment larvae saw a 21-36% decrease in Alcaligenaceae, Bogoriellaceae, and Neisseriaceae. Treatment larvae had 50% more Actinomycetaceae, 62% more Corynebacteriaceae, 44% more Dermabacteraceae, 26% more Microbacteriaceae, and over 100% increases in Flavobacteriaceae and Staphylococcaceae as compared to control larval associated microbial families. At day 10 another shift in abundance was identified. Bogoriellaceae, which was decreased from the previous timepoint, was over 300% more abundant in treatment groups. Families that saw a decrease in treatments from controls at day 10 were: Alcaligenaceae (-66%), Brucellaceae (i21%), Corynebaceriaceae (-87%), Enterobacteriaceae (-40%), Flavobacteriaceae (-74%), Neisseriaceae (-70%) and Staphylococcaceae (-84%).

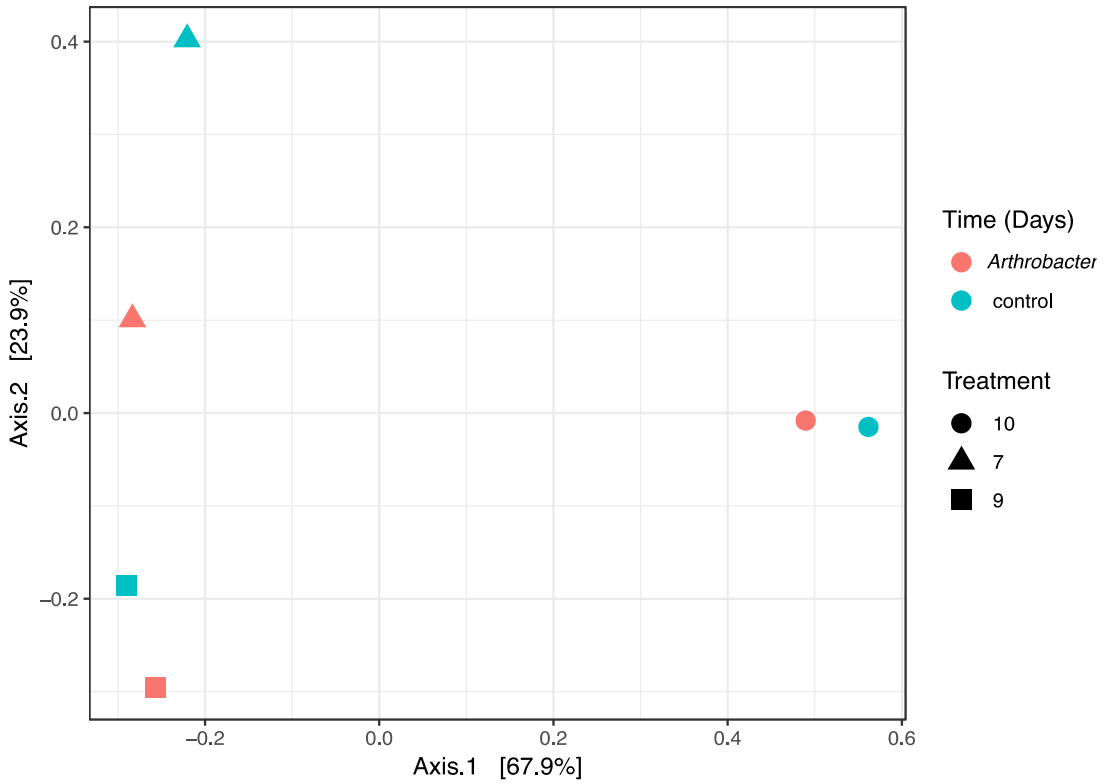

Figure 3.5 Principle coordinate analysis (PCoA) of beta diversity between *Arthrobacter* supplemented and control groups at days 7,9, and 10 using Bray Curtis Distances. The percentage of total variation explained by each PCo axis is shown in the parentheses.

PCoA of β-diversity comparison using Bray Curtis distances revealed significant separation of microbial communities based on time point ($p = 0.03$), Control and treatment samples were similar at days 9 and 10, but the PCoA shows a notable separation at day 7 (Figure 3.5).

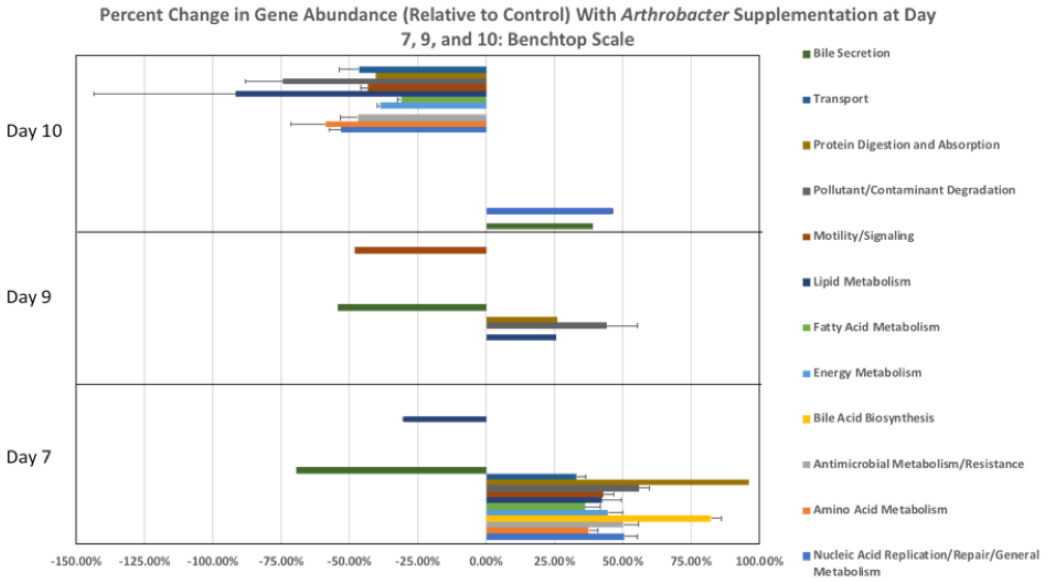

Figure 3.6 Percent differences in predicted functions from microbial metagenomes of *Arthrobacter* supplemented BSFL compared to control BSFL at small scale

Picrust explores relationships between predicted functional gene annotations and identified metagenomes from *Arthrobacter* samples compared to controls. From this, 263 annotated genes were identified, compressed into 12 general gene families and used to compare gene abundance between the two groups. Families with gene abundance within *Arthrobacter* group at or above 25% change from control are shown in Figure 3.6. Percent difference in *Arthrobacter* group gene abundance compared to control at 7 days revealed increases in predicted genes for functions involved in protein digestion and absorption (96.05%), bile acid biosynthesis (82.21%), pollutant/contaminant digestion (55.82%), nucleic acid repair/replication/general metabolism (50.61%), antimicrobial metabolism/resistance (50.26%), motility and signaling (42.73%), genes involved in lipid metabolism (42.39%), energy metabolism (44.47%), fatty acid metabolism (36.17%), amino acid metabolism (37.59%), and membrane transport (33.00%). Decreases in functions for bile secretion (-69.69%) and lipid metabolism (-30.21%) were seen in the supplemented group. At 9 days, compared to control, genes for pollutant/contaminant degradation (43.98%), protein digestion and absorption (25.84%), and lipid metabolism (25.51%) increased, whereas genes for bile secretion (-54.21%) and motility and signaling (-48.15%) were decreased. At day 10 in *Arthrobacter* treated groups, nucleic acid repair/replication/general metabolism (46.18%) and bile secretion (39.05%) increased. Many functional categories within the *Arthrobacter* supplementation group decreased in percent abundance compared to control, including genes for lipid metabolism (-91.59%), pollutant/contaminant degradation (-74.29%), amino acid metabolism (-59.07%), nucleic acid repair/replication/ general metabolism (-52.97%), antimicrobial metabolism and resistance (-47.17%), membrane transport (-46.63%), motility and signaling (-43.15%), protein digestion and absorption (-40.59%), energy metabolism (-38.82%), and fatty acid metabolism (-31.09%).

3.2 *Bifidobacterium breve* small scale study

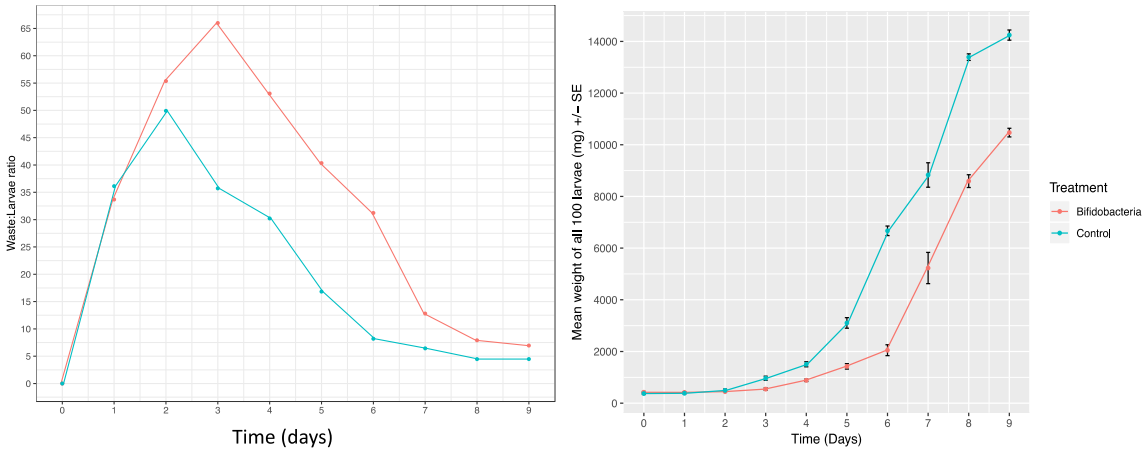

Figure 3.7 Mean weight over time (A) and waste:larvae ratio (B) of *Bifidobacterium breve* supplemented BSFL compared to control

Supplementing with *Bifidobacterium* yielded lower weights over time compared to control BSFL (A). Additionally, supplemented larvae appeared weak, slow, and discolored (data not shown). Also, the treatment BSFL waste:larvae ratio was much lower than control across all timepoints.

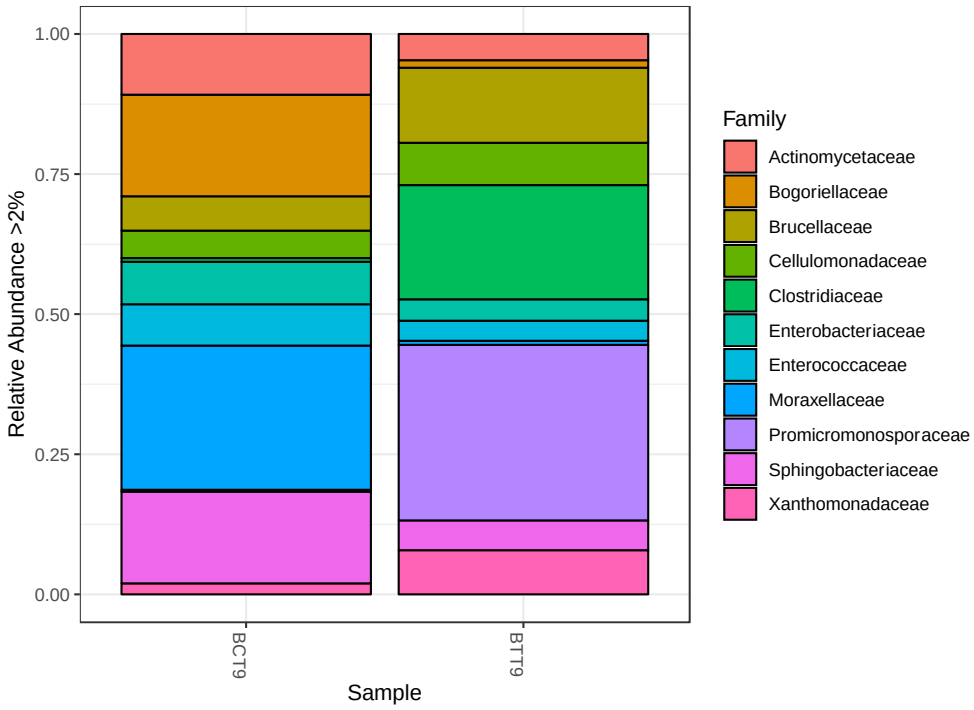

Figure 3.8 Relative abundance of microbial families associated with *Bifidobacterium* supplemented BSFL compared to control on day 9.

Relative abundance of control groups showed an increased amount of Actinomycetaceae (97%), Bogoriellaceae (99%), Brucellaceae (85%), Cellulomonadaceae (89%) *Enterobacteriaceae* (96%), Enterococcaceae (96%), Moraxellaceae (99%), Sphingobacteriaceae (98%), and Xanthomonadaceae (72%) compared to treatment groups. Treatment *B. breve* supplemented BSFL showed an increase in Clostridiaceae (107%) and Promicromonosporaceae (510%) compared to controls at day 9.

3.3 Industrial scale study with *Arthrobacter* and *Rhodococcus*

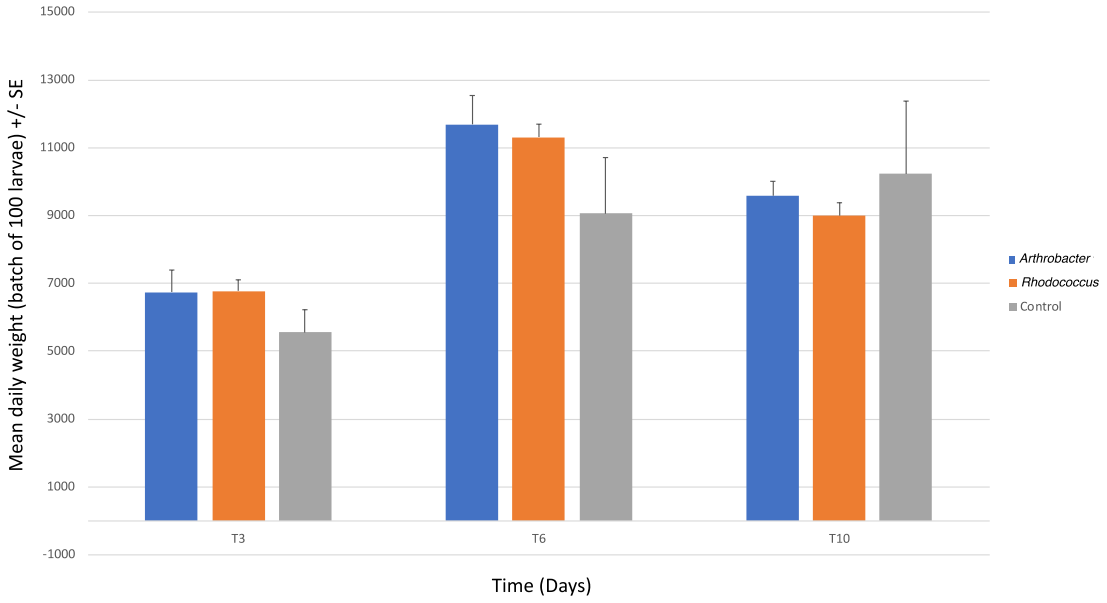

Figure 3.9 Mean larval weight (mg) of 100 *Arthrobacter* and *Rhodococcus* supplemented BSFL compared to control at industrial scale. Standard errors are included as bars above and below bars.

The mean weights of 100 larvae from each treatment are shown in figure 9. *Arthrobacter* and *Rhodococcus* supplemented larvae were not statistically different from each other for the duration of the study. However, both treatment groups weighed statistically significantly more than control larvae at day 3 ($p = 0.02$) where *Arthrobacter* treated larvae were 21.6% larger than controls, and *Rhodococcus* treated larvae were 22.2% larger than controls. At day 6, treatment groups were not statistically different from controls, likely due to large variation in control larvae ($p = 0.06$), though *Arthrobacter* treated larvae were 29% larger than controls, and *Rhodococcus* treated larvae were 25% larger than controls. At day 10, control larvae weighed 6.3% more than *Arthrobacter* treatments, and 12.0% more than *Rhodococcus* treated larvae.

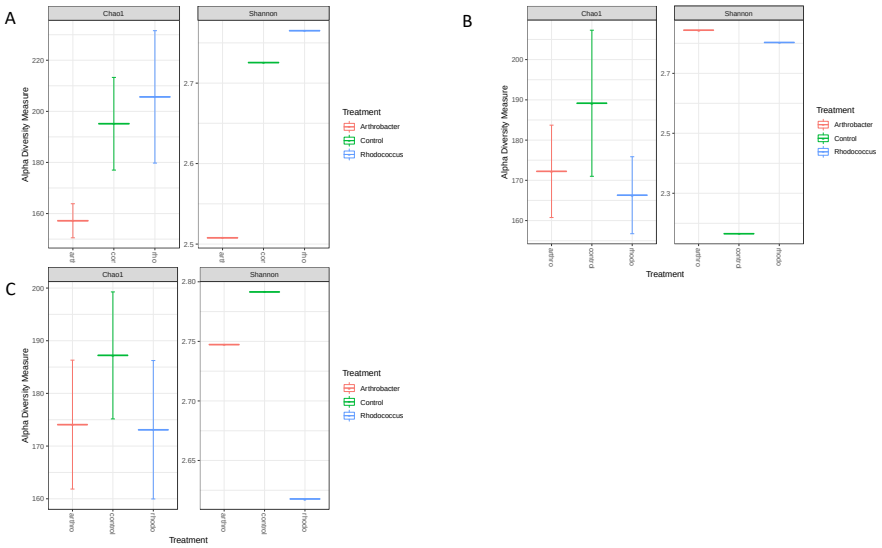

Figure 3.10 Alpha diversity measures of numbers of observed families (Chao1) and abundance and evenness (Shannon) in Arthrobacter and Rhodococcus supplemented larvae compared to control at industrial scale at day 3 (A), 6 (B) and 10 (C).

At day 3, alpha diversity was not significantly different between *Rhodococcus* supplemented larval samples and control, however, *Arthrobacter* was significantly different from both (Figure 10A). At day 6 and 10, the number of observed species was not significantly different between treatment groups and controls. However, at day 6, differences in relative abundance were found (Figure 10B and 10C).

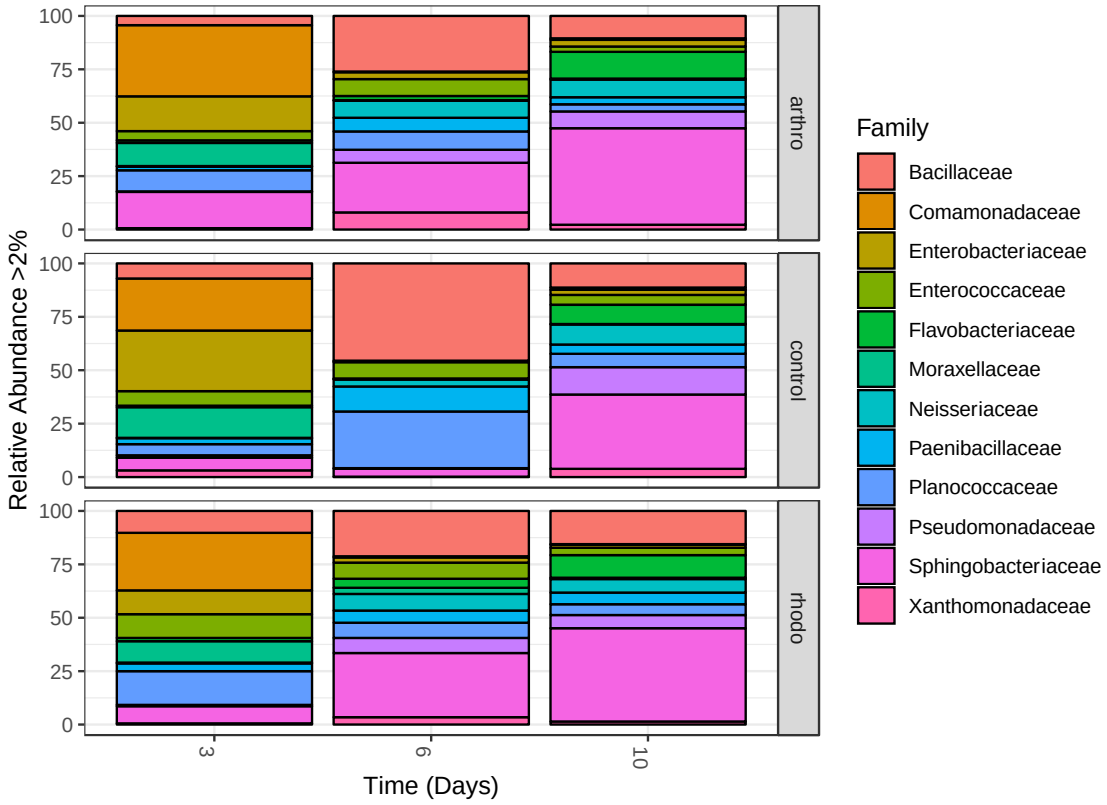

Figure 3.11 Relative abundance of *Rhodococcus* and *Arthrobacter* supplemented larvae compared to control at industrial scale.

At day 3, *Arthrobacter* and *Rhodococcus* larvae had similar relative abundance compared to control (Figure 11). At day 6, compared to control, *Arthrobacter* treated larvae had increases in the abundance of the families Enterobacteriaceae (894%), Enterococcaceae (12%), Flavobacteriaceae (766%) , *Moraxellaceae* (3%), *Neisseriaceae* (157%), Pseudomonadaceae (2295%), Sphingobacteriaceae (564%), Xanthomonadaceae (4794%), and decreases in abundance in Bacillaceae (-38%) Comamonadaceae (-27%), Paenibacillaceae *(-41%)*, and Planococcaceae *(-65%)*. Day 6 *Rhodococcus* treated larvae, when compared to controls had increases in the abundance of Pseudomonadaceae (97%), Sphingobacteriaceae (89%), Flavobacteriaceae (95%), Enterbacteriaceae (24%), Paenibacillaceae (2%), Planococcaceae (10%), Xanthomonadaceae (95%), Bacillaceae (45%), and decreases in Enterobacteriaceae (-87%), Moraxellaceae (-93%), and Neisseriaceae (-5%). At day 10, species richness was similar in all groups. However, differences in relative abundance were noted from day 6 to day 10. For instance, *Arthrobacter* had notable differences at day 10 with less abundance of abundance in Bacilliaceae (-52%), Planococcaceae (-72%), Entercoccaceae (-72%), Paenibacillaceae (-60%), Comamonadaceae (-60%), Enterobacteriaceae (-34%), but an increase in control in abundanc of Moraxellaceae (35%) compared to *Arthrobacter* supplemented larvae (Figure 3.11).

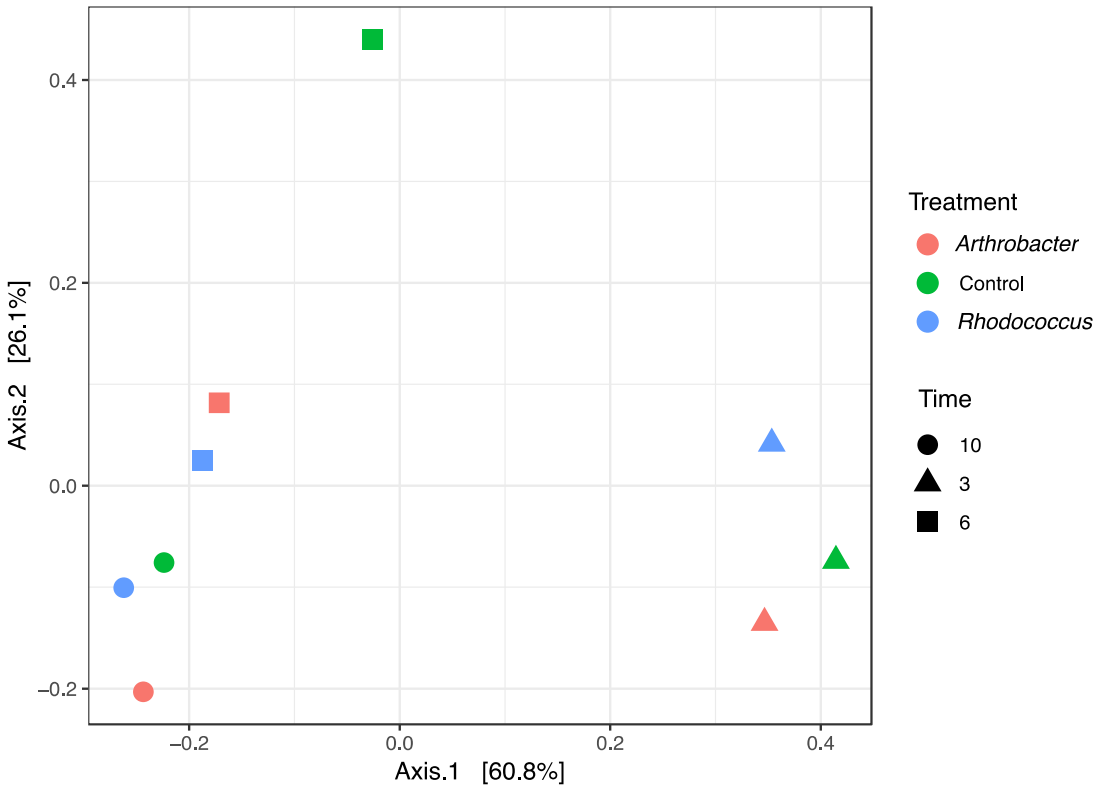

Figure 3.12 Principle coordinate analysis (PCoA) of beta diversity between *Rhodococcus* and *Arthrobacter* supplemented BSFL microbiomes compared to control group microbiomes at days 3, 6, and 10 using Bray Curtis Distances. The percentage of total variation explained by each PCo axis is shown in the parentheses

PCoA of β-diversity comparison using Bray Curtis distances revealed significant separation of microbial communities based on timepoint ($p = 0.03$). Control and treatment samples were similar at days 3 and 10, but the PCoA showed a notable separation at day 6 between the two treatments from the control samples (Figure 3.12).

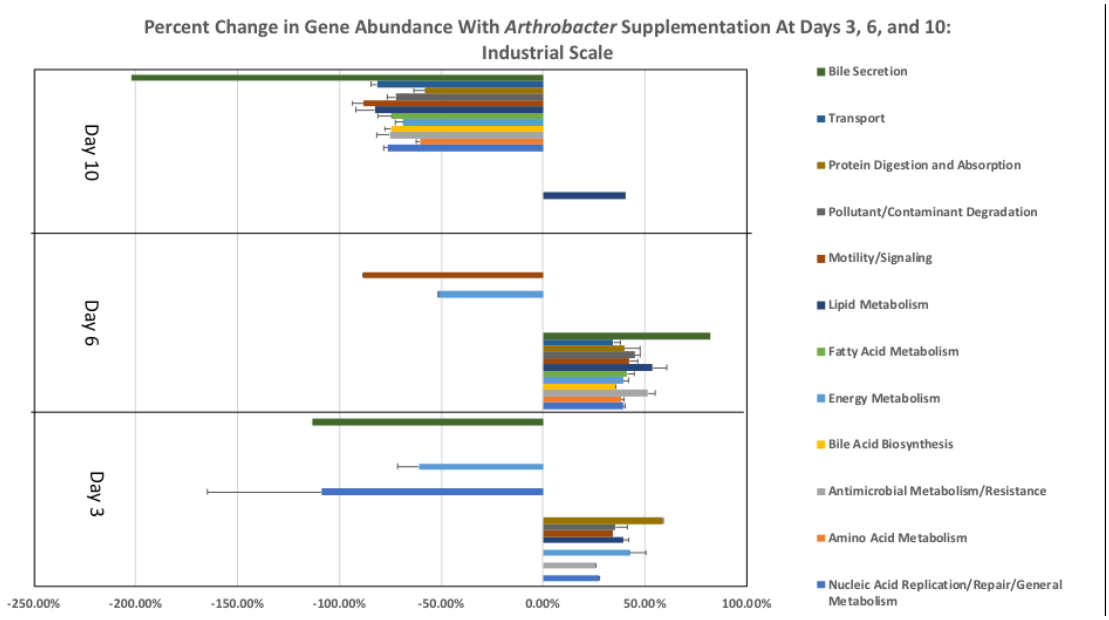

Figure 3.13 Percent differences in predicted functions from microbial metagenomes of *Arthrobacter* supplemented BSFL compared to control BSFL at industrial scale.

Arthrobacter supplemented BSF larvae microbiomes showed increased percent difference in predicted genes compared to control samples for functions involved in protein digestion and absorption (58.93%), energy metabolism (42.77%), lipid metabolism (39.28%), pollutant/contaminant digestion (35.62%), motility and signaling (34.22%), nucleic acid replication/repair/general metabolism (27.19%), and antimicrobial metabolism/resistance (25.97%). Additionally, other genes for energy metabolism (-60.87%), nucleic acid replication/repair/general metabolism (-108.80%), and bile secretion (-113.19) were decreased compared to control (Figure 13). At day 6, *Arthrobacte*r treatments showed enrichment in all general gene families, with the highest percent change from control being bile secretion (82.26%), followed by lipid metabolism (53.91%), antimicrobial metabolism and resistance (51.47%), pollutant/contaminant degradation (45.01%), motility and signaling (42.50%), fatty acid metabolism (41.29%), protein digestion and absorption (39.83%), energy metabolism (39.33%), amino acid metabolism (38.12%), bile acid synthesis (35.73%), and transport (34.42%). Only two gene families were decreased from control at day 6 including motility and signaling (-88.48%) and energy metabolism (-51.53%, Figure 13). At day 10, *Arthrobacter* treatments only had increases in genes functionally predicted for lipid metabolism (40.41%). However, genes associated with all gene families were decreased compared to control. Those included genes for bile secretion (-201.94%), motility and signaling (-88.06%), lipid metabolism (-82.63%), transport (-81.46%), nucleic acid replication/repair/general metabolism (-76.07%), antimicrobial metabolism and resistance (-75.27%), bile acid biosynthesis (-74.67%), fatty acid metabolism (-74.36%), pollutant/contaminant degradation (-72.51%), energy metabolism (-68.67%), amino acid metabolism (-60.46%), and protein digestion and absorption (-57.99%)(Figure 3.13).

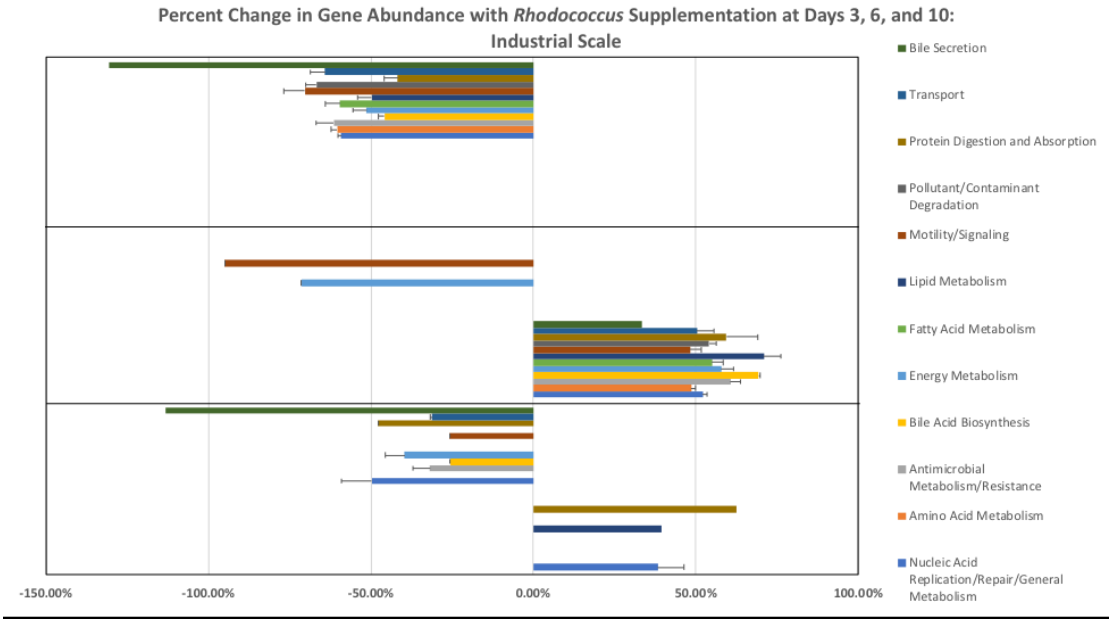

Figure 3.14 Percent differences in predicted functions from microbial metagenomes of *Rhodococcus* supplemented BSFL compared to control BSFL at industrial scale

At day 3, *Rhodococcus* treated BSF larvae microbiomes showed percent increase compared to control in genes involved in protein digestion and absorption (62.69%), nucleic acid replication/repair/general metabolism (38.38%), and lipid metabolism (39.33%). Nine of the twelve gene families were decreased from control including bile secretion (-113.19%), nucleic acid replication/repair/general metabolism (-49.81%), protein digestion and absorption (-47.71%), energy metabolism (-39.69%), antimicrobial metabolism and resistance (-31.75%), transport (-31.15%), pollutant/contaminant degradation (-29.49%), motility and signaling (-25.82%), and bile acid biosynthesis (-25.60%). All gene families were increased compared to control samples, with lipid metabolism being the most increased (71.03%). Following this, bile acid biosynthesis (69.28%), antimicrobial metabolism/resistance (60.74%), protein digestion and absorption (59.38%), energy metabolism (57.88%), fatty acid metabolism (54.96%), pollutant/contaminant degradation (54.12%), nucleic acid replication/repair/general metabolism (52.21%), transport (50.32%), amino acid metabolism (48.63%), motility and signaling (48.20%), and bile secretion (33.29%). Two gene families showed percent decrease compared to control at day 6, including motility and signaling (-95%) and energy metabolism (-71.46%). On day 10, *Rhodococcus* samples showed no increased genes compared to control. Functional genes for bile secretion (-130.46%), motility and signaling (-70.40%), pollutant/contaminant degradation (-66.75%), transport (-64.09%), antimicrobial metabolism and resistance (-61.48%), amino acid metabolism (-60.46%), fatty acid metabolism (-59.57%), nucleic acid replication/repair/general metabolism (-59.06%), energy metabolism (-51.33%), lipid metabolism (-49.69%), bile acid biosynthesis (-45.87%), and protein digestion and absorption (-41.80%).

Table 3.1 Random forest analysis for *Arthrobacter* small-scale study, comparing treatments at day 7 and day 10

Most important classifying taxa	Mean Decrease Gini	% of T7 Day	% of T10 Day
Sphingobacteriaceae	0.210	4.52	0.44
Dermabacteraceae	0.195	1.98	3.31
Bogoriellaceae	0.185	51.99	2.22
Staphylococcaceae	0.185	0.03	18.22
Enterobacteriaceae	0.180	0.42	5.84
Flavobaceteriaceae	0.145	1.59	0.01
Alicaligenaceae	0.135	3.16	0.03
Neisseriaceae	0.130	2.83	0.02
Enterococcaceae	0.115	0.96	43.27
Corynebaceteriaceae	0.090	0.08	13.15

The top ten most important classifying taxa at the family level in this study are shown in table A.1. The percent abundance of that family at the two time points being compared, T7 and T10, are indicated alongside the Mean Decrease Gini value. Of these families, the three with the highest impact on classification are Sphingobacteriaceae, composing 4.52% of T7 day samples and just 0.44% of T10 day samples, Dermatobacteraceae, composing 1.98% of T7 day samples and 3.31% of T10 day samples, and Bogoriellaceae, composing 51.99% of T7 day samples and 2.22% of T10 day samples.

Table 3.2 Random forest analysis for industrial scale study, comparing treatments at day 3 and day 10

Most important classifying taxa	Mean Decrease Gini	% of T3 Day	% of T10 Day
Enterobacteriaceae	0.260	11.04	1.61
Enterococcaceae	0.220	5.98	2.29
Pseudomonadaceae	0.185	0.35	5.27
Planococcaceae	0.175	10.06	3.26
Moraxellaceae	0.165	8.32	0.43
Comamonadaceae	0.160	24.17	0.38
Neisseriaceae	0.155	0.26	5.43
Flavobacteriaceae	0.145	1.06	8.66
Sphingobacteriaceae	0.120	10.17	33.58
Bacillaceae	0.050	5.66	10.14

The top ten most important classifying taxa at the family level in this industrial scale study are shown in table A.2. The percent abundance for the two time points being compared are indicated alongside the Mean Decrease Gini value. Of these top ten taxa, the top three are Enterobacteriaceae, composing 11.04% of T3 day samples and 1.61% of T10 day samples, Enterococcaceae, composing 5.98% of T3 day samples and 2.29% of T10 day samples, and Pseudomonadaceae, composing 5.98% of T3 day samples and 5.27% of T10 day samples.

CHAPTER IV

DISCUSSION

I hypothesized that addition of oleaginous microbes such as *Arthrobacter* AK19 would increase BSFL mass. A previous small-scale study conducted with another oleaginous microbe, *Rhodococcus rhodochrous* 21198, demonstrated this effect (data not shown). *Arthrobacter* AK19 was chosen for additional studies at small scale because the bacterium possesses a high concentration of lipids, usually accumulating greater than 40% lipid in dry biomass[38]. With this high lipid concentration, I expected to see an amplified result in larval weight. In fact, that is what I saw with the daily weights of the *Arthrobacter* supplemented larvae significantly increased from day 3 onward to day 10 compared to no supplementation control larvae. The treatment larvae were tinted orange (*Arthrobacte*r grows bright orange colonies) and just as active as control larvae.

The oleaginous nature of *Arthrobacter AK19* and larval continuous feeding may have allowed the larvae to store energy and nutrients in their fat body. Ingesting such a "fat microbe" may aid in increasing BSFL fat stores. Additionally, *Arthrobacter* could be "pre-digesting" the food for the larvae, allowing an increase in nutrient availability. *Arthrobacter* is a well characterized microbe commonly found in soil and in decomposition environments, and may be breaking down the grain-based diet, making it easier for the larvae to absorb feed associated nutrients. *Arthrobacter* also has commercial uses for the production of L-glutamate, and has been found to be

nutritionally versatile, utilizing a variety of substrates. *Arthrobacter* can reduce a variety of aromatic compounds, herbicides and pesticides, hexavalent chromium and 4-chlorophenol in contaminated soil[57], increasing interest in their use in bioremediation (see http://eawag-bbd.ethz.ch/servlets/pageservlet?ptype=allmicros for a database list of 22 *Arthrobacter* species involved in biodegrading a wide variety of compounds). Most species of *Arthrobacter* are obligate aerobes, but all exhibit a pure respiratory, never fermentative metabolism, and some strains have been found to grow anaerobically, utilizing nitrate as their terminal electron acceptor[58]. *Rhodococcus* is genus related to *Arthrobacter* and has similar biodegradation capabilities (visit http://eawag-bbd.ethz.ch/servlets/pageservlet?ptype=allmicros for a list of 30 *Rhodococcus* species involved in degrading a number of compounds).

A third potential explanation is that *Arthrobacter* is colonizing the gut of the larvae and, like human probiotics, assisting with the digestive process. However, this explanation appears to be poorly supported by these data as few Micrococcacae (less than 2% relative abundance) were detected. This is the family that contains *Arthrobacter*. A more likely explanation is that *Arthrobacter* changes the initial environmental conditions allowing other bacteria to proliferate, and may be maintained in the BSFL waste, particularly as the waste becomes more alkaline. Day 7 of the small-scale study yielded percent increases in all functional groups as compared to control. This is reflective of both the taxa present as well as the relative abundance of those taxa. At day 7, there was an increase in abundance of Actinomycetaceae, Alcaligenaceae, Brucellaceae,

Corynebacteriaceae, Flavobacteriaceae, Neisseriaceae, Sphingobacteriaceae, and Staphylococcaceae.

Amino acid metabolism is responsible for breaking down protein present in feed into basic components of amino acids or di- or tripeptides. Energy metabolism on the other hand is crucial for ATP (adenosine triphosphate) production as well as purine and pyrimidine synthesis, a substrate for nucleic acids. Furthermore, lipid metabolism aids in bacterial cell membranes. An increase in lipid metabolism in the *Arthrobacter* supplement group correlates with an increase in the family Sphingobacteriaceae, which contain high concentrations of cellular lipids[59]. However, there was a decrease in Bogoriellaceae, an obscure bacterial family, and Enterococcaceae, which could be driving the percent decrease in lipid metabolism and bile secretion at day 7. Changes in the conditions of the substrate during larval feeding also likely led to changes in species composition and relative abundance. Species composition and abundance changes in soil communities in response to changes in water availability[60], pH[61], temperature[62], toxicity[63] (contamination by heavy metals), and nutrient availability in the substrate. These factors are also present in the feeding substrate and influence the changes observed throughout the course of larval feeding. Organisms present in high abundance in the *Arthrobacter* supplemented groups appeared to have broad systems for responding to these changes including those for bacterial motility and signaling, antimicrobial resistance and biosynthesis, and pollutant/contaminant degradation. An in-depth look at predicted genes within the functional groups showed enrichment for two component systems, and higher abundance of bacterial motility and flagella proteins and bacterial secretion systems

compared to control, which play important roles in bacterial attachment, colonization, and chemotaxis. Many of the identified microbial families have been found to be involved in gut digestion in mammals and other animals, as well as degradation of organic aromatic compounds and other organic pollutants. Additionally, many of these are known to produce antimicrobial and other secondary compounds.

At day 10, control samples showed an increase in abundance in Corynebacteriaceae, and Staphylococcaceae, while *Arthrobacter* treated groups showed a large increase in Enterococcaceae compared to control. Accordingly, most *Arthrobacter* supplemented BSFL microbiome functional groups showed large percent decreases in abundance compared to controls, with only bile secretion and nucleic acid repair/replication/general metabolism increased. Enterococcaceae and Staphylococcaceae were identified in the random forest analysis as being among the top ten families that help to classify the differences seen between treatment groups at the first timepoint, T7, and the final day of the experiment, T10, suggesting that these families play an important role in the overall changes seen in this experiment.

The change in predicted genes associated with bile acid biosynthesis and bile secretion was an interesting finding across all treatments and experiments. Many bacterial families identified include bacterial species associated with bile acid biosynthesis and digestion in mammals, where clear interactions between bile and gut bacteria have been identified[64]. In mammals, many gut bacteria utilize bile and produce secondary bile acids. Some studies have shown that an increase in bile leads to an increase in gram positive members of Firmicutes, from which families such as

Clostridiaceae, Bacilliaceae, Enterococcaceae and Staphylococcaceae resides. Bile acids have antimicrobial effects on gut microbes, while also aiding in host digestion through emulsifying fats and oils, to allow further nutritional processing. A role of bile and bile acids as hormones and signaling molecules in bacteria have also been identified that act on various physiological functions[65]. And, a dynamic equilibrium has been found to exist between diet-gut microbiome-bile acid pool size and composition, and perturbations in this equilibrium can result in disease states[65–67]. While insects do not produce bile, emulsifiers have been identified in invertebrates that aid in digestion[68]. Some of the microbial families identified in studies presented in this work may be using insect emulsifiers as carbon sources and may be aiding in either insect digestion or lipid storage within the fat body. It is not known what specific enzymes, outside of general lipases, are utilized for lipid digestion and storage in BSFL[69,70]. More work should be done to determine these enzymes, as well as any associated microbially-mediated mechanisms.

Taken together, these small-scale results with *Arthrobacter* are promising and, along with previous small scale *Rhodococcus* data, point toward a potential for industrialization of this process. Larvae that reach harvestable size sooner save industrial BSF production companies money and increase their yield. If the larvae are being used for waste management, their organic material degradation ability can be increased with the aid of these probiotics, helping them to process more waste in a shorter amount of time, with even the possibility of degradation of intractable materials through bacterial supplementation directly, or indirectly through a change in microbial populations toward those with these traits.

The effect that *B. breve* supplementation had on the larvae was unexpected. I hypothesized that addition of this probiotic would have a positive effect on the larvae in some way, just like this probiotic can aid digestion in humans. However, this was not the case. Supplemented larvae appeared discolored, slow, covered in a sticky exudate and overall unhealthy (data not shown). They stuck to each other, to the feeding substrate, and to the container. Healthy control larvae were tan-colored, active and moved through their feeding substrate without issue. The daily mean weight of supplemented larvae was lower than the control larvae, and their waste:larvae ratio was high. *Bifidobacterium breve* did not aid the larvae in converting their food to body mass. Additionally, *Bifidobacterium* treatments showed an increase in Clostridiaceae, and closer inspection revealed an increase in *Clostridium* genera. And despite the increase in Promicromonosporaceae and Cellumonadaciae which contain species with high concentrations of cellulases and xylanases, digestion and frass excretion also appeared stalled. A literature search of studies in other animal models revealed that administration of high fat diets can lead to a decrease in growth among *Bifidobacterium* strains, and also reduction in weight in obese individuals[71]. Additionally, studies have shown that with these same high fat diets, a disproportionate increase in more propionate and acetate producing species, including *Clostridiales*, *Bacteroides*, and *Enterobacteriales* can arise[72]. *Bifidobacterium* has been suggested to be associated with change in inflammatory markers associated with obesity. An additional mechanism hypothesized here includes fat malabsorption and excretion within the insect frass, though more in-depth studies should be conducted to validate this hypothesis. So, in this sense, administration of Bifidobacterium kept the larvae “skinny”,

as has been shown to occur in other animal and human studies. However, in the case of BSFL, this result is not conducive for overall insect health if the goal is to increase growth and waste conversion. However, supplementation with *B. breve* may be useful if the goal is to slow growth and development. Additionally, data from this work suggests a new mechanism for *B. breve*'s role in decreasing obesity. However, more work should be conducted to confirm this, including sampling at earlier timepoints, using differing strains and also at industrial scale.

The industrial scale experiment included both *Arthrobacter* and *Rhodococcus* as treatments. The mean daily weights showed *Rhodococcus* and *Arthrobacter* supplemented larvae were consistently larger than control larvae throughout the study. Control larvae initially had lower weights than both the *Rhodococcus* and *Arthrobacter* treatment, and only overtook them on the very last day. A potential explanation for the decrease in mean weights on the last day could be attributed to pupation. As the larva prepares for pupation it moves into the prepupal stage. In this stage larvae stop feeding and their integument begins to harden. Their digestive system empties, and they exhibit a crawl-off behavior as they seek out a safe place away from the feeding substrate where they can pupate. If the *Arthrobacter* and *Rhodococcus* supplementation was able to accelerate development, then it would undergo this process sooner. When I collected samples to weigh, they would have been in an advanced stage and weighing lighter.

Bacterial supplementation also yielded changes in microbiome species presence and relative abundance, similar to what was found at small scale, with greatest differences found from day 6 samples. At this time point, treatment groups showed increases in

Sphingobacteriaceae and Staphylococcaceae, as well as Pseudomodadaceae, which was absent from the control samples. Predicted genes involved in all functional groups were also present. Planococcaceae and Bacilliaceae were more abundant in controls at this timepoint, and were contributors to the percent decrease in treatments in predicted genes involved in energy metabolism and motility and signaling, particularly with those genes involved in sporulation found in *Bacillus* ssp. At day 10, observed species were the same for all groups, however, relative abundance differed, where control samples showed an increase in relative abundance in Enterococcaceae, Planococcaceae, Pseudomonadaceae, and Xanthamondadaceae, which suggests these are the primary taxa contributing to the percent decrease in predicted gene functions in the treatment groups compared to controls. This suggestion is further backed up by the random forest analysis, which identified all four of these families as important in describing the difference seen between the treated larvae at T10 and T3.

Overall, my results show that bacterial supplementation is beneficial to BSFL growth and waste conversion, though care should be taken toward the appropriate bacterial supplement. Compared to estimated production numbers of prominent BSFL production companies, this method increased production efficiency by about 2000 percent, considering both the shorter development time and the larger body size. Refining and implementing this supplementation method has great potential in further increasing production of soldier fly larvae as a protein source. I showed that bacterial supplementation yielded comparable results at small and large scales, though there was a difference in some bacterial taxa identified among microbiomes from the two experiments. This may be due

to differences in feed batches or larvae initial microbiomes, as the experiments were not conducted at the same time. It will be important to repeat the industrial scale experiments in order to determine consistency in results, particularly if bacteria are to be targeted from the results for further experimentation.

Additional studies important to the field will include the inclusion of a wide variety of food substrates including those such as spent brewer's grain, manure, or food waste, and the inclusion of other potential probiotics. Furthermore, the effectiveness of the combination of *Arthrobacter* and *Rhodococcus* within the same treatment would be interesting. Other important data include transcriptomic data. While useful for this study, PICRUSt is limited in that genes may not be transcribed or translated, limiting the impact of their annotated function. Therefore, conclusions about microbiome function derived from PICRUSt analyses of the metagenomes have been treated as hypotheses that require further in-depth validation through functional assays. Nevertheless, it was quite interesting that changes in functional predictions in these datasets could be related to relative abundance differences across time and treatment, based on gene annotations for a given taxa, giving insight into microbially mediated mechanisms of BSFL feeding and waste conversion. Another interesting finding was the number of taxa with functional potential for pollutant/contaminant degradation. This was an exciting find in that there is further potential of specific bacterial supplementation, particularly many of those enriched within these studies, and manipulation in the BSFL system to allow BSFL to degrade intractable materials and also have potential utility in bioremediation, while also increasing proteins and lipids of value.

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