The effect of *Rhodococcus rhodochrous* supplementation on black soldier fly (Diptera: Stratiomyidae) development, nutrition, and waste conversion

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Abstract

Black soldier fly larvae are mass produced globally for use as livestock, poultry, and aquaculture feed. Efforts are continuously seeking processes optimising larval rate of growth, size, and waste conversion as a means to lower cost and increase output. Manipulating microbes in the larval substrate (i.e. fermentation or supplementation) has been demonstrated as a potential solution. However, identifying appropriate microbes for use in this process has been limited. The objective of this study was to determine whether supplementing black soldier fly larvae with the oleaginous microbe *Rhodococcus rhodochrous* would result in accelerated larval development, increased final larval body size, and increased conversion efficiency. Larvae fed a sterile, or non-sterile, diet treated with *R. rhodochrous* grew 3× faster than the control by the third day and were approximately 2× larger than the control by the conclusion of the experiment. Conversion rate was 2× greater for the treatments indicating less feed would be needed to achieve maximum weight gain. Protein composition of resulting larvae fed diet supplemented with *R. rhodochrous*, was significantly different than the control not receiving the microbial infusion. Larvae provided the microbe had 4.20 and 2.79% greater fatty acid composition and short-chained fatty acids, respectively, but lower monounsaturated fatty acids (1.60%) and polyunsaturated fatty acids (2.4%). Furthermore, larvae provided *R. rhodochrous* produced significantly more proteins related to energy production and storage, as well as muscle development and contraction, while those *sans* microbe, produced proteins related to stress responses (e.g. heat shock proteins). While, this study yielded positive results for the inclusion of *R. rhodochrous* as part of the black soldier fly larval diet, additional research is needed to optimise the dose at an industrial scale.

Keywords: insect farming, bacterial supplementation, *Hermetia illucens*

1. Introduction

Global population and urbanisation pressures mean market demand for food will also continue to grow. In fact, during the first half of the 21st century, food production must increase 70% to address global demand (FAO, 2009). These predictions show a massive increase in animal protein demand needed to satisfy the growth in the human population. The growth in demand for livestock products alone suggests that there will be a consequent rise in demand for animal feed; not only of grains, but other feeds, particularly proteins (Ray *et al.*, 2013). Consequently, alternate strategies for producing protein while reducing environmental pressures are needed.

The black soldier fly, *Hermetia illucens* (L.) (Diptera: Stratiomyidae), represents one example of many insect species (Van Huis, 2013; Van Huis *et al.*, 2013) that can be used for producing protein for use as livestock feed, while also reducing wastes ranging from animal (Myers *et al.*, 2008; Oonincx *et al.*, 2015; Sheppard *et al.*, 1994) and human faeces (Banks *et al.*, 2014) to food (Nguyen *et al.*, 2013, 2015; Salomone *et al.*, 2017). Black soldier fly larvae, in combination with associated microbes, capture and metabolize nutrients within the surrounding medium, such as animal production wastes, to support cellular proliferation and tissue growth, and deposition of energy stores in the form of lipids (Yu *et al.*, 2011). Consequently, microbial conversion of organic compounds to odorous

volatile compounds is decreased (Beskin *et al.*, 2018), as is total manure mass, leaving larvae that are high in fat and protein (Tomberlin, unpublished data). For instance, a recent study showed that soldier flies convert waste coconut endosperm and soybean curd residue to protein and lipids, where 12.4% was the optimum protein constituent in the mixed organic wastes (Lim *et al.*, 2019). Larvae disperse from the feeding site to initiate pupation, a characteristic that can be exploited to facilitate minimum cost, non-mechanized self-harvesting (Sheppard *et al.*, 1994). Studies have also shown that crude oil extracted from black soldier fly larvae biomass can be processed into biodiesel, an attractive alternative fuel source (Li *et al.*, 2011; Surendra *et al.*, 2016).

Symbiotic, host-associated microorganisms are largely responsible for the fermentation of carbohydrates as well as the production of some vitamins and amino acids that their hosts need (Diether and Willing, 2019). These probiotics are non-pathogenic microorganisms that can regulate immune responses and have positive effects on the gut microbiota (Guo *et al.*, 2020; Illiano *et al.*, 2020). Gut-associated microbes rapidly adapt to changes in the insect diet through changes in population and induction of signalling compounds and degradation enzymes that facilitate digestion through absorption and metabolism of complex molecules (Guo *et al.*, 2020; Illiano *et al.*, 2020). Furthermore, gut microbiota aid to prevent pathogenic microorganisms from colonising the gastrointestinal tract (Erdmann, 1987; Erdmann and Khalil, 1986; Greenberg, 1968).

Microbes also contribute largely to the fatty acids used as energy reserves in insect cells serving to aid in vitamin absorption (Berasategui *et al.*, 2016; Engel and Moran, 2013a,b). Many bacterial species possess characteristic fatty acid profiles and abundances. For instance, oleaginous microbes are defined as species with oil contents in excess of 20% biomass weight and include several species of yeast, fungi, and bacteria (Gong *et al.*, 2013; Liang and Jiang, 2013; Liang *et al.*, 2013). *Rhodococcus rhodochrous* is one such oleaginous bacterial species that has previously been investigated as a means of bacterial hydrocarbon synthesis, ultimately to be used in making biofuels (Goswami *et al.*, 2017b; Srinophakun *et al.*, 2017). Additionally, *Rhodococci* are industrially important soil-dwelling, Gram-positive bacteria that are known to be nutritionally versatile, utilising a variety of substrates. *Rhodococcus* can reduce a variety of aromatic compounds, herbicides and pesticides, hexavalent chromium and 4-chlorophenol in contaminated soil, increasing interest in their use in bioremediation (visit Swiss Federal Institute of Aquatic Science and Technology for a list of 30 *Rhodococcus* species involved in degrading a number of compounds¹) (Goswami *et*

al., 2017a). For instance, *Rhodococcus* species can also degrade lignocellulose biomass, including lignin, and lignin-derived aromatics (Brink *et al.*, 2019; Maeda *et al.*, 1995). Lignocellulose biomass comprises the dry, woody plant matter, and is composed of lignin, cellulose, and hemicellulose (Sanderson, 2011).

Research concerning microbes associated with the black soldier fly has been expanding over the course of the past decade (Choi and Jiang, 2014; Shelomi *et al.*, 2020; Wong *et al.*, 2019; Yu *et al.*, 2011; Zheng *et al.*, 2013a,b). The addition of microbes into substrate and methods for substrate fermentation has also been explored (Hasnol *et al.*, 2020; Wong *et al.*, 2020a,b, 2019). However, few data describing the relative nutritional or probiotic value of oleaginous microbes to black soldier fly are available (Gold *et al.*, 2018; Rehman *et al.*, 2019). The objective of this study was to determine the effect of food supplementation with *R. rhodochrous*, on black soldier fly larval growth, survival, food conversion, and nutrition by analysing resulting protein and fatty acid profiles, compared to black soldier fly fed diet alone. Our interest in *R. rhodochrous* in this study was centred on the previously mentioned capabilities, but also on this species' ability to convert agroindustrial wastes into valuable proteins and lipids (Herrero *et al.*, 2018; Le *et al.*, 2019; Mahan *et al.*, 2018). Our rationale being that *R. rhodochrous* would reduce recalcitrant materials within the insect diet, such as lignocellulose, making the diet more nutritionally available, allowing for a more efficient substrate-to-biomass conversion. We also predicted that *R. rhodochrous* metabolism within the diet would aid in digestion and increase valued lipid and protein accumulation within the insect.

2. Materials and methods

Bacteria culturing

R. rhodochrous 21198 was 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.

Black soldier fly rearing

Black soldier fly eggs were collected from a colony maintained in the Forensic Laboratory for Investigative Entomological Sciences (FLIES) Facility at Texas A&M University using methods described by Sheppard *et al.*, (2002). The eggs were collected in three layers of 2 (w) × 2 (h) × 3 (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;

¹ <http://eawag-bbd.ethz.ch/servlets/pageservlet?ptype=allmicros>.

Sheppard *et al.*, 2002). Cardboard was replaced daily. Cardboard containing eggs were placed in a ~1 l deli cup and maintained in an incubator at approximately 70% RH, 27 °C, and 12:12 L:D until hatched.

Experiment design and data collection

Eleven day old larvae were sent to Mississippi State University (Starkville, MS, USA) where allotments of 300 larvae were placed into three separate replicate containers for each of three treatments that were fed daily 6.0 g: (1) Gainesville diet (control) at 70% saturation (with molecular grade water); (2) heat killed Gainesville diet with 10% *R. rhodochrous* supplementation (0.6 g, $\sim 2.5 \times 10^5$ cfu); and (3) non-heat killed Gainesville diet with 10% *R. rhodochrous* supplementation (0.6 g, $\sim 2.5 \times 10^5$ cfu heatkilled). Each replicate had a perforated plastic wrap securing the top to prevent escape and placed in a climate controlled room with 60% relative humidity, 28 °C and 12:12 L:D.

Weight of the total larvae in each replicate was taken in triplicate and recorded at the initiation of the experiment. Larvae were separated from substrate waste (Gainesville diet) and weighed every 24 hours and then returned to their respective cup with fresh substrate. Substrate waste was separated daily from each cup and was dried for three hours in a MyTemp Mini Digital Incubator (Benchmark Scientific, Edison, NJ, USA) set at 50 °C, then weighed as well. After 10 days, the experiment was concluded given larval weight had plateaued. Resulting larvae were sacrificed and analysed as described below.

Nutritional assessment

Four larvae per replicate were randomly selected and pooled for each timepoint at the conclusion of the experiment and were sent to the University of Arizona (Tucson, AZ, USA) for protein analysis, and to Microbial ID, Inc. (Newark, DE, USA) for fatty acid methyl ester (FAME) analysis. Protein analyses were conducted using a Dionex UltiMate 3000 (Thermo Fisher, Waltham, MA, USA) high performance liquid chromatography system coupled with an LTQ Velos Pro (Thermo Scientific) tandem mass spectrometer. Raw mass spectra was analysed using the X!tandem and OMSSA algorithms (Wright *et al.*, 2016). Differential expression analysis of proteins between treatments was performed pairwise using peptide elution profiles as described previously and below (Wright *et al.*, 2016).

Microbial ID conducted FAME analysis using the Sherlock Microbial Identification System (MIS; Microbial ID). This system uses Sherlock Software version 6.3, with a Hewlett Packard HP6890 Gas Chromatograph (Agilent, Santa Clara, CA, USA), with GC ChemStation Rev. B. 04.03 for naming peaks, and associated methods, but was adapted for

black soldier fly (Buyer and Sasser, 2012). Briefly, Sherlock MIS uses fatty acids 9-20 carbons in length. The peaks are automatically named and quantitated by the system. The Sherlock MIS used an external calibration standard developed and manufactured by Microbial ID, Inc. The standard consists of a mixture of the straight chained saturated fatty acids from 9 to 20 carbons in length (9:0 to 20:0) and five hydroxy acids. All compounds were added quantitatively so the gas chromatographic performance could be evaluated at the time the calibration mixture was analysed. The hydroxy compounds are especially sensitive to changes in pressure/temperature relationship and to contamination of the injection port liner. As a result, compounds functioned as quality control checks for the system. Retention time data obtained from injecting the calibration mixture were converted to equivalent chain length data for fatty acid naming. The Sherlock libraries consist of more than 100,000 analyses obtained from experts and collections. Analysis of an unknown sample resulted in an automatic comparison of the composition of the unknown to a database using a covariance matrix, principal component analysis and pattern recognition software. The covariance matrix considers the mole-for-mole relationship of the conversion of one fatty acid to another (e.g. 16:0 to 16:1 due to action of a desaturase), which might occur in relation to a temperature shift or age difference. The pattern recognition software uses calculations of cross terms (e.g. ratios between fatty acid amounts) in addition to the principal component base. The libraries are open ended, and are only limited by Microbial IDentification Inc.'s ability to obtain adequate numbers of samples to make the entries.

Statistical analysis

Data from life history experiments and FAME profiles were analysed using IBM SPSS Statistics for Windows, version 24.0 (IBM Corp., Armonk, NY, USA). Data were first tested for normality then analysed by analysis of variance (ANOVA) to determine significant differences between and within treatments with a Bonferroni's post hoc test when significant effects were observed. Variables for analyses included: treatment (feed substrate type), fatty acids, total residue, waste reduction, and larval weight. Significance was defined as $P < 0.05$. Replicate data variability was represented by error bars on graphs as standard error of the mean.

Differential expression of proteins between groups was performed pairwise using peptide elution profiles as described previously (Wright *et al.*, 2016). Briefly, precursor mass spectra were extracted from the raw data using the MSConvert GUI software from the ProteoWizard toolset (Haas *et al.*, 2006; Schmittgen and Livak, 2008). Peptide precursor m/z values were extracted from protein identifications using Perl software (O'Reilly Media, Inc, Boston, MA, USA). Elution profiles for peptide-spectrum

matches were calculated by parsing each corresponding MS1 file and summing the ion current for that match's m/z value within a 0.25 Da tolerance, effectively integrating the elution profiles. Multiple peptide-spectrum matches with the same precursor m/z were only counted once, ensuring the same integral was not included multiple times. Intensities were summed for each protein on a per-replicate basis. Proteins not identified in a replicate were represented with the average noise level of the replicate's chromatogram for further calculations. Data were normalized using a mode-based technique, where the mode of the protein intensities for each replicate was calculated, representing the most commonly occurring protein intensity, and the intensity per replicate divided by the mode of the same replicate to ensure normalisation was not affected by the minimum and maximum intensities. A permutation analysis was performed for each protein by evaluating the difference in means of the replicates of both conditions. From this permutation, a *P*-value was calculated to indicate the significance of the difference in means. Two additional permutations were performed for each protein, comparing both conditions to their own baselines. These baseline permutations provided a mechanism to further reduce false positives introduced by differences in chromatogram ion current as electron multiplier performance decreases. Proteins were considered to be differentially expressed if the difference in means between conditions resulted in a *P*<0.05 and the difference in means between one of the conditions and its baseline was *P*<0.05.

3. Results

Life-history traits and waste conversion

There was no statistical difference between the weights of the two *R. rhodochrous* treatments during the entire 10 day experiment. However, larval weight from the *R. rhodochrous* + nonautoclaved diet treatment group was significantly higher than the control beginning at day 3 ($F_{2,23}=6.49$; *P*=0.004 Bonferroni post hoc; Figure 1), and larval weights from both *R. rhodochrous* treatment groups were significantly higher than control weights from day 4 through day 10 ($F_{2,23}=14.63$ (day 4), 18.04 (day 5), 36.31 (day 6), 52.08 (day 7), 31.16 (day 8), 14.82 (day 9), 25.53 (day 10), *P*<0.001 for both treatments from days 4 to 10). Additionally, weight of food-waste was divided by the total larval weight in each container to determine conversion rate. The *R. rhodochrous* treatment groups metabolized significantly more food and had a significantly higher waste conversion than control larvae fed Gainesville diet alone beginning on day 5 and day 6 through day 10 for *R. rhodochrous* + nonautoclaved diet treatment group and *R. rhodochrous* + autoclaved diet treatment groups, respectively ($F_{2,23}=31.68$ (day 5), 78.51 (day 6), 128.18 (day 7), 50.00 (day 8), 66.78 (day 9), 15.47 (day 10), *P*<0.001 for all; Figure 2).

Fatty acid composition

The detailed percent fatty acid composition of black soldier fly larvae with or without *R. rhodochrous* supplementation is shown in Table 1. No statistical differences ($F_{1,334}=0.001$, *P*=0.998) were found between fatty acid composition of *R. rhodochrous* with autoclaved or nonautoclaved food; therefore, the two treatments were combined, and the mean taken. Fatty acid composition of larvae fed with *R. rhodochrous* supplementation showed higher saturated fatty acids (79.2%) than those fed Gainesville diet alone (75%). However, larvae fed with *R. rhodochrous* supplementation had lower monounsaturated fatty acids (13.1 vs 14.7%) and polyunsaturated fatty acids than control larvae (7.9 vs 10.3%). *R. rhodochrous* supplemented larvae also showed increased % concentration of short-chain saturated fatty acids when compared to control larvae (65.64 vs 62.85%). Larvae fed *R. rhodochrous* supplemented diet also showed detectable levels of vaccenic (0.88 vs 0.0%) and eicosatrienoic acids (0.35 vs 0.08%), which were absent, or present only in a very small amount, respectively, in control larvae fed diet alone.

Protein expression profiling

The separation profiles of larval proteins with and without *R. rhodochrous* supplementation are shown in Figure 3. The overlay of the sample peaks with 280 nm absorption

Table 1. Comparison of mean % composition of representative fatty acids from larvae fed bacterial supplemented diet compared to diet alone.

Fatty acids ¹		Black soldier fly larvae + bacterial supplemented diet	Black soldier fly larvae + diet alone
Lauric	C12:0	55.2 (2.61)	51.4 (5.65)
Myristic	C14:0	0.34 (0.38)	0.25 (0.54)
Palmitic	C16:0	8.9 (0.73)	9.8 (1.46)
Palmitoleic	C16:1n-7	2.4 (0.51)	2.8 (0.38)
Stearic	C18:0	1.2 (0.22)	1.4 (0.55)
Vaccenic	C18:1n-7	0.88 (0.22)	0.00
Oleic	C18:1n-9	8.7 (0.63)	11.4 (2.12)
Linoleic	C18:2n-6	7.6 (1.24)	10.2 (1.78)
Eicosatrienoic	C20:3n-3	0.35 (0.39)	0.08 (0.10)
Saturated fatty acids ¹		79.2	75.0
Monounsaturated fatty acids ¹		13.1	14.7
Polyunsaturated fatty acids ¹		7.9	10.3

¹ Values are total composition and include all detected fatty acids and standard deviation of replicates is shown in parentheses.

Figure 1. Effect of *Rhodococcus rhodochrous* supplement of autoclaved or non-autoclaved Gainesville diet mean black soldier fly growth over time compared to weights $\pm$ SD over time of black soldier fly larvae fed Gainesville diet alone.

Figure 2. Food-waste:mean larval weight ratio $\pm$ SD over time of *Rhodococcus rhodochrous* supplemented Gainesville diet (autoclaved or non-autoclaved) treatments compared to Gainesville diet treatment.

demonstrates differences in protein profiles associated with *R. rhodochrous* supplementation vs larval proteins fed Gainesville diet alone. Results showed overlap in the initial peaks (6.0-10.0 fractions) from all treatments, though control larval proteins and proteins from *R. rhodochrous* + autoclaved proteins had peaks at higher absorbance as compared to the *R. rhodochrous* + nonautoclaved diet (22.0 vs 20.0 absorbance). The second major peaks were identified for fractions 12.0 through 24.0 and showed differences between *R. rhodochrous* supplemented larval

proteins compared to control larval proteins. *R. rhodochrous* + autoclaved diet had a peak at 31.0 absorbance and *R. rhodochrous* + nonautoclaved diet showed a peak at 29.0 absorbance. However, control larval proteins from these fractions showed three smaller peaks at 12.0, 13.0 and 18.0 absorbance. Protein extracts from the larvae were analysed using mass spectrometry in order to determine relative amounts of major proteins present, and normalized significantly different proteins from expression analysis are shown in Figure 4.

Figure 3. Size exclusion chromatograms (measured by UV absorbance) of proteins extracted from black soldier fly larvae that were fed Gainesville diet alone or that were fed autoclaved or non-autoclaved Gainesville diet supplemented with *Rhodococcus rhodochrous*.

Figure 4. Significantly and differentially expressed proteins of black soldier fly larval replicates that were fed (A) autoclaved Gainesville diet with *Rhodococcus rhodochrous* supplementation; (B) Gainesville diet alone; or (C) non-autoclaved Gainesville diet with *R. rhodochrous*.

Thirty-six proteins were significantly differentially expressed between *R. rhodochrous* treatments and controls. Overall, 18 of the 36 proteins, particularly those involving actin, myosin, and tubulin that are involved in muscle development, contraction, and organisation. Twelve of the 36 differentially expressed proteins are known to be involved in energy production and storage, protein synthesis, and DNA packaging. Two of the 36 proteins were identified as heat shock proteins, and one was identified as calmodulin, a calcium binding protein.

Results show many differentially expressed proteins that are found in black soldier fly larvae fed Gainesville diet alone (Figure 4, panel B), that are absent from those fed with *R. rhodochrous* supplementation (Figure 4, panel A and C). Many of them are involved in muscle development, contraction, and organisation. Notably, heat shock 70 kDa protein cognate 3 and 60 kDa heat shock protein (mitochondrial) were expressed in control black soldier fly larvae fed diet alone, but not expressed in those fed with *R. rhodochrous* supplementation. ATP synthase subunit alpha (mitochondrial), and glyceraldehyde-3 phosphate dehydrogenase 2 were also significantly expressed in the control diet only cohort alone. Additionally, arginine kinase and isoform A of arginine kinase, both energy storage compounds, were found in control larvae fed diet alone. Proteins significantly expressed in black soldier fly larvae fed with *R. rhodochrous* supplementation, while very low or absent in the control, diet alone cohort included histone proteins, H3, H2B (with supplemented, autoclaved diet only), and H4. Additionally, calmodulin was significantly expressed in the *R. rhodochrous* supplement cohorts, but not expressed or expressed below detection limits in the control, diet alone cohort.

4. Discussion

We determined that supplementing diet provided to black soldier fly larvae with *R. rhodochrous* significantly enhanced development as well as protein and fat production. We determined that the one concentration explored in our study resulted in a near doubling (1.7×) in larval weight (Figure 1). Also, larvae reached a mean weight of 120 mg per individual larvae (the target weight for commercial use, F. Yang, personal communication) in the *R. rhodochrous* supplemented treatments between days 8 and 9, whereas mean larval weights in the control treatment did not reach 120 mg per individual larvae during the duration of the study. These data suggest bacterial supplement allowed for greater metabolisation of the diet, and allow for less diet needed to reach optimal weight. This is important when consideration industrial scale and larvae for commercial use.

Analysis of fatty acids also revealed that bacterial supplementation increased the concentration of short-

chain saturated fatty acids, and reduced the amount of unsaturated fatty acids, increasing utility for the biodiesel industry. Moreover, bacteria-supplemented diet also showed detectable levels of vaccenic and eicosatrienoic acids, which are unsaturated fatty acids implicated in potential health benefits (Fan and Chapkin, 1998; Fan *et al.*, 1995; Field *et al.*, 2009). Indeed, probiotics could be used to optimize black soldier fly industrialisation for the production of protein for feed and fat for bioenergy. Doing so could allow for agriculture to more easily meet the needs of billions of people who still lack access to basic, modern energy services while simultaneously meeting food demands and participating in a global transition to clean, low-carbon energy systems. Costs for growing probiotics in industrial scale facilities should be considered for full implementation of the methodology, as purchase of industrial scale bioreactor/fermenters, and other equipment for growing probiotic microbes would be required. This cost would be minimized, however with the implementation of lyophilized *R. rhodochrous*, and other attractive microbes that could be bought commercially for use in industrial facilities (Hasnol *et al.*, 2020).

Black soldier flies are poised for mass production of protein or oils, as they can be produced at the tonnage level daily, and we know more about its biology than any other insect species that could be utilized for this purpose. Black soldier flies can be mass-produced and can convert organic wastes to protein and fat, and reduce dry matter by 50% (Sheppard *et al.*, 1994). Black soldier fly prepupae can be used as feed for a variety of fish (Bondari and Sheppard, 1981; 1987) and could represent a 25 to 50% replacement of current feed used in the aquaculture industry (St. Hilaire *et al.*, 2007a,b). As fish meal and other protein sources for aquaculture feed become more scarce and expensive, insect protein sources offer a much needed alternative (Van Huis, 2013). Protein can also be separated from the chitinous cuticle of the black soldier fly and used as additional feedstuff, leading to greater replacement. Additionally, black soldier flies have recently been approved by the American Association of Feed Control Officials and Canadian Food Inspection Agency for use as feed for salmonid fish species (AAFCO, 2016) and by the Federal Drug Administration for poultry feed, underscoring the importance of black soldier fly utility. However, the system has not been optimized for maximum protein and fat yields, or for waste degradation.

The first step for optimisation is performing experiments on the benchtop, in order to determine variability and efficiency in methodology. It is important, however, to recognize the differences may be found when results at a small scale are compared to those obtained at the industrial scale due to potential differences in nutrient availability, access to food and competition that could influence waste conversion and feeding efficiency.

Remediating waste is of paramount importance to concentrated animal feeding operations (CAFOs), such as dairies (Adhikari *et al.*, 2005). Therefore, an economical method for remediation, while protecting natural resources is essential to the long-term health of CAFOs and the environment. Data presented from this work show that *R. rhodochrous* supplementation allowed black soldier fly larvae to metabolize more of the food than control larvae fed diet alone. These data are promising and suggest the possibility that *R. rhodochrous* would allow black soldier fly larvae to digest higher concentrations of manure. More work is underway to investigate this possibility.

Proteomic analysis of black soldier fly with or without *R. rhodochrous* supplementation led to many significantly differentially expressed proteins, when compared to control black soldier fly fed Gainesville diet alone. Interesting findings from this study showed that control black soldier fly larvae reared without *R. rhodochrous* supplementation expressed a larger number of proteins involved in energy production and storage, as well as muscle development and contraction. Additionally, those control larvae without *R. rhodochrous* supplementation had significantly greater expression of heat shock proteins. And, as previously mentioned, they also weighed less and metabolized less food than those supplemented with the bacteria. We hypothesize black soldier fly larvae fed diet alone were more active in searching for access to key nutrients than those provided the *R. rhodochrous* supplementation, where *R. rhodochrous* could have facilitated increased breakdown of recalcitrant materials within the substrate, allowing for improved access to nutritional needs and richer energy sources. Furthermore, black soldier fly larvae fed with *R. rhodochrous* supplementation also significantly and differentially expressed histone proteins. These proteins are part of the nucleosome and are instrumental in giving structure to DNA. This increase is likely due to an increase in chromatin content and may be an indirect marker for cell proliferation (i.e. growth).

In addition to these data, calmodulin was found significantly expressed in black soldier fly larvae with *R. rhodochrous* supplementation. Calmodulin binds calcium and has been implicated in inflammation, metabolism, and smooth muscle contraction (Nunez *et al.*, 2020). Calcium is also required in many cases for initiation of DNA synthesis (Martelly *et al.*, 1983). The potential of calmodulin as a growth modulator has already been demonstrated in other systems (Nelson *et al.*, 1997; Takuwa *et al.*, 1995a,b) and work is underway to understand the direct impact of calmodulin and calcium in black soldier fly development and waste conversion. Being black soldier fly larvae are known to be high in calcium (Johannsen, 1922), the potential link with our discovery is that bacterial supplementation enhanced calcium deposition in the exoskeleton

Probiotics are live microorganisms that, when administered in adequate amounts, confer a health benefit on the host (Skonieczna-Zydecka *et al.*, 2020). All members of the Animalia kingdom, including humans, have helpful symbiotic microbiota which are extremely important for the proper functioning of the gastrointestinal tract, and now products are being commonly used as dietary supplements. Probiotics are recommended to be added not only to the human diet but also into the forage of different vertebrates as well as invertebrates (Ptaszynska *et al.*, 2016; Skonieczna-Zydecka *et al.*, 2020; Uyeno *et al.*, 2015; Wang *et al.*, 2017).

However, increased scrutiny of human or animal microbiota has led to the identification of novel probiotics, such as in this study, that are not members of the traditional genera (i.e. *Lactobacillus* or *Bifidobacterium* in other systems). The study presented here showed that *R. rhodochrous* should be considered as a candidate probiotic species for black soldier fly because of positive effects on nutrition and development; however, safety and efficacy of these bacteria has to be demonstrated through larger scale, rigorous scientific studies, refining the concentrations, food source (e.g. animal, plant or mixed food waste), and determining efficacy of alternative oleaginous species. Nevertheless, our research is a valid starting point and could give insights into potential mechanisms of probiotic action and resulting host response.

In recent years and in many countries, public concern about the safety of foods of animal origin has heightened due to problems that have arisen with contamination, outbreaks of food borne bacterial infections, and as a result of growing concern about drug residues and microbial resistance to antibiotics. These problems have drawn attention to practices within the feed and livestock industries, and have prompted health professionals and the feed industry to closely examine food quality and safety issues. Notably, black soldier fly larvae present no known harm to the environment or people, as they are a non-pest species and have been found to suppress pathogens in waste (Erickson *et al.*, 2004; Lalander *et al.*, 2015; Liu *et al.*, 2008); however there are still some concerns that these insects could harbour pathogens (Lalander *et al.*, 2015). Experiments from our labs are ongoing to determine the full impact of insect farming on pathogen amplification in digested substrate, or resulting insects, and whether probiotic supplementation will mitigate any such negative effects. Such results will be valuable for informing US and global regulations and best practices.

Our data showed that black soldier fly feed supplemented with *R. rhodochrous* has the potential to increase larval lipid and protein content, feeding efficiency and waste conversion. Our study was conducted at a scale suitable to laboratory experimentation. However, development of a large scale black soldier fly production system will

require scaling up and refinement, so that black soldier fly byproducts will be cycled throughout the system, protein and fat extracted from black soldier fly larvae, and the chitinous cuticle converted into a valuable resource. Oleaginous microbes may also be excellent candidate organisms for the bioprocessing of chitinous waste, as many possess the enzymatic machinery to break down chitin and proteins. For instance, the oleaginous yeast *Cryptococcus curvatus* can produce intracellular lipid using chitin as a carbon source and total lipid content can reach 54% on a chitin rich medium. *R. rhodochrous* was found to use chitin as a carbon source in our study (data not shown).

Another attractive feature is that oleaginous microbes can synthesize and accumulate triacylglycerides, similar in composition to vegetable oils, a primary material for biodiesel production. Altogether, food waste supplementation with oleaginous microbes has the potential to provide added value to the system through increasing black soldier fly lipid and protein content, along with increasing feeding efficiency and waste conversion. Furthermore, the breakdown of chitinous byproducts into lipids and proteins would convert a low value resource to a high value resource for the farmer. Data from our study has contributed to the understanding of the impact and driving mechanisms of food waste supplementation with oleaginous microbes on black soldier fly waste conversion and resulting nutritional composition. These data provide a foundation for future studies utilising these bacterial species at industrial scale to create a self-sustainable systems of optimized protein and oil production for use in both the live feedstock and biodiesel industry.

Conflict of interest

The authors declare no conflict of interest.

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