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High-throughput sequencing of gut microbiota in rainbow trout (*Oncorhynchus mykiss)* fed larval and pre-pupae stages of black soldier fly (*Hermetia illucens*)

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Abstract

Black soldier fly (*Hermetia illucens*) meal is a potential alternative to fishmeal and plant proteins in diets for farmed fish since it can be produced on organic waste substrates, requires little energy and water inputs and contains high levels of essential amino acids. Recent studies have partially replaced fishmeal with black soldier fly meal, however, research on their impact on gut microbiota of fish is limited. In a five week experiment, juvenile rainbow trout (*Oncorhynchus mykiss*) were fed either a reference diet based on fishmeal or three diets with 30% inclusion of black soldier fly meals in the form of pre-pupae, larvae or defatted-larvae. The combined luminal content and mucosa were collected from the distal intestine of three fish per tank with four tanks per diet (n=12) and 16S rRNA gene amplicons were sequenced using the Illumina MiSeq platform. Feeding the insect-based diets increased the alpha-diversity of bacteria and abundance of lactic acid bacteria, which may be due to the addition of dietary chitin. Compared with fishmeal, feeding insects resulted in higher abundance of phyla Firmicutes and Actinobacteria with lower abundance of Proteobacteria. Fish fed the full-fat meals had higher abundance of *Corynebacterium* that was attributed to its ability to produce lipase and the high content of dietary lipids as a substrate. Bacillaceae was increased in fish fed both larvae diets and unchanged in the pre-pupae diet, which indicated that life-cycle stage of the insect influenced the gut microbiota. Based on these results, we found that feeding black soldier flies increased diversity and altered the composition of gut bacteria of rainbow trout, which were further influenced by life-cycle stage and lipid content of the insect meal.

Keywords

Bacterial diversity; Aquaculture; Distal intestine; Fishmeal replacement; Illumina; Insect meal

Highlights

- Gut bacteria of rainbow trout fed black soldier fly meals were identified using high-throughput sequencing for the first time.

- Feeding larvae, pupae and defatted larval meals resulted in three different gut bacteria profiles, indicating that insect life stage and lipid content are decisive factors influencing the gut microbiota in rainbow trout.

- All three insect diets increased bacterial diversity and lactic acid bacteria that may indicate improved gut health of rainbow trout.

1. Introduction

Aquaculture will require more feed resources to produce more fish for a growing human population. Fishmeal and soy are common protein sources in aqua-feeds, but depleted ocean stocks and demand for human consumption has resulted in higher prices and reduced availability of these ingredients (Tacon, Metian, 2008). Low human-interest alternatives that require less water, land and energy resources are needed. A possible alternative is insects since they can convert organic waste substrates with high efficiency, contain high levels of protein and lipids, require low resource inputs for farming, produce low amounts of greenhouse gases and have relatively low interest from human consumers (Henry, et al., 2015; Van Huis, et al., 2013). Insects are a natural part of the diet for wild fish, especially those inhabiting coastal and inland water-bodies (Whitley, Bollens, 2014). Previous studies have found that rainbow trout (*Oncorhynchus mykiss*) fed fat-enriched black soldier fly pre-pupae can replace 25 and 50% of fishmeal without compromising growth performance (Sealey, et al., 2011; St-Hilaire, et al., 2007). Diets with 50% replacement of fishmeal with black soldier fly defatted-larvae have also resulted in similar growth performance, body indices and gut morphology of rainbow trout compared with fish fed the control diet (Renna, et al., 2017). In addition, replacement of 20-85% of fishmeal with black soldier fly has had no negative effects on growth and feed efficiency of Atlantic salmon (*Salmo salar*) (Belghit, et al., 2018; Lock, et al., 2016), turbot (*Psetta maxima*) (Kroeckel, et al., 2012), European seabass (*Dicentrarchus labrax*) (Magalhães, et al., 2017), barramundi (*Lates calcarifer*), Nile tilapia (*Oreochromis niloticus*) (Muin, et al., 2017) and yellow

catfish (*Pelteobagrus fulvidraco*) (Xiao, et al., 2018). However, the impact of feeding black soldier fly meal on the gut microbiota of fish is not well known.

The gut microbiota plays an important role in nutrition, immune system and health of fish (Llewellyn, et al., 2014; Wang, et al., 2018) and the feeding with alternative protein sources such as plants, mussels and microbes (i.e. yeast and microalgae) have been shown to alter diversity and abundance of gut bacteria in salmonid fishes (Desai, et al., 2012; Huyben, et al., 2018; Huyben, et al., 2017; Ingerslev, et al., 2014; Lyons, et al., 2017; Michl, et al., 2017; Nyman, et al., 2017). Recently, a study using gel electrophoresis based sequencing method has found that feeding black soldier fly meal to rainbow trout increased diversity of gut microbiota (Bruni, et al., 2018). A few studies have suggested that chitin, a long-chain polymer of N-acetylglucosamine derived from exoskeleton of insects and crustacean shells, acts as a substrate for chitinase producing bacteria that are not commonly found in the fish gut (Askarian, et al., 2012; Bruni, et al., 2018; Ringø, et al., 2012). Similarly, feeding krill-based chitin has been found to alter the gut microbiota of Atlantic salmon (Askarian, et al., 2012) and Atlantic cod (Zhou, et al., 2013). Rearing substrate, life-cycle stage and lipid content of insects have also been suspected of influencing gut microbiota (Lock, et al., 2016; Sealey, et al., 2011; Xiao, et al., 2018). New advancements in high-throughput sequencing will allow us to identify specific effects of feeding black soldier fly meals on gut microbiota of farmed fish.

The objective of this study was to investigate the effects of feeding three different black soldier fly meals on the gut microbiota of rainbow trout. Specifically, differences between insect life cycle stage (i.e. larval and pre-pupae meals) and lipid content (i.e. commercial defatted-larvae meal) on the abundance and diversity of bacteria in the distal intestine were investigated using high-throughput 16S rRNA gene amplicon sequencing on the Illumina MiSeq platform.

2. Materials and methods

2.1 Fish and facilities

The study was performed in the Aquatic Facility of the Centre for Veterinary Medicine and Animal Science at the Swedish University of Agricultural Sciences (SLU; Uppsala, Sweden). Rainbow trout (201.8 ± 13.9 g; mean ±

standard deviation) were acquired from a commercial producer (Vilstena fiskodling AB, Fjärdhundra, Sweden) and housed indoors in 500 L flow-through tanks. Two weeks before the experiment, 160 fish in total were randomly distributed in each of the 16 experimental tanks (10 fish per tank). The fish were acclimatised to a 12 hr light cycle and fed a commercial diet (3 mm, Efico Alpha 714, BioMar A/S, Brande, Denmark). The flow-through system supplied each 200 L oval, fibreglass tank with municipal freshwater at a rate of 6 L min^{-1}. Water was analysed on a weekly basis for temperature (10.9 ± 0.4 °C) and dissolved oxygen (8.8 ± 0.3 mg L^{-1}) using a portable probe (Hach Lange GmbH, Berlin, Germany) and pH (7.3 ± 0.2) using a pH/redox probe (Oxyguard A/S, Farum, Denmark). At the experimental start, fish were sedated with tricaine methanesulphonate (MS-222; 50 mg L^{-1}) buffered with sodium bicarbonate and weighed. The study was performed in compliance with laws and regulations on the use of animals for research purposes in Sweden, which is overseen by the Swedish Board of Agriculture.

2.2 Diets and feeding

Fish were fed either a fishmeal-based reference diet or one of three test diets where the reference diet was mixed in a ratio of 70:30 with a test ingredient of either pre-pupae, larvae or defatted-larvae meal from black soldier fly, as according to Cho (1979). See Table 1 for the formulation of the reference diet. The larvae and pre-pupae meals were produced by the Environmental Engineering Unit, Department of Energy and Technology, SLU (Uppsala, Sweden) as according to Lalander, et al. (2015), except the rearing substrate was based on food compost from a local restaurant. The defatted-larvae meal was produced by a commercial company (Protix, Dongen, The Netherlands) using a wheat bran substrate. The reference diet was first produced by adding the non-test ingredients, listed in Table 1, in a rotating drum mixer and then, separately, 70:30 of the reference diet was mixed with each test ingredient. Gelatin and hot water were added to each diet as a binder, mixed in a kitchen mixer and pressed through a meat grinder that had a 3.5 mm die (Nima Maskinteknik AB, Örebro, Sweden). Diet strings were air-dried at 55 °C for 24 hr, cut into pellets with a kitchen blender (Kneubühler, Luzern, Germany) and stored at -20 °C until the start of the experiment.

For proximate composition of diets (Table 1), dry matter was analysed after treatment at 103 °C for 16 h and ash was determined after treatment at 550 °C for 3 h followed by cooling and weighing (AOAC, 1995). Total nitrogen (N) was determined using a 2020 Digestor and 2400 Kjeltec Analyser (FOSS Analytical A/S, Hilleröd, Denmark)

and crude protein (CP) was calculated as N × 6.25 (Nordic Committee on Food Analysis, 1976). Crude lipid was determined using a Soxtec System HT 1043 Extraction Unit (FOSS Analytical A/S, Hilleröd, Denmark) according to the manufacturer (ANKOM Technology, Macedon, NY, USA). Neutral detergent fibre (NDF) was determined according to the Amylase Neutral Detergent method (Mertens, 2002). Acid detergent fibre (ADF) was determined after 1 h boiling in a solution of 0.5 M sulphuric acid and 2% cetyl trimethylammonium bromide according to Method 973.18 of AOAC (AOAC, 1995). Chitin was estimated based on the level of ADF solely derived from the 30% inclusion of the insect meals. Chitin was estimated by subtracting the ADF in 70% of the fishmeal reference diet (i.e. 32.4 g/kg; assumed to be cellulose) from the total ADF in the insect-based diets (Finke, 2007).

Over the period of five weeks, fish were fed daily at a rate of 1% body weight (BW) per day via automatic belt feeders (Hølland teknologi, Sandnes, Norway) from 10:00 to 12:00. The four diets were randomly assigned to each of the 16 tanks. Feed waste was collected continuously using a belt collector (Hølland teknologi, Sandnes, Norway), weighed twice per day and then pooled for subsequent dry matter analysis. Feed waste was subtracted from the total feed intake using the recovery method according to Helland, et al. (1996).

2.3 Sampling of the distal intestine

Fish were fed until terminal sampling on the final day of the study (i.e. within 20-24 hours of final feeding). Three fish from each tank (n=12) were euthanised with an overdose of 200 mg L^{-1} MS-222 buffered with sodium bicarbonate and their cervical vertebrae were severed. Under sterile conditions, the midline of each fish was dissected near a flame within a fume hood and the distal intestine (hindgut) was cut and removed between the ileorectal valve and 0.5 cm before the anal opening. The intestine was cut longitudinally and a scalpel was used to scrape and collect 200-400 mg of luminal content and mucosa (combined) into a sterile Eppendorf tube containing 1mL of RNAlater® (Sigma-Aldrich Co, St. Louis, MO, USA). Samples were kept on ice for less than six hours and then stored at -80 °C until later analysis. Both luminal content and mucosa were collected and analysed together in order to show a comprehensive representation of both allochthonous (transient) and autochthonous (adhered) bacteria in the distal intestine.

2.4 Preparation for 16S rRNA gene sequencing

DNA was isolated from intestinal samples and 16S rRNA gene amplicons were generated using a two-step PCR with meta-barcoding. The amplicons were purified with magnetic beads, pooled into a single library and sequenced using an Illumina MiSeq platform, according to Herlemann, et al. (2011) and Hugerth, et al. (2014) with modification by Huyben, et al. (2017). In brief, approximately 200 mg of intestinal content/mucosa in RNAlater® solution were transferred to sterile tubes containing 0.5 g of 0.1 mm silica beads and homogenised in a Precellys homogeniser (Bertin Instruments, Montigny-le-Bretonneux, France) for two cycles of 60 sec at 6000 rpm followed by 5 min rest on ice. The DNA was extracted using a QIAamp Fast DNA Stool Mini Kit (Qiagen Gmbh, Hilden, Germany) according to the manufacturer's instructions. Amplicons were prepared by adding 2 µL of template DNA to sterile tubes containing 1 µL of each primer (10 µM), 8.5 µL nuclease-free water and 12.5 µL of 2x concentrated Phusion® High-Fidelity Master Mix (Thermo Fisher Scientific Inc., Waltham, MA, USA). The V4 region of the 16S ribosomal RNA gene was amplified using the primers 515F (5'-GTGCCAGCMGCCGCGGTAA-3') and 805R (5'-GACTACHVGGGTATCTAATCC-3') (Hugerth, et al., 2014). Conditions for PCR included denaturation at 98 °C for 30 sec, followed by 35 cycles of 98 °C for 10 sec, 60 °C for 30 sec and 72 °C for 10 sec, ending with 72 °C for 2 min. Amplicons were visualised by gel electrophoresis and purified using Agencourt AMPure XP magnetic beads according to the manufacturer's instructions (Beckman Coulter Inc., Bromma, Sweden). The 515F and 805R primers containing Illumina-compatible barcodes (eight nucleotide combinations) with adapters were used to tag each sample individually during the second PCR step. Amplicons of 10.5 µL were added to sterile tubes containing 1 µL of each barcode primer (10µM) and 12.5 µL of 2x concentrated Phusion® High-Fidelity Master Mix (Thermo Fisher Scientific Inc). Conditions for the second PCR step included denaturation at 98 °C for 30 sec, followed by 10 cycles of denaturation at 98 °C for 10 sec, hybridisation at 62 °C for 30 sec and elongation at 72 °C for 5 sec, followed by final elongation at 72 °C for 2 min. The amplicons were purified as before, quantified using a Qubit® 3.0 Fluorometer (Invitrogen, Thermo Fisher Scientific), diluted with elution buffer to 10 nM and then all samples were pooled. The pooled library was quality checked (size and abundance) using qPCR and sequenced using the Illumina MiSeq platform at SciLifeLab AB (Stockholm, Sweden).

2.5 Bioinformatic analysis of sequence data

The bacterial sequence data were processed according to Müller, et al. (2016). In brief, the paired end sequence reads were quality trimmed using the Cutadapt tool (Martin, 2011) in Python version 2.7 (Python Software

Foundation, http://www.python.org) to remove remaining adaptor and primer sequences, bases with quality below 10 from the 3' end, reads containing N bases, reads longer than 300 base pairs and reads not containing primer sequences. Paired end reads were joined using the join_paired_ends.py function according to the SeqPrep method (https://github.com/jstjohn/SeqPrep) in Quantitative Insights into Microbial Ecology (QIIME) version 1.8.0 (Caporaso, et al., 2010b). The joined reads were then used for split libraries and the operational taxonomic units (OTUs) were assigned using the open reference OTU picking strategy at a threshold of 97%, using U-CLUST against Greengenes core set (gg_13_8) (Edgar, 2010; Rideout, et al., 2014). The representative sequences were aligned against the Greengenes core set using PyNAST software (Caporaso, et al., 2010a). The chimeric sequences were removed by ChimeraSlayer (Haas, et al., 2011). Taxonomy was assigned to each OTU using the Ribosomal Database Project (RDP) classifier with a minimum confidence threshold of 80% (Wang, et al., 2007). The alignment was filtered to remove gaps and hypervariable regions using a Lane mask and a maximum-likelihood tree was constructed from the filtered alignment using FastTree (Price, et al., 2010). The final OTU table was further filtered to include OTUs present in at least three samples and to exclude OTUs identified as chloroplasts and mitochondria, since only bacteria were of interest. In addition, the number of reads per sample was normalised (termed subsampled) to equal that in the sample with the lowest number of reads (i.e. 10,238). The 16S rRNA gene sequences were deposited in the NCBI Sequence Read Archive (SRA) as SRA Accession SRP144010 and BioProject PRJNA454155 (https://www.ncbi.nlm.nih.gov/bioproject/PRJNA454155).

2.6 Statistical analyses

All data were analysed using R© version 3.3.1 (R-Core-Team, 2015) with the 'vegan' (Oksanen, et al., 2018) and 'nlme' (Pinheiro, et al., 2014) statistical packages. For α-diversity of bacterial OTUs in the gut, No. of OTUs/taxa, Shannon diversity and Chao-1 richness indices were generated from non-transformed count data using rowSums, diversity and estimateR functions. Significant differences between diets were determined by applying a Linear Mixed Effects (LME) model with diet as a fixed effect and tank as a random effect followed by Least Square Means test (lsmeans package) with Tukey adjustment to account for multiple pair-wise comparisons (Lenth, 2016). A value of $p<0.05$ was considered significant.

For β-diversity, plots of bacterial OTUs were produced based on Non-Metric Multidimensional Scaling (NMDS) with Bray-Curtis index after 2D Wisconsin standardization of square-root transformed data (metaMDS function).

Similarity Percentage Analysis (SIMPER) followed by one way Permutational Multivariate Analysis of Variance (PERMANOVA; adonis function) using Bray-Curtis index at 999 permutations (McArdle, Anderson, 2001; Oksanen, et al., 2018) with diet and tank as factors. Bonferroni adjusted p-values were generated to determine significant differences between diets in terms of composition of gut bacteria. In addition, the LME model and lsmeans test used above were applied to bacterial OTUs at the genus, order and phylum levels that had a mean relative abundance >1% in order to determine effects of each diet on the most prevalent bacterial groups.

3. Results

Illumina Miseq sequencing of the v4 region of the 16S rRNA gene from the gut content/mucosa of rainbow trout fed the fishmeal and insect diets produced a normalised count of 10,238 sequence reads per fish (491,424 in total) that identified to 878 individual OTUs belonging to 109 known taxa (grouped by genus) after data quality filtering. Before filtering and subsampling, the number of sequence reads was 76,875 ± 3,612 (mean ± SE) per sample for a total of 3.7 million sequences. Analysis of OTUs showed that alpha-diversity of gut bacteria increased for fish fed all three insect-based diets compared with the fishmeal diet (Table 2). Fish fed the pre-pupae diet showed the highest diversity for all three indices and was significantly higher than fish fed larvae and defatted-larvae diets for the Shannon and Choa-1 indices (Fig. 1). Compared with fishmeal, fish fed the larvae diet had increased Shannon diversity and fish fed the defatted-larvae diet had higher Chao-1 richness.

Diet had an overall effect on the beta-diversity and composition of bacterial OTUs in the gut (PERMANOVA; $F=24.132$, $R^2=0.633$, $p=0.001$), although there was no significant effect of tank (PERMANOVA; $F=1.300$, $R^2=0.126$, $p=0.161$). Composition of gut bacteria was significantly different between fish fed each diet ($p<0.01$), while least dissimilar between fish fed the larvae and defatted-larvae diets (SIMPER; 71.4%). Compared with fishmeal diet, fish fed the defatted-larvae diet were the most dissimilar followed by fish fed the pre-pupae and larvae diets (SIMPER; 90.3, 87.7 and 82.6%). The NMDS plot (Fig. 2) agreed with the SIMPER analysis as the cluster of fish fed the fishmeal diet were separated from fish fed the insect-based diets, while furthest from the pre-pupae diet.

Relative abundances of bacterial OTUs found in the fish gut were mainly represented by the phyla Firmicutes, Proteobacteria and Actinobacteria (Fig. 3). Compared to fishmeal diet, Firmicutes significantly increased in fish

fed the larvae and defatted-larvae diets (LME, N=12; p=0.002 and <0.001, respectively), Actinobacteria increased in fish fed the pre-pupae and larvae diets (p<0.001 and 0.009, respectively) and Proteobacteria decreased for all the insect-based diets (p=0.002, 0.003 and 0.001, respectively). On the order level, most OTUs were represented by Bacillales, Pseudomonadales, Actinomycetales and Lactobacillales (Fig. 4). Compared to fishmeal diet, Bacillales significantly increased in fish fed the larvae and defatted-larvae diets (p=0.012 and <0.001, respectively), Pseudomonadales decreased for all the insect-based diets (p=0.005, 0.008 and 0.003, respectively), Actinomycetales increased for the pre-pupae and larvae diets (p<0.001 and p=0.009, respectively) and Lactobacillales increased for the pre-pupae and larvae diets (p=0.043 and p<0.001, respectively). On the genus level, OTUs with >1% relative abundance (by decreasing abundance) included *Corynebacterium*, *Pseudomonas*, *Photobacterium*, *Achromobacter*, *Virgibacillus*, *Facklamia*, *Flavobacterium*, *Lactobacillus*, *Brevibacterium* and Lactobacilliceae;Other (unclassified). There was a significant effect of diet (p<0.05) on the OTUs with >1% abundance, except for *Flavobacterium* and *Achromobacter* (p=0.080 and 0.311, respectively). For significant differences between diets, see Fig. 5.

Fish fed the fishmeal, pre-pupae, larvae and defatted-larvae diets had a mean individual weight gain of 74.0, 74.1, 81.8 and 66.1 g (SE = 8.1 g) , respectively, and a mean individual feed intake of 81.5, 88.2, 89.1 and 77.2 g (SE = 2.4 g), respectively. No mortalities were recorded during the experiment.

4. Discussion

4.1 Dietary chitin and chitinase producing bacteria

This study is the first to analyse the effects of feeding black soldier fly meals on the gut microbiota of rainbow trout using high-throughput sequencing (i.e. Illumina next-generation sequencing). This method highlighted the dramatic shift from high Proteobacteria:Firmicutes ratio in the gut of fish fed fishmeal to a low ratio with increased bacterial diversity in fish fed the insect-based diets (Fig. 3, Table 2). Chitin may have acted as a new substrate to increase the proliferation of chitinolytic bacteria, which are mainly represented by the Firmicutes phyla and include many *Bacillus* species (Cody, 1989). Using Sanger sequencing of agar cultured isolates, *Bacillus* spp. were found in the intestine of Atlantic salmon fed a diet with 5% chitin and this group of bacteria showed the highest chitinase activity *in vitro* (Askarian, et al., 2012). Similarly, we found significant increases in Bacillaceae

(the family including *Bacillus*) in our study in fish fed diets with black soldier fly larvae as well as defatted-larvae diets. In addition, previous studies have found that feeding with black soldier fly larvae or krill-derived chitin can significantly change the gut microbiota in rainbow trout (Bruni, et al., 2018) and Atlantic cod (Zhou, et al., 2013). Lastly, supplementation of chitinase enzymes derived from bacteria in chitin-based diets has been shown to increase growth performance of hybrid tilapia (Zhang, et al., 2014). The higher dietary chitin in the insect-based diets may explain the significant change in gut microbiota of fish.

4.2 Increased bacterial diversity and abundance of lactic acid bacteria

High bacterial diversity is considered to have a positive effect on gut health since species-rich communities are thought to out-compete pathogens for nutrients and colonization, consequently resisting pathogen invasion and intestinal infection (Cerezuela, et al., 2013; Levine, D'Antonio, 1999; Yachi, Loreau, 1999). Therefore, fish fed all three insect diets, especially pre-pupae, in our study may have a healthier gut microbiota since their bacterial alpha-diversity was higher compared with the fishmeal diet (Table 2). One possible reason for the highest diversity in fish fed the pre-pupae diet may be a higher content of dietary chitin (Table 1). Previous studies have suggested that more chitin is deposited in the exoskeleton of insects at later life-cycle stages (Xiao, et al., 2018). However, other studies have found similar levels of chitin between life stages, although amino acid composition had changed (Finke, 2007). Chitin is not a typical component in commercial aquafeeds, thus its inclusion may stimulate the colonisation and growth of less common bacteria in the intestine that have the ability to digest chitin as a source of nutrients. In Atlantic salmon, feeding chitin was found to increase lactic acid bacteria (i.e. order of Lactobacillaceae) in the gut as well (Askarian, et al., 2012). Studies have suggested that chitin may be a preferential substrate for lactic acid bacteria in the gut of salmonids (Bruni, et al., 2018), which explains the increased abundance when fish were fed the insect-based diets in the present study (Fig. 4). Increased abundance of lactic acid bacteria has been used as an indicator of a healthy gut since they produce bacteriocins that inhibit pathogens (Dimitroglou, et al., 2011; Merrifield, et al., 2010; Ringø, Gatesoupe, 1998). In addition, several studies have found a decreased abundance of lactic acid bacteria associated with reduced growth or temperature stress in salmonids (Hovda, et al., 2012; Huyben, et al., 2018; Huyben, et al., 2017; Neuman, et al., 2016). However, a recent study found that abundance of lactic acid bacteria increased in Atlantic salmon with soybean meal-induced enteritis (Gajardo, et al., 2017), which challenges this bacterial order as a positive indicator of gut health. The increased bacterial diversity and abundance of lactic acid bacteria indicate that feeding black soldier fly meals

may improve gut health of rainbow trout, although further studies are needed to correlate changes in intestinal bacteria with empirical health indicators, e.g. morphology and gene expression.

4.3 Effect of dietary lipids and insect rearing conditions on fish gut bacteria

The crude lipid content of the insect meals may have altered the gut microbiota in the fish since the full-fat larvae and pre-pupae diets had 71 and 40 g kg^{-1} higher levels than the defatted-larvae diet (Table 1). Insect meal can be defatted in order to reduce the lipid content to maintain feed pellet stability and avoid altering the lipid composition of the fish fillet (Henry, et al., 2015; Sealey, et al., 2011). In our study, OTUs of *Corynebacterium* (including *C. variabile*) were significantly higher in fish fed the full-fat larvae and pre-pupae (Fig. 5) and this bacterium has been reported to produce high levels of lipase (Brennan, et al., 2002). The *C. variabile* has been found in the gut of insects, such as the common fruit fly (*Drosophila melanogaster*) (Storelli, et al., 2011) and predatory mites (*Neoseiulus cucumeris*) (Pekas, et al., 2017), which suggests this bacterium in our study may have originated from the insect meal. This bacterium may also be derived from the insect rearing facility or substrate. The *C. variabile* can tolerate pH values below 4.9 (Brennan, et al., 2002), which may have allowed it to bypass acidic conditions in the fish stomach and colonise the intestine. Fish fed the defatted-larvae meal had very low abundance of *Corynebacterium* (i.e. <1%; Fig. 5), which corresponds to the low lipid content in the diet. These results indicate the lipid composition in the diet and/or bacteria present in the insect meals may have influenced the gut microbiota in these fish, although these aspects need further investigation.

The rearing conditions and microbiota of the insects fed to fish may have influenced the gut microbiota of the fish in our study, especially since the diets were produced via cold-pelleting (opposed to extrusion) that avoids extensive heat inactivation of microbes (Huyben, et al., 2017). In a study that used 454 pyro-sequencing, the microbiota of mealworms and grasshoppers were dominated by the Firmicutes phyla, especially lactic acid bacteria (Stoops, et al., 2016), which is similar to the gut bacteria found in our study (Fig. 3 and 4). The farmed mealworm larvae also had a high abundance of Actinobacteria, which was significantly increased in fish fed black soldier fly larvae and pre-pupae in our study, respectively (Fig. 3). Aside from the effects of dietary lipids, differences in gut bacterial composition of fish fed the full-fat and defatted insects may be due to different insect rearing conditions (i.e. substrate of food compost versus wheat bran). Previous studies have found that different

substrates, such as those enriched with offal trimmings, used to rear black soldier flies can impact growth performance of rainbow trout and Atlantic salmon (Lock, et al., 2016; Sealey, et al., 2011). This may be the case in our study where restaurant compost was used as a substrate to produce black soldier flies included in the larvae and pre-pupae diets compared with a vegetable substrate for the defatted-larvae diet.

5. Conclusions

This study showed the effects of feeding black soldier flies in different life-cycle stages and lipid content on the intestinal microbiota of rainbow trout using Illumina high-throughput sequencing for the first time. The composition of gut bacteria was different for each diet and the pre-pupae diet was the most dissimilar compared with the fishmeal diet. Feeding insects resulted in elevated bacterial diversity and abundance of lactic acid bacteria, which is a potential indicator of improved gut health. Fish fed the insect-based diets had increased abundance of Firmicutes and Actinobacteria with a reduction in Proteobacteria. Fish fed larvae and pre-pupae diets had increased abundance of *Corynebacterium*, which was attributed to its ability to produce lipase and the high content of dietary lipids. For both larvae diets, abundance of Bacillaceae was significantly increased and this was attributed to their ability to produce chitinase and the high level of dietary chitin. These results indicate that feeding black soldier fly alters the gut microbiota of rainbow trout and insects harvested at different life-cycle stages and/or defatted further influence the bacterial communities.

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526 **Tables**

527

528 Table 1. Formulation (g kg^{-1} wet matter basis) and proximate composition (g kg^{-1} dry matter basis) of the
529 reference diet and experimental diets with 30% replacement with larvae, defatted-larvae and pre-pupae of black
530 soldier fly.

Formulation	Fishmeal	Larvae	Defat-Larvae	Pre-Pupae
Fish meal LT	500	350	350	350
Black soldier fly larvae meal[1]	0	300	0	0
Black soldier fly defatted-larvae meal[2]	0	0	300	0
Black soldier fly pre-pupae meal[1]	0	0	0	300
Wheat gluten	50	33	33	33
Wheat meal	80	53	53	53
Wheat starch	80	53	53	53
Fish oil	155	102	102	102
Gelatin	50	50	50	50
α-cellulose	50	33	33	33
Carboxymethyl cellulose	10	7	7	7
Vitamin & mineral premix	10	7	7	7
Monocalcium phosphate	10	7	7	7
Titanium dioxide	5	4	4	4
Proximate composition				
Dry matter	971	950	977	932
Crude protein	507	504	534	515
Crude lipid	191	252	181	221
Crude ash	111	109	100	123
Neutral detergent fibre	67	76	139	98
Acid detergent fibre	46	51	60	62
Chitin	0	19	28	30

531 [1]Larvae and pre-pupae meals produced by SLU (Uppsala, Sweden)

532 [2]Defatted larvae meal commercially produced by Protix (Dongen, The Netherlands)

533

534

535 Table 2. Diversity indices of bacterial OTUs in the distal intestine of rainbow trout fed diets based on fishmeal,
536 larvae, defatted-larvae and pre-pupae of black soldier fly.

	Fishmeal	Larvae	Defat-Larvae	Pre-Pupae	SE	p-value
No. of OTUs/taxa	98^{a}	203^{ab}	215^{ab}	326^{b}	31	0.002
Shannon diversity	1.58^{a}	2.74^{b}	2.16^{ab}	3.72^{c}	0.23	<0.001
Chao-1 richness	128^{a}	264^{ab}	301^{b}	437^{c}	38	<0.001

537 SE; standard error of the mean

538

Figures

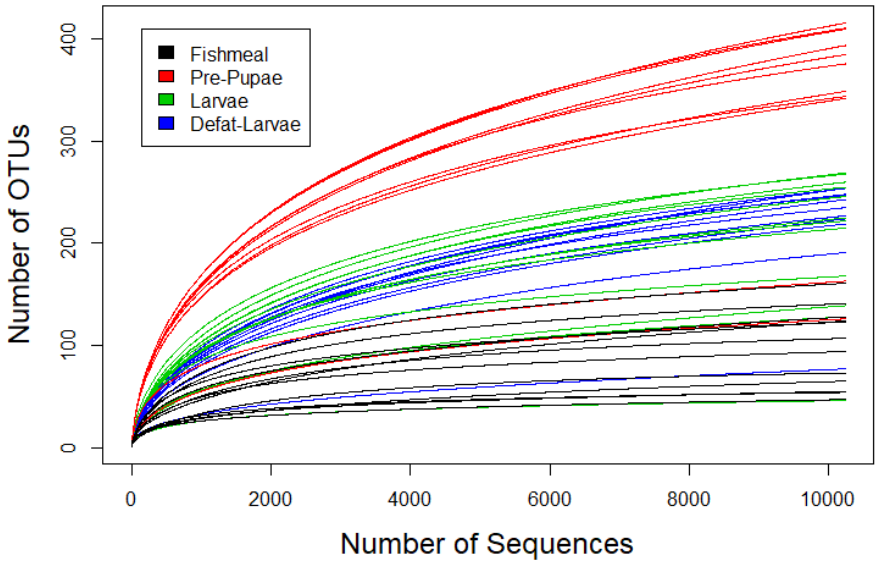
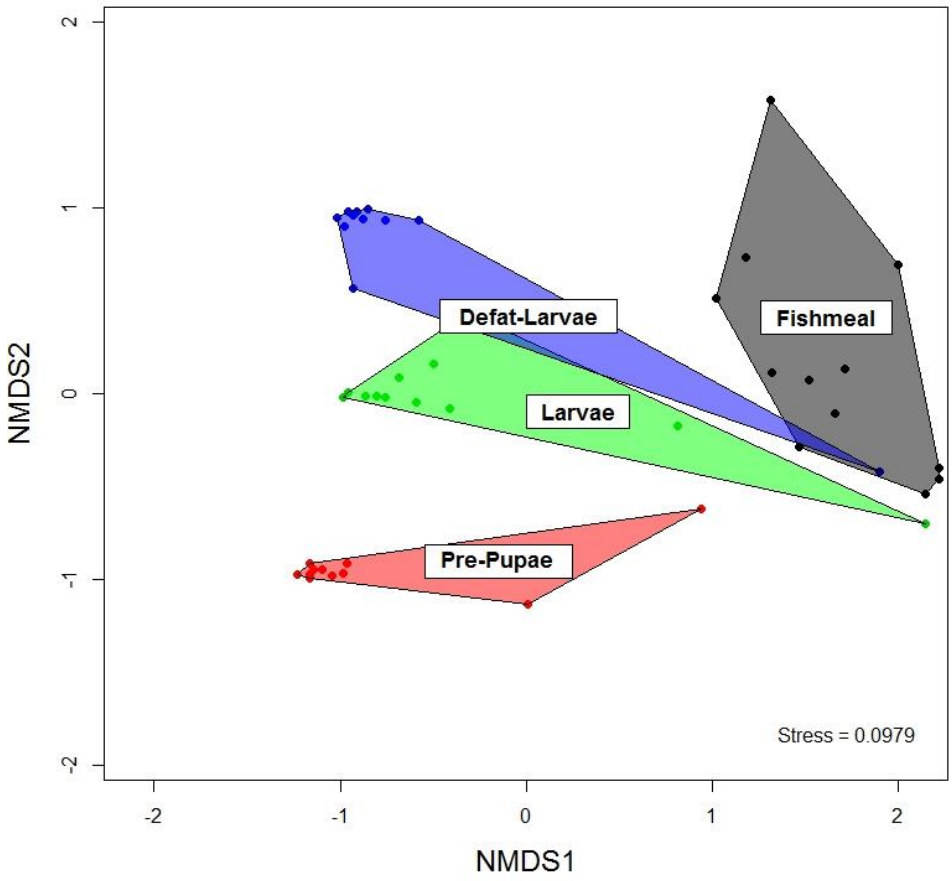


Fig. 1. Rarefaction curves of sequencing bacterial OTUs in the distal intestine of rainbow trout fed diets of fishmeal, larvae, defatted-larvae and pre-pupae of black soldier fly (N=46).



545

546 Fig. 2. Non-metric multidimensional scaling (NMDS) with 2D Bray-Curtis similarity index and after square-
547 root transformation of bacterial OTU counts in the distal intestine of rainbow trout fed diets of fishmeal, larvae,
548 defatted-larvae and pre-pupae of black soldier fly.

549

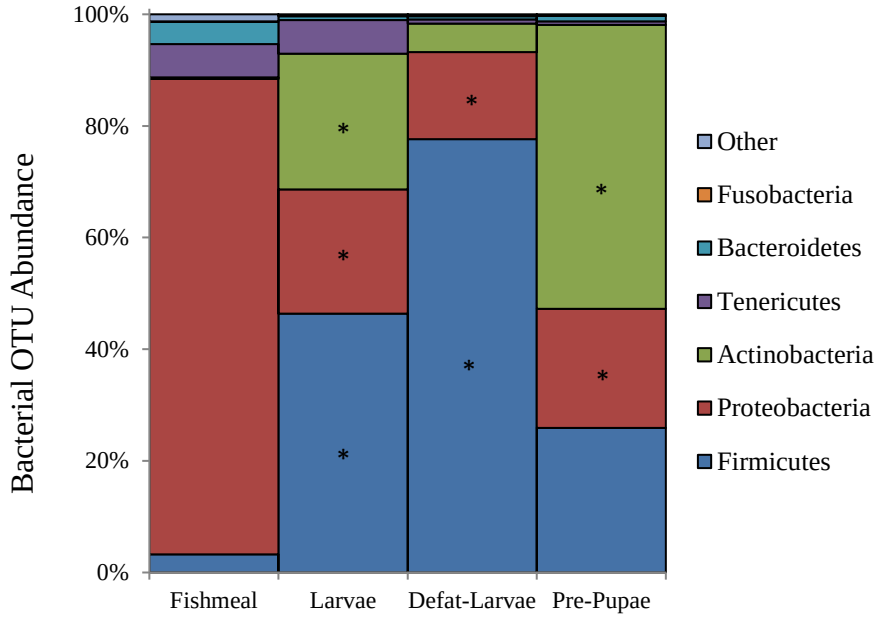


Fig. 3. Mean relative abundance of bacterial OTUs (grouped on phyla level) in the distal intestine of rainbow trout fed diets of fishmeal, larvae, defatted-larvae and pre-pupae of black soldier fly. The * symbol indicates a significant difference compared with the fishmeal diet ($p<0.05$).

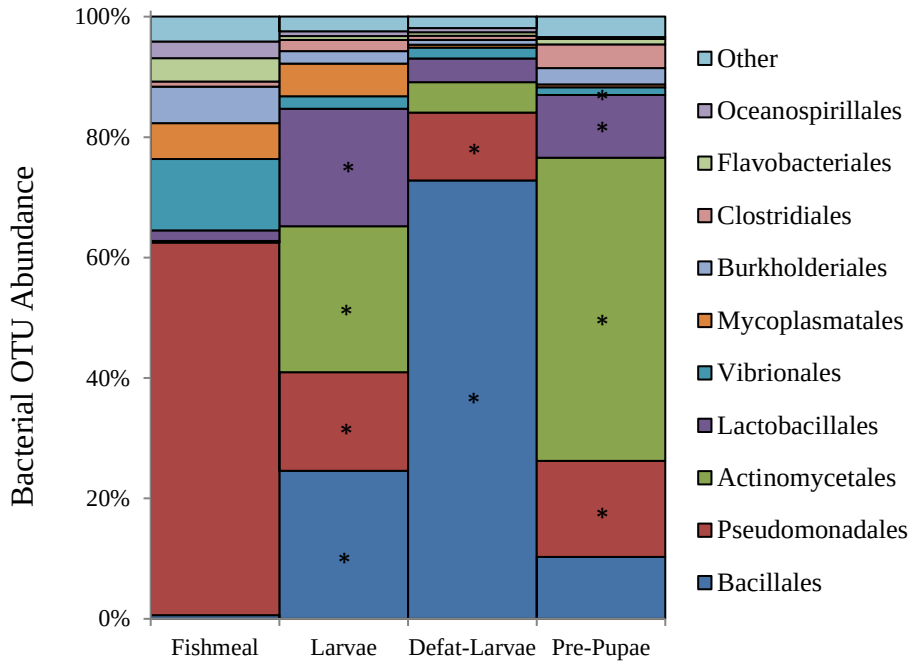


Fig. 4. Mean relative abundance of bacterial OTUs (grouped on order level) in the distal intestine of rainbow trout fed diets of fishmeal, larvae, defatted-larvae and pre-pupae of black soldier fly. The * symbol indicates a significant difference compared with the fishmeal diet ($p<0.05$).

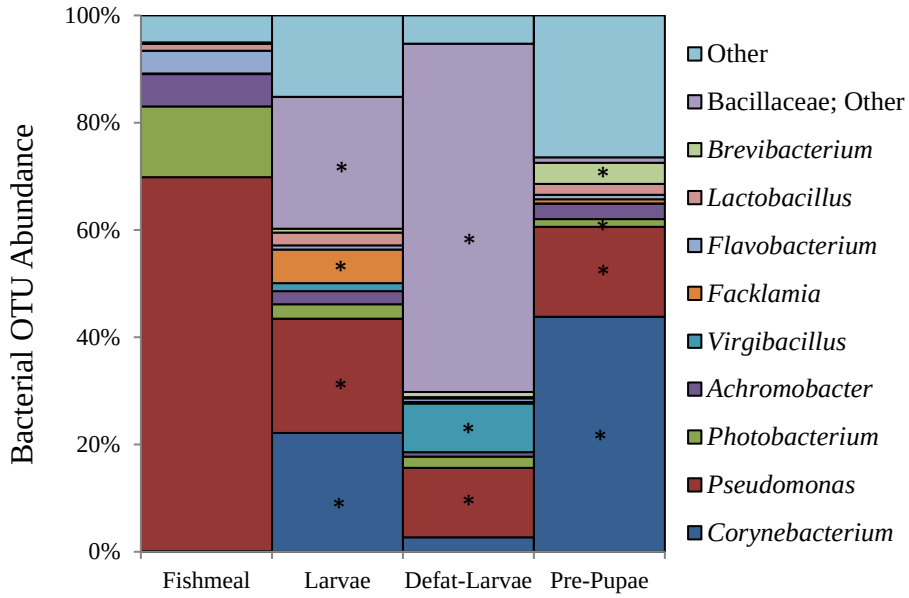


Fig. 5. Mean relative abundance of bacterial OTUs (grouped on genus level) with >1% abundance in the distal intestine of rainbow trout fed diets of fishmeal, larvae, defatted-larvae and pre-pupae of black soldier fly. The * symbol indicates a significant difference compared with the fishmeal diet ($p<0.05$).