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Short title: Intestinal bacteria of rainbow trout fed with *Hermetia illucens*

Full title: Characterisation of the intestinal microbial communities of rainbow trout (*Oncorhynchus mykiss*) fed with *Hermetia illucens* (black soldier fly) partially defatted larva meal as partial dietary protein source

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Abstract

Scientific research has examined the possibility of replacing fishmeal with alternative protein sources in feed for fish. The literature indicates that insects are an eco-friendly nutrient-rich alternative to fishmeal. The purpose of this study was to investigate the effects of including insects in a diet for rainbow trout (*Oncorhynchus mykiss*), by analysing organosomatic parameters, fillet yield and

intestinal bacterial communities. Three experimental diets were formulated: a control diet with fishmeal as main protein source, and diets Hi25 and Hi50 where 25% and 50% of fishmeal, respectively, was replaced with partially defatted meal of larvae of *Hermetia illucens* (Hi; Diptera: Stratiomydae). At the end of the trial, organosomatic parameters and fillet yield were recorded. To profile the complex intestinal mucosa- and digesta-associated bacterial communities (MAB and DAB), denaturing gradient gel electrophoresis was performed on bacterial DNA extracted from intestinal mucosa and digesta, followed by sequencing of selected bands. Rainbow trout fed the alternative diets showed the same organosomatic indices and fillet yields as the control group, but increased bacterial community biodiversity, structure and composition, with ANOSIM $p<0.05$ and $p<0.01$ for MAB and DAB, respectively. The sequencing highlighted a clear prevalence of γ-Proteobacteria in all samples, though α- and β-Proteobacteria and Actinobacteria were also present in MAB of insect-fed fish; DAB of insect-fed fish showed a clear increase in the Firmicutes phylum compared to the control group. The results suggest that *H. illucens* partially defatted larva meal is a valid alternative protein source and can replace up to 50% of fishmeal in rainbow trout feed without impairing organosomatic indices nor fillet yield. The microbiological assays revealed that the intestinal bacterial communities were sensitive to dietary changes, showing modified community structure and increased biodiversity in the Hi-fed groups. We discuss the effects that modified bacterial communities could have on fish biology. There is a good possibility of further studies on the functional role of bacteria.

Key words: rainbow trout, insect, black soldier fly, intestinal microbial community, DGGE, sustainable aquaculture.

Abbreviations

DAB	digesta-associated bacterial community
DISAFA	Department of Agricultural, Forest and Food Sciences

DISPAA	Department of Agri-Food Production and Environmental Sciences
EFSA	European Food and Safety Authority
Hi	*Hermetia illucens*
LAB	lactic acid bacteria
MAB	mucosa-associated bacterial community
OTU	operational taxonomic unit

1. Introduction

Using insect meal instead of fishmeal is becoming more common in the aquaculture sector of many countries. Not only is fishmeal not eco-friendly as principal dietary protein source, but it is also becoming costlier. Issues such as the increasing global demand for fish protein, the impact of fishmeal production on the ecology of fishing grounds, its shortage and its high price have brought attention to the need for alternative dietary protein sources (FAO, 2016, 2014). Animal and fishery by-products as well as plant-derived material are now used as substitutes (FAO, 2014). Regrettably, plant protein derivatives rarely have a balanced essential amino acid (EAA) profile and often contain antinutritive factors (Oliva-Teles et al., 2015). Processed animal protein is considered a valuable alternative as it has a better EAA profile and is more digestible than plant proteins; nevertheless, within the Europe Community, restrictions on the use of certain processed animal proteins persist as protection against transmissible spongiform encephalopathies (Regulation 68/2013/EC, 2013). Insects have recently attracted increasing attention as a sustainable nutrient source for feed, not only in Europe but also around the world. Indeed, insects are a good source of EAA, lipids, vitamins and minerals (Henry et al., 2015; van Huis et al., 2013); they grow and reproduce quickly and easily on low-quality organic waste and manure (van Huis et al., 2013); they have a small ecological footprint and high feed conversion efficiency (Makkar et al., 2014), and can reasonably foster a circular bioeconomy. Finally, the use of processed insects in feed for aquaculture animals was recently allowed by the European Commission (Regulation 2017/893/EC, 2017).

Compared to other insects, the Diptera order and in particular the species *Hermetia illucens* (Hi), also known as black soldier fly, show an EAA pattern very similar to fishmeal (Henry et al., 2015) and are therefore a good alternative protein source. Recent trials substituting up to 40% of fishmeal with Hi meal have shown that it effectively supports fish growth (Lock et al., 2016; Magalhães et al., 2017; Renna et al., 2017). Regarding the hygienic concerns of the European Food and Safety Authority (EFSA) about insect rearing and characteristics (EFSA Scientific Committee, 2015), adults of Hi do not feed, thus diminishing the likelihood of disease transmission to their progeny (Makkar et al., 2014). Insects are rich in chitin, which reduces their digestibility but, as underlined by Karlsen et al. (2017), dietary chitin may select a beneficial intestinal microbiota.

Different diets affect the structure and the composition of fish intestinal bacterial communities in different ways (Hartviksen et al., 2014; Wang et al., 2017; Zhou et al., 2013); the communities in turn affect the digestive functions and immune responses of the host (Ghanbari et al., 2015; Lyons et al., 2017a). The impact of bacteria on digestive functions can be summarised as providing essential nutrient and non-nutrient factors and as increasing the host's ability to harvest nutrients from feed, for instance by producing digestive enzymes that break down chitin or cellulose (Gomez et al., 2013; Ray et al., 2012; Ringø et al., 1995). Additionally, bacteria dwelling in the gastrointestinal tract co-exist in dynamic equilibrium with occasional pathogens; indeed, gut-associated lymphoid tissue sorts microorganisms and responds either with tolerance or an immune response (Merrifield et al., 2010; Nayak, 2010; Pérez et al., 2010; Ringø et al., 2010a). If the composition of the intestinal bacterial community is known and the principles of its assembly and preservation are understood, it can be manipulated to improve the health of the host (Merrifield et al., 2010; Ringø et al., 2010b; Roeselers et al., 2011; van Kessel et al., 2011). The intestinal bacterial community can be considered a key to healthy fish and productive aquaculture plants.

The mucosa-associated bacterial community (MAB) could have a greater impact than the digesta-associated community (DAB) on the biology of the host. It is therefore worthwhile making an effort to examine the MAB. To the authors' knowledge, this is the first study aimed at assessing the changes

in rainbow trout MAB and DAB induced by dietary insect meal. Denaturing gradient gel electrophoresis (DGGE) and band sequencing were used to acquire an overview of these complex communities, to identify dominant bacterial groups, and to glean insights into the effect of the sequenced bacterial strains on fish.

2. Materials and methods

2.1. Experimental diets and growth

The experimental protocol applied in this study was designed according to the ethical standards approved by the current European Directive 2010/63/EU on the protection of animals used for scientific purposes.

The samples analysed in this study were retrieved from the growth trial described in Renna et al. (2017) using meal of partially defatted Hi larvae reared on vegetable by-product substrate purchased from Hermetia Deutschland GmbH & Co. KG (Baruth/Mark, 141 Germany). The Hi larva meal had the following chemical composition: dry matter (DM): 94.18%; crude protein (CP): 55.34 g/100 g DM; ether extract (EE): 17.97 g/100 g DM; ash: 7.12 g/100 g DM; chitin: 5.00 g/100 g DM; nitrogen free extracts (NFE): 14.57 g/100 g DM. It had a gross energy (GE) of 24.37 MJ/kg DM. Three experimental diets were formulated with increasing percentages of Hi larva meal: one control diet (Hi0) and two test diets (Hi25 and Hi50). The diets were formulated to be isonitrogenous (CP: about 45 g/100 g DM), isolipidic (EE: about 15 g/100 g DM), and isoenergetic (GE: about 22 MJ/kg DM). Hi0 contained fishmeal (600 g/kg) as the main protein source, whereas Hi25 and Hi50 had 25% and 50% of the fishmeal replaced by Hi meal, respectively (a total of 20% and 40% of Hi meal in Hi25 and Hi50, respectively).

Rainbow trout (*Oncorhynchus mykiss*) of 178.9 ± 9.81 g initial body weight were fed the experimental diets for 78 days (Renna et al., 2017).

Table 1 shows the ingredients, the chemical composition of the experimental diets containing *Hermetia illucens* larva meal and the main trout growth performance measures as reported in Renna et al. (2017).

2.2. Sampling, fillet yield and organosomatic indices

At the end of the feeding trial followed by one day of starvation, 90 fish (30 per group) were anesthetised with 60 mg/L MS222, killed and immediately transported on ice to the Department of Agri-Food Production and Environmental Sciences (DISPAA), University of Florence (Florence, Italy), where each was weighed and dissected. The fillets (from 90 fish), 30 livers (10 samples per group) and 18 viscera (6 samples per group) were individually weighed to assess fillet yield (FY=weight of fillets with skin×100/BW), visceral index (VSI=total viscera weight×100/BW) and hepatosomatic index (HSI=liver weight×100/BW).

2.3. Sampling for microbiological analysis

On arrival at DISPAA, the abdomen of 18 fish (6 samples per group) was cut open with alcohol-disinfected tools and the intestine, from just after the pyloric caeca to the anus, was removed and placed on tinfoil. Perivisceral fat was removed and the digesta were gently squeezed out into a tube. The intestine was then cut longitudinally, rinsed by flushing in sterile phosphate-buffered saline to remove faecal traces and placed in a tube. Pyloric caeca were not analysed due to the limited resources allocated to the present study and because this segment presents the highest technical difficulties, as stated in the literature (Gajardo et al., 2016). We collected: 6 samples of intestinal tissue for each of the three groups to investigate the mucosa-associated bacterial community (MAB) (18 tubes); 6 samples of digesta for each of the three groups to investigate digesta-associated bacterial community (DAB) (18 tubes). The 36 tubes were stored at -80 °C until microbiological analysis.

2.4. DGGE analyses

The 18 intestine-tissue and 18 digesta samples were processed at the Research Centre for Agriculture and Environment (CREA-AA, Florence, Italy) to assess the biodiversity and composition of MAB and DAB. Each intestine sample was thawed on ice, homogenised with an UltraTurrax® T 25 (IKA-Labor Technik, Staufen, Germany) at 20,500 rpm for 1.5 minutes in an ice bath and immediately processed for total DNA extraction. The DNA of intestinal homogenates and digesta was extracted with the QIAamp®DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the protocol for isolation of DNA from stools for pathogen detection, with minor changes: a) the volume of elution Buffer AE was decreased to 75 µL to obtain a higher concentration of DNA in the final eluate; b) the incubation time of Buffer AE was increased to 5 minutes to increase DNA yield.

The V6-V8 region of the template DNA was amplified with the primer set GC-986F (5'- CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TA -3') and 1401R (5'- CGG TGT GTA CAA GAC CC -3'), as designed by Nübel et al. (1996). This set of primers proved to function well and homogeneously among all samples, as showed in the DGGE profile of bacterial communities (S1 and S2 Figures). Amplification reactions were carried out in a T100™ Thermal Cycler (Bio-Rad) in 25 µL of a mixture containing: 2 µL template DNA, 1× Green GoTaq® Flexi Buffer (Promega Corporation, Madison, WI, USA), 1.5 mmol L^{-1} $MgCl_2$ (Promega), 200 µmol L^{-1} dNTPs (Promega), 10 pmol of each primer and 1U GoTaq® G2 Flexi DNA Polymerase (Promega), under reaction conditions of: denaturation at 95 °C for 2 minutes, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, elongation at 72 °C for 45 s and final elongation at 72 °C for 5 minutes. The amplicons from several amplification reactions were pooled to minimise PCR biases. Amplicon yield was estimated by comparison with the Low DNA mass ladder (Invitrogen, Carlsbad, CA, USA) using a Chemidoc Apparatus (Bio-Rad Laboratories Inc., Hercules, CA, USA).

DGGEs were performed on a DCode™ Universal Mutation Detection System (Bio-Rad), loading 600 ng of the amplicons onto a 6% polyacrylamide gel (acrylamide/bis 37.5:1; EuroClone S.p.A, Pero, Milan, Italy), with a linear denaturing gradient from 45% to 65% (v/v) obtained from a 100%

denaturing solution containing 40% formamide (v/v) (Sigma-Aldrich GmbH, St. Louis, MO, USA) and 7 M urea (Promega). The gels were run for 17 h in 1×TAE buffer at constant voltage (80 V) and temperature (60 °C). At the end, the gels were stained with SYBR® GOLD (Molecular Probes, Eugene, OR, USA) diluted 1:10,000 in 1×TAE buffer. Gel images were digitalised using the Chemidoc apparatus and optimised for analysis by enhancing contrast and greyscale.

2.5. Sequencing

The middle portion of 38 selected DGGE bands was excised and placed in 30 µL distilled water. Given the greater interest in MAB, more bands were sequenced from intestinal than from digesta samples. The PCR products were eluted through freezing and thawing (Throbäck et al., 2004) and reamplified using the primer pairs described above. Amplicons were checked by DGGE for the presence of a single band and then directly sequenced by Macrogen Service (Macrogen Ltd., Amsterdam, The Netherlands, http://www.macrogen.com).

The sequence chromatograms were edited using Chromas Lite software (v2.1.1; Technelysium Pty Ltd; Tewantin, QLD, Australia; http://www.technelysium.com.au/chromas_lite.htm) to verify the absence of ambiguous peaks and to convert them to FASTA format. DECIPHER's Find Chimeras web tool (http://decipher.cee.wisc.edu) was used to uncover chimeras in the 16S rDNA sequences. Representative sequences were deposited in the GenBank database under accession numbers KY270784-KY270810. The Web-based BLASTN tool (http://www.ncbi.nlm.nih.gov/BLAST) was used to find closely related nucleotide sequences. To increase the accuracy of the assignments, different sequence similarity thresholds were used for different taxonomic levels: a similarity ≥97% for species level identification and 95%, 90%, 85%, 80% and 75% for assignment at genus, family, order, class and phylum level, respectively (Webster et al., 2010). Phylogenetic dendrograms were constructed to display the apparent relatedness of the partial 16S rRNA gene sequences of this study to each other and to other sequences of equivalent length recovered from environmental samples and axenic cultures deposited in the GenBank database. Sequence alignment was performed with

ClustalX 2.0.11 software (Larkin et al., 2007); distance analysis was carried out according to Jukes and Cantor (1969) followed by phylogenetic tree construction using the neighbour-joining algorithm (Saitou and Nei, 1987) by TREECON 1.3b (Van de Peer and De Wachter, 1994). The robustness of associations between samples (nodes) was evaluated by bootstrap analysis with 1000 replicates.

2.6. Statistical analysis

Data on fillet yield and organosomatic indices was checked for normality and homoscedasticity, then one-way ANOVA was performed with the diet as independent variable, using the Paleontological Statistics Software Package (PAST; Hammer et al., 2001).

The DGGE banding patterns were normalised and analysed using GelCompar II software v 4.6 (Applied Maths, Sint-Martens-Latem, Belgium). Although amplification products from different bacterial groups can co-migrate, each band was considered to match a single bacterial species or group, and band intensity (the relative surface of the peak compared to the surfaces of all the peaks in the profile) was considered to indicate the relative abundance of the corresponding species or group (Fromin et al., 2002). Bands shared by samples were classified as the same band when they were within a 0.8% range of total pattern length from each other. Phylotype richness (number of DGGE bands), Shannon-Wiener diversity index ($H'=-\Sigma^{R}\times p_i\times \ln p_i$, where R is the number of phylotypes/bands in the sample and p_i is the relative intensity of the ith phylotype/band) and Simpson's evenness index ($\lambda=\Sigma^{R}\times p_i^2$) were calculated with GelCompar II. Simpson data were log-transformed to fit normality. One-way ANOVA followed by a Tukey HSD *post hoc* test was performed on the results of the three indices using PAST.

The banding patterns of the DGGEs were extracted as band-intensity matching tables, then normalised by calculating the relative intensity of each band (ratio of the intensity of each band divided by the sum of the intensities of all bands in the same lane) and finally imported into PAST for further multivariate analyses. Non-metric multidimensional scaling (nMDS) was used to represent the distance between each sample in two-dimensional space. One-way analysis of similarity

(ANOSIM) followed by pairwise comparisons was conducted to determine the extent of differences in MAB and DAB between the three dietary groups. ANOSIM and nMDS were performed using the Bray-Curtis distance measure and 9,999 permutational tests; the accuracy of the nMDS plots was determined by calculating a 2D stress value.

3. Results

3.1. Fillet yield and organosomatic indices

VSI did not highlight differences among treatments and ranged from 7.43 to 7.91 for Hi25 and Hi50, respectively (p-value: 0.50). Similarly, no differences were reported for HSI (1.48, 1.53 and 1.53 for Hi0, Hi25 and Hi50, respectively; p-value: 0.76). As far as fillet yield is concerned, values were above 60% and without differences among treatments (Hi0: 62.4; Hi25: 63.1; Hi50: 61.8; p-value: 0.57)

3.2. Intestinal bacterial communities

The DGGE banding patterns were analysed using biodiversity indices (Table 2). No significant differences were found among the DAB samples; by contrast, the biodiversity of the MAB Hi25 group was significantly higher than the control group ($p<0.05$) and that of the Hi50 group was intermediate between the two; the SEM of richness and Shannon index values seemed higher in the Hi25 and Hi50 groups.

The intensity of staining showed a decreasing trend from the Hi0 to Hi50 groups, especially in MAB samples (S1 Figure).

The nMDS plots showed slight clustering in relation to diet in MAB and DAB samples (Figure 1). In both, the Hi25 samples seemed to form a loose yet separate cluster from the control. The relative positions of the points in the plots was moderately reliable in the MAB (stress=0.1944) and DAB (stress=0.2271) samples.

Again in the case of MAB and DAB samples, ANOSIM tests (Table 3) revealed significant differences between groups ($p<0.05$ and $p<0.01$, respectively). The results of pairwise comparisons

are summarised in Table 4, where differences between all groups were significant ($p<0.01$) in DAB samples, whereas in MAB samples, only the pair Hi0-Hi25 displayed a significant difference ($p<0.05$).

3.3. Sequencing

A total of 29 and 19 bands were retrieved from the MAB and DAB DGGE gels, respectively; of these, 20 and 14 bands from MAB and DAB, respectively, were successfully sequenced; 27 sequences were deposited in the GenBank database. Operational taxonomic units (OTUs) were identified at species level in 24/27, at genus level in 2/27 and at family level in 1/27 sequences (Tables 5 and 6). The OTUs proved related to the phyla Proteobacteria, Firmicutes and Actinobacteria, including eleven genera: *Acinetobacter*, *Aeromonas*, *Brevundimonas*, *Carnobacterium*, *Citrobacter*, *Curtobacterium*, *Delftia*, *Kluyvera*, *Pseudomonas*, *Shewanella* and *Staphylococcus*.

Bands in different lanes at the same height were considered to belong to the same or to a very close phylogenetic bacterial strain. The OTUs from MAB samples were related to eight genera in the Proteobacteria and Actinobacteria phyla: *Curtobacterium* (Actinobacteria); *Brevundimonas* (α-Proteobacteria); *Delftia* (β-Proteobacteria); *Acinetobacter*, *Aeromonas*, *Citrobacter*, *Pseudomonas* and *Shewanella* (γ-Proteobacteria). The core set of OTUs resistant to dietary changes in MAB samples was related to *Pseudomonas* spp. and *Shewanella* spp. OTUs related to *Aeromonas rivipollensis* were only abundant in the control group, while the insect-fed groups were rich in bands related to *Citrobacter gillenii*, *Pseudomonas* spp. and *Delftia acidovorans*; bands related to *Acinetobacter* spp., *Brevundimonas* spp., *Curtobacterium flaccumfaciens* and *Delftia acidovorans* were only recovered from insect-fed fish and were sporadic. The OTUs from DAB samples were related to genera in the Proteobacteria and Firmicutes phyla: *Carnobacterium* and *Staphylococcus* (Bacilli); *Aeromonas*, *Kluyvera* and *Shewanella* (γ-Proteobacteria). The core set of OTUs resistant to dietary changes in DAB samples was linked to *Shewanella schlegeliana* and *Aeromonas rivipollensis*. OTUs related to *Citrobacter gillenii* and *Kluyvera intermedia* (both belonging to the family

Enterobacteriaceae) and *Carnobacterium divergens* were abundant in the insect-fed groups. Control MAB and DAB samples showed very similar DGGE banding patterns, which were more variable in the insect-fed groups.

4. Discussion

The main results obtained by Renna et al. (2017) indicated that substituting up to 50% of fishmeal with a partially defatted Hi larva meal in diets for rainbow trout did not affect survival or growth performance. Differences in apparent digestibility were only found between the Hi25 and Hi50 groups, with the control showing intermediate values. The present study reported that fillet yield and organosomatic performance were not statistically different between control-fed and insect-fed fish. On the other hand, as discussed in detail by Renna et al. (2017), feeding salmonid species with *H. illucens* could negatively affect growth performance (St-Hilaire et al., 2007; Stamer et al., 2014), but the outcome depended on the insect's life stage (larvae vs. prepupae), fat content and rearing substrate (Sealey et al., 2011), on the processing techniques used (Lock et al., 2016) and on the fish life stage. As suggested by Magalhães et al. (2017), detailed information concerning substrate, handling and processing of the insect as well as the composition of each batch of insect meal is needed in order to explain the performance results. Nevertheless, as reported by Oliva-Teles et al. (2015), aquaculture outcome is not defined solely by fish performance, but issues concerning fish welfare also need to be taken into account. These include immunological status, oxidative status and the influence of diets on intestinal microbiota, which directly affect the digestive functions and immune response of the host (Ghanbari et al., 2015; Lyons et al., 2017a) and are therefore considered a key to healthy fish and productive aquaculture plants.

The results of studies on salmonids fed diets containing chitin from various sources are inconsistent. Indeed, chitin is suspected to diminish feed availability and digestibility in fish (Kroeckel et al., 2012; Olsen et al., 2006). Krill meal seemed to depress growth performance of *Oncorhynchus keta* fingerlings (Murai et al., 1980) and rainbow trout (Wojno and Dabrowska, 1984), and chitin sourced

from shrimp (*Pandalus borealis*), included in the diet at a concentration of 2% and 5%, seemed to depress Atlantic salmon performance (Karlsen et al., 2017). However, the results of the present study were positive despite the presence of chitin in the Hi feeds. Moreover, Lellis and Barrows (2000) reported that 6% chitin supplementation enhanced growth in rainbow trout juveniles, and Atlantic salmon, fed a diet in which 50% of fishmeal had been replaced by northern krill (*Meganyctiphanes norvegica*) meal, showed no differences in terms of growth performance (Ringø et al., 2006a). A reason for these contrasting results could be that under certain conditions chitin may counterbalance the negative effects that it itself produces. In fact, some authors have suggested that chitin may be targeted by a positive intestinal microbial community, which improves host organism performance and health status (Karlsen et al., 2017; Ringø et al., 2006a).

Bearing in mind that DGGE only detects dominant bacterial groups (Muyzer et al., 1993), the present study indicates that the fish microbial community is plastic and can be manipulated by addition of insect meal to feed, in line with the majority of studies on other dietary sources (Desai et al., 2012; Hartviksen et al., 2014; Ingerslev et al., 2014; Navarrete et al., 2012; Ringø et al., 2006a; Zhou et al., 2013). Biodiversity parameters of MAB and DAB were increased by dietary administration of the insect meal, in general agreement with studies assessing the effect of dietary krill or inclusion of 5-20% chitin in the diet of salmonids (Askarian et al., 2012; Ringø et al., 2012) but in contrast with studies on the effect of plant proteins (Bakke-McKellep et al., 2007; Reveco et al., 2014). The MAB control group of the present study showed the lowest richness and diversity values, the Hi25 group had the highest while the Hi50 stayed in an intermediate position, in contrast with the hypothesis that a higher content of Hi meal in the diet is accompanied by greater changes in the intestinal microbial community. It is possible that the weak staining of the MAB Hi50 samples was due to less abundant taxa, leading to underestimation of subtle composition variations: hence, the actual diversity could be higher than expressed by the diversity indices. It is commonly recognised that high microbial diversity can compete with pathogens for nutrients and colonization sites (Cerezuela et al., 2013) and that it bestows resilience, a notion Yachi and Loreau (1999) referred to as the 'Insurance hypothesis'. This

concept could explain the similarly good performance recorded by fish fed control and insect diets, although the chitin could have decreased nutrient digestibility.

The diversity indices of the present study for insect-fed trout generally showed a more variable response than for control groups, as indicated by SEM; nMDS plots indicated the same trend. Ordination methods are a quick way to make a 2D plot of trends between groups when many variables are involved, though they unfortunately do not quantify significance that would enable scientists to interpret the plots (Al-Hisnawi et al., 2015; Dimitroglou et al., 2009; Forberg et al., 2016; Heikkinen et al., 2006; Ingerslev et al., 2014). Multivariate tests, an established tool in other disciplines dealing with complex microbial communities and now also used in studies assessing fish gastrointestinal communities (Zhou et al., 2014), back up the results of biodiversity indices and nMDS plots (De Mesel et al., 2004; Lagomarsino et al., 2016; Lam et al., 2008). The ANOSIM tests of the present study showed that most bacterial community structures were diet-specific. Since the high within-group variability indicated the influence of latent factors, experiments including more factors (e.g. farming management, tank and genotype) are required to discriminate the different sources of variation. Similarly, although pooling within-group replicates is a common practice in studies of fish intestinal microbial community (Dimitroglou et al., 2009; Hartviksen et al., 2014; Navarrete et al., 2009; Zhou et al., 2009), it obscures interindividual variability, as indicated by several authors (Desai et al., 2012; Reveco et al., 2014; Ringø et al., 2006b; Yang et al., 2012). Indeed, Navarrete et al. (2012) posited that different host genetics shapes a unique niche that singles out a specific bacterial community. The question of how the host background shapes the community is already on the agenda in other fields of research (Archie and Theis, 2011; McKnite et al., 2012; Spor et al., 2011) and should also be addressed in aquaculture.

Our sequencing results were in line with the general consensus on rainbow trout intestinal bacterial composition, which is principally composed of γ-Proteobacteria and Firmicutes (Desai et al., 2012; Lyons et al., 2017b; Mansfield et al., 2010; Navarrete et al., 2010; Spanggaard et al., 2000). The present study seems to be the first to find the *Acinetobacter*, *Brevundimonas* and *Shewanella* genera

in rainbow trout MAB. In addition, MAB composition differed from DAB, in line with other studies in the literature (Gajardo et al., 2016; Hartviksen et al., 2014; Merrifield et al., 2009a, 2009b). It therefore seems worthwhile analysing the two substrates separately. For instance, in MAB, but not in DAB, of insect-fed fish, α- and β-Proteobacteria and Actinobacteria were also found, coherent with Lyons et al. (2017b). To sum up, like other studies in the literature (Lyons et al., 2017b; Roeselers et al., 2011; van Kessel et al., 2011; Xia et al., 2014), we observed few abundant genera that dominated the community pattern.

Lyons et al. (2017a) indicated that rainbow trout intestinal bacteria could contain genes that could positively influence the host's digestive metabolism. In the present study, OTUs attributable to lactic acid bacteria (LAB, Firmicutes) were only found in DAB samples from insect-fed fish, in contrast with the literature on salmonids (Al-Hisnawi et al., 2015; Askarian et al., 2012; Bakke-McKellep et al., 2007; Lyons et al., 2017b; Merrifield et al., 2009b). Making a hypothesis similar to that of Gajardo et al. (2016), we suggest that chitin was the preferential growth substrate for LAB, which could increase the digestibility of fibre, as found for soybean-based diets (Desai et al., 2012). Several other OTUs retrieved in this study could account for effects on fish physiology. This is why we propose an overview of these hypothetical effects, while bearing in mind that the mere presence of a microbe in the intestine does not necessarily imply a functional role (Zhou et al., 2013).

Shewanella spp. were clearly ubiquitous, especially *S. schlegeliana*. Described by Satomi et al. (2003) for the first time, strains of *S. schlegeliana* have interesting enzyme activities and, above all, their fatty acids contain 18.6% eicosapentaenoic acid. We assume that *S. schlegeliana* contributed to the nutrition of the present rainbow trout. On the other hand, this species has also been found to produce trimethylamine (Satomi et al., 2003), indicating that further studies on the interaction between this species and the host are necessary. *Pseudomonas stutzeri* was part of the core set of OTUs of MAB, with the insect-fed groups particularly rich in these bacteria. *Pseudomonas* spp. are listed as probiotics by Nayak (2010), have antiviral activity (reviewed by Balcázar et al., 2006), together with *Staphylococcus* spp. may promote nutritional processes in Arctic charr (*Salvelinus alpinus* L.) (Ringø

et al., 1995) and Askarian et al. (2012) isolated a *Pseudomonas* sp. strain with amylase, cellulase and lipase activity from the gastrointestinal tract of Atlantic salmon fed a control diet. *Acinetobacter radioresistens* was found in rainbow trout MAB for the first time by the present study; it conceivably played an important role in nutrient digestion since *Acinetobacter* spp. extracted from Atlantic salmon displayed chitinase, amylase, cellulase and phytase activity and seemed to inhibit the growth of the pathogens *Vibrio anguillarum* and *Moritella viscosa* (Askarian et al., 2012). *Aeromonas* spp. strains, observed in the control group MAB and DAB of the present study, are listed as probiotics by Nayak (2010) and known for their cellulase activity (Li et al., 2014). *Carnobacterium divergens* was abundant in DAB samples of the insect-fed groups. Bacteria of the *Carnobacterium* genus are well-known probiotics in salmonids and have several functions: *in vitro* growth inhibition of pathogens, stimulation of non-specific immune response and *in vivo* improvement of disease resistance. Together with *Staphylococcus* species, they may also improve the digestion of proteins and carbohydrates (Al-Hisnawi et al., 2015; Askarian et al., 2012; Balcázar et al., 2006; Mansfield et al., 2010; Ringø et al., 1995, 2010a).

5. Conclusions

Our results indicate that partially defatted *H. illucens* larva meal, a valid alternative protein source to fishmeal for feeding rainbow trout as reported by previous studies, sensitively changed mucosa- and digesta-associated intestinal bacteria communities, showing higher biodiversity in the insect-fed groups. Some bacteria may protect fish from pathogens as well as enhancing digestion, physiological functions and welfare in general. The present results may be useful for future research into salmonid microbial communities and into interactions between host, guest and diet. To delve into the biological consequences of the host-guest interaction, we suggest combining description of bacterial communities with functional methods, such as metagenomics, metabolomics and challenge tests.

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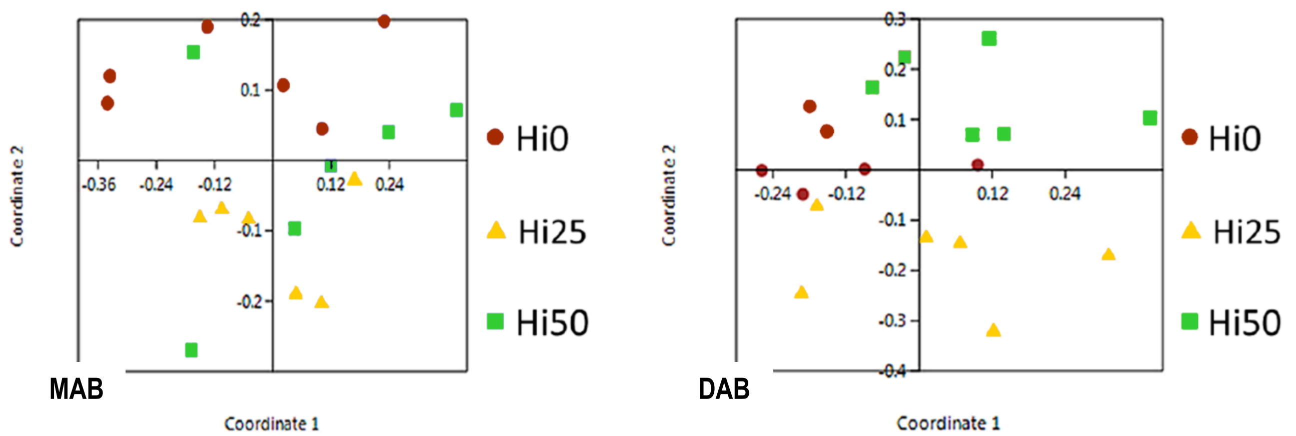
Table 1. Ingredients (% fresh matter), chemical composition (g/100 g DM) of experimental diets containing *Hermetia illucens* (Hi) larvae meal and major performances of trout fed diets (from Renna et al., 2017).

	Hi0	**Hi25**	**Hi50**	**p-value**
Ingredients				
FM	60	45	30	
Hi meal	0	20	40	
Wheat meal	4	4	4	
Wheat bran	9	6	3	
Starch (D500)	15	15	15	
Fish oil	9	7	5	
Vitamin mixture	1.5	1.5	1.5	
Mineral mixture	1.5	1.5	1.5	
Chemical composition				
DM	96.07	94.93	95.63	
CP	45.20	44.86	45.00	
EE	15.86	15.74	15.81	
Ash	11.40	11.43	10.11	
Chitin	0	1.05	2.09	
NFE	27.54	26.92	26.99	
Growth performances (n = 4)				
Weight gain	360.5	366.5	358.9	0.840
Feed conversion ratio	0.90	0.88	0.90	0.739

Captions of Figures

Fig 1. nMDS plots of mucosa- (left, MAB) and digesta-associated bacterial community (right, DAB) constructed on the band intensity matrix of the DGGE pattern using the Bray-Curtis index.

Fig 1.



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