Effects of low level dietary microalgae supplementation on the distal intestinal microbiome of farmed rainbow trout *Oncorhynchus mykiss*

Philip P. Lyons[ab]*, James F. Turnbull[a] Karl A. Dawson[b] and Margaret Crumlish[a]

[a] *Institute of Aquaculture, University of Stirling, Stirling FK9 4LA, United Kingdom*

[b] *Alltech Biotechnology Inc., 3031 Catnip Hill Pike, Nicholasville KY 40356, USA*

Abstract

In this study, high throughput 16S rRNA sequencing was used to investigate the effect of a novel ingredient, dietary microalgae (*Schizochytrium limacinum*), on the distal intestinal microbiome of farmed rainbow trout *Oncorhynchus mykiss*. Dietary microalgae are rich in omega 3 polyunsaturated fatty acids, can be produced sustainably, and have been shown to have beneficial effects on host health. Microbial community profiles were compared between the distal intestinal contents of fish fed a control diet and a treatment diet that partially substituted dietary microalgae for the fish oil component (5% inclusion). The results of this research showed that the microbial communities of both fish populations were composed of similar microbial taxa, however the treatment group fed the microalgae supplement possessed a greater level of microbial diversity than those in the control group. A limited number of bacterial taxa were discriminatory between diets and were significantly elevated in the treatment group, notably operational taxonomic units (OTU's) assigned to the genera *Streptococcus, Leuconostoc, Lactobacillus, Lactococcus* and *Weissella*. However, the overall structure of the intestinal microbiome between control and treatment groups was not found to be significantly different. The treatment group displayed a heavier mean weight and condition factor at the end of the trial period. The results of this study suggest that microalgae can be used as a replacement for a proportion of fish oil in aquafeeds, with minor changes to the intestinal microbiome of farmed rainbow trout, and positive effects on growth.

Keywords: aquaculture, bacteria, intestine, microalgae, microbiome, rainbow trout, 16S rRNA sequencing

*Corresponding author: P Lyons, Institute of Aquaculture, University of Stirling, Stirling FK9 4LA, UK. E-mail: p.p.lyons@stir.ac.uk

1. Introduction

Numerous studies have reported that diet type is a major driver in shaping the GI microflora of both terrestrial and aquatic animals (Ringo & Olsen 1999; Merrifield et al 2010; Merrifield, Dimitroglou, Foey, Davies, Baker, Bogwald, Castex & Ringo 2010; Merrifield et al 2011; Gatesoupe, Huelvan, Le Bayon, Sevre, Aaasen, Degnes, Mazurais, Panserat, Zambonino-Infante & Kaushik 2014; Kormas, Meziti, Mente. & Frentzos 2014; Miyake, Ngugi & Stingl 2015). A number of studies have investigated the impact of different diets on the intestinal microflora of farmed salmonids, mainly focusing on the impact of selected probiotics, prebiotics and immunostimulants included within the diet formulation. The vast majority of these investigations have been undertaken using culture-based and low resolution molecular microbiological techniques that provide an incomplete picture of the intestinal microbiome.

However, more recently, high throughput sequencing technologies have been used to examine the effect of diet on the intestinal microbiome of fish in far greater detail. Desai et al (2012) used 454 pyrosequencing to demonstrate reproducible effects on the intestinal microbiome of farmed rainbow trout fed soybean meal (SBM), noting changes in the ratio of Firmicutes: Proteobacteria as a result of supplementation. Ingerslev, Von Gersdorff, Jorgensen, Lenz Strube, Larsen, Dalsgaard, Boye & Madsen (2014) used the HiSeq® platform to demonstrate changes in the structure of the intestinal microflora of rainbow trout fry fed with marine versus plant-based dietary ingredients. In contrast to these results, Wong, Waldrop, Summerfelt, Davidson, Barrows, Kenney, Welch, Wiens, Snekvik, Rawls & Good (2013) reported that the intestinal microbiome of rainbow trout was largely unaffected by dietary alterations and resulted in only very minor changes to specific microbial community assemblages. Furthermore, substantial inter animal variation in the microbial community structure between individual fish has been reported (Mansfield, Desai, Nilson, Van Kessel, Drew and Hill 2010) suggesting that analysis of pooled samples are not suitable in studies of the intestinal

83 microbiome. The use of pyrosequencing platforms play an important role in this regard,
84 permitting high resolution analysis of individual gut microbiomes, leading to more reliable
85 conclusions regarding the effect of dietary alterations on the structure of the microbial
86 communities in the fish gastrointestinal (GI) tract.

87 It has been widely reported that the gut microflora of aquatic animals is responsible for the
88 digestion of algal cells, the production of both amino acid and short-chain fatty acids, in
89 addition to secreting inhibitory compounds that protect against colonization of the gut by
90 bacterial pathogens (Austin 2006; Nayak 2010; Ghanbari, Kneifel & Domig 2015). Research
91 concerning the impact of microalgae on the structure of the intestinal microbiome however is
92 limited and has hitherto primarily focused on wild herbivorous fish species that consume algal
93 substrates in their natural habitat (Choat & Clements 1998; Ward, Steven, Penn, Methe &
94 Detrich 2009; Smriga, Sandin & Azam 2010) with only a single study examining farmed fish
95 species (Cerezuela, Fumanal, Tapia-Paniagua, Meseguer, Morinigo & Esteban (2012).
96 Conflicting results have been reported in these studies, with some reporting increases and
97 others reporting decreases in microbial diversity as a result of dietary algal consumption. This
98 suggests that whilst diet impacts the diversity of gut microflora identified in fish, the
99 relationship between novel dietary components such as microalgae, and the structure of the
100 intestinal microbiome, is not clear and thus further detailed examination is undoubtedly
101 required.

102 The primary objective of this study was to characterize the intestinal microbiome of farmed
103 rainbow trout fed both a standard control diet, and a treatment diet containing a dietary
104 microalgae supplement (5%), in order to test whether differences in diet composition lead to
105 alterations in the structure of the microbial community. The aquafeed sector recognizes the
106 need to provide dietary alternatives to fish oil which provide comparative health benefits to
107 farmed fish species. Therefore, the secondary aim of this research was to test for any

differences in growth performance between the control and treatment groups and whether or not this could be correlated with the composition of the intestinal microbiome. It was hypothesized that feeding farmed rainbow trout slightly different diets would alter the structure of the intestinal microbiome in these fish.

2. Materials and methods

2.1 Dietary formulation

Two diets, one control and one treatment, were formulated at the Hellenic Centre for Marine Research (HCMR, Anavyssos Attiki, Greece). These diets were similar except that the experimental diet contained a 5% whole cell microalgae ingredient (*Schizochytrium limacinum*; Alltech Biotechnology, Nicholasville USA). The whole cell microalgae principally replaced the fish oil component in the treatment diet (3%), but also partially replaced soybean concentrate and wheat meal ingredients, each at a level of 1% (Table S1). Both diets met or exceeded the guideline nutrient requirements for rainbow trout (National Research Council 2011).

2.2 Experimental design and sampling protocol

Farmed rainbow trout (*O. mykiss*) were obtained from a local trout farm and transferred to the Aquatic Research Facility (ARF) at the University of Stirling Institute of Aquaculture (Stirling, UK). The average weight of the fish on arrival at the ARF was 31.7 ± 2.6g. Fish were quarantined in a communal tank for 10 days, prior to random allocation into twelve 100 L tanks (n=25 $tank^{-1}$) maintained on a flow through system, under a 12h light and 12h dark cycle and an ambient water temperature (14 ± 1^{o}C). All instructions and guidelines set by the UK Home Office under the Animal Welfare Act of 1986 were adhered to throughout this experimental trial. Each tank was randomly allocated the diets, giving four replicates per treatment, and each group was hand fed a ration of approximately 2% of their body weight twice daily.

At the end of the 15 week trial period, a total of three fish from each of four replicates per treatment were randomly removed for sampling. Fish were sacrificed with a lethal dose of anaesthetic benzocaine (Sigma Aldrich®) and swabbed with 100% ethanol before dissection through the ventral surface. The tissues surrounding the visceral fat were removed and the

distal gut contents (~150 mg) were aseptically collected by gently squeezing the tissue with a sterile forceps, and placed into a sterile 2 ml capped microtube (Alpha laboratories ®) containing 1 ml of lysis buffer (Qiagen). The gut was then incised and washed with a sterile 0.85% (w/v) NaCl solution, and the intestinal mucous was carefully removed from the gut wall. This material was placed into the same tube as the gut contents. All tubes were placed on dry ice before DNA extraction later the same day, in order to ensure optimal sample integrity. In addition to the intestinal samples, three pellets from each diet and a sample of the tank biofilm were also processed as described above, to compare the microbial communities of both the diets themselves and of the tank biofilm, with the intestinal microbiome of the trout.

2.3 Growth performance

The length and weight of each fish sampled at the end of the trial period was recorded to measure growth performance, thermal growth coefficient (TGC) and condition factor (K). Final fish weight was measured as the mean final weight of each group ± standard error of the mean (SEM). TGC was calculated using the formula $TGC= (W2^{(1/3)} – W1^{(1/3)})/D^{(0)}$ X 1000 where W2 and W1 are weight at the end and at the start of the trial respectively, and d^0 represents degree days. K was calculated using Fulton's equation $K= (10^5 \text{ x weight})/Length^3$.

2.4 DNA extraction

A total of 150 mg of intestinal content material from each individual fish suspended in 1 ml of buffer ASL (Qiagen) was processed for DNA extraction. A further sample containing only 1 ml of buffer ASL was processed as a negative control. Samples were firstly disrupted using a Mini bead-beater 16 (Biospec Ltd.) at maximum speed for four separate cycles of 35 s each. Samples were allowed to settle, and total genomic DNA was extracted and purified using the QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany), with the following modifications to the manufacturer's protocol: 150 mg starting material in 1ml buffer ASL; suspension heated at

95°C for 10 min to improve lysis of Gram positive bacteria; 0.5 Inhibitex tablet per sample in 700 µl supernatant; final sample elution volume of 50 µl. After extraction, the DNA concentration of all samples was determined both spectrophotometrically (Thermo Scientific NanoDrop™ 1000, DE, USA) and fluorometrically (Qubit® Life Technologies) to ensure optimal DNA purity, and stored at -20°C for subsequent processing.

2.5 16S rRNA PCR and pyrosequencing

A PCR was first carried out using universal eubacterial primers 27F (AGAGTTTGATCMTGGCTAG) and 1492R (TACGGYTACCTTGTTACGACTT) (Weisburg et al 1991) that target the full length bacterial 16S rRNA gene sequence, to confirm the presence of ample microbial community DNA and to rule out the presence of any potential inhibitory compounds. The extraction from buffer ASL was included in the PCR run to check for the presence of microbial DNA in the reagent itself. The PCR conditions for this confirmatory reaction were as follows; denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 94°C for 2 min, annealing at 50°C for 1 min and elongation at 72°C for 2 min; before final elongation at 72°C for 10 min. Products were then visualized on a 1.5% (w/v) agarose gel, run at 100V for approximately 1 h 15 min. The presence of a single strong PCR product of 1500bp was considered to be indicative of the presence of microbial community DNA.

Illumina libraries were prepared following the method described by Caporaso, Lauber, Walters, Berg-Lyons, Huntley, Fierer, Owens, Betley, Fraser, Bauer, Gormley, Gilbert, Smith, & Knight (2012) using the NEXTflex 16S Amplicon-Seq kit (Bio Scientific, Austin USA). A total of 50 ng of template DNA was used for each individual sample and the V4 hypervariable region of the bacterial 16S rRNA gene (length 292bp) was amplified using primers 515F (GTGCCAGCMGCCGCGGTAA) and 806R (GGACTACHVGGGTWTCTAAT) (GATC

Biotechnology Inc., Konstanz). The PCR conditions were as follows; initial denaturation at 95^{o}C for 5 min ; 25 cycles of denaturation at 95^{o}C for 30 s, annealing at 55^{o}C for 30 s and extension at 72^{o}C for 30 s; followed by a final extension step at 72^{o}C for 5 min. All samples were amplified in triplicate and all products purified using Agencourt Ampure XP beads (Beckman Coulter Ltd.). The products of the first PCR served as template for a second PCR with the same conditions as the first, however the number of cycles was reduced to eight, and Illumina sequencing adapters were added to the primers in the reaction mix. Following amplification, PCR products were purified using Agencourt Ampure XP (Beckman Coulter) with a modified 1:1 volume of PCR product to Ampure XP beads. Purified amplicons were quantified with Qubit, pooled in equal concentration and the final quality of the pooled library was validated using a Bioanalyzer 2100 (Agilent Technologies, Waldbronn, Germany). The final library was sequenced using the Illumina MiSeq® NGS system at GATC Biotechnology (Konstanz, Germany).

2.6 Bioinformatics

Demultiplexing was performed with Casava v. 1.8 (Illumina) and reads representing the PhiX or reads not matching indices were removed. The open-source software Mothur (Schloss 2009) was used to process sequences from the demultiplexed 16S rRNA gene libraries. Sequences were firstly merged using the make.contigs command. Reads containing ambiguous bases, homolpolymer runs greater than 8 bases, and sequences of less than 150 base pairs in length were removed from the dataset. Remaining sequences were aligned against mothur's Silva reference database, after customizing the reference alignment to concentrate on the v4 region only (length = 292bp). Further denoising of the dataset was performed using mothur's pre clustering algorithm, allowing for up to two differences between sequences. This sorted sequences by abundance, ordering from most abundant to least and identified sequences within

two nucleotides of each other. If sequences met these conditions they were merged. Chimeric sequences were then removed from the dataset using the UCHIME (Edgar, Haas, Clemente, Quince & Knight 2011) algorithm in mothur as a final denoising step prior to taxonomic classification.

For taxonomic analyses, sequences were annotated using the Bayesian classifier implemented by the ribosomal database project (RDP) Release 11 (Centre for Microbial Ecology, Michigan State University, East Lansing, MI, USA). A minimum confidence bootstrap threshold of 80% was required for each assignment, thus >80% of the classifications returned the same taxonomic assignment for a given read, after one thousand iterations. Sample coverage, rarefaction curves, bias-corrected Chao 1 richness and Simpson's index of diversity were calculated based on assembled OTU's using mothur. Samples were rarefied to the sample with the lowest number of sequences (sample AF6, n=314,961) before performing these diversity analyses, to ensure that any observed differences in diversity were not caused by uneven sampling depth.

2.7 Statistical analyses

A student's t-test was performed to compare the growth performance data between control and treatment groups, and differences were considered significant at $p<0.05$. The similarity of the structure and membership of the microbial communities found in each of the samples was calculated by creating a distance matrix based on the thetaYC (Yue & Clayton 2005) coefficient using the dist.seqs algorithm in mothur. This distance matrix was visualized using principal coordinate analysis, which allowed the intestinal microbial community profiles from the control and treatment groups to be compared. In addition, a dendrogram was created to further describe the similarity of the samples to each other (data not shown). Parsimony (Schloss & Handelsman 2006) and UniFrac (Lozupone and Knight 2005) analyses were

performed to determine whether any observed community structure clustering between diets was statistically significant. Finally, metatstats (White, Nagarajan & Pop 2009), LEfSe (Segata, Izard, Waldron, Gevers, Miropolsky, Garrett & Huttenhower 2011) and Indicator (McCune, Grace & Urban 2002) analyses were performed within mothur, in order to determine whether there were any phylotypes that exhibited a statistically significant representation between the control and treatment samples, and results were considered as significant at two levels, $p<0.05$ and $p<0.01$. The same statistical analyses were also used to compare feed pellet/biofilm samples with the intestinal samples from the control and treatment groups.

3. Results

3.1 Growth performance

All fish consumed both diets readily and upon conclusion of the trial, the weighed individuals from the treatment group had a higher mean weight and condition factor than the control group. The final mean weight and condition factor (± SE) for the treatment group was 136.6 ± 12.1g and 1.44 ± 0.06 whereas these values for the control group were 116.5 ± 9.3g and 1.33 ± 0.04 respectively (Figure S1). A t-test was performed using the Minitab 15 statistical software to test for significant differences between the performance parameters for both groups, however no such differences were found ($p = 0.107$).

3.2 Sequence data and diversity analyses

After quality filtering of sequences, a total of 18,282,541 sequences remained for analysis, which grouped into a total of 660 OTU's. After subsampling to that of the library containing the least number of reads (sample AF6, n=314,961), rarefaction curves generated in mothur showed a trend towards a greater level of microbial diversity in the treatment group with a greater number of overall OTU's being recorded (Figure S2). This trend was reflected in the inverse simpson and Chao1 diversity indices, with the three richest samples (AF7, AF4 and AF6) belonging to the treatment group (Table 1). A very high level of sequence coverage was achieved in the analysis, with all rarefaction curves reaching saturation and Good's coverage estimations reaching >99% for each sample, indicating that the vast majority of microbial phylotypes present were sampled in the analysis.

3.3 Microbial community composition and influence of diets

The overall microbial community composition was similar in both the control and treatment populations of fish. The distribution of OTU's at the phylum level of both the control and treatment libraries is illustrated below (Figure 1). The vast majority of reads were assigned to nine separate bacterial phyla, although an overall total of 13 phyla were recorded. Within these phyla, 13 microbial classes dominated (Figure 2). The mean number of OTU's classified to genus level observed in the control group was 99 (maximum of 177, minimum of 58), whereas in the treatment group the mean was 135 (maximum of 255, minimum of 77) (Table 1), reflecting the trend towards an increased level of microbial diversity in these fish. Considerable variability amongst individuals was noted.

The Tenericutes were the dominant phylum identified in the libraries recovered from both the control and treatment groups, with *Mycoplasma* being the most dominant genus observed in both groups. This suggests that the abundance of *Mycoplasma* was not affected by diet type. The remaining OTU's primarily belonged to the Firmicutes, Proteobacteria and Spirochaetes. OTU's assigned to Bacteroidetes, Actinobacteria, Deinococcus-Thermus, Candidate Division WPS-1 and Fusobacteria were detected at much lower levels of sequence abundances. Within the Firmicutes, the most frequently observed OTU's were *Acetanerobacterium*, *Weissella*, *Catellicoccus, Streptococcus, Leuconostoc,Lactobacillus,Lactococcus,Ornithinibacillus* and *Sediminbaciullus*. *Acetanaerobacterium* represented the second most dominant OTU recorded overall, and was present in higher mean relative sequence abundances in the group fed the control diet. Sequences assigned to the Proteobacteria were observed more frequently in the treatment fish and the most dominant OTU's within this phylum belonged to the γ subclass, and in particular *Acinetobacter*, *Escherischia/Shigella*, *Enterobacter*, *Pseudomonas* and *Pantoea*. The α and β subclasses were also represented and the dominant OTU's recorded from these classes were *Ahrensia* and *Sphingomonas* and *Delftia* and *Pelomonas* respectively. The

Spirochaetes were principally represented by the genus *Brevinema*, however *Sphaerochaeta* was also detected. This microbial class was most abundant in the treatment fish, with an overall mean sequence abundance of 3.1%, versus 0.7% in the control fish. Members of the class Bacteroidetes were infrequently recorded, and the dominant OTU's assigned to this class recorded in this study were *Flavobacterium* and *Cloacibacterium*. Similarly, OTU's assigned to the Fusobacteria were poorly represented within all libraries analysed, with *Fusobacterium* and *Cetobacterium* the principal genera detected in the sequence analysis. One of the most dominant OTU's observed in both control and treatment libraries was assigned to Candidate division WPS-1, an unclassified phylum, indicating that a large portion of the trout microbiome is still yet to be fully characterized.

Principal coordinate analyses, when visualized based on the thetaYC distance matrix comparing similarities in community structure, showed that samples were broadly indistinguishable according to diet, with the treatment and control samples clustering close together (Figure 3). This trend was examined using both the parsimony (Schloss & Handelsman 2006) and unweighted Unifrac (Lozupone & Knight 2005) analyses performed in mothur and confirmed that the microbial community structures were not significantly different between dietary treatments (ParsSig = 0.269, UWSig = 0.49). The community structure between the feed pellets and the intestinal microbiome were however significantly different when analysed statistically (ParsSig = 0.025, WSig = <0.001, UWSig = 0.004). The microbial community structure of the tank biofilm sample was also found to be significantly different from that of the trout intestinal microbiome samples (WSig = <0.001).

Although intestinal community structures were not statistically different between control and treatment fish, metastats (White et al 2009) analyses revealed that a number of OTU's were discriminatory according to dietary treatment and hence were differentially represented according to dietary regime (Table 2). These OTU's were *Leuconostoc* (p = 0.009),

Streptococcus (p = 0.009), *Weissella* (p = 0.048), Candidate Division WPS-1 (p = 0.006), *Lactobacillus* (p = 0.010), *Enterobacter* (p = 0.034), *Lactococcus* (p = 0.046) and *Bacillus* (p = 0.047). Furthermore, sequences representing each of these OTU's were significantly more abundant in the treatment group (Figure 4). Both the LEfSe (Segata et al 2011) and Indicator (McCune et al 2002) statistical algorithms also confirmed the same phylotypes as discriminatory according to diet, with the exception of *Weissella*, where p >0.05 for both metrics. *Acetanaerobacterium* and *Brevinema* were also selected due to obvious differences in overall mean sequence abundances and because of their high prevalence in the sequence libraries, but these phylotypes were not found to be discriminatory according to diet (Figure S3).

4. Discussion

The findings from this study suggest that 5% dietary microalgal supplementation altered levels of bacterial diversity and individual populations of microbes, but not the overall microbial community structure within the intestine of rainbow trout. No significant differences were recorded in growth and condition between control and treatment fish. These results improve our understanding of the interactions between the rainbow trout GI microbiome and novel dietary ingredients such as microalgae in aquaculture. Dietary supplements will undoubtedly continue to be included in future aquaculture feed formulations as the industry's supply of existing sources of fishmeal and fish oil decline. This research found that all of the individual rainbow trout analysed from both test groups possessed broadly similar intestinal microbial community compositions, after fifteen weeks of feeding. However, there were statistically significant differences in the representation of specific bacterial taxa between the control and treatment groups. Within the treatment group a trend towards an increase in microbial diversity was observed, however this pattern was not observed in all fish within this group and consequently was not statistically significant. Nonetheless, the pattern of increased microbial diversity could be indicative of the microbial community within the intestine of these fish responding to the availability of a different dietary ingredient, and perhaps an additional fermentable substrate in the form of the whole cell microalgal supplement.

It has previously been reported that gut microbial diversity increases from carnivorous to omnivorous to herbivorous fish species, a pattern similar to that observed in mammals (Ley, Hamady, Lozupone, Turnbaugh, Ramey, & Bircher 2008). The reason for this pattern is still poorly understood, but may be correlated with the length of the GI tract in each fish species and hence the overall transit time of food through the gut. In carnivorous fish with short digestive systems, such as rainbow trout, food travels quickly through the gut and hence less time is available for microbial fermentation of dietary ingredients. However, in omnivorous

and herbivorous fish, there is a much slower transit time of food through the convoluted GI tract, enabling a greater level of microbial fermentation to occur and precipitating an increase in microbial diversity. Smriga et al (2010) reported that the intestinal microbiome of the herbivorous whitecheek surgeonfish *Acanthurus nigricans*, whose primary diet consists of algae and detritus, exhibited a far greater level of microbial diversity than that of the strictly carnivorous red snapper *Lutjanus bohar*. Similarly the omnivorous yellowbelly rockcod *Notothenia coriiceps* was shown to possess a greater intestinal microbial diversity than the carnivorous blackfin icefish *Chaenocephalus aceratus* (Ward et al 2009). In this study, it is not unreasonable to posit that the changes in microbial diversity observed in the treatment group were indicative of the microbiome adapting to digesting whole cell microalgae and its constituent polysaccharides. Whilst gut transit time was not measured in this study, similar trends towards an increased microbial diversity in the intestine of trout fed plant-based diets have been recorded (Desai et al 2012; Green et al 2013). A high level of microbial diversity in the intestine has been advocated as being beneficial to host health in that it provides a wider range of potential responses to stressful situations or provides individual resilience to acceptance of different dietary ingredients (Backhed, Ley, Sonnenburg, Peterson & Gordon, 2005). The presence of a more diverse microbiome in the microalgae fed fish could therefore represent a reflection of the need for additional plasticity in the structure of the microbiome in these fish, in order to aid digestion and the breakdown of the microalgal meal included in their diet.

The Tenericutes were the dominant microbial phylum in the vast majority of samples, followed by the Firmicutes and Spirochaetes. Within the Tenericutes, the Mollicutes were the most prominent class, with *Mycoplasma* being the dominant genus. This microbe has previously been recorded in the intestinal tract of both marine and freshwater fish species (Kim, Brunt & Austin 2007; Moran, Turner & Clements 2005; Bano, Derae-Smith, Bennett, Vasquez &

418 Hollibaugh, 2007; Holben, Williams, Gilbert, Saarinen, Sarkilahti. & Apajalahti 2002;
419 Suhanova, Dzyuba, Triboy, Nikiforova, Denikina & Belkova 2011; Xing, Hou, Yuan, Liu, Qu
420 & Liu 2013, Carda-Dieguez, Mira & Fouz 2014). More recent analyses employing high
421 throughput sequencing have reported similar findings to those of the present study, in that the
422 Mycoplasmataceae appear to dominate read libraries from the distal intestinal microbiome of
423 Atlantic salmon (Green et al 2013, Zarkasi, Abell, Taylor, Neuman, Hatje, Tamplin, Katouli &
424 Bowman, 2014) and rainbow trout (Lowrey, Woodhams, Tacchi & Salinas 2015; Ozorio,
425 Kopecka-Pilarczyk, Peixoto, Lochmann, Santos, Santos, Weber, Calheiros, Ferrez-Arruda,
426 Vaz-Pires & Goncalves 2015). *Mycoplasma* do not, however, appear to be significantly
427 affected by diet composition, as they were present in all fish sampled in this trial, irrespective
428 of treatment. Furthermore, large numbers of Tenericutes have been documented in the gut of
429 other aquatic animals such as oysters (King, Judd, Kuske & Smith 2012) and in terrestrial
430 animals such as pigs (Leser, Amenuvor, Jensen, Lindecrona, Boye & Moller 2002).

431 The genus *Mycoplasma* are Gram positive bacteria that are closely related to the
432 Bacilli/Clostridium branch of the phylum Firmicutes. These fastidious microbes lack cell walls,
433 have a fermentative metabolism, a high G-C content and possess a genome size (~580Kbp)
434 that is amongst the smallest in self-replicating microorganisms. Owing to this extremely small
435 genome, it is unlikely that they perform many complex metabolic functions within the fish
436 intestine, and may primarily be obligate commensals within the gut ecosystem. However
437 *Mycoplasma* have previously been reported to produce lactic acid and acetic acid as their major
438 metabolites (Freundt & Razin 1958). It is thus also possible that the dominance of *Mycoplasma*
439 in the intestine of trout is a result of a long established symbiosis in which this microbe benefits
440 from easy access to a multitude of fermentable substrates (e.g. cytoplasmic secretions) and the
441 fish benefits from the acetic acid and lactic acid metabolites produced as a result. Extreme

genome reduction in bacterial symbionts residing within terrestrial animal hosts is a well described phenomenon, which may also occur in rainbow trout.

Previous studies analysing the effect of dietary alterations on rainbow trout microflora have reported that whilst slight differences are often observed, these are somewhat negligible in terms of their effect on the 'core' microbial community, and the population structure between control and test populations are usually quite similar (Wong et al 2013 ; Zarkasi et al 2014). However, these authors did report subtle effects of the different diets on the relative abundance of select groups of bacterial taxa. Similarly, the principal coordinate analysis data obtained in this study provides evidence of a very minor effect of different diets on the structure of the microbial community within the intestine of rainbow trout, with only a limited number of taxonomic groups being significantly affected by dietary alteration. Furthermore, analysis of the microbial communities of the diets themselves showed that they were very similar in structure, but were significantly different from the fish intestinal samples. It thus appears to be unlikely that the observed differences in microbiota composition between control and treatment fish could be due to the microbiota structure of the dietary pellets. Therefore, it appears that diet composition only had a minor effect on the intestinal microbiome. Others have also reported that switching dietary regimes, including nutritional substitution, can alter microbial diversity, community membership and/or structure to varying degrees (Ringo and Olsen 1999; Ringo, Sperstad, Myklebust, Refstie, & Krogdahl 2006; Askarian, Zhou, Olsen, Sperstad & Ringo 2012; Sullam, Essinger, Lozupone, O'Connor, Rosen, Knight, Kilham & Russell 2012).

Statistical analyses revealed that *Streptococcus*, *Leuconostoc*, *Weissella*, *Lactobacillus*, *Candidate Division WPS-1* and *Lactococcus* were significantly discriminatory between diets in this study. Each of these genera, most of which are members of the lactic acid bacteria (LAB), were significantly elevated in the microalgae fed fish. LAB are frequently recorded in the intestines of fish, including rainbow trout, albeit at low levels of abundance (Merrifield,

Balcazar, Daniels, Zhou, Carnevali, Sun, Hoseinifar & Ringo 2014). More recent research on the effect of diet on the rainbow trout microbiome using deep sequencing platforms have found that this group appears to be amongst the most responsive to dietary alterations. Ingerslev et al (2014a, b) reported that *Streptococcus*, *Leuconostoc*, *Weissella* and *Lactobacillus* were responsive to dietary shifts, and were significantly elevated in the microbiome of trout fed high levels of plant-based ingredients. Similarly, both Desai et al (2012) and Wong et al (2013) reported that levels of *Lactobacillus*, *Streptococcus*, *Weissella*, *Clostridia* and *Staphylococcus* were discriminatory according to plant based and grain based diets respectively. The same microbial groups, with the exception of *Staphylococcus*, were discriminatory by diet in the present study, indicating the possible development of a distinct trend in the literature towards dietary influences on lactic acid bacterial populations in the rainbow trout intestine, in spite of their perceived rarity within this ecosystem.

These bacterial taxa are generally considered to be beneficial organisms associated with a healthy intestinal epithelium, and many of the genera recorded in this research have been tested elsewhere for their potential probiotic capabilities in rainbow trout aquaculture (Joborn, Olssen, Westerdahl, Conway & Kjelleberg 1997; Irianto & Austin 2002; Panigrahi, Kiron, Kobayashi, Puangkaew, Satoh & Sugita 2004; Kim & Austin 2006, 2008; Balcazar, de Blas, Ruiz-Zarzuela, Vendrell, Girones & Muzquiz, 2007; Vendrell, Balcazar, de Blas, Ruiz-Zarzuela, Girones & Muzquiz 2008; Balcazar, Vendrell, de Blas, Ruiz-Zarzuela & Muzquiz 2009; Merrifield et al 2010; Perez-Sanchez, Balcazar, Merrifield, Carnevali, Gioacchini, de Blas & Ruiz-Zarzuela 2011). LAB are hypothesized to improve the health of rainbow trout in aquaculture by enhancing feed conversion efficiency and conferring protection against pathogenic bacteria via mechanisms of competitive exclusion. In addition, the production of organic acids (e.g. acetic acid, lactic acid) and compounds such as bacteriocins and enzymes can further protect the intestinal epithelium and aid in the digestion of resistant dietary

ingredients (Nayak 2010). The LAB have however been observed to represent only a minor constituent of the fish intestinal microbiome and so potential methods of manipulating and enriching these populations are of great interest in improving intestinal health and consequently fish performance in aquaculture.

Overall, the results presented showed that the inclusion of dietary microalgae did not impair rainbow trout growth or negatively impact the distal intestinal microbiome. The dominance of *Mycoplasma* in the microbial libraries of all fish analysed suggests that this phylotype is well adapted to life in the rainbow trout intestine, and hence further research into its potential functional role is undoubtedly required. The altered microbial diversity observed in the microalgae fed fish suggested a flexibility in the intestinal microbiome of these fish which may represent a response to the breakdown and digestion of this novel dietary ingredient. Whilst the 'global' microbiome structure was similar in both groups, there were statistically significant differences noted in community membership, with distinct microbial groups observed to be discriminatory according to diet, particularly members of the LAB such as *Weissella*, *Streptococcus*, *Lactococcus*, *Lactobacillus* and *Leuconostoc*. This represents a further indication of a possible, albeit subtle, dietary effect of the microalgae on these populations. The potential manipulation of microbial communities through dietary supplementation may represent a promising method for improving gut health and hence nutrient utilization in farmed rainbow trout. Whilst the data presented is certainly supportive of the inclusion of microalgae in farmed rainbow trout diets, further work is required to clarify the optimal level of inclusion to beneficially manipulate the intestinal microflora of these fish.

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6. Conflicts of interest

The authors declare no conflicts of interest

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Figure Legends

Figure 1. Mean relative % sequence abundance of microbial phyla recorded in distal intestine of fish fed a) control and b) treatment diet

Figure 2. Relative % sequence abundance of tank biofilm, diet and intestinal microbial classes in rainbow trout fed a) control and b) treatment diet.

Figure 3. Principal coordinate analysis (PCoA) depicting differences in microbial community structure between control and treatment fish, tank biofilm and feed pellet samples from both diets, based on ThetaYC distance matrix.

Figure 4. Bacterial taxa identified by metastats, LEfSe and Indicator analysis as discriminatory between experimental conditions. The data are plotted as mean percentage relative abundance ± standard error of the mean (SEM). *P<0.05 **P<0.01.

Figure S1. Growth performance data for control and treatment fish populations. Mean final weight and condition factor (K) ± SEM at the end of the 15 week trial period are shown (n=12). Condition factor was calculated according to Fulton's method.

Figure S2. Rarefaction analysis of a) control and b) treatment group sequence libraries. Samples were rarefied according to the library with the lowest number of reads (n=314961, A F6)

Figure S3. Mean relative abundance ± SEM of sequences attributed to *Acetanaerobacterium* and *Brevinema* in the intestinal microbiome of both control and microalgae fed rainbow trout. Metastats, LEfSe and Indicator analyses did not identify these differences as statistically different according to diet administered, despite differences noted in relative sequence abundances between diets.

Figure S4. Heatmap of abundant bacterial genera recorded in this study. Fish are numbered 1-12, green for treatment group, and red for control group. Within the heatmap, RED colours indicate communities that are more similar between samples, whilst **BLACK** indicates dissimilarity between samples, based on ThetaYC distance matrix.

Table 1. Alpha diversity estimates of rainbow trout intestinal microbiomes

			Simpson		Inverse Simpson		Chao 1	
Sample	**OTU**	**Coverage**	**μ**	**σ**	**μ**	**σ**	**μ**	**σ**
C F1	97	0.999917	0.64	0.000054	1.55	0.00013	157.35	2.43
C F2	73	0.999908	0.93	0.000026	1.07	0.00003	140.37	2.17
C F3	150	0.999908	0.35	0.000076	2.87	0.00062	206.02	1.88
C F4	90	0.999905	0.38	0.000125	2.63	0.00085	199.86	4.21
C F5	92	0.999917	0.84	0.000038	1.18	0.00005	178.19	4.03
C F6	58	0.999937	0.90	0.000031	1.10	0.00003	128.80	3.39
C F7	68	0.999914	0.94	0.000025	1.06	0.00003	159.93	4.29
C F8	62	0.999937	0.88	0.000033	1.13	0.00004	117.70	2.17
C F9	94	0.99993	0.89	0.000032	1.12	0.00004	143.69	1.96
C F10	173	0.999838	0.80	0.000017	1.25	0.00002	272.98	1.03
C F11	177	0.999882	0.67	0.000049	1.49	0.00010	237.94	1.89
C F12	62	0.999943	0.93	0.000028	1.07	0.00003	114.63	2.20
MeanC	99.6	0.999911	0.76	0.000045	1.46	0.00016	171.45	2.63
A F1	132	0.999835	0.49	0.000115	2.04	0.00047	284.33	4.73
A F2	87	0.999921	0.89	0.000031	1.12	0.00003	168.74	3.04
A F3	185	0.999851	0.65	0.000052	1.52	0.00012	248.07	1.74
A F4	228	0.999895	0.69	0.000051	1.45	0.00010	318.00	3.90
A F5	87	0.999902	0.85	0.000037	1.17	0.00005	182.45	3.47
A F6	192	0.99981	0.44	0.00004	2.28	0.00001	294.62	2.12
A F7	255	0.999816	0.56	0.000064	1.77	0.00020	332.80	1.93
A F8	77	0.999952	0.88	0.000034	1.13	0.00004	127.85	2.43
A F9	134	0.999886	0.59	0.00006	1.68	0.00017	219.07	2.77
A F10	80	0.999917	0.67	0.000052	1.49	0.00011	141.80	2.69
A F11	86	0.999975	0.38	0.000072	2.61	0.00048	103.96	1.01
A F12	83	0.999927	0.90	0.000033	1.11	0.00004	140.91	2.51
MeanA	135.5	0.999891	0.66	0.000053	1.61	0.00015	213.55	2.69

Normalized mean values (μ) and standard deviations (σ) for the number of OTU's, Sample coverage, Simpson Index, Inverse Simpson Index and Chao 1 richness. Normalized values were obtained by random resampling via rarefaction analysis according to the smallest sample size (n=314961, A F6) and standard errors were obtained by bootstrapping. OTU's are clustered according to a 97% sequence similarity cut-off value. C = Control samples, A= Treatment samples (Algae).

	p value		
Phylotype	**Metastats**	**LEfSe**	**Indicator**
Acetanaerobacterium	0.94	-	0.68
Brevinema	0.41	-	0.39
Streptococcus	0.009	0.009	0.042
Leuconostoc	0.009	0.013	0.046
Weissella	0.043	-	0.12
Candidate division WPS-1	0.006	0.005	0.024
Lactobacillus	0.010	0.007	0.034
Lactococcus	0.046	0.026	0.058
Enterobacter	0.034	0.049	0.078

Table 2. Phylotypes identified as discriminatory according to diet by three separate statistical algorithms within mothur (Metastats, LEfSe and Indicator). Statistical significance was accepted on two levels; $p<0.05$ and $p<0.01$. *Acetanaerobacterium* and *Brevinema* were not discriminatory by diet.

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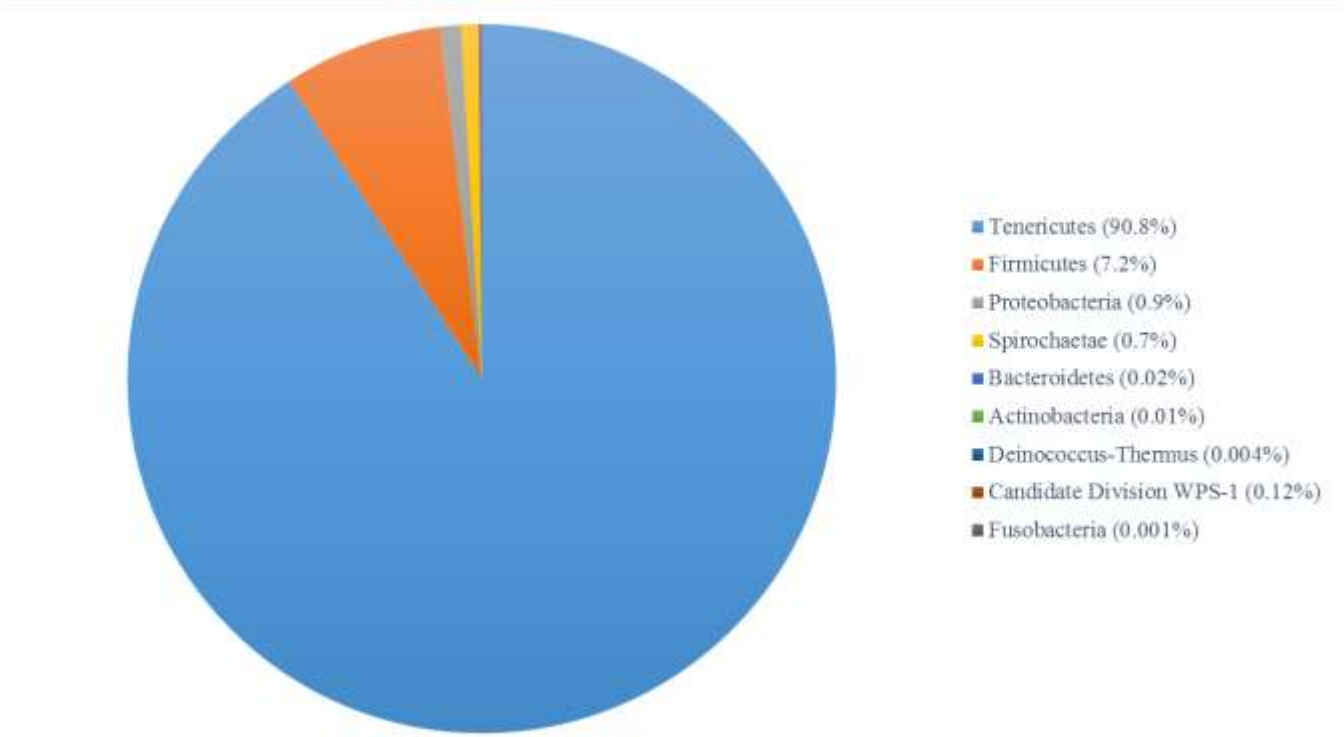
a)


Tenericutes (90.8%)
Firmicutes (7.2%)
Proteobacteria (0.9%)
Spirochaetae (0.7%)
Bacteroidetes (0.02%)
Actinobacteria (0.01%)
Deinococcus-Thermus (0.004%)
Candidate Division WPS-1 (0.12%)
Fusobacteria (0.001%)

b)

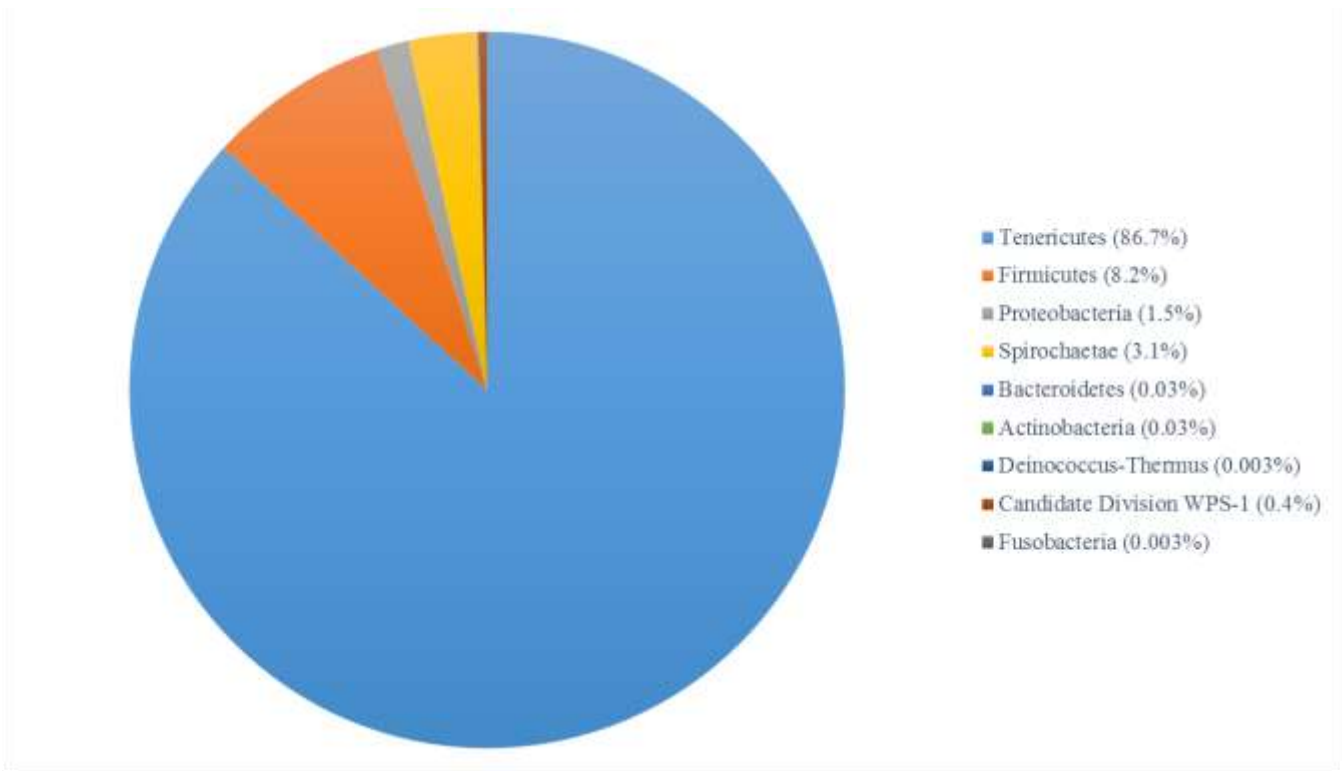
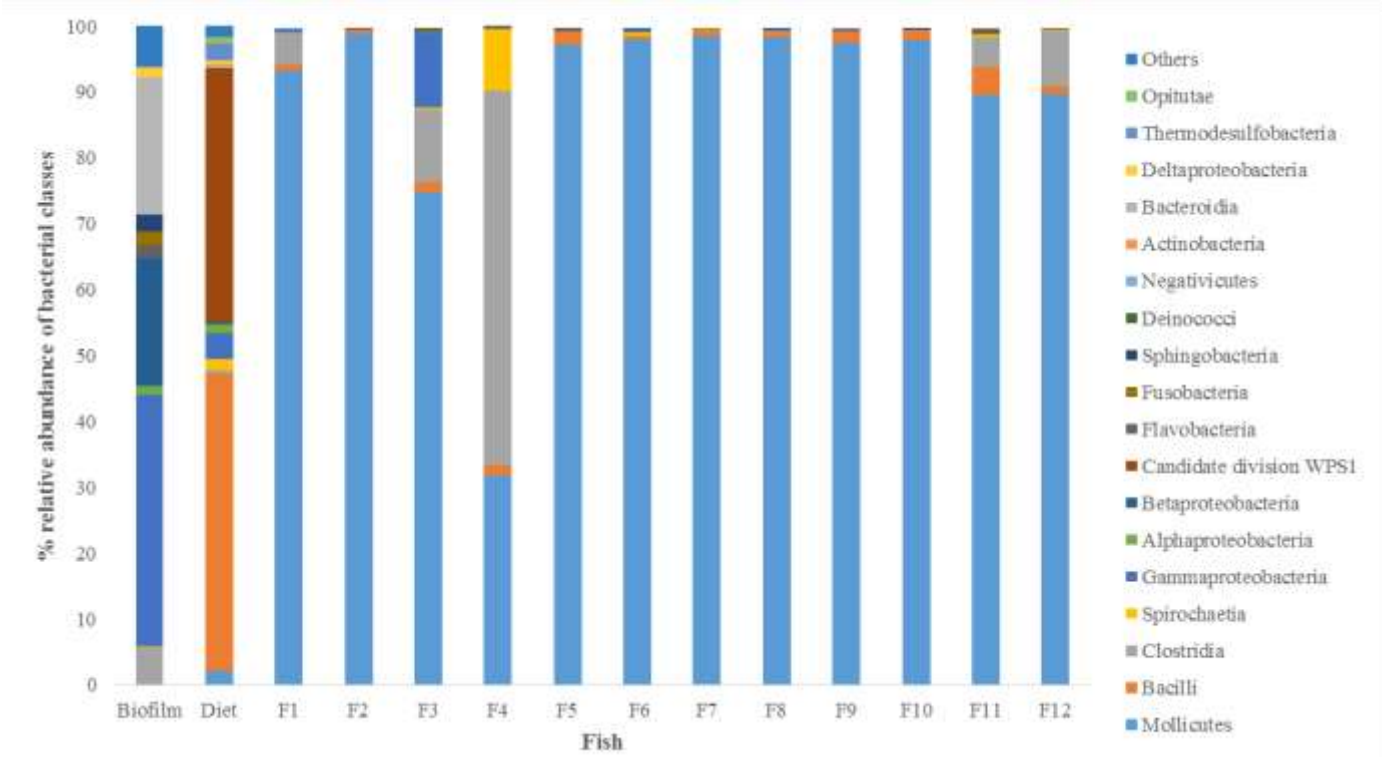

Tenericutes (86.7%)
Firmicutes (8.2%)
Proteobacteria (1.5%)
Spirochaetae (3.1%)
Bacteroidetes (0.03%)
Actinobacteria (0.03%)
Deinococcus-Thermus (0.003%)
Candidate Division WPS-1 (0.4%)
Fusobacteria (0.003%)

Figure 1

a)


% relative abundance of bacterial classes
100
90
80
70
60
50
40
30
20
10
0
Biofilm
Diet
F1
F2
F3
F4
F5
F6
F7
F8
F9
F10
F11
F12
Fish
Others
Opitutae
Thermodesulfobacteria
Deltaproteobacteria
Bacteroidia
Actinobacteria
Negativicutes
Deinococci
Sphingobacteria
Fusobacteria
Flavobacteria
Candidate division WPS1
Betaproteobacteria
Alphaproteobacteria
Gammaproteobacteria
Spirochaetia
Clostridia
Bacilli
Mollicutes

b)

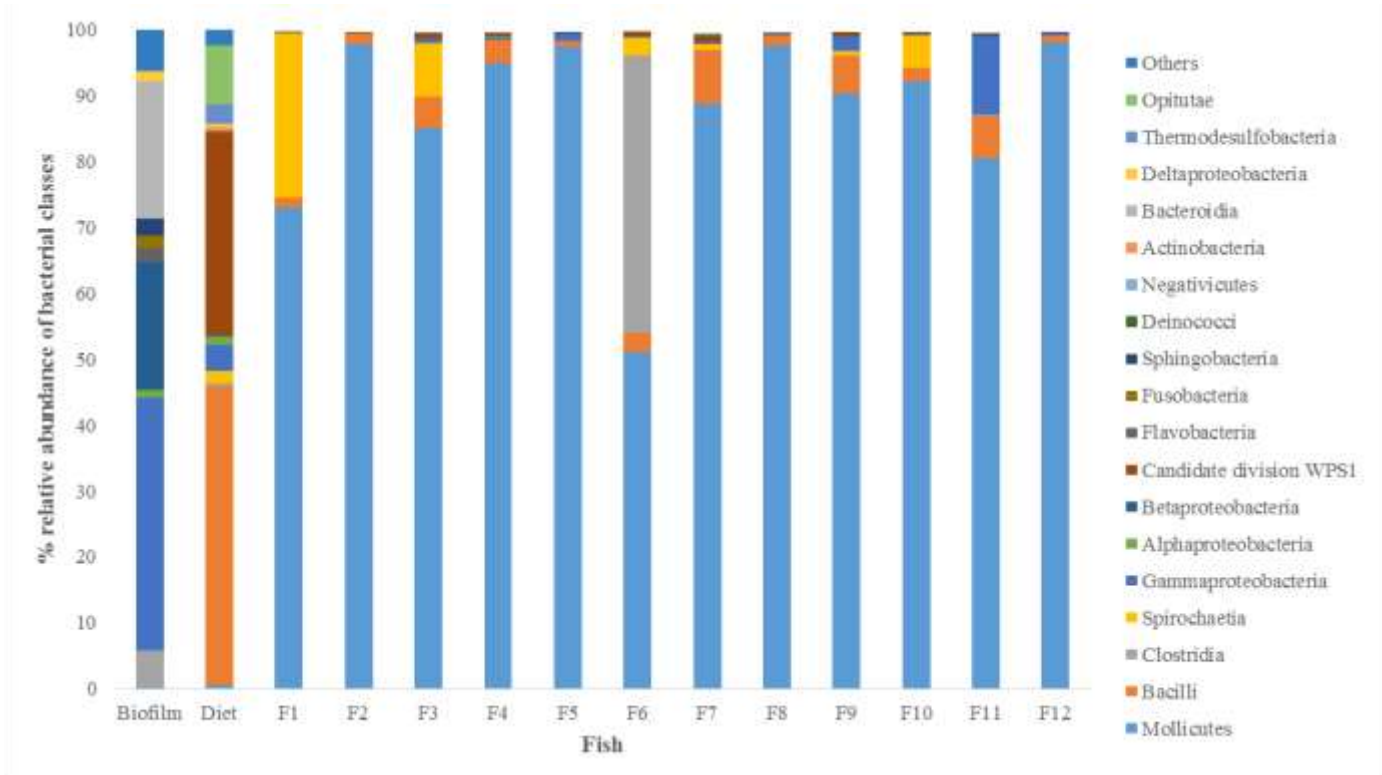
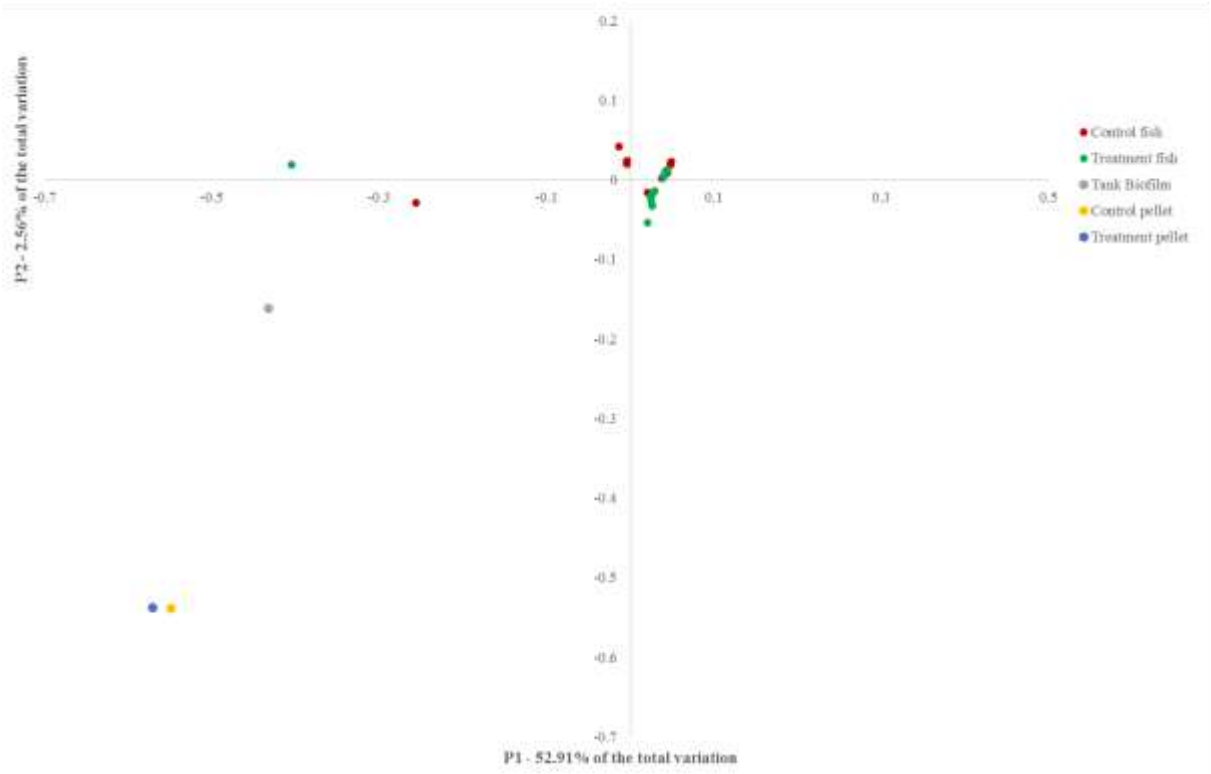

% relative abundance of bacterial classes
100
90
80
70
60
50
40
30
20
10
0
Biofilm
Diet
F1
F2
F3
F4
F5
F6
F7
F8
F9
F10
F11
F12
Fish
Others
Opitutae
Thermodesulfobacteria
Deltaproteobacteria
Bacteroidia
Actinobacteria
Negativicutes
Deinococci
Sphingobacteria
Fusobacteria
Flavobacteria
Candidate division WPS1
Betaproteobacteria
Alphaproteobacteria
Gammaproteobacteria
Spirochaetia
Clostridia
Bacilli
Mollicutes

Figure 2.



802

803 **Figure 3.**

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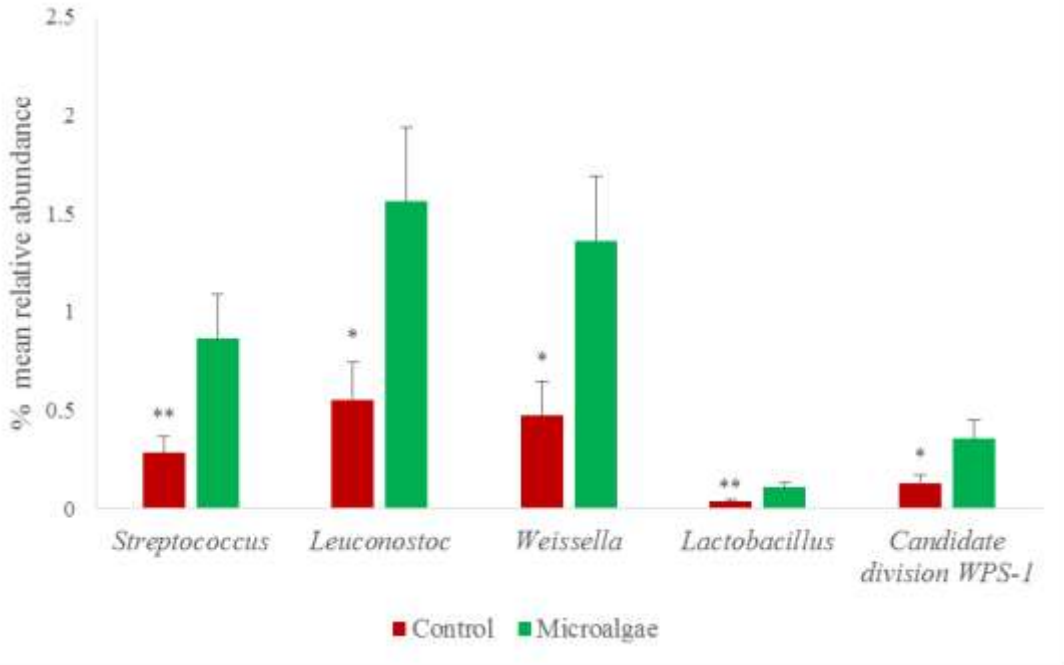

805

806 **Figure 4.**

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808 **Table S1. Ingredient composition and nutrient analysis of diets**

	Diet 1 (Control)	Diet 2 (Treatment)
Ingredient	**% Inclusion**	**% Inclusion**
Fish meal 68	22	22
Wheat meal	15	14
Wheat gluten	10	10
Soybean meal 47	14	14
Soybean concentrate 65	20	19
Alltech algae meal	0	5
Fish oil	15	12
Monocalcium phosphate	1.3	1.3
Mineral and vitamin premix	1	1
Lysine	0.6	0.6
Methionine	0.5	0.5

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Dietary component		
Moisture	6.0	6.1
Protein	45.4	45.2
Fat	18.4	18.3
Ash	5.9	6.0
Fibre	1.1	1.1
NFE	20	19.2

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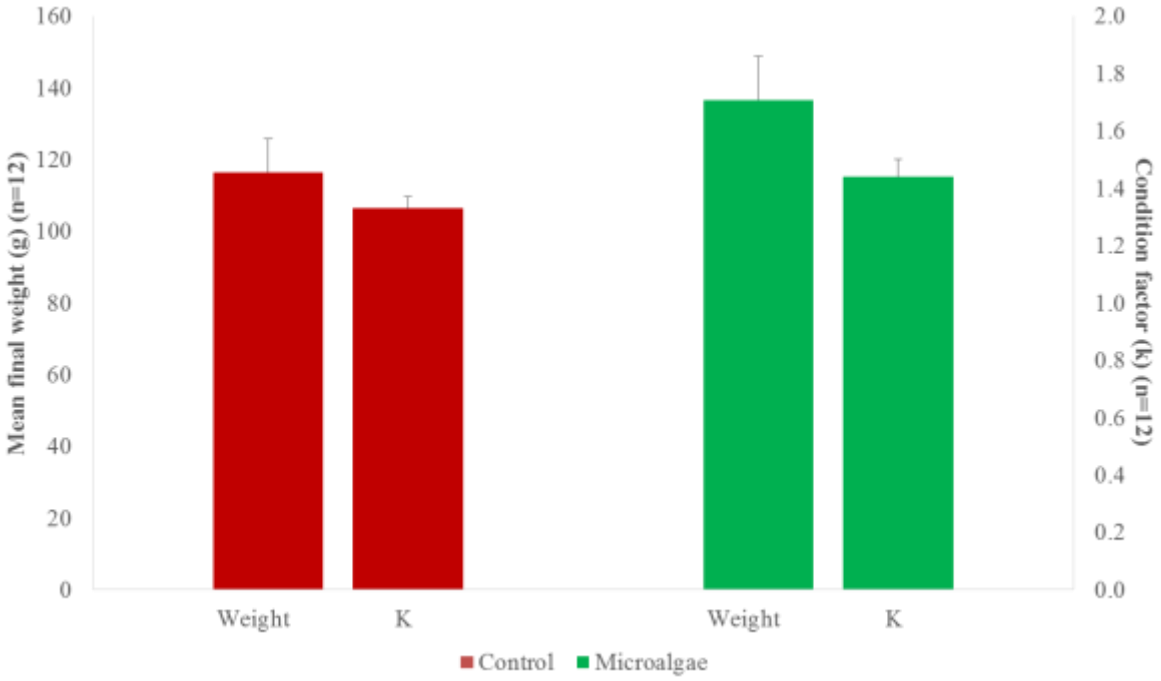
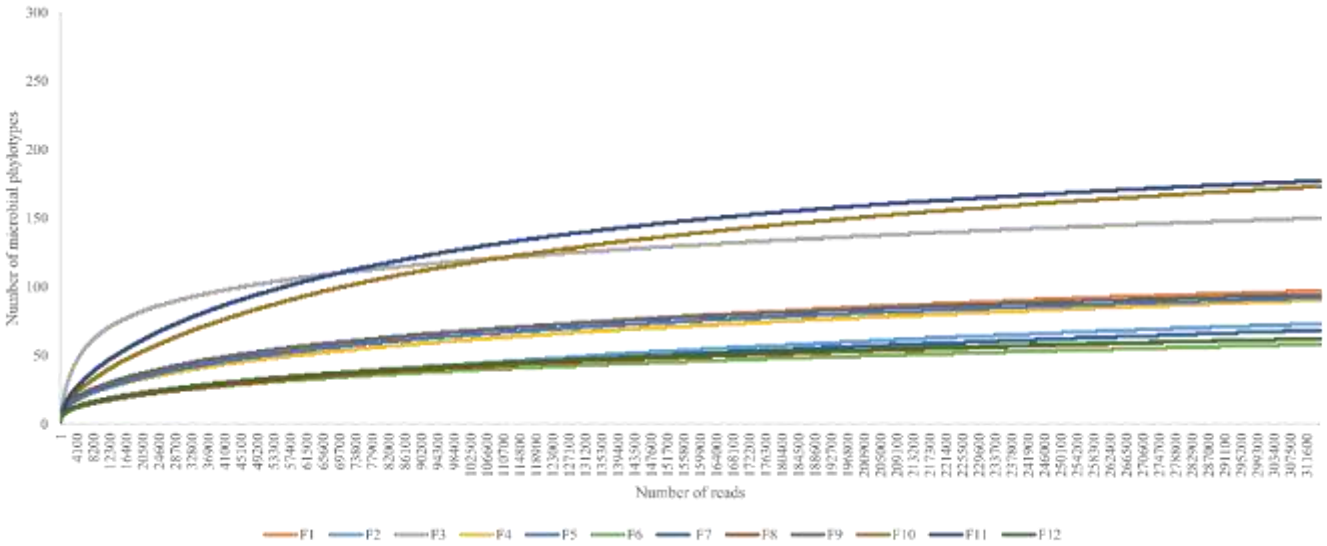

160
140
120
100
80
60
40
20
0
Mean final weight (g) (n=12)
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Condition factor (k) (n=12)
Weight
K
Weight
K
Control
Microalgae

Figure S1

a)



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829 **b)**

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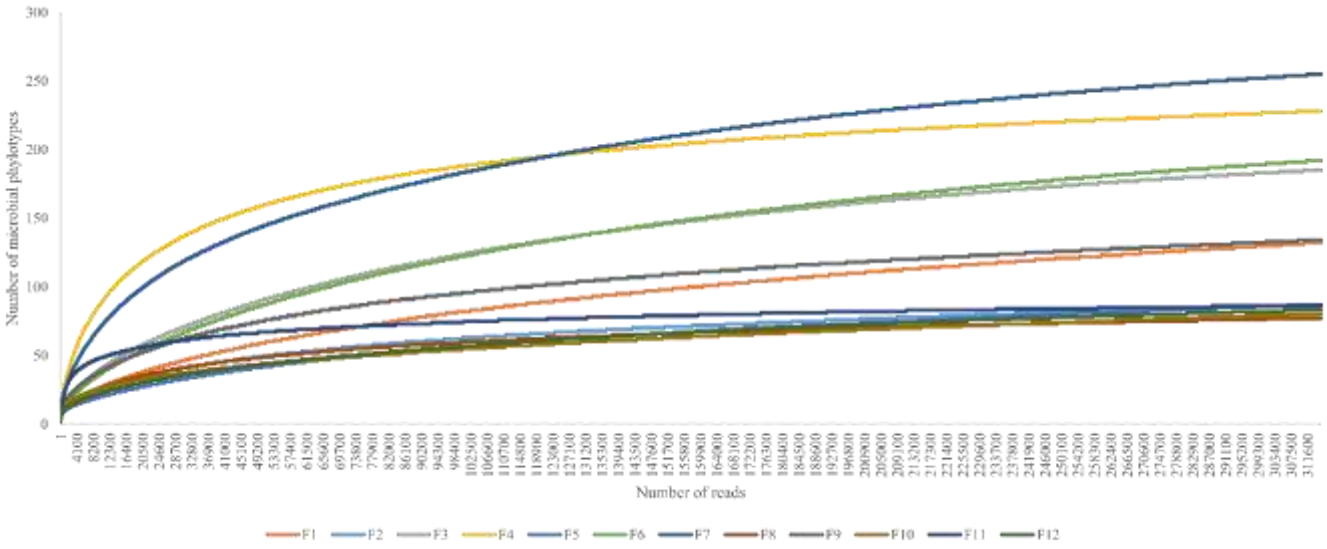

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832 **Figure S2**

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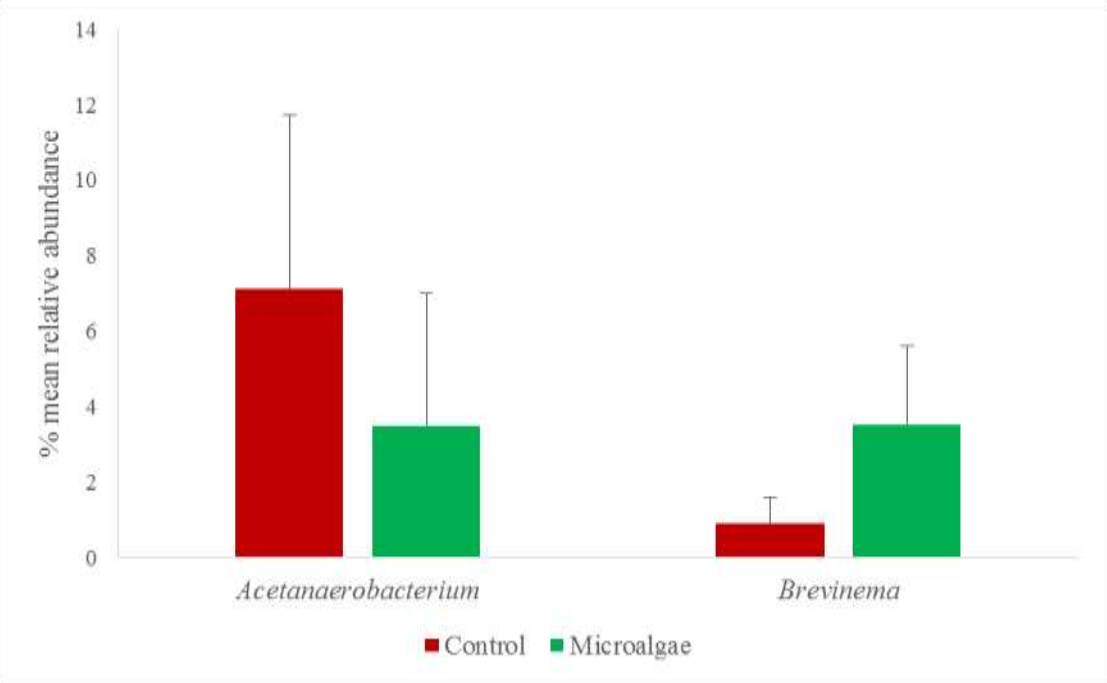
14
12
10
8
6
4
2
0
% mean relative abundance
Acetanaerobacterium
Brevinema
Control
Microalgae

Figure S3

845