Accepted refereed manuscript of:

Zarkasi KZ, Taylor RS, Glencross B, Abell GCJ, Tamplin ML & Bowman JP (2017) In vitro characteristics of an Atlantic salmon (Salmo salar L.) hind gut microbial community in relation to different dietary treatments, *Research in Microbiology*, 168 (8), pp. 751-759.

DOI: 10.1016/j.resmic.2017.07.003

In vitro characteristics of an Atlantic salmon (*Salmo salar* L.) gastrointestinal microbial community in relation to different dietary treatments

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Accepted for publication in *Research in Microbiology* published by Elsevier.

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Abstract

In this study the microbial community dynamics were assessed within a simple *in vitro* model system in order to understand those changes as influenced by diet. The abundance and diversity of bacteria were monitored within different treatment slurries inoculated with salmon faecal samples in order to mimic the effects of dietary variables. A total of five complete diets and two ingredients (plant meal) were tested. The total viable counts (TVC) and sequencing data revealed, there was very clear separation between the complete diets and the plant meal treatments suggesting dynamic response by the allochthonous bacteria to the treatments. Automated ribosomal intergenic spacer analysis (ARISA) results showed the different diet formulations produced different patterns of fragments, with no separation between the complete diets. However, the plant based protein ingredients were clearly separated from the other treatments. The 16S rRNA Illumina-based sequencing analysis showed that members of the genera *Aliivibrio*, *Vibrio* and *Photobacterium* became predominant on all the complete diets treatments. The plant based protein ingredient treatments only sustained weak growth of the genus *Sphingomonas*. *In vitro* based testing of diets could be a useful strategy to determine the potential impact of either complete feeds or ingredients on major fish GI tract microbiome members.

Keywords: Intestinal bacteria; *in vitro* model system; 16S rRNA gene; dietary treatments; Atlantic salmon

Abbreviations: GI – gastrointestinal; DE – digestible energy

1. Introduction

Gastrointestinal (GI) tract microorganisms serve a variety of functions in the nutrition and health of fish by promoting nutrient supply, preventing the colonization of pathogens, and by immunomodulation [1]. Understanding fish GI tract microbiota and how fish physiology is influenced by the gut microbiome, potentially can lead to improvements in the health of fish, productivity of aquaculture systems and aid in industry sustainability. The Atlantic salmon gut microbiome, as in other fish species is highly dynamic due to the open monogastric nature of the GI tract. Short term changes in communities can be forced by diet manipulation [2, 3] and by antibiotic application [4].

We have observed differences in the gut microbiome in relation to faecal consistency [3], which may reflect gastric passage effects as seen in human faecal samples [5]. External influences, such as changes in salinity have been shown to have no effect on the gut community in euryhaline fish [6], thus gut and seawater communities are considered not interconnected. Moreover, the gut microbiome in freshwater systems was actually more reflective of environment than diet [7]. Among fish species, variations in geographical location, the diet composition, and the ecosystem they dwell within has been implicated in shaping the overall gut microbiome [2].

Farmed salmonids generally require a diet containing substantial levels of both fishmeal and fish oil, which contain quite unique protein and lipids nutrient profiles [8]. Numerous studies have supplemented and altered diet composition to observe responses in fish growth performance [9-13]. Comparatively recently there has also been a focus on using diets to improve or assist the stability of GI health, which may improve overall fish health status, feed utilization, growth performance and productivity [14, 15]. However, environmental and management complexity of current Atlantic salmon farming systems,

represents a challenge in devising diet formulations that have predictable and stable effects, and that can at least maintain current expectations of farm-based growth performance under varying environmental conditions.

There has been considerable success in replacing fishmeal and fish oil with other protein or lipid raw materials, such as lupin kernel, soybean, faba beans, field peas, microalgae (as the protein sources), canola oil, sunflower oil and poultry fat [9, 16-23]. The reliance of aquaculture on fishmeal as a protein source and fish oil as a lipid source has been recognised for a long time as a significant risk for the industry [24]. Over the recent past decades there have been a multitude of studies examining a range of different raw materials (both animal and plant) that have potential application in reducing reliance on these resources for aquaculture [19, 22].

Few studies specifically focussed on the effect of a plant protein meal on the fish GI tract microbial communities have been performed in terms of determining potential impacts on community structure [25, 26]. Despite having a high level of carbohydrate and protein content, some plant ingredients can contain anti-nutritional factors, which hinder protein digestion. It is unknown however, whether these ingredients can also affect the gut microbiome. Previous studies have observed that soybean meal inclusion in the diet increased the abundance of the allochontous microbial community and its diversity, even though the effects of this change in the microbiome to the fish health are still need for further study to better understand it potential [12, 18].

The aim of this study was to investigate the effect of different diets on the dynamics of the Atlantic salmon GI tract microbiome community of using a simple *in vitro* model. The study included a range of diet formulations, including some modified formulations where fish meal and fish oil had been largely substituted with poultry and plant meal products to determine if these components promoted a different gut microbiome.

2. Materials and Methods

2.1 Fish diets

Five different diet formulations were prepared including diets with different fish meal and fish oil levels, a low protein (LP), a high protein (HP), and a commercial standard (CS) diet with an intermediate protein and lipid content. The CS diet was modified to yield two different diets. The first formulation had fish meal largely replaced with poultry meal and lupin kernel meal and is referred to as the LM diet. In the second formulation fish oil was replaced with poultry oil and is referred here as the PO diet. In addition to these complete diet formulations samples of ingredients lupin kernel meal (LK) and field pea (*Pisum sativa*) meals (PE) were also tested independently to determine if they are capable of influencing the microbiome. The general composition of each of the diets is shown in Table 1. Each of the diets were manufactured based on methods reported in Glencross *et al.* [27]. Each of the raw materials used (either in the formulation or as a test material) was milled using a Retsch rotor mill with a 750 µm screen to create flour prior to incorporation in the diet mashes.

2.2 Atlantic salmon faecal collection

The faecal samples were collected during November 2013 from Tassal Group Ltd Robert's Point lease located within the D'Entrecasteaux Channel, Bruny Island, Tasmania, Australia. Samples were collected by randomly seining a large group of fish, crowding the fish in the seine to minimize bias and subsequently dip-netting individual fish into 17 ppm Aqui-S anesthetic (Aqui-S, Lower Hutt, New Zealand). The fish were approximately 2-3 kg average weight. The faecal samples were collected from ten apparently healthy salmon by gently squeezing the hind gut into individual sterile plastic zip-lock bags [3]. Samples were

immediately transferred on ice to the laboratory and processed within three hours. Between each sampling the anal region of each specimen was wiped with an ethanol swab to ensure no cross–contamination of skin mucosal microbes.

2.3 In vitro gut model system

In vitro fermentation was conducted in three replicates for each of the diets shown in Table 1 and a negative control (a sample of the inocula in the medium without feed added). The diets were crushed and suspended at 10 g/L as a slurry in the basal growth medium. The basal growth medium contained the following compounds: $NaHCO_3$, 4 g/L; K_2HPO_4, 0.5 g/L; KH_2PO_4, 0.5 g/L; $MgSO_4.7H_2O$, 0.09 g/L; $CaCl_2$, 0.09 g/L; sea salts 30 g/L (Sigma, St Louis, US); resazurin, 0.5 mg/L; hemin, 10 mg/L (MP Biomedicals, Santa Ana, US); and sterile water, 1L. The faecal samples collected from ten individual fish were pooled with equal contributions per fish [28, 29]. Then samples were homogenized and diluted 1:2 (wt/vol) in marine broth (Oxoid, Basingstoke, England). A faecal slurry sample of 1 ml was then aseptically inoculated into the 1200 ml growth medium and incubated at 20ºC, with mixing periodically performed during incubation using large-capacity incubator shaker (Eppendorf, Hauppauge, US). The Anaerogen system produces an atmosphere containing approximately 90:10 N_2:CO_2 with O_2 content reduced below 0.1% within 1 h. The sampling time points of 0, 3, 6, 12 and 24 hours were determined by prior analysis of pH in a trial run where pH was found to decline and stabilize at the 24 h time point, the pH original inoculum was pH 8.10. Samples (5 mL) were taken from the three-replicate growth medium per diet and processed for microbial enumeration and DNA extraction.

2.4 DNA extraction and microbial enumeration

DNA extraction was performed using the QIAamp DNA Mini Kit (Qiagen Sciences, Germantown, MD, US) following the manufacturer's instruction and standard protocols. Samples collected from the *in vitro* fermentation at 0, 3, 6, 12 and 24 hours were serially diluted using marine broth (Oxoid, Basingstoke, England) and plated onto marine agar (MA), thiosulfate-citrate-bile salts-sucrose (TCBS) agar and De Man-Rogosa-Sharpe (MRS) agar (Oxoid, Basingstoke, England) [30]. The plates were incubated at 20°C for 48-72 hours in order to determine the total viable counts. The plates that possessed between 30 and 300 colonies were counted manually to obtain estimates of bacterial numbers (colony forming units/gram wet weight).

2.5 Automated ribosomal intergenic spacer analysis (ARISA)

The bacterial community structure was fingerprinted using ARISA [31]. Polymerase chain reaction (PCR) amplification was performed using primers 1392F (5'-GYACACACCGCCCGT-3') and 23SR (5'-GGGTTBCCCCATTCRG-3') [32]. The PCR conditions consisted of an initial denaturation step at 95°C for 10 minutes, followed by 34 cycles of 94°C for 1 minute, 55°C for 1 minute, 72°C for 2 minutes and final extension at 72°C for 10 minutes and soaking at 15°C. PCR products were purified using UltraClean PCR Clean-Up Kit (MO BIO Laboratories, Carlsbad, CA, US). PCR-amplified fragments were prepared for capillary electrophoresis separation using dsDNA Reagent Kit, 35-1,500 bp (Advance Analytical Technologies, Ames, IA, US) mixed with 2 µL of DNA samples. Capillary electrophoresis was performed using a Fragment Analyzer™ (Advanced Analytical Technologies, Ames, IA, US) following the manufacturer's standard protocols. Electrophoretograms with peaks of different sizes were obtained and each peak represented an operational taxonomic unit (OTU) and was identified by its fragment size. Fragment

171 Analyzer output files were further analyzed by PROSize (Advanced Analytical Technologies,
172 Ames, IA, US).
173
174 ***2.6 MiSeq Illumina-based 16S rRNA gene sequencing***
175 Sequencing of the 16S rRNA gene amplicon was applied to the 42 samples collected
176 from the *in vitro* model system, to examine the microbial communities present in each of the
177 samples, which were collected at the initial time point of 0 h and at 24 h. Sequencing was
178 carried out by Research and Testing Laboratories (Lubbock, Texas, USA) using the Illumina
179 MiSeq platform. Pair-ended PCR amplification of the 16S rRNA gene V3 region was carried
180 using 341F and 519R primers that possessed 12 bp barcode tags. FASTQ files generated were
181 merged using PEAR [33], these were then trimmed to remove the primer, barcode and
182 adapter regions using an internally developed algorithm at Research and Testing Labs
183 (Lubbock, Texas, USA). The seed sequence for each cluster was then sorted by length and
184 clustered with a 3% divergence cut-off to create centroid clusters. Clusters containing only <2
185 sequences or <100 bp in length were then removed. Seed sequences were again clustered at a
186 3% divergence level using USearch to confirm whether any additional clusters appeared.
187 Consensus sequences from these clusters were then accurately obtained using UPARSE [34].
188 Each consensus sequence and its clustered centroid of reads was then analyzed to remove
189 chimeras utilizing UCHIME in the *de novo* mode [35]. After chimera removal, each
190 consensus sequence and its centroid cluster were denoised in UCHIME in which base
191 position quality scores of >30 acted as the denoising criterion. Sequence de-replication and
192 OTU demarcation was further performed in USEARCH and UPARSE to yield OTUs that
193 were aligned using MUSCLE [36] and FastTree [37] that infers approximate maximum
194 likelihood phylogenetic trees. OTUs were then classified using the RDP Classifier [38]

against the curated GreenGenes 16S rRNA gene database [39] utilizing the May 2013 database update.

2.7 Statistical analysis

PRIMER6 and PERMANOVA+ (version 6.1.12 and version 1.0.2; Primer-E, Ivybridge, UK) respectively were used to conduct permutation multivariate analysis of variance (PERMANOVA) [40], and canonical analysis of principal coordinates (CAP) [41] to assess the influence of different diets on community compositions. For this analysis, results data collected from the ARISA and MiSeq Illumina-based 16S rRNA gene sequencing was tabulated with the size bins combined across the samples, square root transformed and a resemblance matrix created by calculation of Bray-Curtis coefficients. PERMANOVA was conducted using default settings with 9999 permutations, while CAP was conducted using default settings. Multiple pairwise comparisons of beta-diversity were also performed. The PERMANOVA derived significance values were considered significant when $P < 0.01$, while $0.01 < P < 0.05$ were considered only marginally significant [3, 42].

3. Results

3.1 Growth responses

Bacterial growth on marine agar and TCBS agar is visualized in Fig. 1. Bacterial growth occurred after 3-6 h adaptation lag phase and reached log 6-7 CFU/ml within 24 h. No growth was observed on MRS agar and samples collected from the negative control. The TVC progression over time was consistent across all complete diets for marine agar and TCBS agar with growth poorer by approximately 1 log unit on the plant meals.

3.2 ARISA profiles

The different diet formulations produced different patterns of ARISA fragments ($P < 0.01$) but the results also indicated a significant effect of sampling time (0 h vs 24 h, $P < 0.01$), however the interaction between diet and sampling time was not significant ($P = 0.15$, Supplementary Table S1) indicating bacteria growing within the system inevitably become predominant. Further analysis using pairwise tests showed that populations varied either significantly ($P < 0.01$) or marginally significant ($0.01 < P < 0.05$) among the several diets tested (Supplementary Table S2). No clear separation was observed between the HP and CS diets ($P = 0.75$), CS and LP diets ($P = 0.27$), CS and LM diets ($P = 0.08$) or between the LP and LM diets ($P = 0.08$). These differences are illustrated in canonical analysis of principal coordinates (CAP) plots which show that clustering can be readily correlated on the basis of diets. The microbial profiles emerging from cultures based on purely plant based protein ingredient material (LK and PE) were clearly separated from the other diets (Fig. 2).

3.3 Composition of the microbial community grown in the in vitro system

Three replicates *in vitro* samples from different diets were analysed for bacterial composition by using the Illumina Mi-Seq platform. The community observed in the inoculum was more diverse but was dominated by members of family *Vibrionaceae* (Supplementary Table S3). The bacteria present also included those likely associated with the feed itself [43] including representatives of the genera *Sphingomonas*, *Paenibacillus*, *Pectobacterium* and *Methylobacterium*. These taxa are non-marine, mainly aerobic species that are relatively abundant due to the heavy dilution of faecal material (cells added <104 CFU/ml) against a high feed background. The taxa observed are consistent with possible DNA extraction reagents though the feed as a source cannot be ruled out at this stage. Other bacteria may originate from the skin (*Propionibacterium, Staphylococcus*) or are other

members of the gut microbiome of fish (*Carnobacterium*, *Escherichia*, *Obesumbacterium*, *Holophaga*) (Supplementary Table S3).

Following 24 h fermentation all complete diet samples were dominated by sequences affiliated with the family *Vibrionaceae* (*Aliivibrio, Photobacterium* and *Vibrio*) making up >90% of total reads (Fig. 3). Feed associated bacteria did not grow and represented a very small proportion of reads after 24 h (Supplementary Table S3). The CS diet formulation supported mainly growth of *Aliivibrio finisterrensis* (77% of total normalized reads), *Vibrio tasmaniensis* (15%), *Photobacterium phosphoreum* (3%), and *Aliivibrio fischeri* (4%). The LP diet cultures were dominated by *Aliivibrio finisterrensis* (86%) and *Aliivibrio fischeri* (12%). Diet HP, however, was dominated by the salmon derived species *Vibrio tasmaniensis* (43% of total reads) *Aliivibrio finisterrensis* (16%), *Photobacterium phosphoreum* (17%), *Vibrio ichthyoenteri/V. scophthalmi* (13%), and *Aliivibrio fischeri* (3% of total reads) (Fig. 3). By comparison, the diets PO and LM differed in containing mainly unclassified *Vibrio ichthyoenteri/V. scophthalmi* (making up 36-54% of reads), *Aliivibrio finisterrensis* (30-45% of reads), and *Vibrio tasmaniensis* (8-14% of reads). Other bacterial species that grew in the HP, CS, LP, PO and LM diets based on the reads and TVCs increasing relative to the inoculum included *Pseudoalteromonas* spp., *Vibrio aestuarianus*, *Photobacterium leiognathi*, and unclassified *Photobacterium* spp. (Fig. 3). Lactic acid bacterial species were not observed to grow in the *in-vitro* system, however it should be noted these bacteria had low abundance in the starting inoculum (Fig. 3). Strict anaerobes also were not detected. CAP analysis of the sequence data (Fig. 2) reiterated the outcomes of ARISA analysis showing essentially similar statistical relationships between samples.

3.4 In-vitro fermentation of plant based protein ingredients

The 100% plant based protein ingredient treatments (LK and PE) did not support the growth of most of the bacteria originating in the faecal inoculum including any members of the family of *Vibrionaceae*. Most of the bacterial reads detected were classified as *Sphingomonas* species and represented 99% (diet LK) and 98% (diet PE) of the total bacterial sourced reads respectively (data not shown). This was against an overwhelming plant DNA background with most 16S rRNA reads classified as chloroplast 16S rRNA. This result correlates with the finding from the PERMANOVA and CAP analysis (Supplementary Table S2, Fig. 2 and Fig. 4).

4. Discussion

This study investigated and analysed the growth responses of Atlantic salmon gastrointestinal tract associated bacteria within different diet formulations using a simple *in vitro* fermentation system. Though this system does not attempt to replicate the salmon GI tract the experiments are based on the principal that bacterial growth is controlled largely by several basic criteria: temperature, nutrient availability, O_2 availability and pH. TVCs obtained (10^6-10^7 CFU/ml) after 24 h were lower than the bacterial populations in the inocula (typically 10^7 to 10^9 CFU/g of faeces wet weight). This is due to dilution, bacteria having to adapt to the diet slurries since the nutrient regime being different to *in situ* GI tract (distal intestine) conditions. In *in situ* GI tract, bacteria are exposed to gut secretions, mucous and different nutrient profiles due to the prior absorption that predominantly occurs in the stomach than the diets directly [44]. The endpoint (24 h) microbial community was influenced to a degree by specific diets according to the CAP and PERMANOVA analysis (Supplementary Table S1, Fig. 2) with the composition also dictated by the starting inoculum community. The rapid growth of *Vibrionaceae* in diet slurries seems to reflect the Atlantic

294 salmon GI microbial community since this group of bacteria has found previously abundant
295 in Tasmanian Atlantic salmon [3, 28, 29, 42, 45]. *Vibrionaceae* appeared to predominate in
296 most faecal samples (>70%) reaching densities of 10^8-10^9 CFU/g, however other bacteria can
297 become predominant for reasons that cannot yet be explained [3].

298 Amongst the complete diets, the HP, CS and LP diets produced similar outcomes
299 suggesting that the differences in protein to lipid ratio were not significant enough to have a
300 marked effect on growth of different species in the *in vitro* system. The low fish meal (LM)
301 and low fish oil (PO) diets have qualitatively similar species structure though individual
302 species abundances change. The alteration of these components though disparate appears to
303 lead to a similar outcome that could be coincidental and determined by stochastic forces.
304 Overall, the manipulation of protein and lipids did not have a demonstrably major effect on
305 the outcomes of the experiment since it can be presumed the *Vibrionaceae* are able to grow
306 on the fish meal and oil present in all diets.

307 The results are also very likely affected by the high level of *Vibrionaceae* (mean 54%
308 of reads) in the starting faecal inocula. This level is however typical of the *Vibrionaceae*
309 composition in faeces from Tasmanian salmon [28, 29, 42, 45]. This would inevitably
310 provide a large advantage to this group of species given they have fast growth rates. The lag
311 phase and 24 h time frame of the experiment, meant that to enclose the mean time for gastric
312 passage in salmon during summer in Tasmania, was possibly also not long enough for some
313 taxa to adapt and grow, such as the lactic acid bacteria. These bacteria were only at low
314 abundance in the starting inoculum.

315 *Aliivibrio finisterrensis* was one of the most abundant bacterial species through all the
316 complete diet culture results and was most promoted on the low (LP) and intermediate (CS)
317 fish meal content diets. This bacterial species was originally isolated from the Manilla clam
318 (*Ruditapes phillipinarum*) [46], and has been found to predominate in the intestinal tract of

Tasmanian farmed Atlantic salmon [3, 28, 42]. *Aliivibrio finisterrensis* also predominates during the warmer months based on data obtained to date [28]. In the HP, PO and LM diet formulations the most abundant bacterial species were *Vibrio ichthyoenteri/V. scophathalmi*, together with *Aliivibrio finisterrensis*. *Vibrio* spp. such as *Vibrio tasmaniensis*, *V. ichthyoenteri/V. scophthalmi*, *V. aestuarianus* and *V. splendidus*, appear to be normal microbiota in the salmon GI tract, since they have also been observed in the Northern hemisphere [30]. Other bacterial species detected in this study *Photobacterium phosphoreum*, *Photobacterium* spp., *Pseudomonas* spp., *Pseudoalteromonas* spp., *Sphingomonas* spp., and *Aliivibrio fischeri* are common bacteria that can be found and previously isolated from the salmon GI tract [3, 21, 28, 29, 42, 47].

Since the LK and PE treatments were purely plant derived ingredients materials, the lack of response by most of the detected microbes after 24 h suggests that the nutrients in the lupin kernel and pea meals are either not accessible or the meals contain inhibitory substances [48]. These could include phytogenic substances, mainly essential oils and flavonoids that usually have generalised antimicrobial properties [49]. The phytogenicity of the plant meals, if any, used here is uncertain, however since the slurry only consisted of the meals the effect would have been concentrated relative to what would be a typical situation where the lupins were a component of a more complex formulated diet (e.g. in diet PO). Only *Sphingomonas*, present initially at high levels in the inocula (average 16% of reads) was able to be detected. This aerobic genus was common in faecal samples analysed in Zarkasi et al. [42] is known to possess extensive detoxification and xenobiotic degradative capabilities as well as an ability to grow under conditions of nutrient stress [50]. It is unclear whether plant meal diet supplements have any capacity to select for this particular genus of bacteria in complete diets though the data raises this possibility. Further analysis is required to better understand the

343 metabolic properties of the *Sphingomonas* detected and any role it may play in the gut
344 microbiome of farmed salmon.

345 According to Kotzamanis et al. [51] the manipulation of fish meal by replacement
346 with fish protein hydrolysates (FPH) appeared to boost bacterial proliferation, and specific
347 families of bacteria such as *Vibrio* spp., could be favoured by high doses of FPH. Besides, it
348 is likely that the processing conditions used in the extrusion of the feed also sterilise the
349 resultant pellets [27]. Factors that also potentially favour *Vibrionaceae* included the high salt
350 content (3% w/v) of the basal medium. Future experiments should examine some alternatives
351 to the methodology used in the *in vitro* system established here, including testing lower
352 temperatures, altering the atmosphere CO_2 and H_2 content, inoculum preparation and amount
353 added, application of mixing, overall culture volumes, predigestion of diets via enzymes,
354 additives such as bile salts, and pH control.

355 The study presented here examined the potential of different nutritional treatment on
356 bacterial community members of Atlantic salmon gastrointestinal tract using a simple *in vitro*
357 system. A critical extension of the present study would be to correlate microbial observations
358 with diet digestibilities and other nutritional performance criteria [52]. With further
359 improvements additional experiments could be implemented in the testing of different diet
360 formulations and the use of other diet additives, including probiotics, prebiotics, phytogenic
361 additives, activated carbon and different forms of the core ingredients (non-heat treated
362 versus heat-treated). The results obtained suggest that such a system could provide an option
363 for screening specific diet formulations as to how they influence the GI tract community
364 structure. The data revealed salmon GI tract bacterial community members were influenced
365 and dynamic in the presence of different nutritional treatments. Beside, the data may be
366 useful in developing a more predictive basis of the impact of feed ingredients on GI tract

367 microbiomes of farmed fish species, and more studies need to be conducted for further
368 understand it potential for aquaculture industry.
369
370 ## Conflict of interest
371
372 The authors have declare no conflict of interest.
373
374 ## Acknowledgements
375
376 Thanks are extended to the Australian Seafood Cooperative Research Centre, Tassal
377 Group and Skretting Australia for in-kind support and research funding (project 2011/701),
378 and to the Commonwealth Scientific and Industrial Research Organisation (CSIRO) for in
379 kind support in diet formulation and extrusion. Thanks also extended to University of
380 Tasmania animal ethic committee for the ethic approval (A12001; 19 August 2011). The
381 authors would also like to thank David Ratkowsky for statistical advice and David Blyth of
382 CSIRO (Bribie Island, Queensland) for extruding the diets used in this study.
383
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Figure Legends

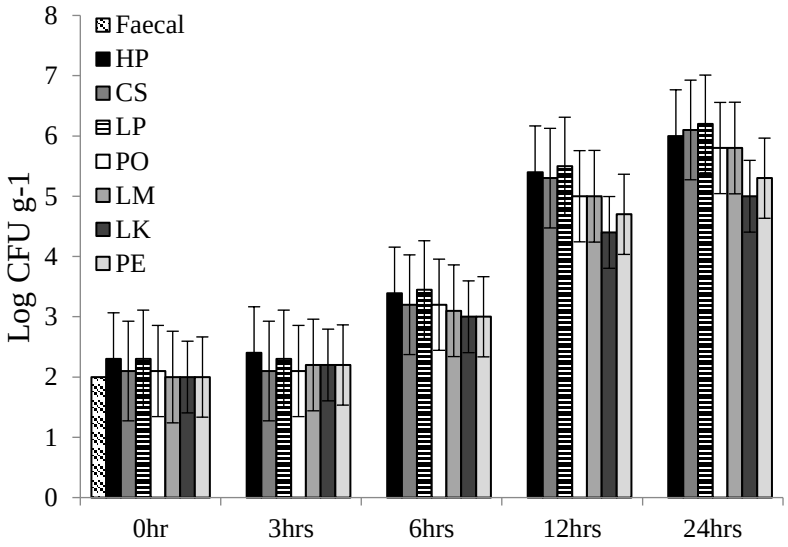
Fig. 1. Total viable counts (TVC) in the *in vitro* model system experiment according to the time of sampling. TVC are derived from the colony numbers appearing on a) marine agar and b) TCBS agar (see Table 1 for abbreviations).

Fig. 2. Canonical analysis of principal coordinates plots showing faecal community similarity on the basis of diet (see Table 1).

Fig. 3. Relative abundance of the bacterial species present in the *in vitro* model system shown as average percentile values with standard deviations. Community composition was determined by Illumina MiSeq 16S rRNA gene amplicon analysis.

Fig. 4. CAP plot of showing comparisons of salmon faeces-derived bacterial assemblages analysed by 16S rRNA amplicon sequencing arising on a range of diets and dietary ingredients within an *in vitro* model system at 20ºC (see Table 1).

a)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CS
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LK
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Log CFU g-1
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b)

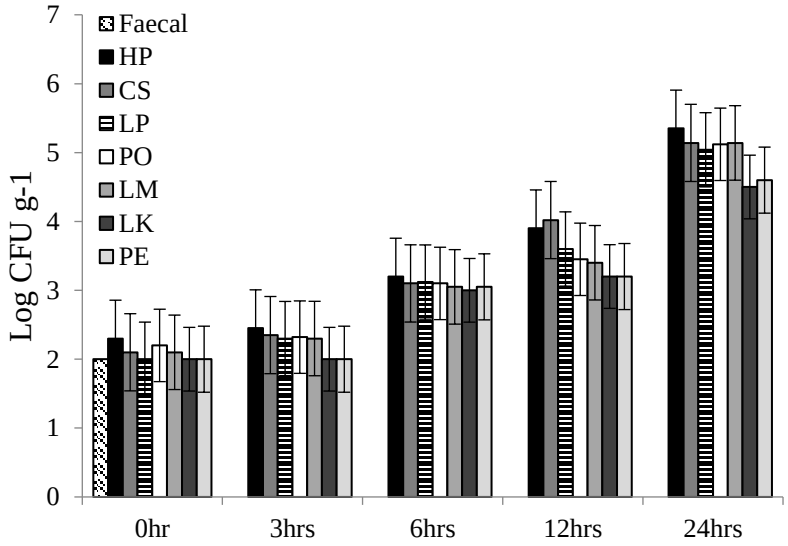
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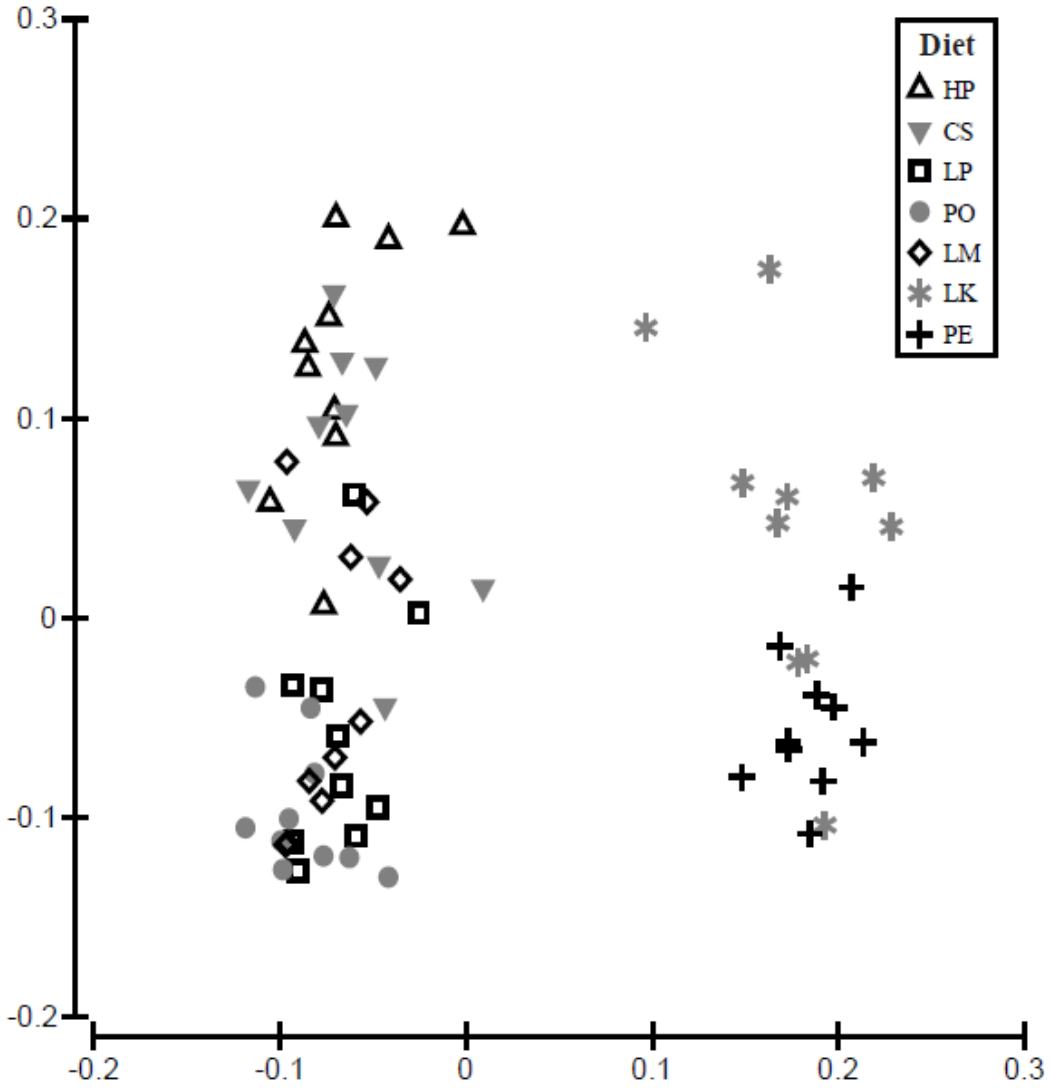
Figure 1



571

572 Figure 2

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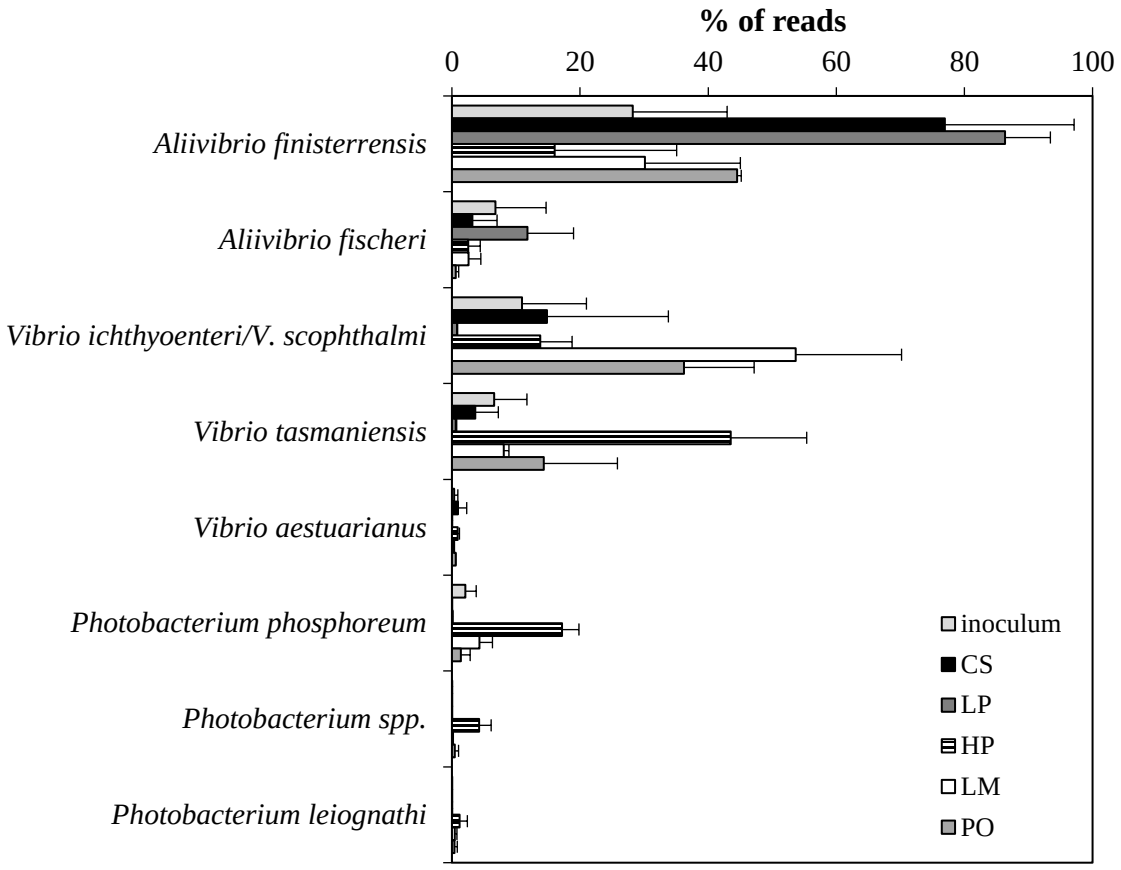
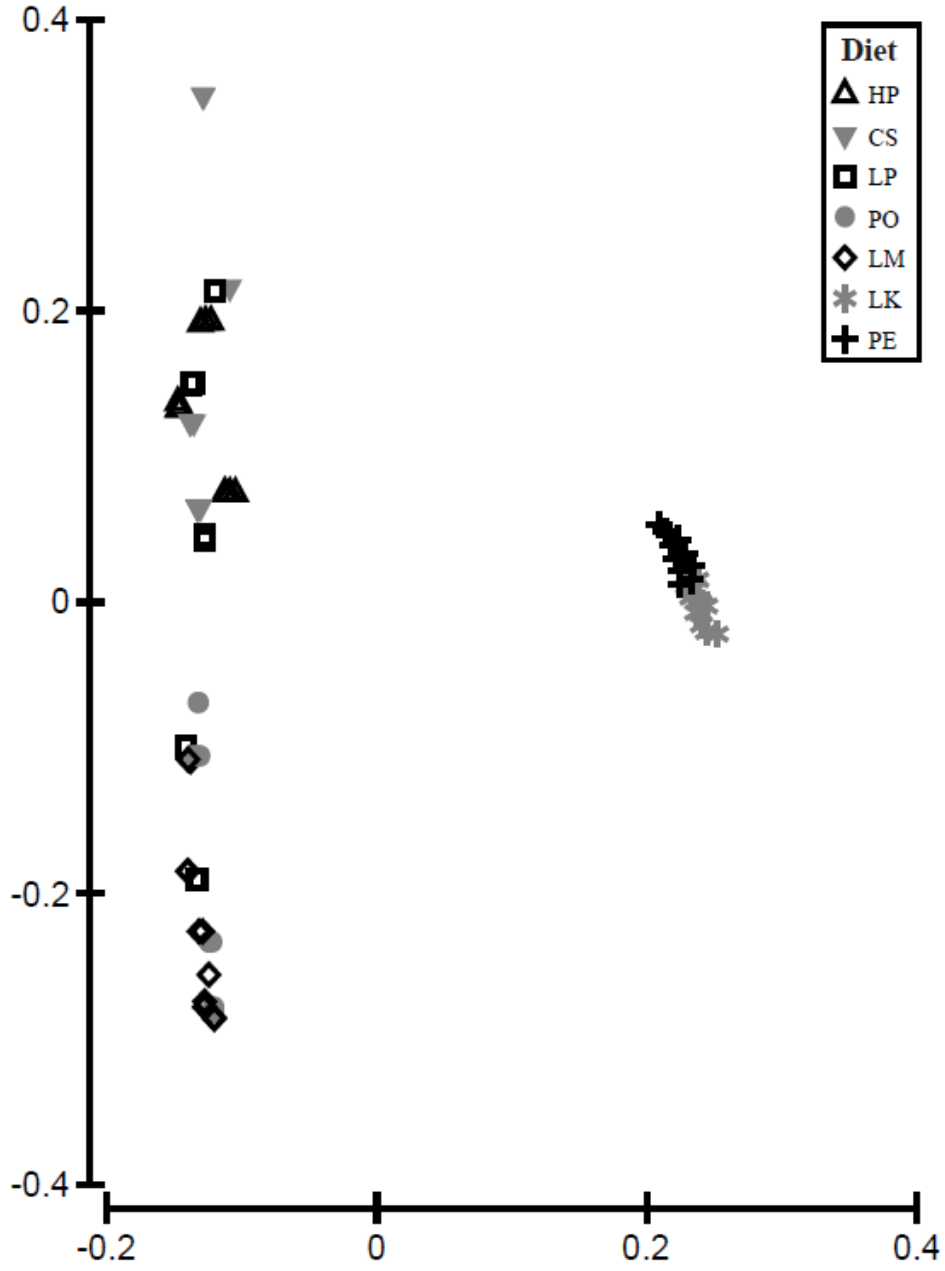


Figure 3 Relative abundance of the bacterial species present in the *in vitro* culture system shown as average percentile values with standard deviations. Community composition was determined by Illumina MiSeq 16S rRNA gene amplicon analysis. The faecal inoculum composition was determined from 10 samples per diet. The composition after 24 h at 20°C were determined in duplicate (see Table 1 for abbreviations).



584

585 Figure 4

586

587 **Table 1** The composition of diet formulations and ingredients utilised in this study.

Diet group	HP	CS	LP	PO	LM	LK	PE
Composition and energy:							
Protein (%)	50	45	40	45	45	37	24
Lipid (%)	20	25	30	25	25	8	1
Digestible energy (Mj/kg)	18.0	18.8	19.6	18.8	18.4	13.2	ND
Protein to digestible energy ratio	27.7	23.9	20.4	23.9	24.4	28.0	ND
Ingredients:							
Fishmeal (%)	71.2	63.5	55.8	63.5	16	0	0
Fish oil (%)	13.4	63.5	24.8	0	18.1	0	0
Wheat flour (%)	14.8	19.1	18.8	16.8	12.3	0	0
Wheat gluten (%)	0	16.8	0	0	0	0	0
Lupin kernel meal (%)	0	0	0	0	10	100	0
Poultry meal (%)	0	0	0	0	40	0	0
Poultry oil (%)	0	0	0	19.1	0	0	0
Vitamin/minerals premix (%)	0.5	0.5	0.5	0.5	0.5	0	0
Yttrium oxide (%)	0.1	0.1	0.1	0.1	0.1	0	0
Pisum sativa meal (%)	0	0	0	0	0	0	100

588 ND: no data available for Atlantic salmon.
589