Atlantic salmon (*Salmo salar* L.) gastrointestinal microbial community dynamics in relation to digesta properties and diet

Kamarul Zaman Zarkasi[1,2], Richard S. Taylor[3], Guy C.J. Abell[3], Mark L. Tamplin[1], Brett D. Glencross[4], John P. Bowman[1]

[1]Tasmanian Institute of Agriculture, Food Safety Centre, University of Tasmania, Hobart, Tasmania, Australia

[2]School of Biological Sciences, Universiti Sains Malaysia, Penang, Malaysia

[3]CSIRO Agriculture, Hobart, Tasmania, Australia

[4]CSIRO Agriculture, Brisbane, Queensland, Australia

Corresponding author: Kamarul Zaman Zarkasi

Address: Tasmanian Institute of Agriculture, Food Safety Centre, University of Tasmania, College Road, Sandy Bay, Hobart, Tasmania 7005, Australia

Phone: +61 3 6226 2621

Fax: +61 3 6226 7444

E-mail: kamarul.zarkasi@gmail.com

Abstract

To better understand salmon GI tract microbial community dynamics in relation to diet, a feeding trial was performed utilising diets with different proportions of fish meal, protein, lipid and energy levels. Salmon gut dysfunction has been associated with the occurrence of casts, or an empty hind gut. A categorical scoring system describing expressed digesta consistency was evaluated in relation to GI tract community structure. Faster growing fish generally had lower faecal scores while the diet cohorts showed minor differences in faecal score though the overall lowest scores were observed with a low protein, low energy diet. The GI tract bacterial communities were highly dynamic over time with the low protein, low energy diet associated with the most divergent community structure. This included transiently increased abundance of anaerobic (*Bacteroidia* and *Clostridia*) during January and February and facultatively anaerobic (lactic acid bacteria) taxa from February onwards. The digesta had enriched populations of these groups in relation to faecal cast samples. The majority of samples (60-86%) across all diet cohorts were eventually dominated by the genus *Aliivibrio*. The results suggest that an interaction between time of sampling and diet is most strongly related to community structure. Digesta categorization revealed microbes involved with metabolism of diet components change progressively over time and could be a useful system to assess feeding responses.

Keywords: Atlantic salmon; intestinal bacteria; diet formulations; 16S rRNA gene; digesta properties

Abbreviations: GI – gastrointestinal, DE – digestible energy, SGS- Summer Gut Syndrome

Introduction

Aquaculture of Atlantic salmon (*Salmo salar* L.) in Tasmania is currently the highest volume and highest value fishery in Australia [1] and is rapidly expanding. The South-eastern region of Tasmania (43°S 147°E) has been subject to intermittent unseasonably warm summers. This occurrence results in a still ill-defined problem that has been referred to as "Summer Gut Syndrome" (SGS) featuring salmon populations that have poor feed utilisation and poor or variable growth performance, especially when water temperatures exceed 17ºC [2]. The cause of SGS has been anecdotally linked to; i) water temperature, ii) diet and iii) husbandry and farm management. Though not necessarily causally related, observations suggest changes in the gastrointestinal health of affected populations, suggesting the condition could be linked to GI tract homeostasis.

The rapid development of salmon aquaculture over the past three decades has required a parallel increase in global fish feed production [3] despite competition for pelagic sourced marine proteins and oils from terrestrial animal production, human consumption, industrial and pharmaceutical sectors. Salmon diets have changed markedly over this time, trending to higher energy and higher oil: protein ratios while incorporating an increasing range of alternative ingredients obtained from marine, animal and plant sources [4]. The makeup of commercial diets is dictated by seasonal price and availability of alternative ingredients which are blended with the aim of meeting essential amino acid and fatty acid requirements. Although this approach continues to lower the dependence of farmed salmon on marine forage fish, there is potential to inadvertently impact fish health and performance as non-marine inclusion levels increase. Internal environmental factors, including the fish's nutritional state [5] may affect the magnitude of the stress response. Therefore, the

susceptibility to limiting nutritional factors is likely to be expressed in response to seasonal environmental stress.

The Atlantic salmon gastrointestinal (GI) tract system like other fish is not as complex as mammalian systems and thus more influenced by external factors such as temperature [6, 7]. Understanding the connectivity between environment, diet, animal physiology and their GI tract microbiota has gained substantial attention in recent years due to the association of gut microbiomes with host health [8-11]. Though only limited knowledge is available on how GI tract microbes contribute to piscine physiology [12, 13] it can be assumed that there are interactions that can be both beneficial and negative [14, 15].

The main interest in native GI tract microbial communities is that they likely maintain GI tract homeostasis [16-18]. Gut homeostasis essentially refers to the role bacteria (amongst other influences) play in stimulating the immune system and other physiological responses via end-products of metabolism or through interactions with the epithelial layers. An appropriate level of homeostasis would be assumed to be associated with optimal feed conversion efficiency. Homeostatic changes to gut function could be influenced by probiotic bacteria, natural bioactive products, and dietary components [19-25]. In this respect such an optimal state is required for maximal economic return in mariculture operations since output is determined biomass production relative to the feed input. To date the main focus of Atlantic salmon GI tract microbial communities and physiological response research has been performed in relation to diet [26-33].

Since feed costs are one of the important factors in the economic success of Atlantic salmon mariculture, a focus has been placed on understanding how valuable feed resources can be best used [3]. Thus there is a need to better understand how alternative protein and lipid substitutes and other feed additives potentially positively or negatively affect salmon health and productivity [23, 34-37]. Standard commercial diets have been continually

redesigned to maximize salmon growth in relation to developmental and husbandry management regimes. Such manipulations to diets tend to impact on growth performance [38] but there is a dearth of knowledge of whether there is a consistent effect on GI tract microbiota. An important question is whether GI tract microbiota can be linked to growth performance and whether manipulation of microbial communities can be achieved to optimise health and/or production.

This study explores the relationship between diet and GI tract community composition within the larger context of commercial mariculture diets. This study was performed between spring and autumn to cover the risk period of SGS and included the application of feeds of different protein:lipid ratios, digestible energy (DE) and fish meal levels to investigate the link between dietary energy, fishmeal replacement and GI tract microbial communities. As part of this study an assessment of the faecal digesta properties, using a ordinal 1 to 5 scoring system, was instigated to determine if it could be used as a measure of "gut health" since production of very liquid excreta and faecal casts (pseudofaeces), which consist of sloughed intestinal mucous cells and enterocytes, has been sometimes associated with GI tract immune system dysfunction [39, 40]. This scoring system was assessed in tandem with community structure analysis to determine if GI tract microbial populations could be linked to a concept of "gut health" as well as to diet-linked changes.

Materials and Methods

Caged fish trial design and feeding

Mixed-sex Atlantic salmon smolt (n=2359) that had previously been PIT (passive integrated tag, Sokymat, Switzerland) tagged in June 2010 were stocked within a single 10 × 10m sea

cage at Meads Creek, Dover, Tasmania on 4th August 2010 (mean weight 169 ± 48 g). The fish were fed with commercial feed (Skretting Australia) using an automated feeder (Aquasmart Pty. Ltd, Glenorchy, Australia) to ensure optimum growth rates. Three weeks after marine input the fish were treated under veterinary supervision with trimethoprim for seven days due to a low incidence of *Yersinia ruckeri* related mortalities in a neighbouring pen of fish. At the commencement of the experimental trial in early November 2010 the fish population was split evenly (by random pre-allocation of PIT tag numbers) into four 5 × 5m pens that were conjoined two per side to a central walkway. Within two days the fish were provided 6 mm pellet trial diets representing a range of protein:energy ratios, one test diet per pen (Table S1).

Four different diets were tested, commercial standard (IntPro), low fish meal (LFM), high protein:DE ratio (HiPro) and low protein:DE ratio (LoPro) (Table S2). The low fish meal (LFM) diet included poultry meal with fish meal level reduced to 10% of the content. All feeds contained lupin kernel and faba bean meal. Feeds were supplied via a corner-mounted 50 kg spreader (AGK Kronawitter GmbH, Wallersdorf, Germany). Feeding reactions was routinely monitored to ensure fish were fully satiated. Mortalities were removed every 5 days and the PIT tag number recorded. Throughout the trial, the populations were routinely assessed for signs of amoebic gill disease (AGD) by fortnightly subsampling of 40 fish and determining the range of gill score [41]. These checks triggered two freshwater baths at normal commercial bathing thresholds prior to the trial and a further three during the trial (Table S1). The trial was terminated in April 2011 by lethal anaesthesia (100 ppm Aqui-S). The maturation status of each surviving animal was assessed by inspection of the gonads.

Faecal sample collection and assessment

Samples of hind gut faeces were obtained 5 times across a 5 month period (Table S1). Throughout the trial, faecal sampling was performed 10-14 days after each freshwater bathing process. This ensured AGD impact was minimised throughout the trial and reduced the direct effect of freshwater bathing on GI tract microbial communities. At each faecal sampling event fish were fed until crowding, then dip-netted in small batches into 17 ppm Aqui-S anaesthetic (Aqui-S, Lower Hutt, New Zealand). All fish were individually weighed and hind-gut faecal content scored on a scale of 1 to 5 (solid faeces to no faeces, Table S3). Faeces were collected from 10 overtly immature fish from each diet cohort by gently squeezing faecal samples into sterile plastic vessels. The process of taking faecal samples and ten fish per group were chosen to account for size variation within a population, in line with previous studies [42, 43]. All faecal samples were frozen in liquid nitrogen, transported to the laboratory, and stored frozen at -80°C. Water temperature was obtained from a data logger located at a depth of 5 m (Scielex Australia) and compared against the long term average (LTA) temperature for the farm (LTA data since 1995).

Animal Ethics

All animal handling procedures were approved by the Department of Primary Industries, Parks, Water and Environment (DPIPWE) Animal Ethics Committee (Project 30/2009-10) under the guidelines of the Australian Code of Practice.

Faecal score analysis

All faecal samples collected were qualitatively scored on the basis of appearance and consistency using the categorical system shown in Table S3. The distance and physiological significance between the individual score categories is undefined with the scores used purely as a relative comparison of digestive status. This follows the assumption that a low score is

favourable for performance and suggests normal GI tract function while a high score suggests poor feeding rates.

Total faecal DNA extraction and 16S rRNA gene sequencing

Total bacterial DNA was extracted directly from the faecal samples using the QIAamp DNA Stool Mini Kit (QIAGEN Sciences, Maryland, US) following the manufacturer's instructions. To examine the microbial communities present in the faecal samples, 16S rRNA gene tag pyrosequencing was applied to 217 samples collected during the study that yielded sufficient levels of faeces and subsequently DNA. Samples of each cohort and time included an even contribution of each faecal score (1 to 4), score 5 (empty gut) did not produce a sample. Tag-encoded FLX amplicon pyrosequencing of the region covered by application of the 341F and 907R primers [44] was carried out by Research and Testing Laboratories (Lubbock, Texas) using a Roche 454. FLX instruments with Titanium reagents as previously detailed by Dowd et al. [45]. Approximately 3000 raw reads were obtained per sample. Sequences were de-noised and chimera-filtered through a bioinformatics pipeline [46]. Briefly, all sequences were organised by read length and de-replicated using USearch [47]. The seed sequence for each cluster was then sorted by abundance and then clustered again with a 1% divergence cut-off to create consensus sequences for each cluster. Clusters containing only one sequence or <250 bp in length were then removed. Seed sequences were again clustered at a 5% divergence level using USearch to confirm whether any additional clusters appeared. Once this process was completed any reads that failed to have a similar or exact match to seed sequences (typically poor quality reads) were removed. Chimeras were also removed from the clustered sequences created during denoising by using UCHIME in the *de novo* mode [48]. Sequences that yielded high score matches of <75% were discarded. Singleton sequences were not assessed.

Statistical analysis of faecal score

Calculation of Relative Growth Index (RGI) between measures allows individual growth 'success' to be compared without size bias, for each tagged animal, the 'expected' growth was calculated from known start weight and actual daily average water temperature using an 'industry standard' model. Actual growth achieved is expressed as a proportion of modelled growth so that animals growing slower than model are at RGI <1 (100%). Animals that lose weight are expressed at RGI <0.

The primary variables assessed were body weight, RGI and Condition Factor (CF) in relation to factors "type" and "cohort". Gender (males, female, uncertain) and maturation level (mature, immature) were considered together as "type" (mature male, immature female etc.) with fish that died before the end of the trial removed from the data. The effect of pen and feed treatment (IntPro, LFM, HiPro and LoPro) was assessed together as "cohort" since due to the experimental design the independent effect of the pens could not be independently separated. Over the 5 time points stepwise regression analysis using Genstat v14.1 (VSN International) utilising 2-way interactions between variables and factors was used to rank the effect of the variables. Differences between variables in relation to the different factors were tested using one-way ANOVA with cohort, maturation and cohort × maturation representing fixed terms.

Diversity and multivariate analysis of GI tract microbial diversity

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) [49], and canonical analysis of principal coordinates (CAP) [50]. For this analysis sequence read data organised at the genus-level was normalised as percentages,

square root transformed and a resemblance matrix created by calculation of Bray-Curtis coefficients. PERMANOVA was conducted using an unrestricted permutation of the data (n=9999), fixed terms summed to zero, and utilizing partial sum of squares since the data is effectively an unbalanced data layout due to a high proportion of GI tract taxa frequently not observed owing to detection limitations, and the inherent nature of bacterial growth and eco-physiology. CAP was conducted using default settings. The PERMANOVA derived significance values were considered significant when $P < 0.01$, while $0.01 < P < 0.05$ were considered marginally significant. PRIMER-6 was used to calculate alpha diversity indices utilising Fisher's α-diversity and Pielou's evenness (J'). Overall species richness was calculated with Chao2 [51]. Beta diversity and multivariate dispersion (IMD) [52] was assessed using PERMDISP in Primer 6 and with the updated PERMDISP2 version [52] in order to determine whether trial sampling time, diets or faecal scores are important factors in determining community overlap and scatter.

Results

Fish environmental conditions

Temperatures experienced by experimental salmon populations were comparatively mild with a peak of 16.8°C and 16°C being exceeded from early January to early March 2011 (Fig. S1). This was on average 1°C lower than the long term average [53]. Oxygen saturation was on average 91% (±6%) with only 3.2% of readings <80%. AGD was effectively controlled by 5 freshwater baths (Table S1). The experimental series thus essentially assessed dietary responses in conditions where temperature stress and AGD pressures are not likely to be substantial, at least within the context of standard commercial operations.

Faecal score trends

At the first faecal measure (December 2010) few fish produced casts or showed signs of empty hind-gut (scores 3.5, 4 and 5; 3.5% total of fish assessed, Table S1). As the trial progressed faecal scores rose substantially (Fig. S2). Interpretation of this data is necessarily constrained due to the single cage per diet and the limitation of faecal scoring large numbers of fish. During a sampling day (two pens of fish) there was a tendency for faecal score to increase as feed progressed through the gut over time (data not shown). It became necessary to include at least a day of undisturbed feeding between sampling days because the presence of people on the cage walkway reduced feed intake on the two un-sampled pens (data not shown). The primary variables of consequence affecting faecal scores included cohort, RGI, type, and to a lesser extent weight.

The importance of these variables varied over time but in general the effect of fish type (gender and maturation status) and RGI strengthened while cohort declined as the trial progressed. This can be explained by immature fish having lower faecal scores by the end of the trial indicative of a greater level of overall feeding and weight gain compared to maturing fish progressing from mid-summer to early autumn. This trend can be attributed to the process of sexual development which is typified by faster growth in spring and early summer and lower feeding rate as gonads and secondary sexual characteristics develop [54]. Immature fish rapidly caught up in terms of body weight. Thus faecal scores must be interpreted in line with the maturation status of specimens. The faecal scores associated with fish fed the LoPro ratio diet were significantly lower (on average 0.5) than those from the LFM (p=0.015) and HiPro ratio diets (p=0.019) at the final measure (April 2011). Overall the IntPro diet was slightly lower (0.34) compared to the LoPro diet (p=0.034).

Diet cohort faecal microbial community dynamics and relation to faecal score

Faecal samples for community analysis were taken from sexually immature fish. A global view of faecal microbial populations and the effect of time, diet, and correlation to faecal score was analysed by PERMANOVA (Table S3) and illustrated by CAP (Fig. 1). All of these factors have significant overall effects on GI tracts communities (Fig. 1, 1b, 1c). Main (Table S3) and pair-wise (P <0.006) tests indicated the communities were ceaselessly dynamic with all times significantly different from each other and this was reflected in the CAP analysis (Fig. 1a) where 67-84% of samples were correctly categorised to a given sample time (m=50). There was a significant shift in the community on the basis of diet (Table S3, Fig. 1b). The largest shifts from the trial start point were observed for the LFM, HiPro and LoPro ratio diets (Fig. 1b). From pair-wise analysis the HiPro ratio diet was not significantly different from either the LFM or LoPro diet (P=0.45) while the LFM diet showed greater separation from the LoPro ratio diet (P=0.008). Community structure separated between low scores (1 to 3) and samples containing casts (score 3.5, 4) (Fig. 1c) in the CAP analysis where 77-80% of samples were correctly classified to the two main groups.

The overall temporal dynamism in community structure is consistent to that observed previously by Zarkasi et al. [55] in which samples were obtained through a farm production cycle. However, altering diet energy levels and the fish meal levels shifted the microbial community to a much greater degree than the communities achieved with diet regimes containing commercial standard levels of fish meal and DE as tested in the Zarkasi et al. [55] paper. From the statistical analysis faecal score-associated differences in the GI tract communities are largely independent of diet (P=0.406) while interactions are only weakly associated with sampling time (P=0.045) suggesting the responses are associated with communities being consistently different between the digesta in comparison to excreted cast-rich material. This result was consistent with the findings related to faecal scores of immature

fish, which had different feeding and growth rates throughout the trial whereby cohort groups did not otherwise have large differences in faecal scores.

Diet influenced diversity changes in GI tract communities

From the 217 samples about 4100 filtered sequence reads were obtained on average per sample with 23.1% of all reads being *Salmo salar* 18S rRNA sequences, 11.4% chloroplasts or algae and the remainder bacteria. Chloroplast and algal sequences were excluded in the aforementioned statistical analysis though inclusion had virtually no effect on the outcomes (data not shown). Bacterial 16S rRNA reads were classified into 1507 OTUs mostly grouped in the phyla *Bacteroidetes, Firmicutes* and *Proteobacteria* (Fig. S3). Chao-2 species richness across a conglomeration of all 217 samples was 3167 (±171) suggesting diversity on par with GI tracts of mammals though individual specimen diversity was much lower and highly variable (4 to 229 $OTU_{0.98}$ per individual, average 48). A relatively high proportion of reads could be ascribed to "species groups" since the 16S rRNA sequence region (V3 to V6) obtained was limited in its ability to demarcate species within certain clades.

Diversity was assessed to examine time, diet and faecal score influenced patterns. Fisher's α-diversity was affected over time with a transiently pronounced increased diversity observed for the LoPro cohort and similar weaker responses for the IntPro and HiPro cohorts (Fig. 2). The LoPro ratio diet had the highest overall diversity and the IntPro cohort the lowest (68 vs 33 OTUs on average). The LFM diet showed a slow trend of increasing diversity while for the isoenergetic IntPro cohort it declined. A sharp reduction in evenness occurs after the first month of the trial though this was temporarily reversed during the summer period.

PERMDISP/PERMDIP2 provided an assessment of multivariate spread in the datasets and via pairwise analysis in Primer 6 an indication of overlap by measuring relative

distances to a group centroid value (the point that minimises the sum of squared distances to the points within a given group) [56]. The sampling time showed a trend of increased dispersion in summer (F=5.87, df=4, 212, *P*=0.001) (Fig. 3), which correlates with the increased of diversity from the December to March sampling periods. The trend was weaker for cohort (F=4.18, *P*=0.02) and negligible for faecal score. The LoPro cohort stood out in being distinctly different (Fig. 3) likely related to the higher species diversity compared to other cohorts (Fig. 3, *P*<0.02).

Ecogroup dissection to assess GI tract β-diversity

Given the salmon gut is an open system and thus readily influenced by environmental factors an approach was used to dissect community dynamics and β-diversity by examining the GI microbiota in relation to oxygen requirement and to a predicted ecosystem source, the latter defined by the typical habitats the identified species are most often associated. This is based on the types of taxa observed which ranged from anaerobic to aerobic and had either marine or non-marine origins. This breakdown resulted in six major "ecogroups": i) GI tract anaerobes (abbreviated as GAN) typically associated with GI tracts, primarily class *Bacteroidia*, class *Clostridia* and class *Fusobacteria*); ii) GI tract facultative anaerobes (GFA), mainly non-spore-forming lactic acid bacteria (class *Bacilli*) and class *Mollicutes*; iii) facultatively anaerobic marine species (MFA) dominated by members of the family *Vibrionaceae* of class *Gammaproteobacteria*; iv) marine aerobes (MA) mainly belonging to major seawater clades including the *Roseobacter* clade (class *Alphaproteobacteria*) and class *Flavobacteriia*; v) facultatively terrestrial anaerobic (TFA), and vi) aerobic bacteria (TA) normally associated with plants, fresh water, and soil. Respectively, the terrestrial originated groups consisted primarily of genus *Bacillus* and its spore-forming relatives (class *Bacilli*) and members of the proteobacterial families *Comamonadaceae*, *Sphingomondaceae*, and

Methylobacteriaceae. Minor levels of anaerobes bacteria from marine and terrestrial ecosystems were also observed though abundances were 1 to 3-orders of magnitude lower than the other groups.

Diet and seawater derived bacteria transiting the Atlantic salmon hindgut

The proportions of marine aerobes and the terrestrial facultative anaerobes both declined from initially high levels in all four cohorts (Fig. 4). Based on the taxa present the marine species are derived from seawater imbibed during feeding and the predominance at the beginning of the trial owes to low bacterial numbers present initially. Thus the marine bacteria passing into the hind gut seem to become less predominant as other bacteria grow and colonise digesta in the GI tract. Spore-forming bacilli which made up most of the terrestrial facultative anaerobes included substantial presence of the thermophilic genera *Aeribacillus*, *Anoxybacillu*s and *Geobacillus*.

These bacteria are diet derived, present as "contaminants" since minimum and optimum growth temperatures of these taxa (minimum temperature for growth 30-35°C, optimum rate at 55-65°C) are well above that of the *in situ* temperature (≤17°C). Analysis of feeds using an initial heat exposure of 80°C for 10 minutes followed by plating onto Brain-Heart infusion agar and incubation at 25, 37 and 55°C revealed 10^3 to 10^5 CFU of putative spore forming bacteria per gram of feed. The kilning treatment used in feed manufacture likely selects for these bacteria which are able to survive on stored feeds.

Salmon GI tract anaerobes are mostly confined to the digesta

The proportional level of salmon 18S rRNA reads was doubled in high faecal score samples consistent with casts including sloughed intestinal cellular material. Chloroplast sequence levels were markedly more abundant (7-10-fold) in low faecal score samples and thus

associated with the digesta (Fig. 5). The vast majority of chloroplast 16S rRNA sequences were derived from the diets (11.1%) and included sequences identified as lupin (*Lupinus*), faba bean (*Vicia*), wheat flour (*Sativa*/*Triticum*) and pea (*Pisum*). *Nannochloropsis* was the source of most of the algae-derived chloroplasts. Based on distributions between high and low faecal scores anaerobic taxa typically associated with GI tracts seem to be predominant in the digesta while the proportion of reads is 6-fold lower in samples containing casts (Fig. 4). This phenomenon did not apply to the marine (and non-marine) associated facultative anaerobic taxa consistent with the concept that the bulk of the digesta represents an anoxic domain allowing strictly anaerobic bacteria to grow. There was a high diversity observed amongst the anaerobic taxa, contributing about one-third of observed OTUs, and populations were very dynamic (Fig. 5). Thus changes in anaerobe populations seem influential on the diversity within the faecal samples since the shape of the anaerobic diversity curves (Fig. 4a) matches overall diversity curves (Fig. 3).

In all diets, most obviously for the LoPro diet, anaerobes peaked in the summer months and then declined in cooler months of March-April. This change contributed strongly to the increase and then subsequent decrease in α-diversity observed for this cohort and correlated to sampling time dispersion as indicated above. Community structure appraisal suggests that initially members of genus *Bacteroides* predominate amongst the anaerobes (>50% of GI tract anaerobe reads) but are progressively replaced by members of genus *Porphyromonas* towards the end of the trial. *Clostridium*, *Veillonella* and *Fusobacterium* spp. were also quite common. *Bacteroide*s spp. typically are saccharolytic while porphyromonads are proteolytic mainly fermenting amino acids [57] thus the progressive enrichment of *Porphyromonas* may occur due to the high protein content of the diets.

The LoPro diet transiently promotes growth of facultative anaerobes including lactic acid bacteria.

The community component consisting of GI tract facultative anaerobes consisted (in order of predominance) of the Gram-positive genera *Brochothrix*, *Streptococcus*, *Carnobacterium*, *Lactobacillus* and *Lactococcus.* All of these genera are classic aerotolerant strictly fermentative anaerobes that are grouped collectively as the "lactic acid bacteria", forming lactic acid as a major end-product of metabolism. Members of the genus *Cloacibacterium*, which belongs to the *Chryseobacterium* clade in family *Flavobacteriaceae,* were also comparatively abundant. An unclassified *Mycoplasma* OTU was the predominant community member in 5% of specimens by the end of the trial similar to observations made by Zarkasi et al. [55].

As with the anaerobic taxa, populations of facultative anaerobes peak in the summer in the LoPro diet and then decline. The levels in the other diets by comparison show a gradual downward trend. These results suggest that the availability of substrates and lack of oxygen is potentially most optimal when the LoPro diet is applied. However, the reasons for this are unclear, but could be associated with diet: metabolic interactions within the GI tract. Many of the lactic acid bacterial taxa are known to form bacteriocins [58] that potentially provide them a temporary advantage against the growth of other bacteria, however if this is the case the effect is clearly only transient. Distribution between samples lacking casts and cast-rich faecal samples shows that lactic acid bacteria are slightly more enriched in the digesta (Fig. 5). Lactic acid bacteria could also be present as adherent populations on the gut epithelium [24, 43] but the exact localisation of these bacteria is still poorly defined.

Marine facultative anaerobes eventually predominate regardless of diet.

All diets by the end of the trials became dominated by marine-derived facultatively anaerobic species (Fig. 4), especially those belonging to the genus *Aliivibrio*. The primary *Aliivibrio* taxon (making up 33.2% of reads) was nearly identical to the species *A. finisterrensis*, originally isolated from the Manilla clam (*Ruditapes phillipinarum*) [59]. In some samples it made up >95% of reads. Other members of the family *Vibrionaceae* were also significant, especially *A. fischeri* (3.3% of reads), *A. sifiae* (0.9%), an unclassified *Aliivibrio* species (0.2%), *Vibrio atypicus* (2.1%), *V. vulnificus* species complex (0.6%), *V. ichthyoenteri* (0.2%), and *V. scophthalmi* (0.4%). In the first month of the trial rapid growth of these species occurs in all cohorts. In the IntPro and HoPro diet the levels reach a peak of 65-70% of bacterial reads before slightly declining to 55-65%. In the LFM diet the level reached an average of 78% of reads at the end of the trial. The LoPro diet showed a pronounced lag before the proportion of the marine facultative read group rose to approximately 70% at the end of the trial.

High abundance of non-halophilic aerobes in the salmon intestinal tract

It was observed that taxa typically of non-marine origin with a strictly aerobic oxidative metabolism made up 5-20% of reads throughout the trial peaking in the March 2011 samples. The major taxa include *Sphingomonas paucimobilis*, *Acidovorax ebreus*, and *Methylobacterium* spp. (mainly *M. extorquens*). This surprising result could suggest these taxa are diet "contaminants", however the populations persist at significant levels suggesting they may have footholds in the salmon GI tract. Diet did not have much effect on their distribution nor was there any form of digesta distribution (Fig. 4 and Fig. 5) consistent with anaerobes preferring the largely anoxic digesta.

Discussion

Manipulations of diet attributes such as protein and oil, supplementation with prebiotic additives, plant extracts and probiotic agents can potentially stimulate immune system responses possible as a consequence of the alteration of GI tract microbial populations [19, 60, 61]. However determination of how microbial community adjustments generate a farm level effect such as increased growth rate or body weight is challenging due to experimental system complexities and lack of knowledge on what constitutes a salmon core GI tract microbiome and how it connects with salmon physiology. Many factors impact what that the core species suite could be composed of including ecosystem inputs and influences [7]; scale and methods of sampling communities [62]; the nature of the specimen cohorts being tested (wild; domesticated; maturation stage; sex; ploidy) and their management; and the dietary regimes relative to what is considered a commercial standard being tested. That said, the process to realise this form of knowledge within commercial production system settings requires first some clear concepts of what can be achieved from observations of GI tract community changes.

In this study we used a relatively high resolution community analysis approach, substantial replication (10-20 samples per treatment,) and also examined faecal samples in an ordination system in order to study GI tract community patterns that might be subsequently related to performance outcomes. In a previous study Zarkasi et al. [55], where 6 specimens each from two separate diet groups per time point were analysed we observed considerable specimen-to specimen variability and though we could discern temporal trends any differences between diets were more difficult to separate, if different community responses were indeed present. Therefore, to increase the power of the analyses we increased replication to 10-20 specimens in the current study.

From the 4 diet cohorts tested we could observe different community structure dynamics and a range of faecal scores. This was done at the *fish specimen level* only though such experiments would also be needed at whole farm level to better account for environmental and temporal variables. Nevertheless, we could show that the LoPro diet, which resulted in growth performances at the end of the trial distinguishable within non-mature and mature subpopulations also possessed pronounced albeit transient differences in the abundance and diversity of strict anaerobe, lactic acid bacteria, and *Vibrionaceae* community components. However, it is still unknown if these transient changes in the communities relative to the other diets have casual links to final growth performance outcomes, for example via differential effects on immune system modulation or via more efficient digestion. Also how reproducible these transient phenomena are is unknown and for greater confidence would require instigation of experiments where there is tighter control on the community structure. The open nature of the salmon GI tract challenges this proposition especially within a commercial or experimental farm setting since local conditions are potentially highly influential and could potentially change over the long term affecting all facets of the biology, for example changes in surface water temperatures [53].

We observed a wide variety of microbes in the faecal samples and could show conclusively differences in community structure relative to the nature of the samples, diets and on a temporal scale as well as the inherent variability within treatment groups. Based on the typical bacterial densities in digesta (10^5 to 10^9 cells/g) bacterial taxa could be resolved with pyrosequencing with a 1000-fold dynamic range (effectively 10^2 to 10^6 cells per gram). We observed high specimen to specimen variability, however this was not entirely consistent. At the commencement of the trial the individual variability was relatively constrained (49 ± 15 OTUs, n=19) however as the trial progressed through summer it seems GI tract communities of individual fish diverge as confirmed by dispersion analysis. A similar

situation has been observed in wild Atlantic cod populations sampled in the one region. Individuals within these wild cohorts were found to have very poorly conserved β-diversity [63]. In order to adequately assess salmon GI tract microbial communities high resolution procedures seem necessary, coupled to sufficient biological replication. The variability between specimens could be to some extent be partly understood by applying the faecal scoring system since variations in some community components could be observed systematically. In human microbiome studies large datasets have been used in order to link metabolic dysfunctions to GI tract diversity as well as to make correlations with marker metabolites [64]. In order to make better links between diets or other treatment criteria with farm level outcomes replicated cohorts would need to be studied since individual fish variation may mask subtle trends.

Rapid fingerprinting procedures for community structure analysis (for example ARISA analysis [65] could be useful for delineating large numbers of specimen samples followed by more focused sequencing and other forms of analysis. Older methods such as DGGE and TRFLP seem to be less appropriate being costly, labour intensive, non-quantitative and given to gel-based and PCR-based biases [66] and only with limited means for sequence interrogation (e.g. DGGE gel band analysis). Statistically testing community structure changes with multivariate approaches seems to be also an essential requirement to understand community structure changes in relation to other factors. Doing this made it possible to show populations are dynamic over time as well as affected by diet leading to an interaction effect (Fig. 1, Table S3) and observations that digesta has high chloroplast content correlates to high strict anaerobe population levels suggesting these bacteria can play a substantial role in feed digestive processes even in the open fish digestive tract (Fig. 5). The data suggests that the microbes that predominate in digestion roles changes over time and may comprise anaerobes, *Vibrionaceae*, *Mycoplasma* and/or lactic acid bacteria. This implies

the types of metabolites formed during feed metabolism plus other benefits (vitamin synthesis) could also change and thus impact on fish growth performance.

Temperature washighly influential in this study. Bacterial growth is fundamentally controlled by temperature and can be accurately modelled [67]. The fact *Vibrionaceae* predominates in most samples could be related to the relatively fast-growing, psychrotolerant [59, 68] and bile tolerant [69] nature of these species. Since many *Vibrionaceae* share similar ecophysiological traits it is likely specific species can get supplanted by other species. In other Tasmanian salmon surveys it was observed *A. fischeri*, *Photobacterium phosphoreum*, *Vibrio scophthalmi* and *V. ichthyoenteri* could predominate [2, 55, 70, 71] instead of *A. finisterrensis*. Further analysis of salmon strains may reveal biological facets that could have significance to salmon physiology and health, though data suggests that *A. finisterrensis* and its relatives seem to be neutral in the relation to fish growth rates and CF as they are comparatively predominant across all diets. Furthermore their abundance is equivalent between low and high faecal score samples suggesting that this group is not specifically predominant in digesta and may also congregate or interact with the intestinal epithelium.

It must be noted the predominance of *Vibrionaceae* did not apply to all specimens. By the end of the trial 14-40% (dependent on the cohort) of fish had different predominant OTUs present including species belonging to the genera *Bacteroides*, *Sphingomonas*, *Litoreibacter*, *Mycoplasma* and *Brochothrix*. The reasons for this lack of homogeneity between fish is unknown but could relate to a host of factors such as hierarchical dominance and maturation resulting in different fish body size and feeding rates; stochastic colonisation processes; and/or differential affects caused by the farm conditions and husbandry including anti-AGD baths destabilizing microbial communities.

One might speculate that these differences in dominant microbiota and overall individual variability could collectively comprise the population wide effects influencing

averaged performance outcomes. However, greater understanding of how GI tract microbiota functionally influence salmon physiology including the redundancy [72] of such influences is needed to determine this. This would require utilisation of metagenomic technologies for assessing salmon gene expression and protein abundances [73] coupled to bacterial-mediated processes including enzymatic and metabolomic characteristics [13]. Indeed, recent transcriptome studies of Atlantic salmon exposed to chronic high temperature and hypoxia indicate down-regulation of transcripts encoding proteins involved in the protection against oxidative stress and a metabolic rate suppression that ultimately results in reduced growthOur data also suggests aerobic microbes are found in the digesta and also associated with sloughed off intestinal cast material suggesting possibly some level of GI tract epithelium association or interaction though this remains to be clearly defined. The presence of aerobes in GI tract systems is not unprecedented as the mouse colon has a substantial population of aerobic taxa colonizing epithelium crypts [74], with oxygen supplied by the cell layer. The population most enriched in crypts versus the gut lumen were members of classes *Betaproteobacteria* and *Gammaproteobacteria*. It was hypothesized these bacteria may contribute to gut biology in homeostatic and protective capacities based on their inherent ability to consume oxygen radicals and xenobiotic compounds though this remains to be determined in any detail.

The types of non-marine aerobic taxa detected in this study mainly belonged to classes *Alphaproteobacteria* and *Betaproteobacteria* could also have capacity for detoxification since the main genera found (*Sphingomonas*, *Methylobacterium* and *Acidovorax*) are strong catalase producers, have diverse metabolic pathways for xenobiotic catabolism and in the case of *Methylobacterium* spp. can utilise methanol and convert toxic formaldehyde to CO_2. The fact that an array of aerobic bacteria are present in the salmon GI tract, which unlike mammals lacks crypts raises the possibilities of some bacterial role in

providing protection and homeostasis as observed in the mouse model. Confirmatory analysis of the epithelium layer is required to determine if stable populations are indeed present and also determine their biological activities *in situ*. Higher resolution studies of the GI tract epithelial layer via careful biopsies coupled with other methods such as fluorescent in situ hybridisation could confirm spatial localisation of these and other important bacteria that can be potentially linked to gut homeostasis and immune system modulation [75]. This would be important in connecting GI tract communities with salmon physiological and immune system responses and eventually broader performance outcomes.

We conclude that time (incorporating seasonal changes in temperature) and diets contribute to how faecal microbial communities are structured. Categorization of the digesta also revealed that microbes are different in relation to digesta properties, especially anaerobic bacteria. We hypothesize these community shifts could lead to the formation of different levels of metabolites and/or immune system stimulation that and could influence overall salmon physiology though we are still far from establishing mechanistic links between microbial communities and farm-level performance outcomes.

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Figure Legends

Figure 1: CAP plots comparing factors affecting GI tract community structure: a) time of sampling; b) diet cohorts (Table S1, Table S2) with the first sampling time in November 2010 designated "initial" and representing prior starved specimens that only had recently fed; c) faecal scores (Table S3).

Figure 2: Diversity (based on Fisher's α-diversity) and evenness (Pielou's J) index trends for diet cohorts over time, a) IntPro; b) LFM; c) HiPro; d) LoPro. The same information is provided for faecal score categories for each of the diet cohorts, e) Fisher's α-diversity; and f) Pielou's evenness.

Figure 3: Boxplots showing distances of a) time, and b) treatment subgroups from the centroid inferred from PERMDISP2. Asterisks indicate outlier values. The line in the box indicated the mean centroid distance value for the subgroup. The letters above the box indicate significance ($P<0.02$) determined by permutation in PERMDISP2 ($n= 999$) with the designation of the same letter denoting non significance.

Figure 4: Changes in the relative abundance of GI tract microbial components within each diet cohort categorised as "ecogroups". The taxonomic groups a) GI tract anaerobes (GAN); b) GI tract facultative anaerobes (GFA); c) facultatively anaerobic marine species (MFA); d) marine anaerobes (MA); e) facultatively anaerobic (TFA); f) aerobic bacteria (TA).

Figure 5: The enrichment of sequence groups on the basis of faecal scores. Bars indicate the proportion of reads making up all reads accumulated for the community or sequence component. The symbols above the bar indicate the proportional ratio of reads between low (1 to 3) and high (3.5 and 4) scores for each community or sequence subcomponent.

Supporting information

Table S1: Sequence of freshwater bathing and faecal scoring events. Sampling order denotes the order in which cohorts were handled during each faecal measure event.

Date	Event	Comments	Cohort	Sampling order		Faecal Score (%)					
					Pop.	1	2	3	3.5	4	5
4 Aug 2010	Marine Input	10x10m pen, Spirit Feed			2359						
22 Sep 2010	AGD Bath 1	Bathed			2236						
21 Oct 10	AGD Bath 2	Split and bathed to two 5x5m pens			2215						
2-3 Nov 2010	Faecal Measure 1	Starved pre-sample; split to four 5x5m pens	IntPro	2	553	63.9	28.9	5.6	0	0.5	1.1
			LFM	1	553	74.6	18	5.6	0	0.5	1.3
			HiPro	3	550	51.1	34.7	8.9	0	1.8	3.5
			LoPro	4	557	48.9	33.8	11.9	0	1.3	4.2
1-2 Dec 2010	AGD Bath 3	Bathed									
14-15 Dec 2010	Faecal Measure 2	Starved from evening pre-sample	IntPro	3	526	13.1	15.2	12	8.2	20	31.6
			LFM	2	532	5.6	18.4	2.6	25.4	29.3	18.6
			HiPro	1	528	16.9	25.8	6.1	19.4	18.2	13.7
			LoPro	4	524	5.3	6.7	3.2	14.9	37.8	32.1
12-13Jan 2011	AGD Bath 4	Bathed									
25-27 Jan 2011	Faecal Measure 3	Fed until sample	IntPro	2	453	39.7	24.3	7.5	4.6	8.8	15
			LFM	3	470	22.6	24	21.7	3.2	15.1	13.4
			HiPro	4	465	23.7	27.1	12.9	7.7	13.8	14.8
			LoPro	1	485	24.7	29.2	12.3	12.8	14.2	6.8
23-25Feb 2011	AGD Bath 5	Bathed									
4-7 Mar 2011	Faecal Measure 4	Fed until sample	IntPro	3	424	17.1	21.6	28.6	7	16.4	9.2
			LFM	1	420	18.8	22.6	16.9	10	18.8	12.9
			HiPro	2	405	13.6	19.6	11.4	9.4	28.7	17.3
			LoPro	4	426	14.8	17.9	23.8	12.8	22.1	8.6
4-6 Apr 2011	Faecal Measure 5	Fed until sample	IntPro	3	384	20.2	23.9	17.8	9.4	26.2	2.4
			LFM	1	388	17.3	13.9	14.4	12.9	32.7	8.8
			HiPro	2	379	19.5	21.6	13.2	12.1	25.3	8.2
			LoPro	4	416	26	22.1	14.2	18.8	14.2	4.8

Table S2: Diet specifications utilised in feed cohorts

Ingredients:	commercial standard (**IntPro**)	low fish meal (**LFM**)	high protein:DE ratio (**HiPro**)	low protein:DE ratio (**LoPro**)
(g/kg)				
Protein: lipid ratio	45:25	45:25	50:20	40:30
Fish meal (%)	35	10	35	35
Digestible energy (DE) (MJ)	20.4	20.4	19.7	21.4
Protein:DE ratio (g/MJ)	22.1	22.1	25.4	18.7

Table S3: Multivariate Statistical comparison between Atlantic salmon GI tract microbial communities on the basis of sampling time, diet and categorised faecal properties.

Factor	**df**	**MS**	***F***	***P* (Perma-nova)**	**Unique permu-tations**	**P (Monte Carlo)**
Time	3	14432	6.0811	0.0001	9872	0.0001
Diet	3	13051	5.4991	0.0001	9891	0.0001
Faecal Score	4	8464.2	3.5665	0.0001	9866	0.0001
Time × Diet	9	3910.6	1.6478	0.0008	9799	0.001
Time × Faecal Score	12	2968.1	1.2506	0.0459	9783	0.0485
Diet × Faecal Score	12	2422.6	1.0208	0.4058	9805	0.4107
Time × Diet × Faecal Score	36	2661.6	1.1215	0.0798	9666	0.0924
Residuals	133	2373.3				
Total	216					

Figure S1: Salmon growth and water temperature during the feeding trial. Average weight at each measure (Measure1 in November 2010 to Measure5 in April 2011), showing immature (circles) and maturing (triangles) sub-populations within each 'Cohort'; IntPro (Green), LFM (Yellow), HiPro (Red), and LoPro (blue). Error bars show average least significant difference (0.5 LSD above and below each point). LSD bars that are not overlapping can be considered as depicting significantly different averages (P <0.001). Temperatures are daily 5 metre readings. Long term average (LTA) temperatures for Meads Creek are shown for comparison.

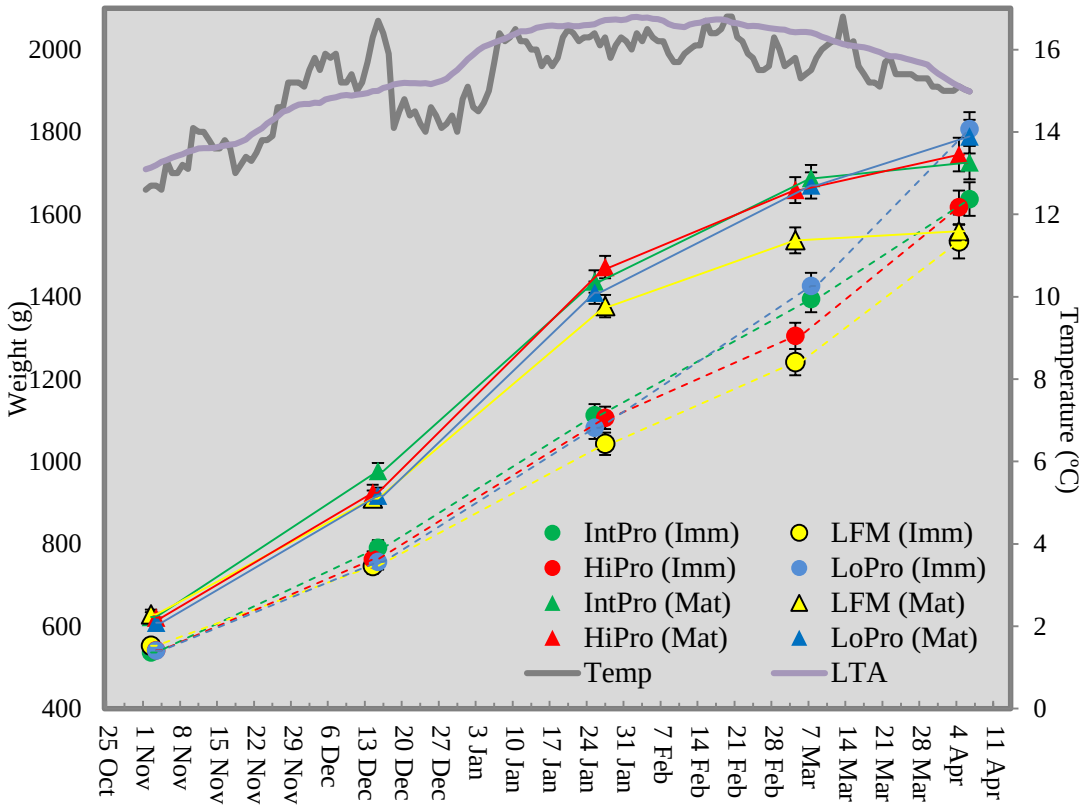


Figure S2: Faecal score progression during the feeding trial. Showing immature (circles) and maturing (triangles) sub-populations within each 'Cohort'; IntPro (Green), LFM (Yellow), HiPro (Red), and LoPro (blue). Error bars show average least significant difference (0.5 LSD above and below each point). LSD bars that are not overlapping can be considered as depicting significantly different averages (P <0.001).

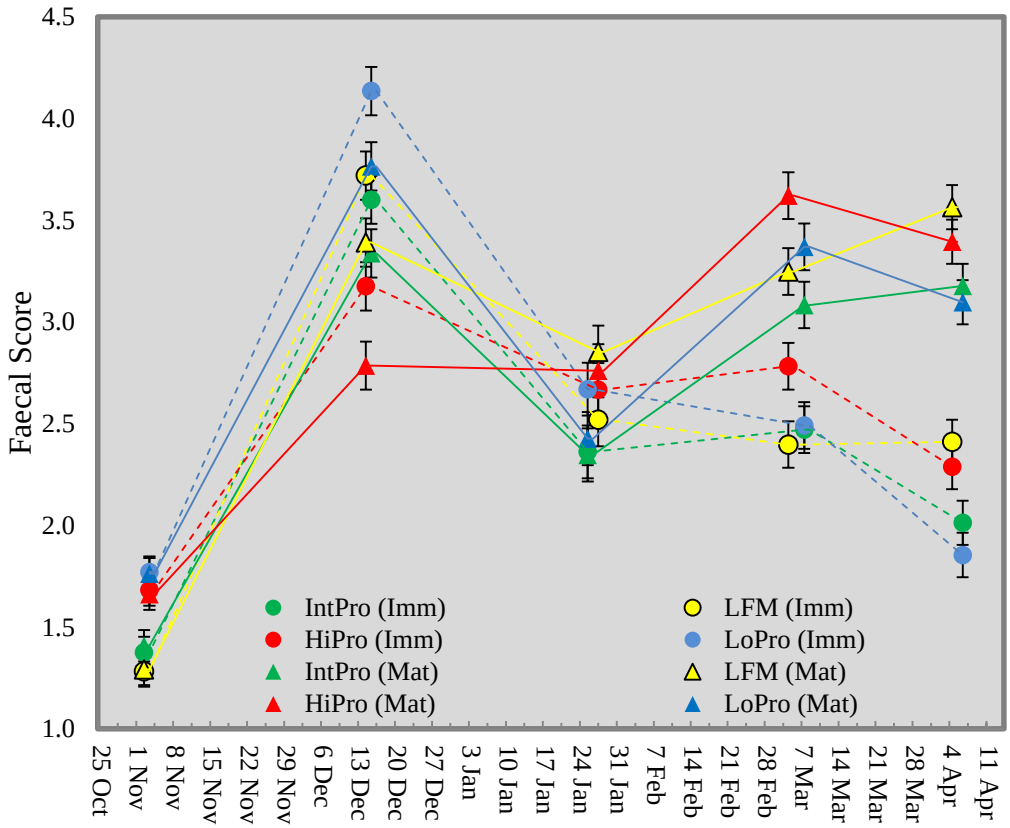
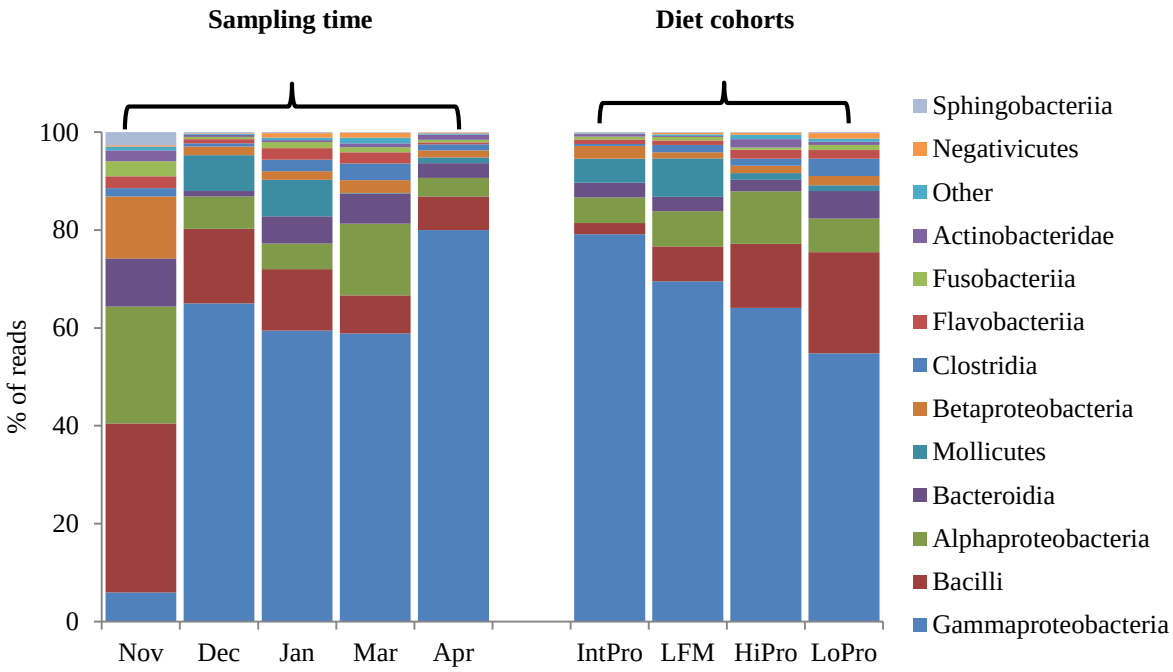


Figure S3: Bacterial diversity at the class level as defined by a) sampling time and diet cohort; and b) faecal score factors. Faecal scores that are low (1 to 3) and high (3.5 and 4) are shown for comparison since communities were statistically distinguishable.

a)



b)