SalmoSim: the development of a three-compartment in vitro simulator of the Atlantic Salmon GI tract and associated microbial communities

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Abstract

Background

Atlantic salmon are a fish species of major economic importance. Innovative strategies are being sought to improve salmon feeds and feed additives to enhance fish performance, welfare, and the environmental sustainability of the aquaculture industry. There is still a lack of knowledge surrounding the importance and functionality of the salmon gut microbiome in fish nutrition. *In vitro* gut model systems might prove a valuable tool to study the effect of feed, and additives, on the host's microbial communities. Several *in vitro* gut models targeted at monogastric vertebrates are now in operation. Here, we report the development of an Atlantic salmon gut model, SalmoSim, to simulate three gut compartments (stomach, pyloric caecum and midgut) and associated microbial communities.

Results

The gut model was established in a series of linked bioreactors seeded with biological material derived from farmed adult marine phase salmon. In biological triplicate, the response of the *in vitro* system to two distinct dietary formulations (fish meal and fish meal free) was compared to a parallel *in vivo* trial over forty days. Metabarcoding based 16S rDNA sequencing, qPCR, ammoniacal nitrogen and volatile fatty acid measurements were undertaken to survey the microbial community dynamics and function. SalmoSim microbiomes were indistinguishable (p=0.230) from their founding inocula at 20 days and the most abundant genera (e.g. *Psycrobacter, Staphylococcus, Pseudomonas*) proliferated within SalmoSim. Real salmon and SalmoSim responded similarly to the introduction of novel feed, with majority of the taxa (96% Salmon, 97% SalmoSim) unaffected, while a subset of taxa (e.g. a small fraction of *Psychrobacter*) were differentially affected across both systems. Consistent with a low impact of the novel feed on microbial fermentative activity, volatile fatty acids profiles were not significantly different in SalmoSim pre- and post-feed switch.

Conclusion

This study represents an important step in the development of an *in vitro* gut system as a tool for the improvement of salmon nutrition and welfare. This system aims to be utilised as a pre-screening tool for new feed ingredients and additives, as well as being used to study antimicrobial resistance and transfer, and fundamental ecological processes that underpin microbiome dynamics and assembly.

Background

In the last 50 years, per capita fish consumption has almost doubled from 10 kilograms in the 1960s to over 19 kilograms in 2012 [1]. This increase in the demand for fish protein has put wild fish stocks under pressure. The aquaculture sector now accounts for almost 50% of all fish for human consumption and is anticipated to provide 62% by 2030 [2]. The Atlantic salmon (*Salmo salar*) is the leading farmed marine finfish and, in economic terms, the ninth most important aquaculture fish species farmed globally [1].

Atlantic salmon are carnivores and wild pelagic fish stocks from reduction fisheries are an important protein source (fish meal (FM)), as well as the principal lipid source (fish oil FO), exploited to feed farmed salmon. Reduction fisheries negatively impact the marine ecosystem, and feeding farmed salmon on FM/FO ingredients is unsustainable as well as expensive [3,4]. To address these issues, farmed salmon feed composition has changed considerably during the relatively short history of intensive salmon farming, for example in Norway, reducing the ratio of the marine origin components within salmon feed from around 90% in 1990 to 30% in 2013 [5]. However, there is evidence that non-marine dietary ingredients can result poor fish growth, altered gut health alongside a modified fish gut microbial community composition and activity [6–8]. For instance, Atlantic salmon feed supplementation with dietary soybean protein concentrate can induce intestinal disorder [9]. Concomitant alterations in gut microbiota can result in the undesirable fermentation of various feed components [9,10]. In view of all this, considerable interest lies around the development of novel ingredients that have comparable performance to marine ingredient-based feeds in terms of their impact of the host and its associated microbes.

To study the impact of novel feed ingredients on gut microbial communities (e.g. Gajardo et al., 2017), as well as the supplements (e.g. pre-biotics, pro-biotics) tailored to modify microbial community diversity and function (e.g. Gupta et al., 2019), *in vivo* trials are widely deployed in salmonid aquaculture. Although physiologically relevant, *in vivo* trials have several scientific, ethical and practical disadvantages. In salmonids, for example, gut sampling is terminal, preventing the generation of time series data from individual animals/microbial communities. Furthermore, microbial impacts on feed ingredients cannot be subtractively isolated from host enzymatic/cellular activity. From an ethical perspective, *in vitro* models offer the opportunity to reduce harm via replacement of *in vivo* models [12]. Practically, *in vivo,* testing of novel feed ingredients is both time consuming and costly. A reliable *in vitro* model could offer advantages in this respect. To the best of our knowledge, there is only one other gut system in place simulating a generalised teleost gut, (‘fish-gut-on-chip’ [13]) The ‘fish-gut-on-chip’ system exploits microfluidic technology and is based on the reconstruction of the rainbow trout's intestinal barrier by culturing only intestinal cell lines in an artificial microenvironment and currently does not involve microbial communities isolated from the fish's gut.

Prior to deploying an *in vitro* gut microbiome simulator to perform biological experiments, several criteria must be met. Firstly, steady-state microbial communities need to be established prior to the experimental procedure to ensure that results due to experimental treatments are not confounded with bacterial adaptation to the *in vitro* environment [14]. Secondly, physicochemical conditions within the artificial gut simulator and the gut of the target species should be similar. Thirdly, the bacterial communities need to be gut compartment-specific and representative of (if not identical to) the *in vivo* situation [15]. Finally, the *in vitro* gut simulator should be validated against a parallel *in vivo* experiment, to establish the degree to which the results from the experimental protocol within the artificial gut are generalisable to the *in vivo* situation [16]. Towards this end, several molecular techniques can then be deployed to analyse microbial populations within the gut. Multiplex quantitative PCR (qPCR) coupled with taxon-specific primers can rapidly detect and quantify the bacterial consortia within a large population [17]. Whilst shotgun

metagenomics and amplicon sequencing approaches can provide a detailed taxonomic assessment of the microbial composition of the gut, they may be less useful for day-to-day monitoring of specific taxa [18].

In view of the above criteria, the aim of the current study is to develop a synthetic, continuous salmon gut microbial fermentation simulator, representative of generalised marine lifecycle stages of the Atlantic salmon. Salmonids are gastric fishes [19], with their guts characterised by a clearly defined stomach followed by a pylorus with attached blind vesicles called pyloric caeca, as well as a relatively short and non-convoluted posterior (mid and distal) intestine leading to the anus [20]. Our experimental gut system simulates the stomach, the pyloric caeca, and the midgut regions of the gastrointestinal tract of farmed Atlantic salmon. To validate the system, we compare the performance of the simulator with *in vivo* modulation of the gut microbial community during a feed trial that ran in parallel. We aimed to first assess microbial community dynamics within SalmoSim over time; then compare the SalmoSim microbiome to that found in real salmon; and finally, to evaluate the response to the two systems (SalmoSim and real salmon) to a change in diet.

Methods

Experimental set-up and sample collection in an aquaculture setting

The Atlantic salmon (*Salmo salar*) *In vivo* feed trial was performed by MOWI ASA at their research site in Averøy, Norway. Prior to commencement of the feed trial, salmon were fed on a fish meal diet (FMD) until they reached *circa* 750 grams in mass. Fish were separated into 5x5 meter marine pens (150 randomly distributed fish per pen) in 4x4 modular design. Four pens were randomly assigned to each of the trial diets. This study focused on eight pens housing fish fed on FMD and fish meal free diet (FMF) (Supplementary Table 1, Figure 1D). The feed trial was conducted over five months (November 2017 - March 2018). For *in vivo* samples recovered from actual salmon, three randomly selected fish were collected at the end of the feed trial for two different feeds (N=3 fish/feed) and sacrificed by MOWI employees. After, samples from three salmon gut compartments were collected (stomach (N=3/feed), pyloric caeca (N=3/feed) and mid gut (N=3/feed) (approximately 20 cm from the vent)), placed into 1.5 ml cryovials and kept on ice before long term storage in -80^{o}C conditions. For *in vitro* initial inoculum samples (the founding community for SalmoSim runs), a further three fish fed on FMD were sacrificed and samples from three distinct gut compartments were collected (Stomach (N=3), pyloric caecum (N=3) and midgut (N=3)), transferred to 15 ml Falcon tubes containing 30% glycerol and kept on ice before long term storage in -80^{o}C conditions. Details of samples collected from farmed Atlantic salmon have been described previously [21].

In vitro feed trial within SalmoSim system

Physicochemical conditions within Atlantic salmon gastrointestinal tract and microbiome sampling

Physicochemical conditions (temperature, pH, dissolved oxygen) were directly measured in adult Atlantic salmon from Mowi salmon farm in Loch Linnhe, Scotland (Supplementary Figure 1A-C). Bacterial inoculums were prepared for the *in vitro* trial from the different gut compartments sampled from individual fish (three biological replicates, three gut compartments per fish – stomach, pyloric caecum and midgut) collected at the start of the *in vivo* feed trial in Averøy, Norway. Prior to SalmoSim inoculation, inoculums that were stored in 15 ml falcon tubes containing 30% glycerol solution in -80^{o}C freezer were dissolved in 1 ml of autoclaved 35 g/L Instant Ocean® Sea Salt solution. Distinct individual fish collected in Averøy formed the founder community for each distinct replicate of the *in vitro* trial (i.e., a true biological replicate (Figure 1)).

In vitro system 'feed' preparation

In vitro system feed media was prepared by combining the following for a total of 2 litres: 35 g/L of Instant Ocean® Sea Salt, 10 g/L of the FMD or FMF used in the MOWI feed trial (Supplementary Table 1), 1 g/L freeze-dried mucous collected from the pyloric caecum and 2 litres of deionised water. This feed was then autoclave-sterilised, followed by sieving of the bulky flocculate, and finally subjected to the second round of autoclaving.

In vitro system preparation

Three 500 ml Applikon Mini Bioreactors were filled with four 1cm^3 cubes made from sterile aquarium sponge filters used as a surface for biofilm formation, assembled by attaching appropriate tubing and probes (redox, temperature, and dissolved oxygen, Figure 1A), and autoclaved. Bioreactor preparation was followed by attachment of reactor vessels to the Applikon electronic control module, connection of feed and acid and base bottles (0.01M hydrochloric acid and 0.01M sodium hydroxide solutions filtered through 0.22µm polyethersulfone membrane filter unit (Millipore, USA)). Nitrogen gas was periodically bubbled through each vessel to maintain anaerobic conditions and dissolved oxygen continually monitored. The bioreactors were then allowed to fill with 400 ml of feed media. Once the system was set up, media transfer, gas flow and acid/base addition occurred for 24 hours in sterile conditions (without microorganisms present in the system) in order to stabilise the temperature, pH, and oxygen concentration with respect to levels measured from adult salmon.

Initial pre-growth period during *in vitro* trial

In order to allow bacterial communities to proliferate in the *in vitro* environment without washing through the system, the microbial populations within the inoculum from real salmon were pre-grown inside the SalmoSim system for four days. During this phase, the system was filled with FMD media preparation and inoculum, and no media transfer occurred.

Performing feed trial within SalmoSim system

After the initial pre-growth period, each validation experiment was run for 20 days while supplying SalmoSim system with FMD. After 20 days, SalmoSim was run for 20 additional days while supplying FMF food. During the full 44-day experiment (4-day pre-growth period, 20-day system fed on FMD, and 20-day system fed on FMF) physiochemical conditions within three SalmoSim gut compartments were kept similar to the values measured in real salmon: temperature inside the reactor vessels were maintained at 12^{o}C, dissolved oxygen contents were kept at 0% by daily flushing with N_2 gas for 20 minutes, and pH was kept stable in each bioreactor by the addition of 0.01M NaOH and 0.01M HCl (stomach pH 4.0, pyloric caecum pH 7.0, and mid intestine pH 7.6). During this experiment (apart from the pre-growth period) the transfer rate of slurry between reactor vessels was 238 ml per day (5% of the total bioreactor volume - the maximum volume of sampling without disturbing microbial community structure). Finally, 1 ml of filtered salmon bile and 0.5 ml of sterile 5% mucous solution were added daily to the reactor simulating the pyloric caecum compartment. The schematic representation of SalmoSim system is visualised in Figure 1A and full feed trial within SalmoSim is visually summarised in Figure 1B and C.

Sampling was performed in several steps. First, samples from initial inoculums were collected for each gut compartment. Once SalmoSim main experiment started, the sampling from each bioreactor vessel was performed every second day throughout the 40-day run period (20 samplings in total). The SalmoSim samplings entailed collecting 30 ml of the bioreactor contents into 50 ml falcon tubes, centrifuging them for 10 minutes at 5000 rpm speed, and freezing the pellets at -20^{o}C for storage.

Measuring nitrogen metabolism within the SalmoSim system

At each sampling point, the protein concentration in each chamber of the system was measured using Thermo Scientific™ Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, USA) and the ammonia concentration using Sigma-Aldrich® Ammonia Assay Kit (Sigma-Aldrich, USA) to assay the bacterial community activity. Both methods were performed according to manufacturer protocol by using The Jenway 6305 UV/Visible Spectrophotometer (Jenway, USA).

Measuring Volatile Fatty Acid (VFA) production in SalmoSim

The last two time points for each diet were selected from the SalmoSim system (for all three gut compartments) for VFA analysis: 18 and 20 for FMD; and time points 38 and 40 for FMF, respectively, to ensure that the bacterial communities had enough time to adapt to SalmoSim system (for FMD time points) and the feed change (for FMF time points). During runs, 1ml of supernatant from SalmoSim bioreactors was frozen in -80°C which, was then used for VFA extraction. The protocol involved combining 1ml of supernatant with 400µl of sterile Phosphate-buffered saline (PBS) solution (Sigma Aldrich, USA) and vortexing the mixture for 1 minute. The sample was then centrifuged at 16,000 g for 30 minutes, followed by two rounds of supernatant removal, before centrifuging at 16,000 g for 30 minutes. Finally, the supernatant was then filtered through 0.2µm Costar SpinX centrifuge tube filters (Corning, USA) at 15,000 g for 2 minutes until clear. The extracted VFAs were sent for gas chromatographic analysis at MS-Omics (Denmark). In order to determine if the VFA concentrations were statistically

different between SalmoSim fed on FMD and FMF diets, measured VFA values dataset were subjected to statistical analysis using linear mixed effect models (See Supplementary methods 2). Results are shown in supplementary Figure 6.

In vivo phenotypic fish performance fed on two different feeds

Phenotypic performance data (fish length, weight, gutted weight, carcass yield, gonad, and liver weights) was collected and provided at the end of the *in vivo* feed experiment by MOWI. The differences between each feed group (n=32 fish per feed) for each phenotype were visualised and statistical analysis was applied (independent two-sample t-test) to identify statistically significant differences between the two feed groups.

Measuring bacterial population dynamics in SalmoSim

Genomic DNA extraction

The DNA extraction protocol followed was previously described by [21]. In short, samples were subjected to a bead-beating step for 60 seconds by combining samples with MP Biomedicals™ 1/4" CERAMIC SPHERE (Thermo Fisher Scientific, USA) and Lysing Matrix A Bulk (MP Biomedicals, USA). Later, DNA was extracted by using the QIAamp® DNA Stool kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol [22].

NGS library preparation and sequencing

In the first instance, microbial population dynamics in SalmoSim were measured in near real-time using a set of qPCR primers including published and custom sequences to enable the stability of the system to be monitored (See supplementary Methods 1 and data Supplementary Figure 3). Subsequently 16S rRNA sequencing was deployed to provide a fuller picture community dynamics. The commonly used 16S ribosomal hypervariable region 4 primers were shown to cross-amplify *Salmo salar* 12S ribosomal gene sequences [21,23] and hence were not used in this study. Rather, amplification of the 16s V1 hypervariable region was adopted as an alternate approach [24]. Amplification of 16S V1 hypervariable region from diluted DNA samples was achieved using redundant tagged barcode 27F and 338R at final concentration of 1 pM of each primer. Primer sequences are summarised in Supplementary Table 3. First-round PCR was performed in triplicate on each sample and reaction conditions were 95°C for ten minutes, followed by 25 cycles at 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds, followed by a final elongation step of 72°C for 10 minutes. After the triplicate reactions were pulled together into one, their concentration was measured using a Qubit® fluorometer (Thermo Fisher Scientific, USA), and all of them were diluted to 5ng/µl using Microbial DNA-Free Water (Qiagen, Valencia, CA, USA). The second-round PCR, which enabled the addition of the external multiplex identifiers (barcodes), involved only six cycles with otherwise identical reaction conditions to the first. The detailed composition of second-round PCR primers is summarised in Supplementary Table 4. This was followed by the DNA clean-up using Agencourt AMPure XP beads (Beckman Coulter, USA) according to the manufacturers' protocol. The

cleaned DNA was then gel-purified by using the QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA) and then quantified by using Qubit® (Thermo Fisher Scientific, USA). All the PCR products were pulled together at 10nM concentration and sent for sequencing using HiSeq 2500.

Bioinformatic analysis of 16S rRNA gene amplicon sequencing data

Sequence analysis was performed with our bioinformatic pipeline as described previously with slight modifications [21]. First, quality filtering and trimming (>Q30 quality score) was performed on all the reads of the 16s rRNA V1 hypervariable region using Sickle version V1.2 software [25]. Second, read error correction was performed using the BayesHammer module within SPAdes V2.5.0 software to obtain high-quality assemblies [26]. Third, paired-end reads were merged (overlap length 50bp) using PANDAseq v2.11 software with simple_bayesian read merging algorithm [27,28]. After overlapping, paired end reads merged reads were dereplicated, sorted, and filtred chimaeras using GOLD SILVA reference dataset [29] and singletons were removed by using VSEARCH version 2.3.4 tool [30]. Merged pair-end filtered reads were clustered in operational taxonomic units (OTUs) using VSEARCH software at 97% identity followed by a decontamination step by mapping OTUs against the host (*Salmo salar*) reference genome (available on NCBI) DNA using bwa aligner implemented in DeconSeq v0.4.3 tool [31]. Taxonomic assignment of OTUs was achieved using the Naïve Bayesian Classifier [32] implemented in the QIIME 2 platform using SILVA 132 database [33,34]. Phylogenetic trees of OTUs were generated using FastTree software after using MAFFT for multiple sequence alignment [35]. The resultant OTU table was converted to a biological observation matrix (BIOM) format for the post-OTUs statistical analysis [36].

Post-OTUs statistical analysis: diversity metrics and community structure and composition analysis

All statistical analysis of the OTU table was performed by using R v 4.0.1 and RStudio v 1.3.959 [37]. Alpha diversity analysis was performed using Rhea pipeline [38], supplemented by microbiomeSeq [39], and PhyloSeq [40] for ANOVA and visualisation steps. Two alpha diversity metrics were calculated: microbial richness (estimated number of observed OTUs) and Shannon diversity (an estimate of balance of the community using effective Shannon index [41,42]. Before calculating effective microbial richness, proportional filtering was performed at a relative abundance of 0.25% in each community to minimise the inflation in microbial richness caused by the very low abundant OTUs. Afterwards, a one-way ANOVA of diversity between groups was calculated with the p-value threshold for significance (p-value <0.05) represented using boxplots.

To investigate the effect of time on the bacterial community stability, beta diversity analysis was performed using different phylogenetical distances metrics to assay phylogenetic similarities between samples (weighted, generalised, and unweighted UniFrac). To compare communities isolated from various sources (SalmoSim, inoculum and real salmon), only samples fed on FMD were included as initial inoculum were collected from fish fed on FMD alone. Furthermore, only SalmoSim samples from the last 3 time points fed on FDM were selected as they are considered stable time points (once bacterial communities had over two weeks to adapt and grow within SalmoSim system). In short, the resulting dataset contained: real salmon samples fed on FMD, all inoculum samples and stable SalmoSim time

points fed on FMD (days 16, 18, and 20). This dataset was then subdivided into several smaller datasets that included OTUs, shared by various percentage of samples (60%, 50%, 40% and 30% of samples), with the aim of minimising the impact of rare OTUs (low prevalence) on comparisons and only focusing on prevalent OTUs between samples (see details in Supplementary Table 4).

To analyse the response of microbes to the diet change (see supplementary Table 1 for feed formulation) in real salmon and SalmoSim, in addition to the full dataset (*in vivo* and *in vitro* samples); three different full dataset subsets were used to perform beta diversity analysis: samples from *in vivo* study, all samples from SalmoSim (all data points), and samples only from SalmoSim once it had achieved stability (the last 3 time points fed on FMD (days: 16, 18, and 20) and FMFD (days 36, 38, and 40). These datasets were used to compute ecological (Bray-Curtis and Jaccard) and phylogenetic (unweighted, weighted, and generalised UniFrac) distances with vegdist() function from the vegan v2.4-2 package and GUniFrac() function (generalised UniFrac) from the Rhea package [38,43] Both ecological and phylogenetical distances were then visualised in two dimensions by Multi-Dimensional Scaling (MDS) and non-metric MDS (NMDS) [44]. Finally, a permutational multivariate analysis of variance (PERMANOVA) by using calculated both distances was performed to determine if the separation of selected groups is significant as a whole and in pairs [44].

To provide an overall visualisation of microbial composition across all samples, a principal Coordinates Analysis (PCoA) was performed using the microbiomeSeq [39] package based on phyloseq package [45] with Bray-Curtis dissimilarity measures calculated and visualised for four different subset-datasets: the full dataset (real salmon, inoculum and all SalmoSim samples), and, three different subsets each containing only one of the free biological replicate samples from SalmoSim (Fish 1, 2, or 3), along with all real salmon and inoculum samples.

Differential abundance was calculated by using microbiomeSeq based on DESeq2 package [39,45]. BIOM generated OTU table was used as an input to calculate differentially abundant OTUs between selected groups based on the Negative Binomial (Gamma-Poisson) distribution.

Results

Stabilisation of microbial communities within the SalmoSim system

Effective richness (Figure 2 A) indicates that within the stomach and midgut compartments the initial inoculum contained the highest number of OTUs compared to later sampling time points from SalmoSim system: in the stomach compartment, effective richness was statistically different between time point 0 (initial inoculum) and time points 16, 30, 36 and 38, and within midgut compartment number of OTUs within initial inoculum (time point 0) was statistically different from time points 2, 4, 6, 16, 34, 36, 38, and 40. However, within the pyloric caeca compartment, only one-time point (time point 34) had a significantly different number of OTUs from initial inoculum (time point 0).

Our results reveal that within the stomach compartment over time (including initial inoculum), the effective Shannon diversity was stable with a downwards but non-significant trend over the course of the experiment (Figure 2 B). A similar downwards trend was observed in the pyloric caecum, with significant differences between later time points, but no significant differences were noticed between the inoculum and SalmoSim. Within the midgut compartment Shannon diversity was statistically lower than the inoculum (time point 0) over most time points (sampling days 2-40).

Taken together, diversity and richness metrics suggest some loss of microbial taxa as a result of transfer into SalmoSim in the pyloric caecum and midgut, but not in the stomach. Subsequently, richness and evenness were then stable over the time course of the experiment in stomach and mid gut compartments (some instabilities seen only between initial inoculum and later time points), whilst much more instability within alpha diversity metrics were detected in the pyloric caecum compartment.

To assess the compositional stability of the system, comparisons over time were undertaken with reference to pairwise beta-diversity metrics. Significant differences in composition between time points represent instability in the system. Figure 3 visually summarises between time point comparison of beta-diversity metrics within the SalmoSim system: unweighted UniFrac (Figure 3 A), generalised UniFrac (Figure 3 B), and weighted UniFrac (Figure 3 C). Irrespective of the metric used, microbial community composition appeared to stabilise in all gut compartments over the course of the 40-day experiment, with little-observed impact of introducing the different feed at day 20. This trend was supported by our qPCR data, suggesting increasing stability over the course of the 40-day experiment (Supplementary Figure 3).

Microbial identity and diversity compared between SalmoSim, founding inocula and real salmon.

To compare different samples types (inoculum, real salmon, SalmoSim) sample sizes were first balanced by examining a reduced dataset that contained: real salmon samples, all inoculum samples and stable SalmoSim time points fed on FMF (days 36, 38, and 40). Alpha diversity comparisons between inoculum, real salmon and SalmoSim are shown in Figure 2.

Compositional comparisons between different samples types (inoculum, real salmon and SalmoSim) were made using several pairwise beta-diversity metrics (phylogenetic (unweighted, generalised, and weighted UniFrac and ecological distances (Bray Curtis and Jaccard)) (Table 1). Ecological metrics could not distinguish between SalmoSim and inoculum. In contrast, metrics that incorporate phylogenetic differences between taxa (i.e., Unifrac) did identify significant differences, indicating that there is variability between the inoculum and SalmoSim, but the taxa involved are closely related. To explore the impact of rare OTUs when accounting for observed differences between sample types, the dataset was partitioned and analysed. Partitioned datasets indicated that progressive removal of rare OTUs increased the compositional similarity of the inoculum and SalmoSim via all metrics (Table 1). Between real salmon (*in vivo*) and SalmoSim, and between real salmon and inoculum, however, statistically significant differences were found in the majority of cases using all metrics regardless of inclusion of rare OTUs. These observations are consistent with the fact that SalmoSim and inoculum samples originated from

the same individuals, while real salmon samples were, by necessity, collected from different individuals during the *in vivo* trial.

Effect of changing diet on the microbiome of real salmon (*in vivo*) and SalmoSim (*in vitro*).

The impact of diet on the abundance of individual taxa: In response to the change of diet, the relative abundances of individual taxa in salmon vs SalmoSim also revealed some differences, as well as multiple similarities in response of the two systems (Figure 4). In this respect, the abundance of the vast majority of OTUs (SalmoSim – 97%; Salmon – 95%; Figure 4 C) were unaffected by the change in feed; these included 161 OTUs shared by SalmoSim and the real salmon assayed. For OTUs whose individual abundance was impacted by feed across the two systems, only a single common OTU changed in the same way in both Salmon and SalmoSim (Figure 4 A). qPCR-based estimates of taxon abundance variation in response to diet (Supplementary Table 6), and corresponding data for the same taxa from 16S OTU profiles (Figure 4D) show several similarities and differences between SalmoSim and real salmon. Again, however, the overall pattern is that of limited change in both *in vivo* and *in vitro* systems in response to the change in diet. Invariance observed in the microbiome in response to feed were reflected in estimates of physical attributes of fish in response the change in feed formulation. As such, no statistically significant differences in various phenotypic measurements (fish length, weight, gutted weight, carcass yield, gonad, and liver weights) were noted in salmon fed on the two different diets used in the experiment (see Supplementary Figure 5). Invariance was also observed in VFA production data, in which no significant differences were observed in SalmoSim between the fish meal and fish meal free diets (See Supplementary Figure 6).

Microbial composition in SalmoSim and real salmon fed different feeds: Most gut compartments for real salmon, SalmoSim, and the salmon used to inoculate SalmoSim were dominated by *Pseudomonas*, *Psychrobacter* and *Staphylococcus* genera, suggesting that genera present in the marine phase salmon are generally maintained in SalmoSim (Figure 5). In terms of change in alpha diversity, the only statistically significant difference in response to the switch in feed was observed in the pyloric caeca compartment of the SalmoSim compartment based on the Shannon diversity metric (Supplementary Figure 4), where a slight decrease alongside the FMF occurred. Otherwise, the change in feed formulation did not impact alpha diversity in any gut compartment, either in real salmon, or in SalmoSim.

To provide an overview of microbial composition and variation in the experiment, a PCoA (Principal coordinates Analysis) based on Bray-Curtis distance was performed and plotted (Figure 6 A-D). Biological replicate (the fish providing the founding inoculum of each SalmoSim run) appears to be a major driver of community composition in the experiment (Figure 6 A). Taxonomic composition represented in stacked bar plots in Figure 5 also supports this observation. Once individual SalmoSim runs (biological replicates) are visualised separately changes to microbial communities in response to the feed become apparent (Figures 6 B-D). Statistical comparisons based PERMANOVA show there is a clear effect of feed on microbial composition in both salmon and SalmoSim (Table 2), however, based of OTU differential abundance data (above) the effect seems to be small. Samples from real salmon fed on the different

diets also diverge from one and other (supported by Table 2, Figure 5), however, not necessarily along the same axes as each SalmoSim replicate. This divergence is potentially indicative of an effect of the biological replicate (i.e., inter-individual variation). Consistent with Figure 5, inoculum for the respective SalmoSim replicates cluster among SalmoSim samples for the fish meal diet in each case.

Discussion

Our findings suggest a loss of microbial taxa diversity and richness as a result of transferring initial inoculums from real salmon into the SalmoSim system in the pyloric caeca and mid gut compartments. Several lines of evidence from our core OTU analysis suggest that low prevalence (rare) OTUs make up most of the taxa lost, and progressive removal of rare OTUs increased the compositional similarity between inoculum and SalmoSim samples using both phylogenetic and ecological distances. A general trend was observed, in which all gut compartments became increasingly stable throughout the 40-day of experiment, with little-observed impact of introducing the different feed at day 20. Comparison of real salmon and SalmoSim samples at the microbial level showed significant differences using both ecological and phylogenetic metrics. These differences may be explained by the fact that samples used for real salmon and SalmoSim originated from different individuals, whereas initial inoculum and SalmoSim samples for a given run originated from the same fish. Correspondingly, we observed that the biological replicate (the founding inoculum of each SalmoSim run) was the major driver of community composition in the experiment. This could be explained by the fact that feed used in the *in vitro* study was sterile, thus the only bacterial communities within the SalmoSim system originated only from real salmon inoculums. Once the individual runs were separated, phylogenetic and ecological distances suggested that changing feed was the driver of community composition in both real salmon and SalmoSim. However, the vast majority of OTUs remained unchanged by the switch in feed in both systems and no changes were noticeable in the bacterial activity (VFA production) within the system after the introduction of plant-based feed, nor in phenotypic performance of Atlantic salmon fed on two different feeds (fish length, weight, gutted weight, carcass yield, gonad, and liver weights) in *in vivo* trial.

Many of the microbes we detected, and cultured, from the salmon gut microbiome have been reported previously in this species. For example, gram-negative *Pseudomonas* and *Psychrobacter*, the most abundant genera we observed, are among the core bacterial taxa known to reside within the real salmon gut [24,47,48]. *Staphylococcus* genera have also been reported widely in fresh-water and marine farmed salmon [49]. SalmoSim was able to maintain these species in culture throughout the experimental run, and although some diversity was lost, no statistical differences could be detected between the composition of SalmoSim and that of the fish gut communities used to found the different biological replicates via ecological metrics. Notable by their scarcity were mycoplasma OTUs, which occurred at relatively low abundance in both the *in vivo* and *in vitro* systems in this study. *Mycoplasma* OTUs were recovered from most SalmoSim gut compartments at low abundances (see supplementary Table 7), suggesting that these fastidious microbes can survive in the bioreactors. Our group and several others have widely reported *Mycoplasma* species from marine and freshwater stage of wild and farmed Atlantic salmon (higher abundance in farmed salmon), where many proliferate intracellularly in the gut epithelial

lining [21,50–52]. Establishing whether mycoplasma can actively proliferate in SalmoSim would require the use of founding communities rich in these organisms in a future experiment, and we found, as has been reported in a number of other microbiome studies (e.g [53], that inter-individual variability (in our case affecting the initial inoculum) was a main driver for gut microbial composition divergence.

We identified that a change in feed resulted in an overall shift in microbial community structure in both real salmon and SalmoSim system, as has been found to be the case in many previous studies [7,54,55]. The direction of this shift, and the microbial taxa involved, were not equivalent in SalmoSim and real salmon, although no overall trend was observed at higher taxonomic levels in either system. Importantly, it is also the case that the vast majority of OTUs within both real salmon and SalmoSim were not affected by the switch in feed. Furthermore, it was found that change in feed did not affect VFA production in the SalmoSim system. As such, it is not clear whether any relevant functional shifts occurred in the microbiome of SalmoSim or real salmon as a result of the treatment. Furthermore, we did not identify any phenotypic changes (fish length, weight, gutted weight, carcass yield, gonad, and liver weights) within *in vivo* trial of Atlantic salmon fed on two different feeds. This lack of change is not unexpected, considering the plant-based feed was developed to have similar macronutrient composition to a Fish meal-based feed. One difference is a slightly higher crude fibre (fermentable substrate) proportion in Fish meal free diet, which could explain higher microbial diversity in *in vivo* samples fed on plant-based feed [56].

The use of *in vitro* systems to study and model the microbial communities of monogastric vertebrates is becoming increasingly widespread, with systems simulating: *Sus scrofa* (pig) [57], *Gallus gallus* (chicken) [58], *Canis lupus* (dog) [59] and other vertebrate guts. Using *in vitro* gut simulators is also a widely accepted approach to study the human gut microbiome [15,60,61]. One of the most established systems is the Simulator of the Human Intestinal Microbial Ecosystem (SHIIME) that mimics the entire gastrointestinal tract incorporating the stomach, small intestine and different colon regions [16]. This system was used to study the effects of many different dietary additives on human microbiome [62,63]. The value of *in vitro* simulators in providing genuine insights is limited only by the research question and the corresponding level of sophistication required. The host component of the system, for example, is often poorly modelled, although cell lines, artificial mucosae and digestion / absorbance systems can be included, which can provide specific physiological and metabolic insights [60,64]. As we found, inter-individual variability may be an important consideration, and adequate biological replication is necessary to enable reliable interpretation of results, a consideration that can be overlooked by even the most sophisticated systems. Prior to the current study, only one other attempt was made to study the effect of diet on Atlantic salmon gut microbial composition *in vitro* [65]. In this preliminary study a simple *in vitro* system was used to assess the impact of different feed formulations on the microbial communities of faecal slurries prepared from live salmon. However, no direct comparison was made with a true *in vivo* trial; nor were the different gut compartments present in salmon modelled in any detail and the predictive value for such simple *in vitro* systems in not immediately clear. Nonetheless, the work provided an important catalyst for the development of more sophisticated systems.

Conclusions

Our results indicate that SalmoSim can not only maintain stable microbial communities from real salmon, but also demonstrates similar responses in experimental treatments as those seen in real salmon. These results are encouraging, however, the nature of the treatment applied in this study: a switch between two similar feeds that had little effect on the gut microbiota *in vivo*, suggests that further experimentation with SalmoSim would be beneficial. For example, the survival and influence of probiotics within the system or the influence of known prebiotics could also be assessed, as has been previously studied in other *in vitro* gut systems [59]. Gut models such as SalmoSim could provide a powerful role in aquaculture, where there is considerable interest associated with the development of feed and feed additives [66–68], but where the capacity for *in vivo* trials is limited. The aim of such systems could be to provide pre-screening tool for new feed ingredients and additives with the aim of reducing the cost and scale of *in vivo* testing. In parallel, an *in vitro* gut model for salmon could also be exploited to understand questions of public health importance (e.g. antimicrobial resistance and transfer [58]), as well as the fundamental ecological processes that underpin microbiome dynamics and assembly.

Declarations

Ethics approval and consent to participate

Animals sampled in the study were euthanised by authorised MOWI employees under Home Officer Schedule 1 of the Animals (Scientific Procedures) Act 1986.

Consent for publication

Not applicable

Availability of data and material

Sequence data have been deposited alongside metadata to the NCBI Short Read Archive

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

RK and ML conceived the experiment, and RK, CH, JR and AK performed the *in vitro* experimental procedure and sampling. RK performed the DNA extraction and molecular biology experiments including libraries preparation, quantification and qPCR. RK prepared samples for VFA analysis and analysed the results. RK and BC produced and analysed the NGS results and performed functional diversity analysis. RK and ML wrote the manuscript. All authors reviewed, edited and approved the final draft of the manuscript.

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References

1. FAO. The State of Fisheries and Aquaculture in the world 2018. Fao. 2018.
2. Moffitt CM, Cajas-Cano L. Blue Growth: The 2014 FAO State of World Fisheries and Aquaculture. Fisheries. 2014;
3. Worm B, Barbier EB, Beaumont N, Duffy JE, Folke C, Halpern BS, et al. Impacts of biodiversity loss on ocean ecosystem services. Science (80-). 2006;
4. Cashion T, Tyedmers P, Parker RWR. Global reduction fisheries and their products in the context of sustainable limits. Fish Fish. 2017;
5. Ytrestøyl T, Aas TS, Åsgård T. Utilisation of feed resources in production of Atlantic salmon (Salmo salar) in Norway. Aquaculture. 2015;448:365–74.
6. Ingerslev HC, Strube ML, Jørgensen L von G, Dalsgaard I, Boye M, Madsen L. Diet type dictates the gut microbiota and the immune response against Yersinia ruckeri in rainbow trout (Oncorhynchus mykiss). Fish Shellfish Immunol. 2014;
7. Gajardo K, Jaramillo-Torres A, Kortner TM, Merrifield DL, Tinsley J, Bakke AM, et al. Alternative protein sources in the diet modulate microbiota and functionality in the distal intestine of Atlantic salmon (Salmo salar). Appl Environ Microbiol. 2017;
8. Beheshti Foroutani M, Parrish CC, Wells J, Taylor RG, Rise ML, Shahidi F. Minimizing marine ingredients in diets of farmed Atlantic salmon (Salmo salar): Effects on growth performance and muscle lipid and fatty acid composition. Soengas JL, editor. PLoS One [Internet]. 2018 [cited 2020 Feb 3];13:e0198538. Available from: http://dx.plos.org/10.1371/journal.pone.0198538
9. Green TJ, Smullen R, Barnes AC. Dietary soybean protein concentrate-induced intestinal disorder in marine farmed Atlantic salmon, Salmo salar is associated with alterations in gut microbiota. Vet Microbiol. Elsevier; 2013;166:286–92.

10. Michl SC, Beyer M, Ratten JM, Hasler M, LaRoche J, Schulz C. A diet-change modulates the previously established bacterial gut community in juvenile brown trout (Salmo trutta). Sci Rep. 2019;
11. Gupta S, Fečkaninová A, Lokesh J, Koščová J, Sørensen M, Fernandes J, et al. Lactobacillus dominate in the intestine of atlantic salmon fed dietary probiotics. Front Microbiol. 2019;
12. Payne AN, Zihler A, Chassard C, Lacroix C. Advances and perspectives in in vitro human gut fermentation modeling. Trends Biotechnol. 2012. p. 17–25.
13. Drieschner C, Könemann S, Renaud P, Schirmer K. Fish-gut-on-chip: Development of a microfluidic bioreactor to study the role of the fish intestine: In vitro. Lab Chip. 2019;
14. Possemiers S, Verthé K, Uyttendaele S, Verstraete W. PCR-DGGE-based quantification of stability of the microbial community in a simulator of the human intestinal microbial ecosystem. FEMS Microbiol Ecol. 2004;49:495–507.
15. Van Den Abbeele P, Grootaert C, Marzorati M, Possemiers S, Verstraete W, Gérard P, et al. Microbial community development in a dynamic gut model is reproducible, colon region specific, and selective for bacteroidetes and Clostridium cluster IX. Appl Environ Microbiol. 2010;76:5237–46.
16. Molly K, Woestyne M Vande, Smet I De, Verstraete W. Validation of the simulator of the human intestinal microbial ecosystem (SHIME) reactor using microorganism-associated activities. Microb Ecol Health Dis. 1994;
17. Postollec F, Falentin H, Pavan S, Combrisson J, Sohier D. Recent advances in quantitative PCR (qPCR) applications in food microbiology. Food Microbiol. 2011. p. 848–61.
18. Malla MA, Dubey A, Kumar A, Yadav S, Hashem A, Allah EFA. Exploring the human microbiome: The potential future role of next-generation sequencing in disease diagnosis and treatment. Front. Immunol. 2019.
19. Aas TS, Sixten HJ, Hillestad M, Sveier H, Ytrestøyl T, Hatlen B, et al. Measurement of gastrointestinal passage rate in Atlantic salmon (Salmo salar) fed dry or soaked feed. Aquac Reports. 2017;
20. Lkka G, Austb L, Falk K, Bjerkås I, Koppang EO. Intestinal morphology of the wild atlantic salmon (Salmo salar). J Morphol. 2013;
21. Heys C, Cheaib B, Busetti A, Kazlauskaite R, Maier L, Sloan WT, et al. Neutral processes dominate microbial community assembly in Atlantic salmon, Salmo salar Appl Environ Microbiol [Internet]. 2020;AEM.02283-19. Available from: http://aem.asm.org/content/early/2020/02/03/AEM.02283-19.abstract
22. Claassen S, du Toit E, Kaba M, Moodley C, Zar HJ, Nicol MP. A comparison of the efficiency of five different commercial DNA extraction kits for extraction of DNA from faecal samples. J Microbiol Methods. 2013;
23. Werner JJ, Koren O, Hugenholtz P, Desantis TZ, Walters WA, Caporaso JG, et al. Impact of training sets on classification of high-throughput bacterial 16s rRNA gene surveys. ISME J. 2012.
24. Gajardo K, Rodiles A, Kortner TM, Krogdahl Å, Bakke AM, Merrifield DL, et al. A high-resolution map of the gut microbiota in Atlantic salmon (Salmo salar): A basis for comparative gut microbial research.

Sci Rep [Internet]. Nature Publishing Group; 2016 [cited 2020 Aug 27];6:1–10. Available from: www.nature.com/scientificreports/

25. Joshi N, Fass J. sickle - A windowed adaptive trimming tool for FASTQ files using quality. (Version 133). 2011;
26. Nikolenko SI, Korobeynikov AI, Alekseyev MA. BayesHammer: Bayesian clustering for error correction in single-cell sequencing. BMC Genomics. 2013;
27. Masella AP, Bartram AK, Truszkowski JM, Brown DG, Neufeld JD. PANDAseq: Paired-end assembler for illumina sequences. BMC Bioinformatics. 2012;
28. Schirmer M, D'Amore R, Ijaz UZ, Hall N, Quince C. Illumina error profiles: Resolving fine-scale variation in metagenomic sequencing data. BMC Bioinformatics. 2016;
29. Mukherjee S, Stamatis D, Bertsch J, Ovchinnikova G, Katta HY, Mojica A, et al. Genomes OnLine database (GOLD) v.7: Updates and new features. Nucleic Acids Res. 2019;
30. Rognes T, Flouri T, Nichols B, Quince C, Mahé F. VSEARCH: A versatile open source tool for metagenomics. PeerJ. 2016;
31. Schmieder R, Edwards R. Fast identification and removal of sequence contamination from genomic and metagenomic datasets. PLoS One. 2011;
32. Pedregosa F, Varoquaux G, Gramfort A, Michel V, Thirion B, Grisel O, et al. Scikit-learn: Machine learning in Python. J Mach Learn Res. 2011;
33. Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, et al. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat. Biotechnol. 2019.
34. Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, et al. The SILVA ribosomal RNA gene database project: Improved data processing and web-based tools. Nucleic Acids Res. 2013;
35. Katoh K, Standley DM. MAFFT multiple sequence alignment software version 7: Improvements in performance and usability. Mol Biol Evol. 2013;
36. Douglas GM, Maffei VJ, Zaneveld J, Yurgel SN, Brown JR, Taylor CM, et al. PICRUSt2: An improved and extensible approach for metagenome inference. bioRxiv. 2019;
37. Rstudio Team. RStudio: Integrated development for R. RStudio, Inc., Boston MA. RStudio. 2019.
38. Lagkouvardos I, Fischer S, Kumar N, Clavel T. Rhea: a transparent and modular R pipeline for microbial profiling based on 16S rRNA gene amplicons. PeerJ. 2017;
39. Ssekagiri A, T. Sloan W, Zeeshan Ijaz U. microbiomeSeq: An R package for analysis of microbial communities in an environmental context. ISCB Africa ASBCB Conf. 2017;
40. McMurdie PJ, Holmes S. Phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. PLoS One. 2013;
41. Jost L. Entropy and diversity. Oikos. 2006.
42. Jost L. Partitioning diversity into independent alpha and beta components. Ecology. 2007;
43. Oksanen J, Blanchet FG, Kindt R, Legendre P, Minchin PR, O'Hara RB. Package vegan. R Packag ver. 2013;

44. Anderson MJ. A new method for non-parametric multivariate analysis of variance. Austral Ecol. 2001;
45. Love M, Anders S, Huber W. Analyzing RNA-seq data with DESeq2. Bioconductor. 2017;
46. Sprockett D, Fukami T, Relman DA. Role of priority effects in the early-life assembly of the gut microbiota. Nat. Rev. Gastroenterol. Hepatol. 2018.
47. Navarrete P, Espejo RT, Romero J. Molecular Analysis of Microbiota Along the Digestive Tract of Juvenile Atlantic Salmon (Salmo salar L.). Microb Ecol. Springer-Verlag; 2009;57:550–61.
48. Webster TMU, Consuegra S, Hitchings M, de Leaniz CG. Interpopulation variation in the Atlantic salmon microbiome reflects environmental and genetic diversity. Appl Environ Microbiol. 2018;
49. Dehler CE, Secombes CJ, Martin SAM. Environmental and physiological factors shape the gut microbiota of Atlantic salmon parr (Salmo salar L.). Aquaculture. Elsevier; 2017;467:149–57.
50. Llewellyn MS, Boutin S, Hoseinifar SH, Derome N. Teleost microbiomes: The state of the art in their characterization, manipulation and importance in aquaculture and fisheries. Front. Microbiol. 2014.
51. Cheaib B, Seghouani H, Ijaz UZ, Derome N. Community recovery dynamics in yellow perch microbiome after gradual and constant metallic perturbations. Microbiome. 2020;
52. Holben WE, Williams P, Saarinen M, Särkilahti LK, Apajalahti JHA. Phylogenetic analysis of intestinal microflora indicates a novel Mycoplasma phylotype in farmed and wild salmon. Microb Ecol. 2002;
53. Jones RB, Zhu X, Moan E, Murff HJ, Ness RM, Seidner DL, et al. Inter-niche and inter-individual variation in gut microbial community assessment using stool, rectal swab, and mucosal samples. Sci Rep. 2018;
54. Egerton S, Wan A, Murphy K, Collins F, Ahern G, Sugrue I, et al. Replacing fishmeal with plant protein in Atlantic salmon (Salmo salar) diets by supplementation with fish protein hydrolysate. Sci Rep. 2020;
55. Michl SC, Ratten J-M, Beyer M, Hasler M, LaRoche J, Schulz C. The malleable gut microbiome of juvenile rainbow trout (Oncorhynchus mykiss): Diet-dependent shifts of bacterial community structures. Prunet P, editor. PLoS One [Internet]. Public Library of Science; 2017 [cited 2020 Sep 4];12:e0177735. Available from: https://dx.plos.org/10.1371/journal.pone.0177735
56. Gajardo K, Jaramillo-Torres A, Kortner TM, Merrifield DL, Tinsley J, Bakke AM, et al. Alternative protein sources in the diet modulate microbiota and functionality in the distal intestine of Atlantic salmon (Salmo salar). Appl Environ Microbiol. 2017;
57. Tanner SA, Berner AZ, Rigozzi E, Grattepanche F, Chassard C, Lacroix C. In vitro continuous fermentation model (PolyFermS) of the swine proximal colon for simultaneous testing on the same gut microbiota. PLoS One. 2014;
58. Card RM, Cawthraw SA, Nunez-Garcia J, Ellis RJ, Kay G, Pallen MJ, et al. An in Vitro chicken gut model demonstrates transfer of a multidrug resistance plasmid from Salmonella to commensal Escherichia coli. MBio. 2017;8.

59. Duysburgh C, Ossieur WP, De Paepe K, Van Den Abbeele P, Vichez-Vargas R, Vital M, et al. Development and validation of the Simulator of the Canine Intestinal Microbial Ecosystem (SCIME). J Anim Sci. 2020;
60. Déat E, Blanquet-Diot S, Jarrige JF, Denis S, Beyssac E, Alric M. Combining the dynamic TNO-gastrointestinal tract system with a Caco-2 cell culture model: Application to the assessment of lycopene and α-tocopherol bioavailability from a whole food. J Agric Food Chem. 2009;57:11314–20.
61. Kim HJ, Li H, Collins JJ, Ingber DE. Contributions of microbiome and mechanical deformation to intestinal bacterial overgrowth and inflammation in a human gut-on-a-chip. Proc Natl Acad Sci [Internet]. 2016;113:E7–15. Available from: http://www.pnas.org/lookup/doi/10.1073/pnas.1522193112
62. Sánchez-Patán F, Barroso E, Van De Wiele T, Jiménez-Girón A, Martín-Alvarez PJ, Moreno-Arribas MV, et al. Comparative in vitro fermentations of cranberry and grape seed polyphenols with colonic microbiota. Food Chem. 2015;
63. Giuliani C, Marzorati M, Innocenti M, Vilchez-Vargas R, Vital M, Pieper DH, et al. Dietary supplement based on stilbenes: a focus on gut microbial metabolism by the in vitro simulator M-SHIME®. Food Funct [Internet]. 2016;7:4564–75. Available from: http://xlink.rsc.org/?DOI=C6FO00784H
64. Van den Abbeele P, Roos S, Eeckhaut V, Mackenzie DA, Derde M, Verstraete W, et al. Incorporating a mucosal environment in a dynamic gut model results in a more representative colonization by lactobacilli. Microb Biotechnol. 2012;5:106–15.
65. Zarkasi KZ, Taylor RS, Glencross BD, Abell GCJ, Tamplin ML, Bowman JP. In vitro characteristics of an Atlantic salmon (Salmo salar L.) hind gut microbial community in relation to different dietary treatments. Res Microbiol. 2017;
66. Encarnação P. Functional feed additives in aquaculture feeds. Aquafeed Formul. 2016.
67. Kristiansen M, Merrifield DL, Vecino JLG, Myklebust R, Ringø E. Evaluation of prebiotic and probiotic effects on the intestinal gut microbiota and histology of Atlantic salmon (Salmo salar L.). J Aquac Res Dev. 2011;
68. Hartviksen M, Vecino JLG, Ringø E, Bakke AM, Wadsworth S, Krogdahl Å, et al. Alternative dietary protein sources for Atlantic salmon (Salmo salar L.) effect on intestinal microbiota, intestinal and liver histology and growth. Aquac Nutr. 2014;

Tables

Table 1 Beta diversity comparisons of microbial composition between different samples (real salmon, inoculum and SalmoSim).

Test		Data		Salmon vs SalmoSim	Salmon vs Inoculum	SalmoSim vs Inoculum
UniFrac	Unweighted (0%)	All		0.001 ***	0.002 **	0.002 **
		Subset		0.001 ***	0.002 **	0.001 ***
		Core OTUs	60%	0.04 *	0.032 *	0.143
			50%	0.001 ***	0.001 ***	0.033 *
			40%	0.001 ***	0.003 **	0.244
			30%	0.001 ***	0.001 ***	0.005 **
	Balanced (50%)	All		0.001 ***	0.003 **	0.001 ***
		Subset		0.001 ***	0.001 ***	0.003 **
		Core OTUs	60%	0.138	0.059	0.12
			50%	0.002 **	0.019 *	0.041 *
			40%	0.002 **	0.062	0.132
			30%	0.001 ***	0.005 **	0.008 **
	Weighted (100%)	All		0.012 *	0.007 **	0.003 **
		Subset		0.012 *	0.007 **	0.004 **
		Core OTUs	60%	0.381	0.063	0.125
			50%	0.008 **	0.217	0.078
			40%	0.023 *	0.467	0.122
			30%	0.021 *	0.014 *	0.06
Bray-Curtis		All		0.001 ***	0.001 ***	0.23
		Subset		0.001 ***	0.001 ***	0.273
		Core OTUs	60%	0.009 **	0.004 **	0.079
			50%	0.001 ***	0.008 **	0.394
			40%	0.001 ***	0.002 **	0.327
			30%	0.001 ***	0.001 ***	0.388
Jaccards		All		0.001 ***	0.001 ***	0.147
		Subset		0.001 ***	0.001 ***	0.161
		Core OTUs	60%	0.002 **	0.003 **	0.073
			50%	0.001 ***	0.002 **	0.386

40%	0.001 ***	0.002 **	0.22
30%	0.001 ***	0.001 ***	0.254

The table summarises different beta-diversity analysis outputs calculated by using different distances: phylogenetic (unweighted, balanced and weighted UniFrac) and ecological (Bray-Curtis and Jaccard's), between different samples (data from all gut compartments combined): real salmon (**Salmon**), SalmoSim inoculum from the real salmon (**Inoculum**) and SalmoSim (only stable time points: 16, 18 and 20 fed on Fish meal diet, and 36, 38 and 40 fed on Fish meal free diet). A permutational multivariate analysis of variance (PERMANOVA) by using phylogenetic and ecological distances was performed to determine if the separation of selected groups is significant as a whole and in pairs. Numbers represent p-values, with p-values <0.05 identifying statistically significant differences between compared groups. The comparisons are shown for 3 different datasets: All (completed data set containing all the OTUs sequenced), Subset (containing OTUs that appear only in more than 3 samples and contribute to 99.9% of abundance within each sample), and core OTUs (containing OTUs that appear in 60%, 50%, 40% and 30% of the samples). The stars flag the levels of significance: one star (*) for p-values between 0.05 and 0.01, two stars (**) for p-values between 0.01 and 0.001, and three stars (***) for p-values below 0.001.

Table 2 Beta diversity analysis for various samples fed on different feeds.

		Fish meal vs Fish meal free diets		
		Salmon	**SalmoSim**	**Stable SalmoSim**
UniFrac	**Unweighted (0%)**	0.001 ***	0.011 *	0.062
	Generalised (50%)	0.001 ***	0.01 **	0.251
	Weighted (100%)	0.016 *	0.004 **	0.288
Bray-Curtis		0.008 **	0.001 ***	0.126
Jaccards		0.01 **	0.001 ***	0.053
Number of differentially abundant OTUs		18	32	28

Table summarises different beta-diversity analysis outputs calculated by using different distances: phylogenetic (unweighted, balanced, and weighted UniFrac) and ecological (Bray-Curtis and Jaccard's), between samples fed on Fish meal or Fish meal free diets. Numbers represent p-values, with p-values <0.05 identifying statistically significant differences between compared groups. The comparisons are shown for three different subset-datasets: Salmon (containing sequenced samples from real salmon), All SalmoSim (containing all samples from SalmoSim system), and Stable SalmoSim (containing samples only from stable time points: 16, 18 and 20 fed on Fish meal (once bacterial communities adapted to SalmoSim system), and 36, 38 and 40 fed on Fish meal free diet (once bacterial communities adapted to

feed change). The stars flag the levels of significance: one star (*) for p-values between 0.05 and 0.01, two stars (**) for p-values between 0.01 and 0.001, and three stars (***) for p-values below 0.001.

Figures

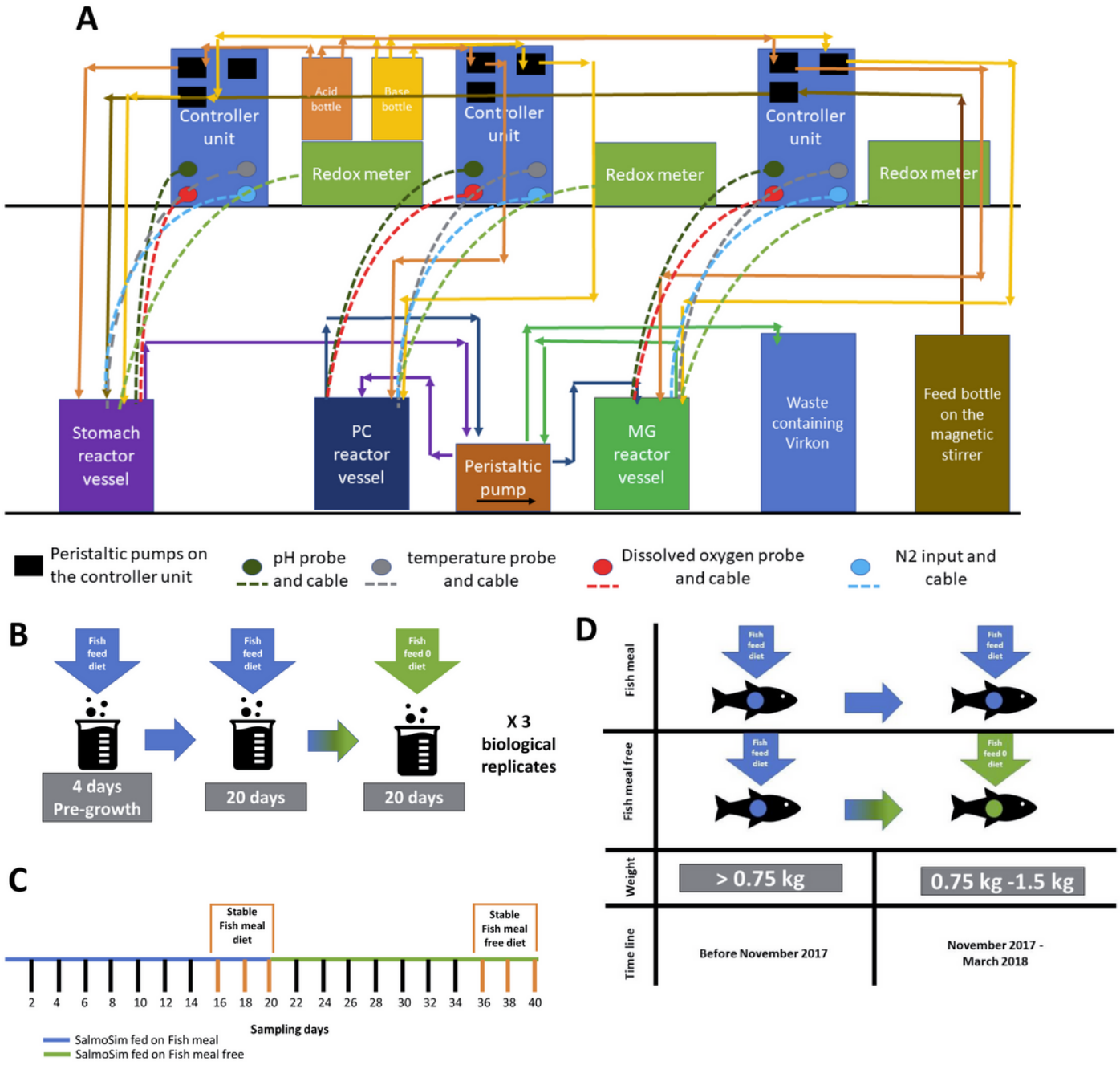


Figure 1

Salmon gut in vitro simulator. Schematic encompasses the artificial gut model system set-up, in vivo and in vitro feed trial set up. 1A is a schematic representation of SalmoSim system; 1B SalmoSim feed trial design; 1C SalmoSim sampling time points, which include definition of stable time points (days 16, 18, and 20 for Fish meal (once bacterial communities had time to adapt to SalmoSim system), and days 36,

38, and 40 for Fish meal free diet (once bacterial communities had time to adapt to change in feed); 1D in vivo feed trial design.

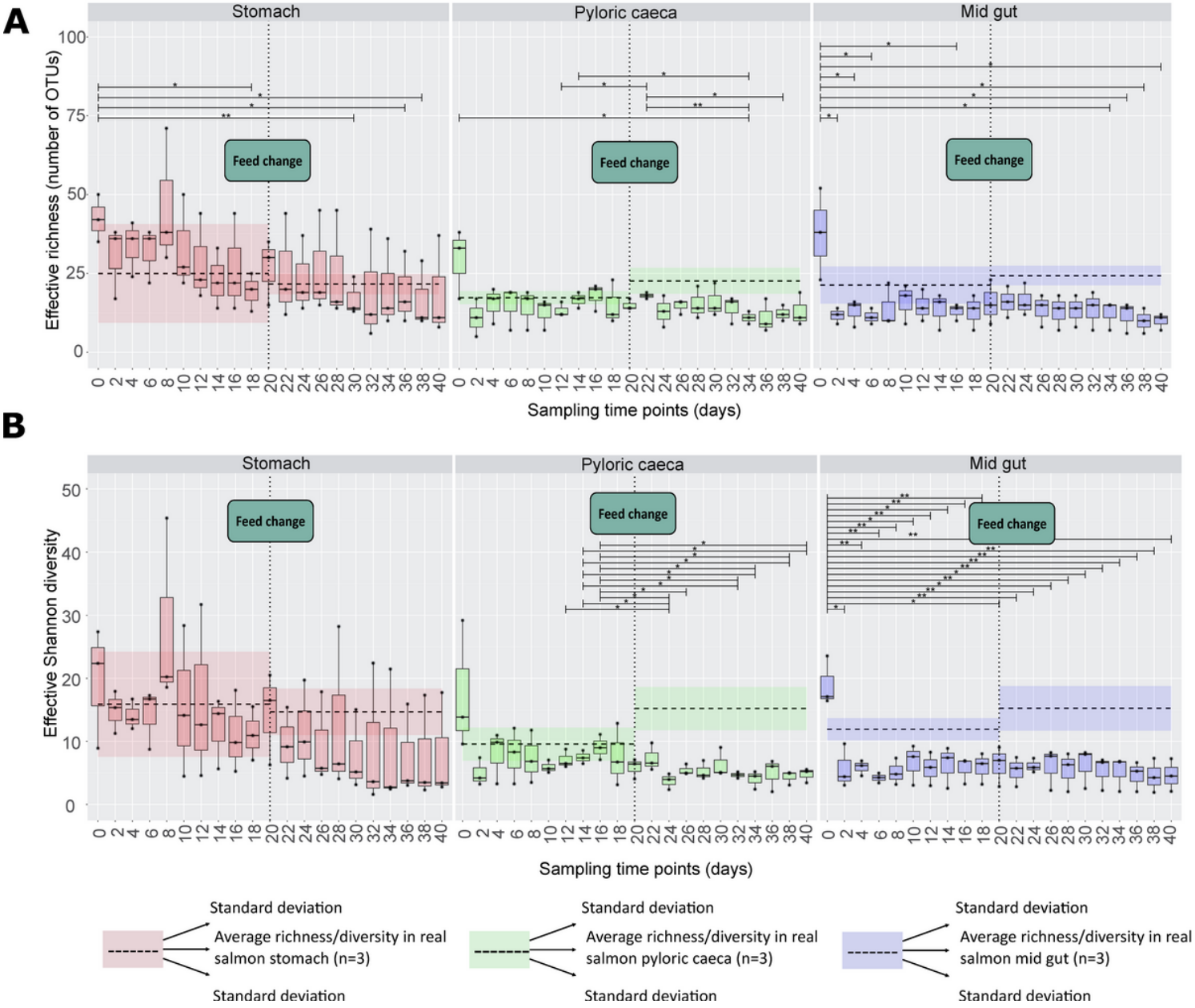


Figure 2

Calculated alpha-diversity metrics within SalmoSim system over time. The figure represents different alpha diversity outputs at different sampling time points (days) from SalmoSim system. Time point 0 represents microbial community composition within initial SalmoSim inoculum from the real salmon, time points 2-20 identifies samples from SalmoSim system fed on Fish meal diet, and time points 22-40 identifies samples from SalmoSim system fed on Fish meal free diet. The dotted vertical line between days 0-20 represents average alpha diversity values measured in real salmon fed on Fish meal diet and dotted vertical line between days 22-40 represents average alpha diversity values measured in real salmon fed on Fish meal free diet. Finally, the horizontal dashed lined represent average effective richness (A) and effective Shannon diversity (B) in real salmon individual gut compartments fed on

different diets (n=3 fish/feed and gut compartment) and shaded region around the horizontal dashed line represents the standard deviation of the values measured within real salmon samples fed on the different diets. A visually represents effective richness (number of OTUs) and B represents effective Shannon diversity. The lines above bar plots represent statistically significant differences between different time points. The stars flag the levels of significance: one star (*) for p-values between 0.05 and 0.01, two stars (**) for p-values between 0.01 and 0.001, and three stars (***) for p-values below 0.001.

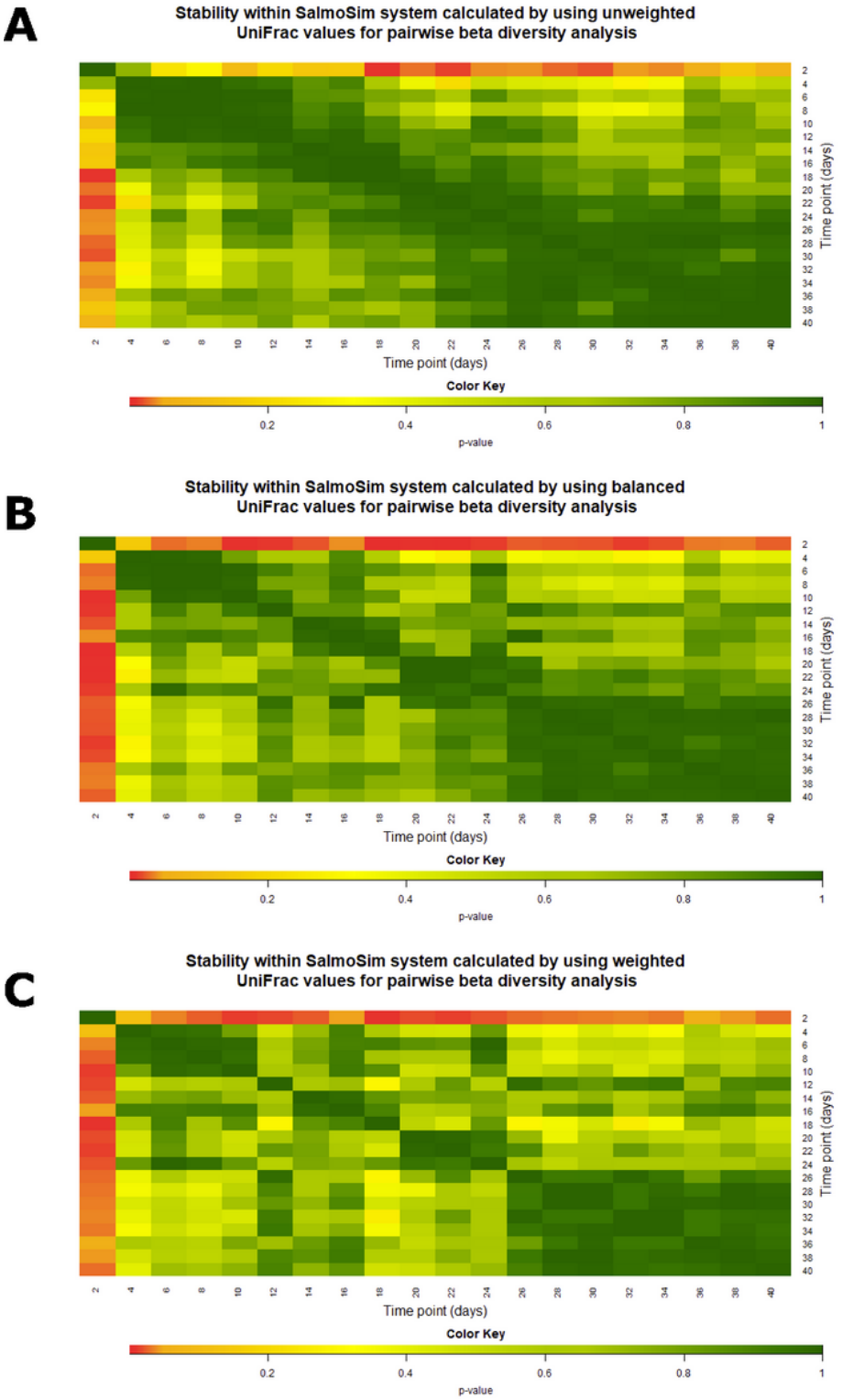
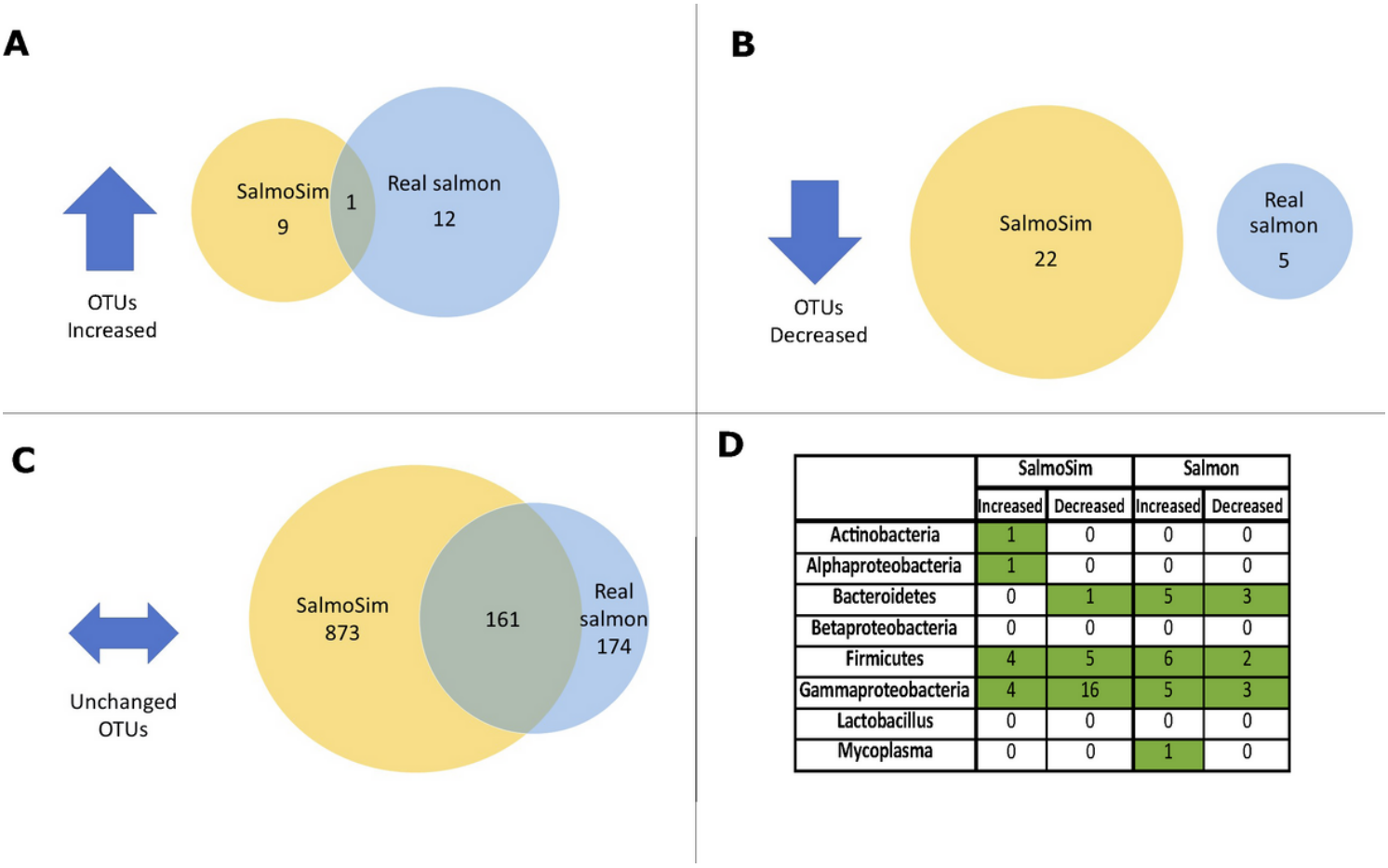


Figure 3

Stability within SalmoSim system calculated by using different UniFrac values for pairwise beta diversity analysis. The figure represents microbial stability within the SalmoSim system (data from all gut compartments combined) as the pairwise beta diversity comparison between different sampling time points (days), calculated by using A balanced (50%), B unweighted (0%) and C weighted (100%) UniFrac as a distance measure. A small p-value indicates that the two time points are statistically different, and p>0.05 indicates that two time points are not statistically different. The colour key illustrates the p-value: red end of spectrum denoting low p values (distinct compositions between time points) and dark green indicating high p values (similar compositions between timepoints).



	SalmoSim		Salmon	
	Increased	Decreased	Increased	Decreased
Actinobacteria	1	0	0	0
Alphaproteobacteria	1	0	0	0
Bacteroidetes	0	1	5	3
Betaproteobacteria	0	0	0	0
Firmicutes	4	5	6	2
Gammaproteobacteria	4	16	5	3
Lactobacillus	0	0	0	0
Mycoplasma	0	0	1	0

Figure 4

Differential abundance of OTUs within the real salmon and SalmoSim samples fed on Fish meal and Fish meal free diets. A: Venn diagram representing number of OTUs that were upregulated in both SalmoSim and real Salmon samples once the feed was switched, B: Venn diagram representing number of OTUs that were downregulated in both sample after the feed change, C: Venn diagram representing number of OTUs that did not change (relative abundance did not change) within SalmoSim and real salmon samples despite feed switch, D: table summarising number of OTUs that increased/decreased after feed change in real salmon and SalmoSim samples within different bacterial groups (that same that were analysed by using qPCR approach). Green colour indicates the values that are higher than 0.

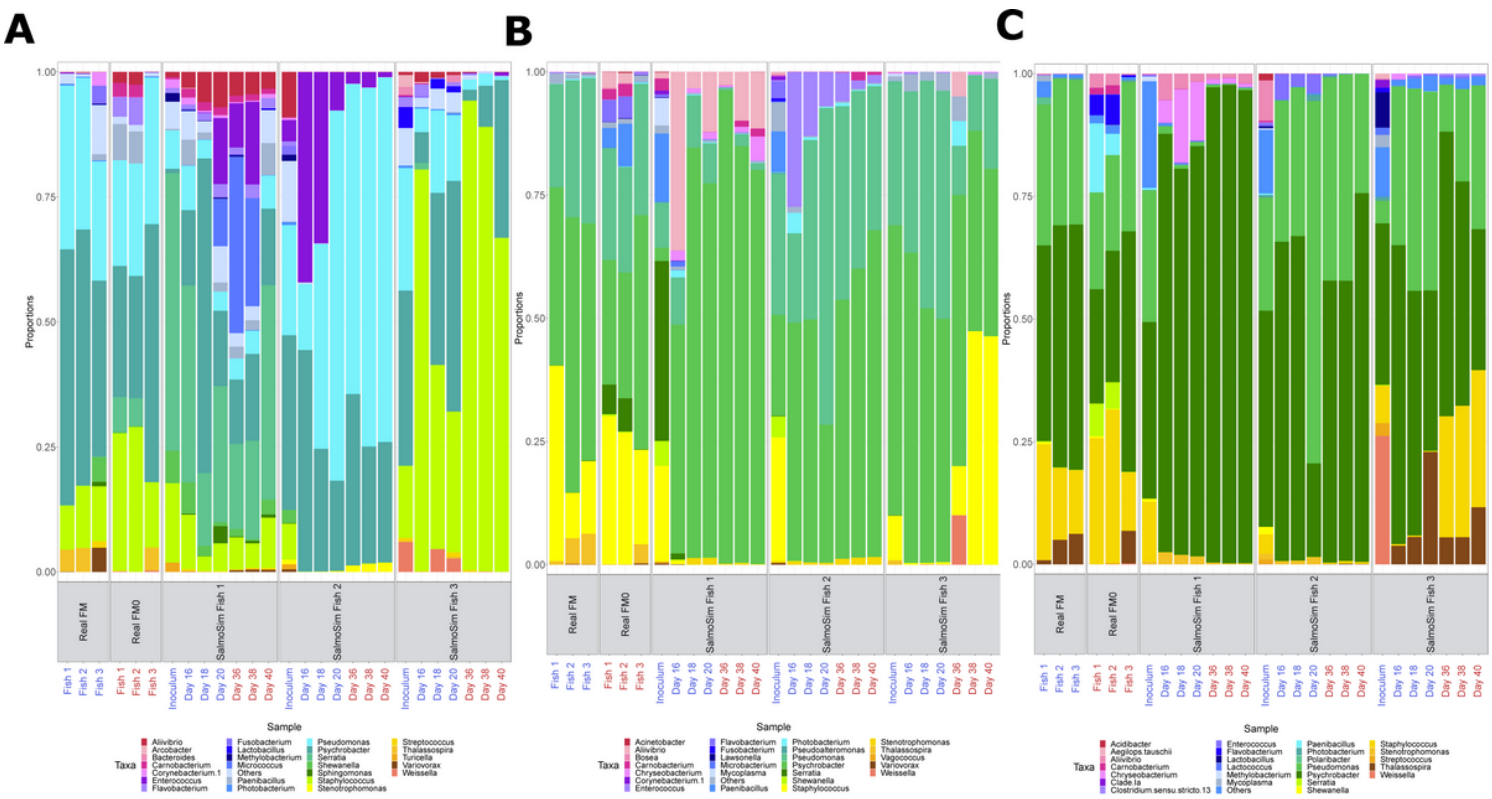


Figure 5

Microbial composition (25 most common genus + others) amongst sample types and feeds. A: microbial composition within stomach compartment, B: microbial composition within pyloric caeca compartment, and C: microbial composition within midgut compartment. The different sample types are represented by the labels on the x-axis: Real FM (real salmon fed on Fish meal), Real FM0 (real salmon fed on Fish meal free diet), SalmoSim Fish 1-3 (SalmoSim biological replicate runs 1-3). Labels in blue represent samples fed on Fish meal diet and in red samples fed on Fish meal free diet. For SalmoSim only stable time points for each feed were selected: time points 16-20 for Fish meal diet, and time points 36-40 for Fish meal free diet.

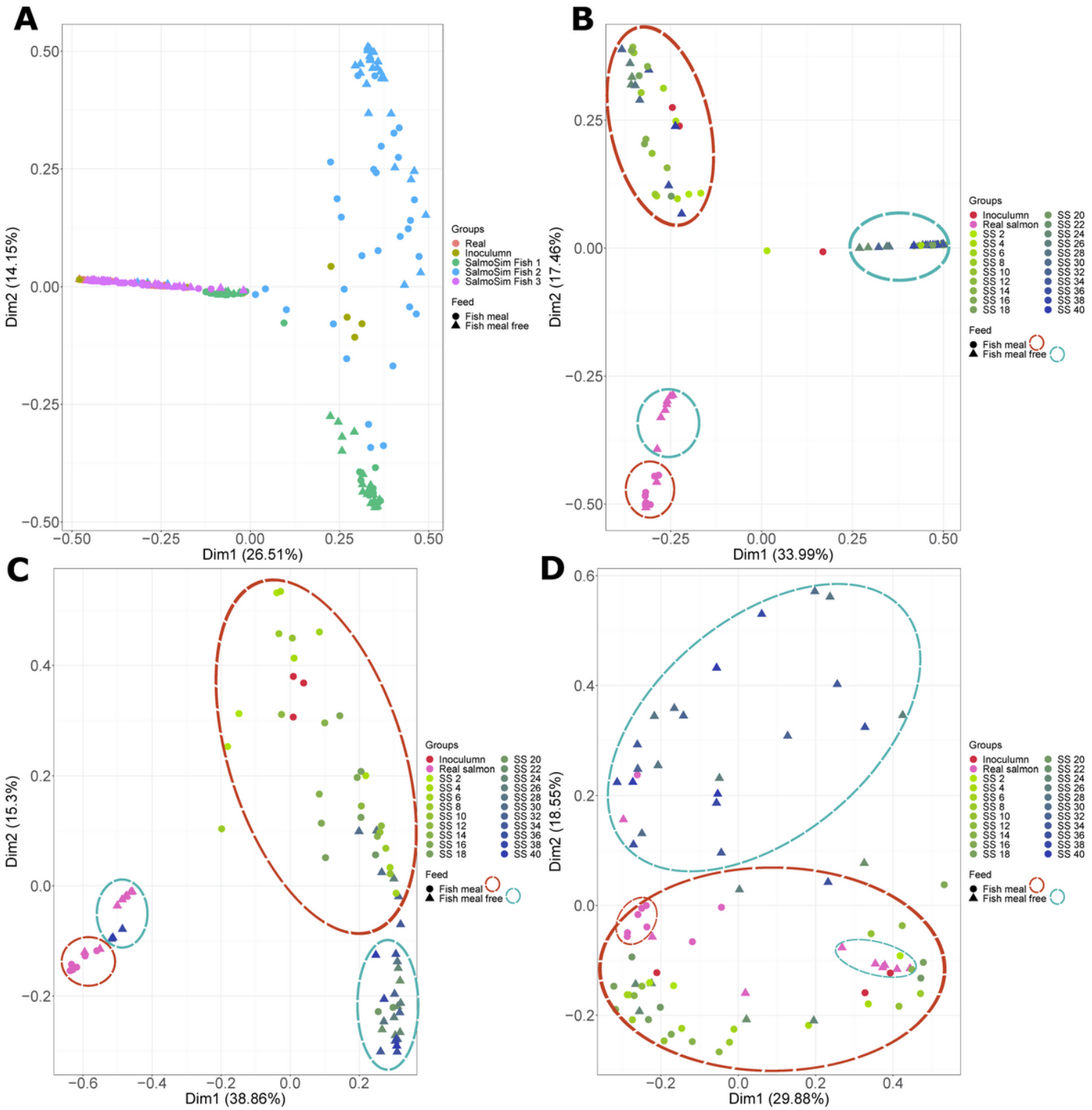


Figure 6

PCoA analysis for various samples fed on different feeds. Figure visualises four principal-coordinate analysis (PCoA) plots for Bray-Curtis dissimilarity measures for different samples (Inoculum, real salmon and SalmoSim), different sampling time points from SalmoSim system, different biological replicates and different feeds. A represents all sequenced data together (all real salmon, inoculum and all 3 biological SalmoSim runs) in which different colours represent different samples (real salmon, inoculum and 3 different SalmoSim biological replicates (Fish 1, Fish 2, Fish 3)) and different shapes represent

different feeds; while B-D (subsets of Figure 6A) represent sequenced data together for real salmon, inoculum and different biological replicates of SalmoSim (B: Fish 1, C: Fish 2, D: Fish 3). In figures B-D different colours represent different samples (inoculum, real salmon and different sampling points of SalmoSim), different shapes represent samples fed on two different feeds, and samples fed on same feeds were circled manually in dotted circles. Dim 1 is principal coordinate 1, and Dim 2 is principle coordinate 2.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfile1Supplementarymethods.docx
- Additionalfile2TableS1.xlsx
- Additionalfile3TableS2.xlsx
- Additionalfile4TableS3.xlsx
- Additionalfile5TableS4.xlsx
- Additionalfile6TableS5.xlsx
- Additionalfile7TableS6.xlsx
- Additionalfile8FigureS1.pdf
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