

Gut microbiome analysis of fast- and slow-growing Rainbow Trout (*Oncorhynchus mykiss*)

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Abstract

Background Diverse microbial communities colonizing the intestine of fish contribute to their growth, digestion, nutrition, and immune function. We hypothesized that the gut microbiome of rainbow trout could be associated with differential growth rates observed in fish breeding programs. If true, harnessing the functionality of this microbiome can improve profitability of aquaculture. To test this hypothesis, four full-sibling families were stocked in the same tank and fed an identical diet. Two fast-growing and two slow-growing fish were selected from each family. Five different extraction methods were used to obtain DNA from feces for 16S rRNA microbiome profiling. These methods were Promega-Maxwell, phenol-chloroform, MO-BIO, Qiagen-Blood, Qiagen-Stool. Methods were compared according to DNA integrity, cost, feasibility and inter-sample variation based on non-metric multidimensional scaling ordination (nMDS) clusters. Results Differences in DNA extraction methods result in significant variation in identification of bacteria that compose the gut microbiome. Promega-Maxwell had the lowest inter-sample variation and was therefore used for the subsequent analyses. The gut microbiome was different from that of the environment (feed and water). However, feed and gut shared a large portion of their microbiome suggesting significant contribution of the feed in shaping the gut microbiota. Beta diversity of the bacterial communities showed significant variation between breeding families but not between the fast- and slow-growing fish. An indicator analysis determined that cellulose, amylose degrading and amino acid fermenting bacteria (*Clostridium*, *Leptotrichia* and *Peptostreptococcus*) as indicator taxa of the fast-growing fish. In contrary, pathogenic bacteria (*Corynebacterium* and *Paeniclostridium*) were identified as slow-growing indicator taxa. Conclusion DNA extraction methodology should be taken into account for accurate profiling of the gut microbiome. Although the microbiome was not significantly different between the fast- and slow-growing fish groups, some bacterial taxa with functional implications were indicative of fish growth rate. Further studies are warranted to explore how bacteria are transmitted and potential usage of the indicator bacteria of fast-growing fish for development of probiotics that may improve fish health and growth.

Introduction

The efficiency and profitability of industrial aquaculture depends in part on the growth rate of farmed fishes. Growth in farmed fishes is a complex process that is directly dependent on host genetics, food quality and availability, and environmental conditions[1]. Selective breeding is one strategy that can be used to improve important phenotypic traits and help in understanding the genetic architecture and the role of molecular factors causing genetic variation among different fish [2]. Family-based selection procedures have been undertaken by the United States Department of Agriculture (USDA), National Center for Cool and Cold-Water Aquaculture (NCCCWA) to improve growth rate, fillet quality and disease resistance of rainbow trout [3]. A growth-selected line was developed starting in 2002, and since then yielded a genetic gain of approximately 10% in improved growth performance per generation[4].

Microorganisms may also contribute to the productivity of farmed fishes. Microorganisms making up the fish microbiome reside on the fish skin, gills, and gastrointestinal tract and likely play a crucial role in the growth rate, metabolism, and immunity of the fish host [5, 6]. While host genetics has a profound role in determining the gut microbiome of humans and other mammals, it is not well studied in fish [7-9]. On the other hand, feed and water in which fish are reared have vital roles in shaping the gut microbiome. For example, plant and animal-based meal can widely alter the composition of the host microbiota since fish acquire their microbiome from the first-feed they eat [10, 11]. Sharp *et al.* reported that microbiota of the marine species can be directly inherited from ancestors and passed from generation to generation [12]. The gut in particular features a diverse microbiome contributing to the weight gain, immune development, pathogen inhibition, and various metabolic activities of the hosts [13]. Resident gut microbes are beneficial for hosts either by inhibiting pathogenic bacteria with dedicated toxins or by secreting enzymes that breakdown indigestible polysaccharides in host gut to simple monosaccharides and short chain fatty acids [14]. Gut microbes can supply compounds such as vitamin B and K to host which may improve the host energy metabolism [15].

An accurate census of bacteria from fish may allow investigation of the positive effects of the microbiota. However, profiling of the gut microbiome is directly influenced by many factors including the experimental design, sample collection, and processing. DNA extraction is particularly important, since microbiome analysis requires adequate quality and quantity of DNA isolated for a true representation of the host microbiome [16]. Many protocols have been commercialized for DNA extraction and previous reports demonstrate that microbiome diversity varies with different DNA extraction methods [17]. It is difficult to determine the most appropriate extraction method for the downstream microbiome analysis of a particular species. Each method has its own merits and drawbacks; for example, standardized kits are typically designed for ease of use and efficiency, but a more labor-intensive method such as phenol-chloroform extraction, despite its risk of inconsistency or contamination, can potentially produce a higher yield with better quality if performed by an experienced researcher.

In this study, we investigate how the gut microbiome of rainbow trout correlates with differential growth rates. Therefore, one objective of this research was to characterize the gut microbiome of rainbow trout using high-throughput DNA sequencing. In order to achieve this objective, we considered the effect that DNA extraction methodologies play in characterization of different microbial communities in the gut of rainbow trout. The specific objectives of our study were to determine differences in community structure of the gut microbiomes between fast- and slow-growing rainbow trout and to determine if genetics plays a role in determining the gut microbiome profile. Our results highlight differences of the gut microbiome between fish family and the bacterial taxa indicative of fast- and slow-growing rainbow trout.

Methods

Fish population

Fecal samples were collected from 15 fish representing four different genetic families. The parents of these families originated from a growth-selected line at NCCCWA (year class 2014) that was previously described [4, 18]. Families were produced and reared until ~18 months post-hatch. Briefly, full-sibling families were produced from single-sire × single-dam mating events. All sires were siblings from a single family while dams exhibited low relatedness (coefficient of relatedness < 0.16). Eggs were reared in spring water, and water temperatures were manipulated between approximately 7-13 °C to synchronize hatch times. Each family was reared separately from hatch through approximately 20 g (7 months post hatch) when 15 fish per family were uniquely tagged by inserting a passive integrated transponder (Avid Identification Systems Inc., Norco, CA) into the peritoneal cavity. Tagged fish were comingled for the remainder of the grow-out period. Fish were fed a commercial fishmeal-based diet (42% protein, 16% fat; Ziegler Bros Inc., Gardners, PA) using automatic feeders (Arvotec, Huutokoski, Finland). Feed was provided at or just below satiation for the entire grow-out period. This study includes four families with high variance in adult body weight. From each family, four fish were selected, two that were considered fast-growing (>1952 g) and two that were slow-growing (<1572 g). Of the 16 fish selected for sampling, one slow-growing fish from family two exhibited morphological signs of disease during sample collection and was excluded from analysis, reducing the total number of samples to 15. The statistical significance of the rank body mass between the two groups was tested by a one-way Mann-Whitney U test with an alpha of $p < 0.001$ (GraphPad Software, Inc., La Jolla, CA).

Sample collection

To characterize the gut microbiome and compare it to the surrounding water and food source, samples were collected from fish feces, water and feed. For fecal sampling, fish were anesthetized with tricaine methanesulfonate (150 mg mL^{-1}) (Tricaine-S, Western Chemical, Ferndale, WA) and then manually stripped to collect the fecal samples in sterile Eppendorf tubes (Eppendorf, Hauppauge, NY). Water samples of 1.5-liter volume were collected from both the inlet water source and tank ($n = 4$ total samples), then filtered twice through a clean sterile membrane filter with pore size $0.45 \mu\text{m}$. DNA was isolated from filters to sample bacteria present in the environment. Feed samples were also collected by taking 100 g of feed and storing it in a Ziploc bag. All samples were stored at $-80 \text{ }^\circ\text{C}$ until DNA extraction.

DNA isolation and sequencing

For comparison of extraction methods, fecal samples from 8 fast-growing and 7 slow-growing fish were pooled together and DNA extraction was done in triplicate using five different extraction methods including PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc., West Carlsbad, CA), Promega Maxwell DNA Isolation Kit (Promega Corporation, Madison, WI), Qiagen Blood and Tissue, Qiagen Stool (Qiagen, Germantown, MD) and phenol-chloroform (Phenol: Chloroform 5:1, SIGMA) extraction method [19]. Three of the mentioned DNA extraction methods were chosen to study the gut microbiome of fast-growing versus slow-growing trout: MO BIO kit, Promega Maxwell, and phenol-chloroform extraction. More detail of the DNA extraction methods is provided in Additional file 1. Once DNA was extracted, concentration and quality were measured, and integrity of genomic DNA was checked by gel electrophoresis. All DNA extractions were stored at $-80 \text{ }^\circ\text{C}$ until library preparation.

Before library preparation, concentrations of all DNA samples were normalized to $2 \text{ ng}/\mu\text{L}$ for PCR amplification using a Qubit fluorometer (v3.11) (Invitrogen, Carlsbad, CA). The primers 515F and 926R (Integrated DNA Technologies) (EMP; <http://www.earthmicrobiome.org/emp-standard-protocols/16s/>), were used to target the 16S rRNA marker gene using polymerase chain reaction (PCR). The final PCR reaction consisted of $5 \mu\text{L}$ buffer, $1.5 \mu\text{L}$ 50 mM MgCl_2 , $2 \mu\text{L}$ 10 mM dNTP, $0.2 \mu\text{L}$ Taq polymerase, $3 \mu\text{L}$ Kb extender, $1 \mu\text{L}$ $10 \mu\text{M}$ primer, $5 \mu\text{L}$ DNA template and $7.3 \mu\text{L}$ nuclease-free water. PCR amplification and sample indexing was performed according to the standard earth microbiome project protocols [20]. The amplification conditions were $94 \text{ }^\circ\text{C}$ for 45 sec, $50 \text{ }^\circ\text{C}$ for 60 sec, $72 \text{ }^\circ\text{C}$ for 90 sec for 35 cycles. Amplification was preceded by a 10-minute preheating step at $94 \text{ }^\circ\text{C}$ and followed by a 10-minute elongation step at $72 \text{ }^\circ\text{C}$. Amplification of each sample was performed in triplicate and combined to a final volume of $75 \mu\text{L}$. The indexed samples were then normalized ($240 \text{ ng}/\text{reaction}$) and pooled for sample purification purposes. The pooled amplicon was purified using Promega PCR purification kit (Promega Corporation, Madison, WI) and visualized on a 1.5 % agarose gel stained with ethidium bromide. A DNA fragment of size approximately 370 bp for each sample was excised from the DNA gel with a clean, sharp scalpel and collected in nuclease-free sterile tubes. QIAquick gel extraction kit was used to purify DNA from the resulting gel slice (Qiagen, Germantown, MD) according to the manufacturer's recommendation. The concentration of the gel-extracted library was assessed with a Qubit fluorometer (Invitrogen, Carlsbad, CA) and fragment size was determined using an Agilent 2100 Bioanalyzer (Agilent, Santa Clara, California). Final qPCR-based quantification of the library was done using a KAPPA quantification kit (Roche, Pleasanton, CA). Sequencing was done using 250bp-paired end sequencing using a 300 cycle V2 reagent cartridge on an Illumina MiSeq flow cell (Illumina, Inc., San Diego, CA) according to manufacturer's instructions (MiSeq System Guide) [21]. The output file was demultiplexed and converted to fastq on the Illumina MiSeq (Illumina, Inc., San Diego, CA).

Bioinformatics analyses

Sequencing data (3,972,613 raw sequences reads) were analyzed using Mothur (v.1.40.2, www.mothur.org) according to the Mothur Illumina Miseq standard operating procedure (SOP) [22] with several modifications. After forming contigs, we determined the median length (371 bp) of the sequences. *Chops.seqs* was used to keep the first 371 bp of each sequence[23]. Sequences with ambiguous base pairs were removed by using the *screen.seqs* command. The *split.abund* command was used to keep abundant sequences with greater than two reads[24]. Sequences were aligned to the SILVA v123 database and sequences that failed to align, or classified as Archaea, chloroplast, eukaryotic mitochondrial, or unknown sequences, were excluded from the analysis. Sequences detected by UCHIME as chimeric were removed from the analysis. The remaining sequences were clustered using VSEARCH[25] at a threshold of >97% sequence similarity. The *remove.rare* command was used to remove operational taxonomic units (OTUs) having less than ten reads among shared samples. Two samples (one fast-growing extracted using Promega Maxwell method and one slow-growing fish extracted using phenol chloroform method) were excluded from the analysis because sequences in these samples did not pass the quality control and filtering steps. The parameters and the command used to analyze the data are included in additional file 2.

Statistical analysis

To study the effect of DNA extraction methods on microbial community profiling, Bray-Curtis distances were compared and nMDS ordination was used for visualization. To test for a significant effect of extraction method, we used Permutational Multivariate Analysis of Variance (PERMANOVA) on the basis of Bray-Curtis dissimilarity matrices by considering extraction technique as a fixed effect and using type III sum of squares and unrestricted permutation of data with 999 permutations. SIMPROF (Similarity Profile) was performed to test the inter-sample variation on the replicate samples with a significant cut off value of 0.5 (95% similarity).

Beta diversity of the gut and environmental samples were calculated using Bray-Curtis dissimilarity matrices representing pairwise (sample to sample) distances to test the variation among gut and environmental samples (feed and water). Non-metric multidimensional scaling ordination (nMDS) was used to explore the microbial communities in the fast-growing and slow-growing fish by considering the dissimilarity distance matrices among the samples. One-way PERMANOVA was used to assess the effect of sample type (feces, feed and water) as predictive of the microbiome.

To understand the effect fish growth rate on the microbiome, values from Bray-Curtis dissimilarity matrices were compared and visualized using nMDS ordination. A one-way PERMANOVA was used to determine if the growth rate or fish breeding family, both considered as fixed effects, were predictive of the microbiome.

An indicator analysis was done in Mothur in order to statistically and independently select bacterial taxa that are indicative of fast-/slow-growing fish or fish breeding family. Taxa with indicator values greater than 40 and a p-value (<0.05) were considered as indicative of fish growth rate or breeding family. All data files for reproduction of the bioinformatics and statistical analyses are included in additional files 3 – 8.

Results

Mean weight difference between fast and slow-growing fish

The mean weight of the fast-growing fish was 2123.9 ± 105.57 g, whereas, the mean weight of the slow-growing fish was 988.6 ± 297.65 g. The mass of the fast-growing fish was significantly greater than that of the slow-growing fish when compared using one-way Mann-Whitney U test ($p > 0.05$) as shown in Figure 1.

B-diversity of the fecal samples, feed and water

A total 1,988 OTUs were identified from all gut, feed and water samples. To determine if the gut microbiome differs either from environment or feed, Bray-Curtis distance matrices were compared and the results indicated the overall gut microbiome was significantly different from that of the environment (water and feed) ($F_{3,48} = 2.29$ and $p < 0.05$, $R^2 = 39\%$) Fig. 2 A).

Microbiome analysis of gut, feed and water

Data analysis of the microbial communities using the Promega extraction technique revealed a total of 385 OTUs in gut, whereas feed and water samples consisted of 236 OTUs and 122 OTUs, respectively. Only 17 OTUs were shared among all samples whereas 150 OTUs were shared among feed and gut (Fig. 2 B).

The overall gut microbiota was composed of 5 phyla, 10 classes and 130 genera, while the feed consisted of 5 phyla, 11 classes and 90 genera, and the water samples consisted of 6 phyla, 36 classes and 56 genera as shown in Table 1. These numbers do not include the taxa that were considered as unclassified and were not assigned a specific class or genus. Phylum Firmicutes was the most abundant phylum in the gut and was the most shared phylum among gut and feed samples. Proteobacteria was the most abundant phylum in the water samples. The most abundant phylum in the feed was Firmicutes. Detailed information about the microbiota classification is included in additional file 9.

Comparison of different DNA extraction methods

To test if profiling of the gut microbiome is directly influenced by DNA extraction method, three replicate pools of the fish fecal samples were sequenced and analyzed using five different extraction methods. Within non-metric dimensional scaling ordination plots, the three-replicate samples extracted with Promega clustered tightly, whereas, replicate samples of the four other extraction methods were relatively more heterogeneous (Fig. 3). PERMANOVA confirmed that DNA extraction method is predictive of the microbiome ($F_{4,13}=2.4234$, $p<0.05$, $R^2=51\%$).

To further investigate the effects of DNA extraction methodology on microbiome profiling, three different methods were chosen for microbiome sequencing from individual (non-pooled) fecal samples of all available fish in the study. PERMANOVA results confirmed the significant effect of extraction technique on predicting microbial communities (Fig. 4 A; $F_{2,42}=10.467$, $p<0.05$, $R^2=34\%$). Comparative analysis of the three extraction methods revealed that phenol-chloroform had the highest OTU richness with 649 OTUs. A total of 119 OTUs overlapped between all three DNA isolation methods (Fig. 4 B). Comparing the abundance of Gram-positive and Gram-negative bacteria, it was clear that the abundance of the Gram-positive is higher than that of the Gram-negative in all three DNA extraction techniques (Fig. 4 C) with the Promega kit showing the most drastic gram-positive bias. The SIMPROF test for statistically significant cluster and it showed that the Promega method had 95% similarity within the replicate samples forming the tightest cluster (p -value < 0.05).

Beside heterogeneity and abundance biases, other factors including yield, integrity, time durations for sample processing, amount of hazardous waste liberated were also considered during extraction comparison. Phenol-Chloroform gave the highest yield, but it is tedious, time-consuming, requires individual handling and released more hazardous waste. Whereas, Promega is semi-automated method, easy to perform in large-scale production, and shows the least inter-sample variation among the replicate samples, results release of least hazardous waste as shown in (Table 2). We decided to choose Promega for our downstream analysis of the gut microbiome.

Gut microbiome analysis of fast- and slow-growing fish

Both nMDS ordination and PERMANOVA results indicated that the microbial communities did not significantly differ between the fish of different growth rates ($p>0.05$, Fig. 5 A). Both fast- and slow-growing fish possessed unique sets of OTUs and also overlapping taxa (Fig. 5 B). An indicator analysis predicted that 10 OTUs were found as indicative of growth rate (Table 3, $P < 0.05$). All fast-growing indicator taxa belonged to phylum Firmicutes, whereas, the slow-growing indicator taxa belonged to the Actinobacteria and Firmicutes (Table 3).

PERMANOVA results indicated that fish breeding family was predictive of the microbiome ($F_{3,13}=2.1673$, $p<0.05$, $R^2=39\%$) (Fig 5 C). The Venn-representation depicted 106 OTUs shared among all the families with family 2 having the most unique OTUs (Fig. 5 D). An indicator analysis of each fish family predicted that 6 OTUs were identified as indicative of family 1, 3 OTUs for family 2, and 1 OTUs for family 4 (Table 4, $P<0.05$).

Discussion

The salmonid aquaculture industry can benefit from development of fast-growing fish. Selective breeding is one strategy that can be used to improve fish growth and help in understanding its genetic architecture[26]. On the other hand, the environmental factors, particularly diet, have immense role in improving this phenotypic trait. Microbiota of the fish gut also have a significant role in determining host health and, in some instances, growth rate[27]. The microbiota and its host have an intimate and sometimes mutually beneficial relationship. The environmental conditions and host diet have profound role in shaping the gut microbiota [28]. In addition, recent studies have illustrated that host genetics have direct impact on gut microbial composition in many species including human and rodents [9] and to some extent chicken[29]. Influence of the host genetics in determining the microbiota of a fish has yet to be characterized[9, 30].

In this study, the DNA extraction methodology comparison was performed to optimize the extraction methodology and apply this to the comparison of fast- and slow-growing fish gut microbiomes. Five different extraction techniques including bead beating and semi-automated

methods were included. The effects of the DNA extraction methods were assessed on the basis of DNA quantity, quality and the inter-sample variation in microbial communities between replicates. The concentration and the quality of the DNA varied significantly between the DNA extraction techniques. The MOBIO, Qiagen blood and Qiagen stool gave relatively low yield, whereas Promega Maxwell kit that uses automated method resulted in a higher yield in comparison to the other three kits which is consistent with previous reports[31]. In comparison, phenol-chloroform, being a robust method, uses a stringent lysis step and produced the highest DNA yield and highest microbial diversity. This is likely due to this method being able to effectively lyse the cell walls of both the Gram-positive and Gram-negative bacteria. However, the phenol-chloroform method resulted in higher inter-sample variation, was the most labor-intensive, and produces more hazardous waste when compared to the Promega method. It has been proven that the bead-beating methods result in identification of greater microbial diversity than non-beating methods[32]. MOBIO method involves bead beating to physically lyse cell wall of bacteria and increase the number of the microbial species identified but showed relatively high inter-sample variation among replicates. Promega Maxwell, semi-automated method seems biased toward the Gram-positive bacteria, perhaps, due to addition of lysozyme enzymes which induces lysis of the Gram-positive bacterial cell wall or decreased lysis of Gram-negative bacteria. The Promega method showed the least inter-sample variation among replicates. Similar is the case with Qiagen-stool, Qiagen-Blood and Tissue kit, since both kits gave sufficient yield and integrity but resulted higher inter-sample variation among replicates.

This study showed that the fish gut microbiome differs from that of the feed and environment (tank water and inlet water). Regardless of the DNA extraction method, most of fish microbiomes clustered together separately from that of the water samples. Conversely, microbiomes of the feed samples clustered closer to those of the gut samples and shared higher number of OTUs compared to that of the inlet and tank water samples. Various studies have been done in rainbow trout to see the influence of feed on shaping the gut microbiome, and it has been shown that the diversity and abundance of microbiomes in rainbow trout increases gradually after the first feed they eat. For example, plant-based feed may increase the abundance of phylum Firmicutes whereas marine based feed increases the abundance of phylum Proteobacteria[33]. These findings suggest that fish may acquire at least some of their microbiome from the feed they eat[10, 33, 34]. Regarding the water samples, the gut samples shared relatively higher number of OTUs with the tank water compared to that shared with the inlet water. The tank water likely contains bacteria that has leached from feces, or fecal particulates, which may explain the higher OTUs shared among gut and tank water in comparison to inlet water source. Fish were also reared in partial reuse water (60% recycled) which may contribute to the sharing of OTUs between the water and gut samples.

We found that certain taxa were indicators of the fish growth rate and fish breeding family. The indicator taxa associated with slow growth rate seem to be harmful/pathogenic bacteria whereas the indicator taxa of fast-growing fish seem to have a mutual beneficial relationship with the host. *Corynebacterium* and *Paenibacillus* which are known pathogens [35] were more prevalent in slow-growing fish. The toxins produced by these bacteria cause swelling and abdominal discomfort due to fluid accumulation and sometimes also lead to development of circumscribed lesions and lethargic behavior[36]. Conversely, bacteria belonging to phylum Firmicutes; *Lactobacillus*, *Lactococcus*, family *Propionibacteriaceae* and phylum *Bacteroidetes* were significantly more abundant in the fast-growing fish. These bacteria produce microbial metabolites such as short-chain fatty acids during glucose fermentation[37]. Such fatty acids can improve growth rate of rainbow trout[3]. These bacteria, perhaps, can be used as probiotics since they produce enzymes for fatty acid degradation, help in breakdown of food and produce valuable nutrients and energy[38-41]. These microbiomes also induce mucus production which acts as a barrier for pathogenic bacteria and sometimes also leads to production of antimicrobial peptides. In addition, the *Bacteroidetes* produce inhibitory substances like bacteriocin which initiates pathogenic bacterial cell lysis or growth inhibition[38]. *Lactobacillus* has been proven to inhibit the pathogens and, therefore, used as preservatives for food storage since they can induce the barrier function in the host epithelium against pathogens[42].

Moreover, families *Lachnospiraceae*, *Planococcaceae*, *Leptotrichiaceae*, and *Peptostreptococcaceae* belonging to the phylum Firmicutes were indicator taxa for the fast-growing fish in this study. The high abundance of phylum *Firmicutes* in host is associated to obesity or increase body weight in host as the bacteria belonging to this phylum can take part in lipoprotein degradation, fatty acid accumulation in adipose tissue, in addition, these bacteria consists of nutrient transporter gene[43-45]. Bacteria belonging to class *Lachnospiraceae* reside in digestive tract, produce butyric acid, aid in amino acid fermentation, protein digestion, absorption of fatty acids, associated with weight gain and prevention of different diseases due to microbial and host epithelial cell growth[46, 47]. On the other hand, bacteria like *Sellimonas*, *Clostridium*, *Peptostreptococcus* in fast-growing fish can take part in fermentation of different amino acids, lactates and sugars. *Clostridium* are more likely to produce cellulase enzyme and result in degradation of the cellulolytic fibers, whereas other bacteria like *Sellimonas* produce propionate which initiates gluconeogenesis and also produces xylosidase enzymes that aid in digestion of the animal feeds. The most widely prevalent and statistically significant indicator taxa of the fast-growing fish, *Peptostreptococcus* and *Clostridium*, are more likely be involved in amino acid fermentation that ultimately leading to amino acid absorption in host gut. *Leptotrichia*, the most abundant taxa in gut of all the fast-growing fish are cellulose-degrading bacteria, therefore, amylase and cellulase activity are expected to be more prominent in the host inhabiting this bacteria[48]. Similarly, the class *Enterobacteriaceae* was found to be significantly abundant taxonomical class in most of the fast-growing fish. *E. coli* belonging to class *Enterobacteriaceae* group has proven to be associated with weight gain in human infants[49].

Strategies such as manipulation of plant and animal sources for nutrition have implications for the gut microbiome of fish[50, 51]. For example, plant-based feed may constitute different microbiota in comparison to animal-based feed. Many enzymes producing bacteria residing inside gastrointestinal tract produce enzymes like amylase, lipase, cellulase aid in the digestion of the different metabolites[52]. The most abundant phylum *Firmicutes* found in gut are more importantly associated to digest polysaccharide such as cellulose, hemicelluloses and xylan. On the other hand, *Bacteroidetes* and other Lactic Acid Bacteria induce production of antimicrobial agents to inhibit the growth of other pathogenic bacteria in gut[53] improving the host immune system and metabolism.

Finally, although most of microbiota were shared among the fish families, some unique taxa were characteristic for each family which suggests that genetics is a contributing factor that affecting the gut microbiome. Unique taxa included *Trueperiolla*, *Kocuria*, *Lactobacillus*, *Propionibacteriaceae*, and *Lactococcus*. *Kocuria* has been reported to induce the protective immune system in rainbow trout by inhibiting pathogenic bacteria like *Vibrio* [54]. Differences in microbiota among the families suggest that genetics affects the susceptibility of the fish to colonize specific members of the microbiota from the environment. However, it should also be acknowledged that early periods of development occurred in different tanks specific to each family. Although all four tanks were positioned sequentially, utilized the same water source (inlets came originated from the same pipe), and consumed identical feed, it is unknown if the microbial communities within each tank differed and, if so, how they could have persisted through the subsequent 12-month grow-out period. It is also unknown if there is vertical microbiome transmission from the parents to progeny or if maternal fecal contamination of eggs during manual egg stripping contributes to the offspring microbiome. Further research is needed to validate familial differences and determine the contribution of genetic and environmental factors to development of the gut microbiome.

Conclusions

This study showed that DNA extraction methodology should be taken into account in accurate profiling of the gut microbiome. The fish gut microbiome is different than that of the environment (feed and water). However, feed and gut shared a large portion of their microbiome suggesting significant contribution of the feed in shaping the gut microbiota. Some bacterial taxa were found to be significantly different between fish families, perhaps due to host genetics, unique early rearing environments, or vertical microbiome transmission. Although population-level microbiome differences were not found to be significantly associated with the fish growth rate, several indicator taxa were determined in the fast- and slow-growing fish. For future studies, some of these important taxa can be investigated for potential use as probiotics to improve the gut microbiota of rainbow trout.

Declarations

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Availability of data and materials

All data are provided in additional files

Authors' contributions

Conceived and designed the experiments: PC, BA, BC, MS. Performed the experiments: PC, BA, BC, DW, MS. Analyzed the data: PC, BA, DW, MS. Wrote the paper: PC, DW, MS. All authors reviewed and approved the publication.

Ethics

Institutional Animal Care and Use Committee of the United States Department of Agriculture, National Center for Cool and Cold Water Aquaculture (Leetown, WV) specifically reviewed and approved all husbandry practices used in this study (IACUC approval #098).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Additional Files

Additional file 1	Detailed description of the DNA extraction methods
Additional file 2	Mothur Standard operating procedure
Additional file 3	Mothur Analysis log file
Additional file 4	Metadata file for Fast and Slow, Extraction Technique
Additional file 5_a 5_b	Mothur final output file; a) Shared file of fast and slow fish, b) Shared file of Extraction techniques
Additional file 6	Primer analysis file of fast and slow
Additional file 7	Primer analysis file of the extraction techniques
Additional file 8_a and 8_b	Mothur output file: a) Taxonomy file of fast and slow, b) Taxonomy file of the extraction techniques
Additional file 9	Taxa information of gut, feed and water samples using Promega extraction technique

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Tables

Table 1. Number of phyla, classes and genera in gut, feed and water samples using the Promega DNA extraction method.

Samples	Phylum	Class	Genus
Gut	5	10	130
Feed	5	11	90
Water	6	10	58

Table 2. Comparison of five different DNA extraction methods for microbiome analysis on the basis of cost, concentration, time duration for sample processing

Extraction Kit	Manufacturer	Principle	Bead Beating	Concentration(ng/ul)	A260/230	Cost per sample	Time duration	Hazardous waste
Power Soil	MoBio	Manual	Yes	6.49±9.09	1.78±0.18	\$6.48	6 hrs	Moderate
Maxwell	Promega	Automated	Yes	28.76±12.44	1.72±0.17	\$7.40	1.5 hrs	Least
Phenol:Chloroform	Sigma	Manual	No	257.1±285.0	1.73±0.08	\$4.50	2 days	High
Qiagen_Stool	Qiagen	Manual	No	25.1±10.07	1.92±0.16	\$5.60	5 hrs	Less
Qiagen_Blood and tissue	Qiagen	Manual	No	35.2±2.7	1.72±0.01	\$4.20	5 hrs	Less

Table. 3: Indicator analysis of the taxa for growth rate using Mothur. P-value indicates the significant taxa to act as the indicator of the fast-growing or slow-growing fish

Growth	Phylum	Class	Order	Family	Genus	Abundance	Indicator Value	p-value
Fast	Firmicutes	Clostridia	Clostridiales	Clostridiaceae_1	Clostridium_sensu_stricto_1	1589	86	<0.001
	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Sellimonas	1265	66	0.03
	Fusobacteria	Fusobacteriia	Fusobacteriales	Leptotrichiaceae	Leptotrichia	940	75	0.03
	Firmicutes	Clostridia	Clostridiales	Clostridiaceae_1	Clostridium_sensu_stricto_18	761	78	0.04
	Firmicutes	Clostridia	Clostridiales	Family_XI	Tepidimicrobium	456	77	0.03
	Firmicutes	Bacilli	Bacillales	Planococcaceae	Planococcaceae_unclassified	388	79	0.01
	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Lachnospiraceae_unclassified	357	78	0.02
	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	Peptostreptococcus	139	80	0.01
Slow	Actinobacteria	Actinobacteria	Corynebacteriales	Corynebacteriaceae	Corynebacterium_1	10033	74.07	0.01
	Firmicutes	Clostridia	Clostridiales	Peptostreptococcaceae	Paeniclostridium	958	65	0.04

Table. 4: Indicator analysis of the taxa for fish families using Mothur.

Due to technical limitations, Table 4 has been placed in the Supplementary Files section.

Figures

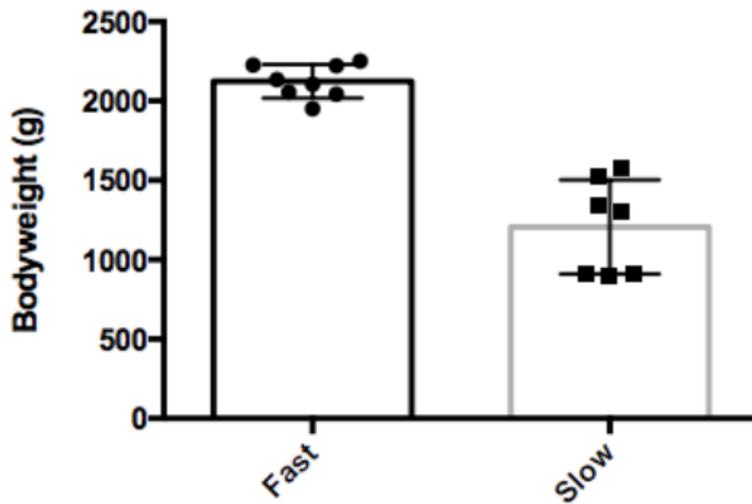


Figure 1

Mean weight of fast-growing and slow-growing fish. The error bars indicate standard deviation.

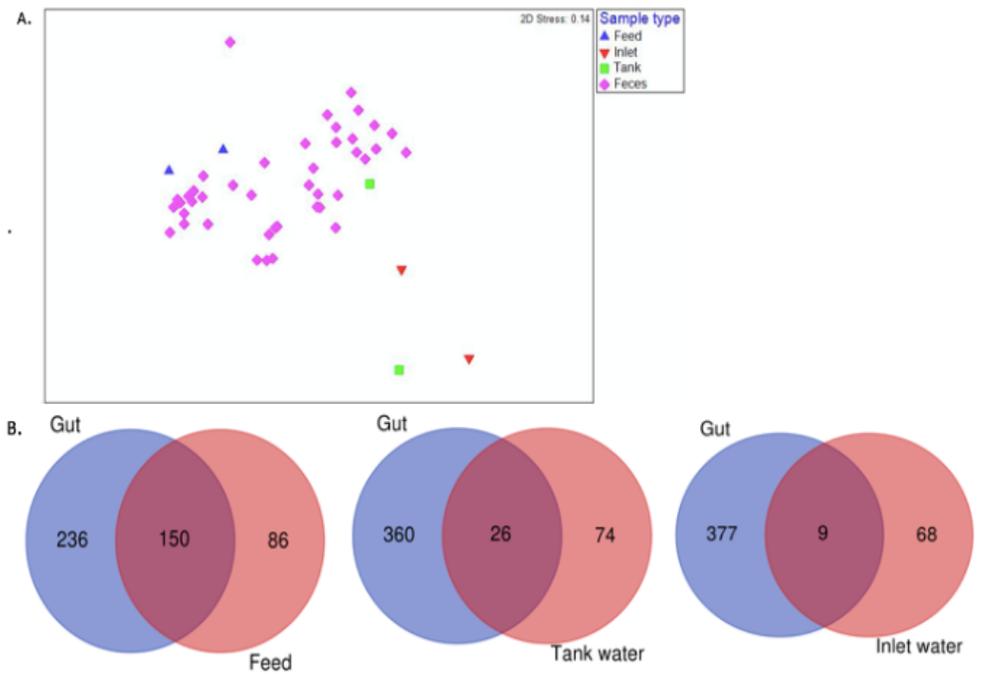


Figure 2

A) nMDS representation of bacterial community of fish gut, feed and water samples using all three extraction methods (stress value=0.14). Feed sample clustered together with fecal samples, whereas most of the water samples clustered apart from fecal samples. B) Venn-diagram of common and unique OTUs in gut, feed and water samples.

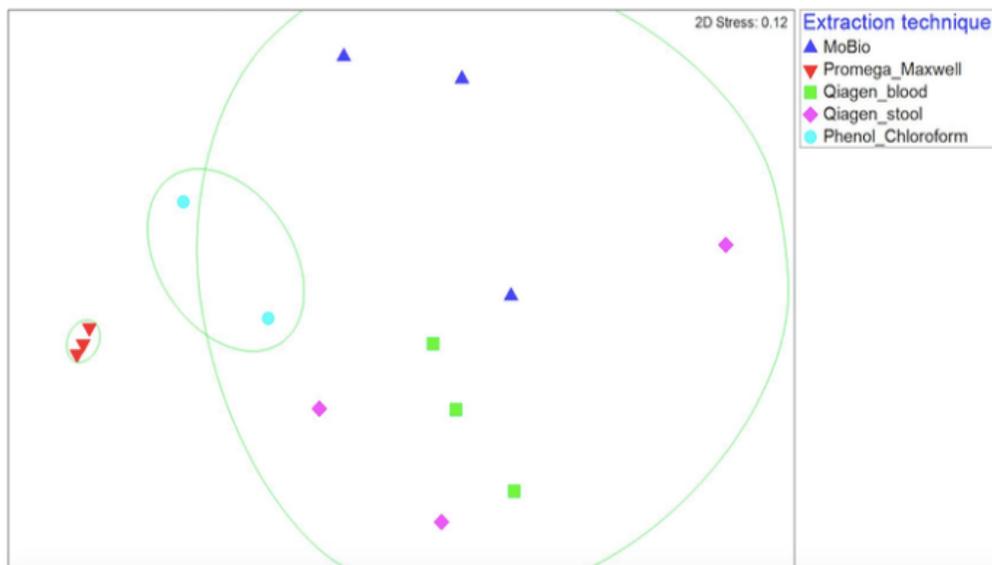


Figure 3

nMDS representation of three replicate pooled samples using 5 different extraction methods (stress value=0.12). Each extraction method is significantly different ($p < 0.05$). SIMPROF analysis tested for significant distinct cluster. One of the phenol-chloroform samples did not pass the QC and was excluded from the analysis.

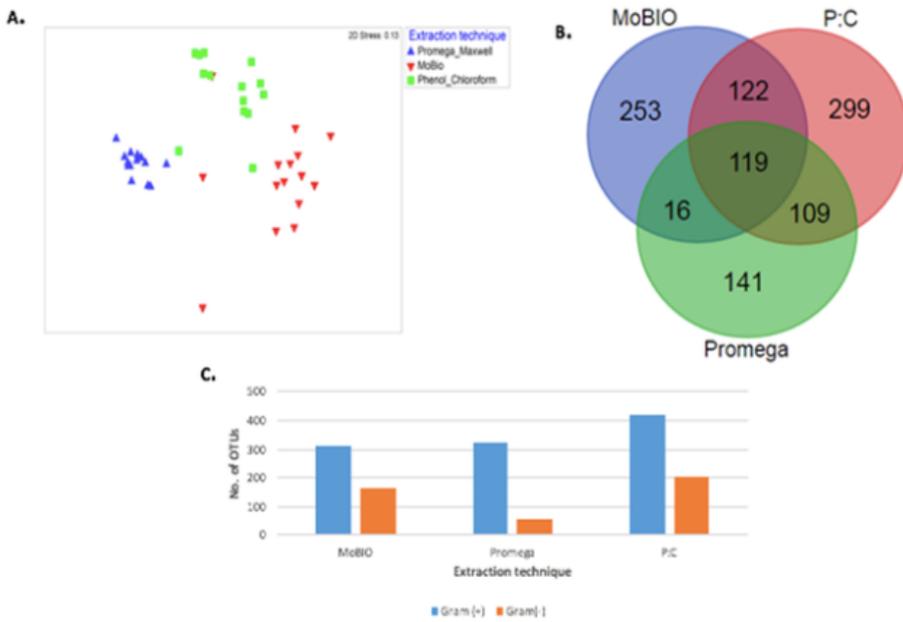


Figure 4

A) nMDS representation of the fecal samples using three different extraction methods. Samples were clustered on the basis of Bray-Curtis distance matrices (stress value=0.13). B) Venn Diagram depicting the common and unique OTUs in three different extraction methods, P:C indicates phenol chloroform C) Abundance of Gram-positive and Gram-negative bacteria on rainbow trout gut using three different extraction methods.

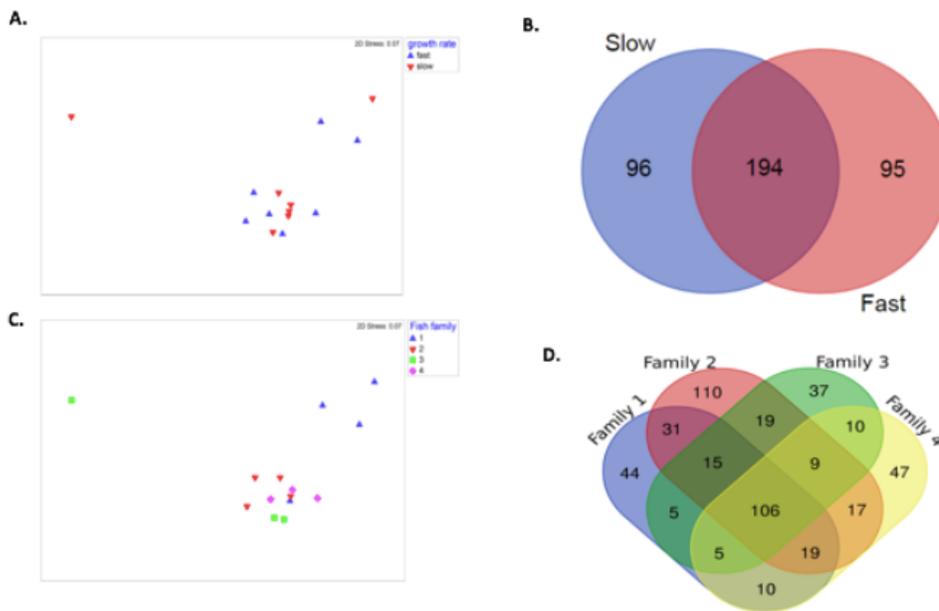


Figure 5

A) nMDS representation of the fast- and slow-growing fish using Promega extraction method (stress value=0.07). B) Venn-diagram depicting the common and unique OTUs in fast-growing and slow-growing rainbow trout C) nMDS representation of the fish family on the basis of dissimilarity matrices (stress value=0.07). Most of the samples from family 1 were clustered apart from families 2, 3 and 4. D) Venn representation of the common and unique OTUs among four different families.

Supplementary Files

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