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Distinct Microbial Assemblages Associated with Genetic Selection for High- and Low- Muscle Yield in Rainbow Trout

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Research article

Keywords: Aquaculture, Gut microbe function, Microbiota, Selective Breeding, ARS-FY-H, ARS-FY-L

Posted Date: May 15th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-26816/v1

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Version of Record: A version of this preprint was published on November 23rd, 2020. See the published version at https://doi.org/10.1186/s12864-020-07204-7.

Abstract

Background Fish gut microbial assemblages play a crucial role in the growth rate, metabolism, and immunity of the host. We hypothesized that the gut microbiota of rainbow trout was correlated with breeding program based genetic selection for muscle yield. To test this hypothesis, fecal samples from 19 fish representing an F2 high-muscle genetic line (ARS-FY-H) and 20 fish representing an F1 low-muscle yield genetic line (ARS-FY-L) were chosen for microbiota profiling using the 16srRNA gene. Significant differences in microbial population between these two genetic lines might represent the effect of host genetic selection in structuring the gut microbiota of the host.

Results Tukey's transformed inverse Simpson indices indicated that ARS-FY-H samples have higher microbial diversity compared to those of the ARS-FY-L (LMM, $\chi2(1) = 14.11$, $p < 0.05$). The fecal samples showed distinct clusters with significant differences in microbial assemblages between the genetic lines ($F_{1,36}= 4.7$, $p < 0.05$, $R^2 = 11.9\%$). Further, Tax4Fun analyses predicted characteristic functional capabilities of the microbial communities in the ARS-FY-H and ARS-FY-L samples.

Conclusion The significant differences of the microbial assemblages between ARS-FY-H and ARS-FY-L indicate an effect of genetic selection on the microbial diversity of the host. The functional composition of taxa demonstrates correlation of the function in improving the muscle accretion in the host, probably, by producing various metabolites and enzymes that might aid in digestion. Further research is required to elucidate the mechanisms involved in shaping the microbial community through host genetic selection.

Background

Aquaculture is one of the fastest-growing industries and plays a vital role in fulfilling the global requirements for human protein consumption [1]. Growth rate and muscle yield are key traits affecting the profitability of aquaculture. Understanding the mechanisms for fast and efficient muscle growth is beneficial for developing strategies that improve these characteristics. Muscle growth in farmed fish is influenced by host genetics, and factors such as nutrition and environmental condition [2]. Traditional phenotype-based genetic selection is used to improve fish production traits. However, it is not possible to apply this muscle-yield selection strategy on potential breeding candidates since measuring this trait requires sacrificing the fish prior to sexual maturation [3]. Family-based selection procedures have been undertaken by the United States Department of Agriculture at the National Center for Cool and Cold Water Aquaculture (NCCCWA) to improve growth rate and muscle yield in rainbow trout. The 5th-generation fast-growth line families were the base population for the fillet yield selection lines [4]. A family-based selection for muscle yield in a closed, pedigreed population was used to develop high muscle-yield (ARS-FY-H), randomly mated control (ARS-FY-C), and low muscle-yield (ARS-FY-L) genetic lines.

The gastrointestinal compartments of fish contain large microbial communities that play an essential role in homeostasis, physiology, and gut development [5–7]. Microbiota residing in the host gut act as a barrier for the colonization of pathogenic bacteria [8]. These bacteria can produce vitamins B and K,

short-chain fatty acid, butyric acid, and different antimicrobial metabolites, which may improve the host growth rate and muscle percentage [9]. Host genetics also play a crucial role in shaping the gut microbiome [10]. In addition to host genotype, diet alteration (plant- and animal-based meal) can change the population of the host microbiota as fish subsequently obtain their microbiota from the first-feed they eat [11, 12]. In humans, previous studies showed influence of gut microbiota in muscle fitness and degradation [13, 14]. Symbiotic microbial population residing in humans supply short-chain fatty acids (SCFAs) to the skeletal muscle resulting in improved muscle percentage and fitness, whereas dysbiosis (imbalance in microbiota) results in muscle degradation in host due to increased intestinal permeability and liberation of endotoxin into circulation [14–16]. Muscle constitutes about 50–60% of the fish body weight [17] and plays a significant role in the regulation of nutrient metabolism, growth, and inflammation in humans as well as in fish [18–20]. Similarly, Lahiri *et al.* reported a correlation between the gut microbiota and the skeletal muscle mass in mice. Mice lacking gut microbiota showed muscle atrophy, decreased expression of insulin-like growth factor 1, and reduced transcription of genes associated with skeletal muscle growth and mitochondrial function [21] suggesting a potential role of the gut microbiota in improving muscle yield and reducing muscle atrophy.

Microbiota transmit nutrient signals to the host, which might shape the gut microbiome in every stage of life based on diet intake, behavior change, and environmental influence [22]. Research had shown that transplantation of gut microbes comprising materials in an animal may improve the muscle mass percentage and function and, to some extent, reduce muscle atrophy markers [21]. Few studies have been done to investigate the correlation of gut microbial composition in muscle development and metabolic profile in fish. Therefore, the overall objective of our research was to study the gut microbiota in two genetic lines of rainbow trout selected for high- and low-muscle yield. We postulated that microbial diversity is associated with genetic selection for improved muscle yield.

Results And Discussion

Divergent selection was practiced for fillet yield to develop ARS-FY-H, and ARS-FY-L genetic lines of rainbow trout. The two fish groups used in this study were collected after two generations of selection and were statistically different in their average muscle yield as indicated by a one-way Mann-Whitney U test ($p < 0.05$; Fig. 1). The mean muscle yield of the ARS-FY-H was 0.53 ± 0.01%, and that of the ARS-FY-L was 0.51 ± 0.02%.

Comparison of gut assemblages in high- and low-muscle yield genetic lines

Fish were reared and harvested under identical conditions; however, there was a significant difference in gut microbes between the two harvest days in the ARS-FY-H ($F_{1,15}= 8.24$, $p < 0.05$, $R^2 = 37.06\%$) but not ARS-FY-L ($F_{1,17}= 0.85$, $p > 0.05$). Therefore, harvest day was treated as a random effect in all models to test for the main effect of genetic line. Using a linear mixed model, we tested for differences in gut alpha

diversity between fish genetic lines and found that diversity was higher in the ARS-FY-H (LMM, $\chi2(1) = 14.11$, $p < 0.05$, Fig. 2) when controlling for the harvest day effect. Both nMDS ordination and PERMANOVA results ($F_{1,36} = 4.7$, $p < 0.05$, $R^2 = 11.9\%$) indicated that the muscle-yield genetic line was predictive of gut microbial assemblages in rainbow trout (Figure. 3A). On running the beta dispersion analysis, there were no significant differences in multivariate dispersion between gut assemblages of ARS-FY-L and ARS-FY-H samples. In nMDS ordination, the ARS-FY-H samples formed two distinct clusters, partly due to the harvest day effect, and the samples had higher homogeneity within each cluster. Distantly, the ARS-FY-L samples were scattered. A total of 468 OTUs were shared between the two genetic lines (Fig. 3B). However, the ARS-FY-H samples had almost double the number of unique OTUs compared to the ARS-FY-L.

Together these results indicate that the muscle-yield genetic lines are predictive of gut microbial assemblages and suggest that host genetic breeding might select for particular gut microbial assemblages. This notion is supported by recent studies in tilapia, showing host genetic selection effects for thermal tolerance on the microbiome composition [23]. Similarly, studies in stickleback fish identified association between gut microbial differences and host genetic divergence [24]. Besides, previous work from our lab group revealed significant variation in beta diversity of the bacterial communities of rainbow trout families showing variation in growth rate [25]. Together, these studies indicate a substantial impact of host selection or genetics in predicting the host-associated microbial assemblages.

Taxonomy and functional diversity correlate with selection for fish muscle yield

To understand taxonomic groups of organisms that are significantly different between the two genetic lines, we ran a taxonomic profile comparison based on a Kruskal Wallis test. A total of 8 phyla, 13 classes, 36 families and 64 genera had significant differences in abundance between the two genetic lines ($p < 0.05$, additional file 1). Phyla Bacteroidetes, Fusobacteria, Deniococcus, Acidobacteria, Patescibacteria, and Nitrospora had higher abundance in ARS-FY-H. In contrast, phylum Tenericutes had higher relative abundance in ARS-FY-L (Fig. 4). Using a genus-level comparison, some unclassified genera belonging to family Burkholderiaceae and Gammaproteobacteria had higher abundance in ARS-FY-H. Similarly, Genera *Bacteroides, Deniococcus, Lutelibacter, Nitrosomonas, Pasteurella*, and *Negativibacillus* were present only in ARS-FY-H.

The significant higher abundance of phyla Bacteroidetes, Fusobacteria, Deniococcus in ARS-FY-H might be associated with the higher muscle percentage as most of the bacteria in these phyla are known symbionts and produce metabolites such as SCFAs and enzymes that are beneficial to the host [26, 27]. For example, genera in the phylum Bacteroidetes are associated with degradation of protein complex polymers and these are responsible for the formation of SCFAs like succinic acid, propionic acid, and acetic acid as the end products [28]. Besides, they produce bacteriocin, a toxin that causes pathogenic bacterial cell lysis [29]. Similarly, genera in the phylum Fusobacteria, the dominant phylum in freshwater fish guts, produce butyrate which supply energy to gastrointestinal cells and also inhibit pathogens in

freshwater [30]. Similarly, bacteria in phylum Deinococcus aid in sugar degradation and metabolism in the host gut [31]. Conversely, genera in the phylum Tenericutes had higher abundance in the ARS-FY-L samples. This phylum was found in Zebrafish gut microbiota [32]. The functional role of this phylum is not well explained yet in fish. However, a study on crabs showed that this phylum was correlated with Hepatopancreatic necrosis disease [33].

Tax4Fun analyses predicted differential functional capabilities of microbial communities in the ARS-FY-H and ARS-FY-L samples. Specifically, bacterial functional pathways related to calcium signaling, pentose and glucuronate interconversions, synthesis and degradation of ketone bodies, linoleic acid metabolism, lysine degradation, and arachidonic acid metabolism were enriched in most of the ARS-FY-H samples. Microbial pathways involved in fatty acid metabolism are known to supply energy to muscle cells, which is essential for muscle growth [34]. Genus Bacteroides belonging to phylum Bacteroidetes that showed significant differences in abundance between the ARS-FY-H and ARS-FY-L are associated with fatty acid metabolism, producing SCFAs that mediate glucose metabolism in skeletal muscle [35]. Similarly, taxa belonging to the same phylum may modulate lysine degradation in ARS-FY-H, resulting in the production of precursors of fatty acid metabolism and lipid synthesis that might enhance the muscle percentage in a host [36]. Calcium signaling pathways are associated with skeletal muscle development, maintenance, and regeneration [37]. Microbial synthesis and degradation of ketone bodies (KB), identified in the ARS-FY-H samples, were reported as associated with increased muscle mass in humans [38, 39]. Ketone bodies are an energy substrate that supply energy to the brain and muscles, contributing to the maintenance of energy homeostasis through regulation of lipogenesis [38]. Arachidonic acid metabolism is essential for the functions of skeletal muscle and the immune system, which might be associated with increased muscle mass and health in the host [40, 41]. On the other hand, bile secretion, pyruvate metabolism, amino acid metabolism, folate biosynthesis, glycosphingolipid biosynthesis, glyoxylate and dicarboxylate metabolism, adipocytokine signaling pathway and two-complement system were enriched in most of the ARS-FY-L samples (Fig. 5). Glycosphingolipids act as negative regulators of the skeletal muscle differentiation and growth [42]. Bile secretion is associated with lipid digestive functions [43] and may reduce adiposity in hosts, which might result in lower muscle mass.

Conclusion

In this study, the gut microbial assemblage (alpha and beta) diversity correlated with selectively- bred muscle yield genetic lines. Microbial differences between the two genetic lines could be observed as a host genetic selection signature on the gut microbiota. Differences in taxonomic groups of microbes and their functional predictions between the two genetic lines correlated with muscle yield. The functional composition of taxa suggests that the unique microbial profile associated with each genetic line may provide unique metabolites that contribute to differences in muscle growth and ultimately the fillet yield phenotype. The significant differences in the relative abundance of different taxa in the two different genetic lines hypothesize a role of specific taxa in improving the host muscle growth and metabolism. However, further research is required to test this hypothesis.

Methods

Fish husbandry and harvest

Fish were produced at the NCCCWA as a part of our ongoing selective breeding program. Briefly, full-sibling families (ARS-FY-H = 99; ARS-FY-L = 23) were produced from single-sire × single-dam mating events. Each family was reared separately from hatch through approximately 30 g (4 months post-hatch) when 8 fish per family were anesthetized (100 mg/L tricaine methanesulfonate, M-222) and uniquely tagged by inserting a passive integrated transponder (Avid Identification Systems Inc., Norco, CA) into the peritoneal cavity. After tagging, fish were comingled and reared in a single, 1,800-L tank that also housed contemporary fish (n = 118) from the ARS-FY-C line. The tank received identical water from a partial re-use system and water temperature was maintained at approximately 13 degrees C for the entirety of the grow-out period. Fish were split at random into a total of two 1,800-L and two 3,800-L tanks as they grew to maintain biomass densities below 100 kg/cubic meter. At approximately 13 months of age, fish used in the current study were split into three replicate 800-L tanks (n = 46 fish per tank). One week before harvest, the fish were split at random into four 800-L tanks (34–35 fish per tank) to allow the harvest of two complete tanks on each of two successive days and thus minimize netting-associated stress associated with harvesting of a partial tank. Fish were fed a commercial diet (Finfish G, 42% protein, 16% fat; Ziegler Bros Inc., Gardners, PA) using automatic feeders (Arvotec, Huutokoski, Finland) that provided feed at a daily ration that was considered as slightly below satiation.

At approximately 15 months post-hatch, fish were euthanized using an overdose of anesthetic (300 mg/L MS-222) and processed for analysis of the muscle yield trait. Fish were not fed the day prior to and the day of harvest. Twenty families were pre-selected from the ARS-FY-H and ARS-FY-L lines (40 families total within four tanks; n = 1 fish per family) for fecal collection at harvest; selection was based on divergent mid-parent breeding values and to maximize genetic diversity within each line. Due to a mortality of a ARS-FY-H fish, 39 fecal samples were collected for this study, 19 from the ARS-FY-H genetic line (11 and 8 samples from the first and second harvest dates, respectively) and 20 representing the ARS-FY-L line (13 and 7 samples from the first and second harvest dates, respectively). Fecal samples were stripped manually into sterile Eppendorf tubes (Eppendorf, Hauppauge, NY), then stored in a -80 °C freezer until analysis. Fish were eviscerated and the carcasses were placed on ice and held in a 4 °C refrigerator overnight for analysis of muscle yield the following day.

DNA extraction, library preparation and sequencing

To extract DNA, fecal samples from 19 ARS-FY-H and 20 ARS-FY-L fish were subjected to DNA isolation using a Promega Maxwell DNA Isolation Kit (Promega Corporation, Madison, WI), as we previously described [25] with a minor modification where 20 µL of lysozyme was added in samples to facilitate cell wall lysis. Briefly, 200 mg of fecal sample was added to a microtube containing 160 µL of incubation buffer, 20 µL proteinase k solution, and 20 µL lysozyme. The mixture was incubated at 70 °C overnight,

and after incubation, 400 µL of lysis buffer was added to the mixture and the sample was vortexed briefly. The samples were then subjected to the Maxwell 16 Automated DNA purification machine and the DNA was collected in a 50-µL elution buffer.

Library preparations and sequencing were done based on 16srDNA sequencing strategy using the Illumina 16S Metagenomic Sequencing Library Preparation Guide. Briefly, 10 µM of 515F and 10 µM of 806R primers amplifying V4 regions were used to target 16srRNA gene using McLAB HiFi master mix using polymerase chain reaction (PCR). The final PCR reaction consisted of 12.5 µL 2x HiFi, 1 µL of 10 µM 515F primer and 1 µL of 10 µM 806R primer, 5 µL DNA and 5.5 µL sterile nuclease-free water. The PCR product was then subjected to size selection using a magnetic bead capture kit (Ampure; Agencourt). After the first PCR clean up, dual indexed primers were used to amplify the V4 region as described by Kozich *et al.* [44]. After indexing, samples were again size selected using a magnetic bead capture kit (Ampure; Agencourt). PCR products were quantified after amplification and indexing using a Qubit fluorometer (Invitrogen, Carlsbard, CA) and fragment size (approximately 450 bp) was visualized on a 1.5% gel electrophoresis stained with SYBER safe, then samples were normalized to 4 nM. Samples were loaded onto an Illumina MiSeq flow cell and sequencing was done using 250 bp-paired end sequencing using a 500 cycle V2 reagent cartridge (Illumina, Inc., San Diego, CA) according to the manufacturer's instructions (Miseq System Guide) [45].

Bioinformatics Analysis

A total of 28,518,046 paired-end raw sequences were obtained during the Miseq run. Sequencing data were analyzed using Mothur (v.1.40.2, www.mothur.org) according to the Mothur Illumina Miseq standard operating procedure (SOP) [44, 46] with several modifications. After forming contigs, the total number of sequences was 11,020,368, and *pcr.seqs* command was used to trim primers and adaptors to the V4 region. The median length of the sequences was determined as 253 by using the *summary.seqs* command [47]. *Screen.seqs* command was used to remove sequences with length > 254 bp and < 251 bp containing homopolymers of > 8, and with ambiguous base calls. The *split.abund* command was used to keep sequences with more than two reads [48]. The SILVA v123 database [49] was used to align the sequences and sequences that failed to align, or classified as Archaea, chloroplast, eukaryotic mitochondrial, or unknown sequences were excluded from the analysis. Chimeric sequences were detected by *chimera.vsearch* and removed from the analysis. The remaining sequences were clustered using *cluster.split* [50] at a threshold of > 97% sequence similarity. Operational Taxonomic Units (OTUs) with relative abundance < 10 across all samples were removed from the analysis by using the *remove.rare* command [51, 52]. The final data set was subsampled at 2420 sequences to normalize the data set for statistical analyses. DNA extraction and library preparation blanks were included during sequencing and bioinformatics, and all OTUs within these samples were removed from the final analysis. The code used during bioinformatics analysis, taxonomy file, and shared file are all included in additional files 2, 3, and 4, respectively.

Statistical analysis

To test for the statistically significant differences of the muscle yield between the two groups, a one-way Mann-Whitney U test (Prism, GraphPad Software, Inc., La Jolla, CA) was performed. Statistical analyses (Alpha diversity, Beta diversity, microbial functional profiling pathways) were performed in R version 3.5.2 using the packages *vegan* [53] *plyr* [54] *dplyr* [55], *ggplot2* [56], *lmerTest* [57], *pheatmap*, *MuMIn* [58], *lme4* [59], *Tax4Fun* [60], *DEseq2* [61], *rcompanion* [62], *grid* [63], and *TidyVerse* [64].

Alpha and Beta diversity analysis of fecal samples between high- and low- muscle yield genetic lines

Sixteen fecal samples (that passed QC during bioinformatics analysis) from the ARS-FY-H and 18 fecal samples (that passed QC) from ARS-FY-L were used for this analysis. A Tukey's ladder of power transformation was performed to fit inverse Simpson values to a Gaussian distribution. Alpha diversity between the genetic lines was compared using a linear mixed-effect model (LMMs) with the genetic line as a fixed effect and harvest day set as a random effect (package *lme4)* [65].

Beta diversity was calculated to test if the gut microbiota was predictive of muscle yield genetic lines. To do this, a Bray-Curtis dissimilarity matrix was generated using the *vegdist* function in the Vegan package [66]. The *betadisper* function in Vegan was used to test for the homogeneity of multivariate dispersion between gut assemblages from ARS-FY-H and ARS-FY-L genetic lines. The *metaMDS* function in Vegan was used to generate non-metric multidimensional scaling ordination (nMDS) values, which were then plotted using *ggplot2* [67]. The *adonis* function in Vegan was used to perform PERMANOVA on Bray-Curtis dissimilarity values to determine if the genetic line was predictive of gut assemblages while controlling for a harvest day effect (harvest day as strata, 999 permutations). An indicator species analysis was performed in Mothur to determine the microbial assemblages that were explanatory of muscle-yield genetic lines [29]. In addition, to determine the significant differences in relative abundances of taxa between the genetic lines, Kruskal test was performed. The nMDS ordination showed a pattern suggesting a 'harvest day' based effect; therefore, we subset our samples into two data frames based on independent harvest days. Both data frames had nearly equal numbers of gut microbial samples from the two genetic lines (10 ARS-FY-H and 11 ARS-FY-L - in harvest day 1, and 6 ARS-FY-H and 7 ARS-FY-L- in harvest day 2). Separate Bray-Curtis dissimilarity matrices were generated for each data frame, followed by nMDS ordination values calculated and plotted in *ggplot2.* PERMANOVA was used to test for differences in microbial assemblages with genetic line set as a fixed effect.

Functional annotation of 16srRNA sequence data

To investigate the microbial functional and metabolic capacities of the microbial assemblies, phylotype based OTU clustering and classification was performed using the *phylotype* command in Mothur. The

shared file was then converted to the *biome* format using the *make.biom* command in Mothur. The Tax4FUN package in R was used to predict the microbial functional and metabolic capacities by linking 16srRNA gene-based taxonomic profiles to KEGG metabolic reference profile references [60]. The normalized KEGG pathway output was used to investigate the enrichment of microbial pathways between the genetic line samples using DESeq2. Important pathways associated with host-microbiome interactions and with an average FTU score of 0.55 and an adjusted p-value less than 0.001 were selected for heatmap visualization using the *pheatmap* R package[68]. The R code used during the analysis have been included in Additional file 5, and statistical results for all analysis are included in Additional file 6.

Abbreviations

ARS-FY-H: High muscle genetic line

ARS-FY-L: Low muscle genetic line

OTU: Operational Taxonomic Unit

PERMANOVA: Permutation multivariate analysis of variance

KEGG: Kyoto encyclopedia of genes and genomes.

nMDS: non-metric Multidimensional Scaling

SCFAs: Short-chain fatty acids

KB: Ketone Bodies

Declarations

Ethics approval and consent to participate

The 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).

Acknowledgments

The authors acknowledge technical and animal caretaking contributions from Josh Kretzer and Kyle Jenkins. Mention or use of trade names does not represent endorsement by the USDA. The USDA is an equal opportunity provider and employer.

Availability of data and materials

All data are provided in additional files

Funding

No funding was obtained for this study.

Authors' contributions

Conceived and designed the experiments: PC, BC, TL, MS. Performed the experiments: PC, BC, TL, DW, MS. Analyzed the data: PC, DW, MS. Wrote the paper: PC, DW, MS. All authors read and approved the final manuscript.

Consent for publication

Not applicable.

Competing interests

Mohamed Salem is a member of the editorial board of BMC Genomics. Otherwise, the authors declare that they have no competing interests.

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Figures

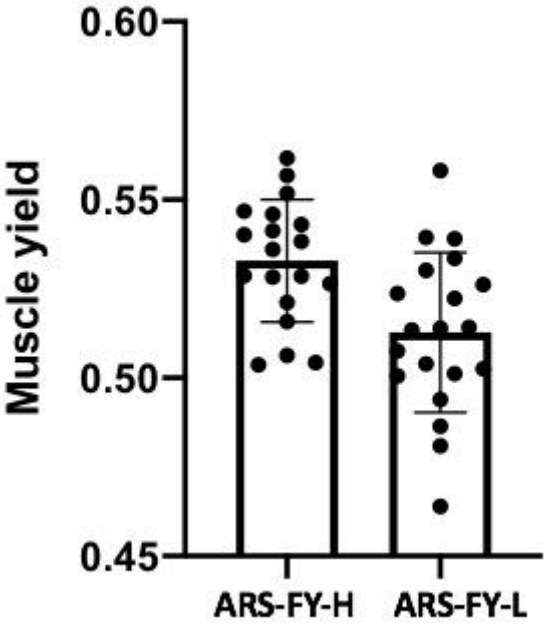

Figure 1

Significant differences in the muscle yield among samples collected from the ARS-FY-H and ARS-FY-L rainbow trout genetic lines. The statistical significance of the differences in muscle yield between the two groups was tested by a one-way Mann-Whitney U test (p=0.0029).

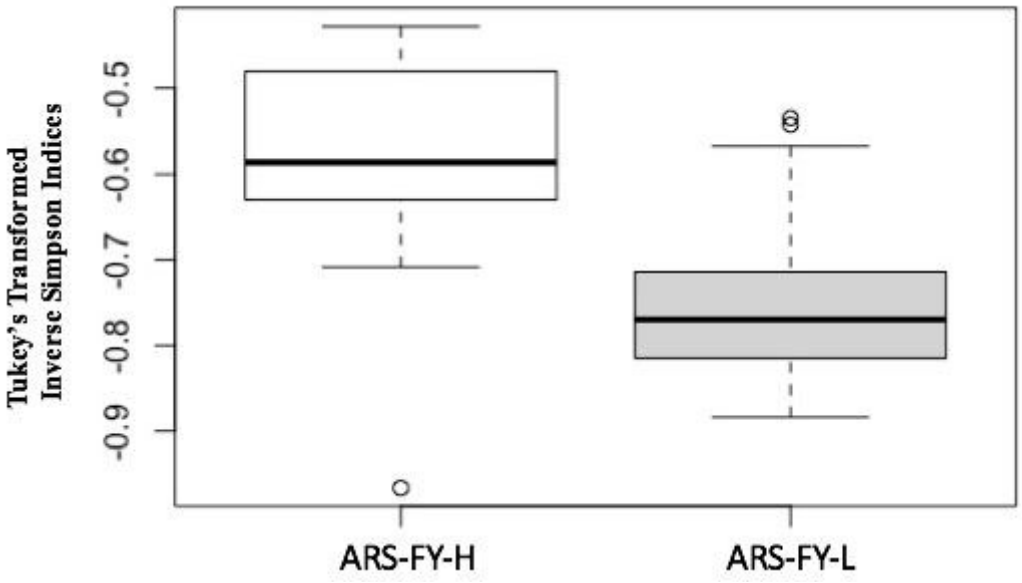

Figure 2

Alpha diversity analysis of ARS-FY-H and ARS-FY-L genetic lines using Tukey's Power transformed data. The box plots indicate higher microbial diversity in the ARS-FY-H samples ($p<0.05$). Boxplots show the median value as a bold black bar, the upper and lower limits of the box being the third and first quartile of the data, the whiskers extend up to 1.5 times the interquartile range, and open circles are outlier points.

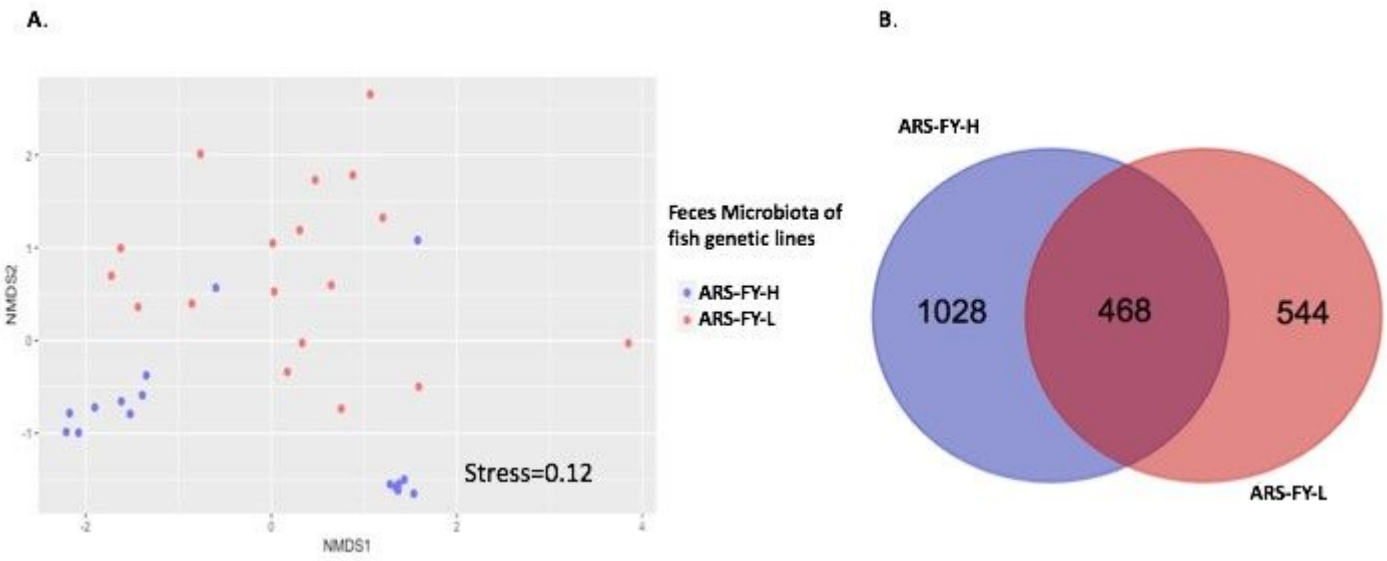

Figure 3

A). nMDS representation of the microbial diversity in the ARS-FY-H and ARS-FY-L genetic lines. A) The ARS-FY-H samples formed two distinct clusters, partly due to harvest day effect, and the samples had higher homogeneity within each cluster. Distantly, the ARS-FY-L samples were scattered. PERMANOVA results indicate significant differences between ARS-FY-H and ARS-FY-L genetic lines. B) Venn-diagram showing numbers of shared and unique OTUs between the two genetic lines.

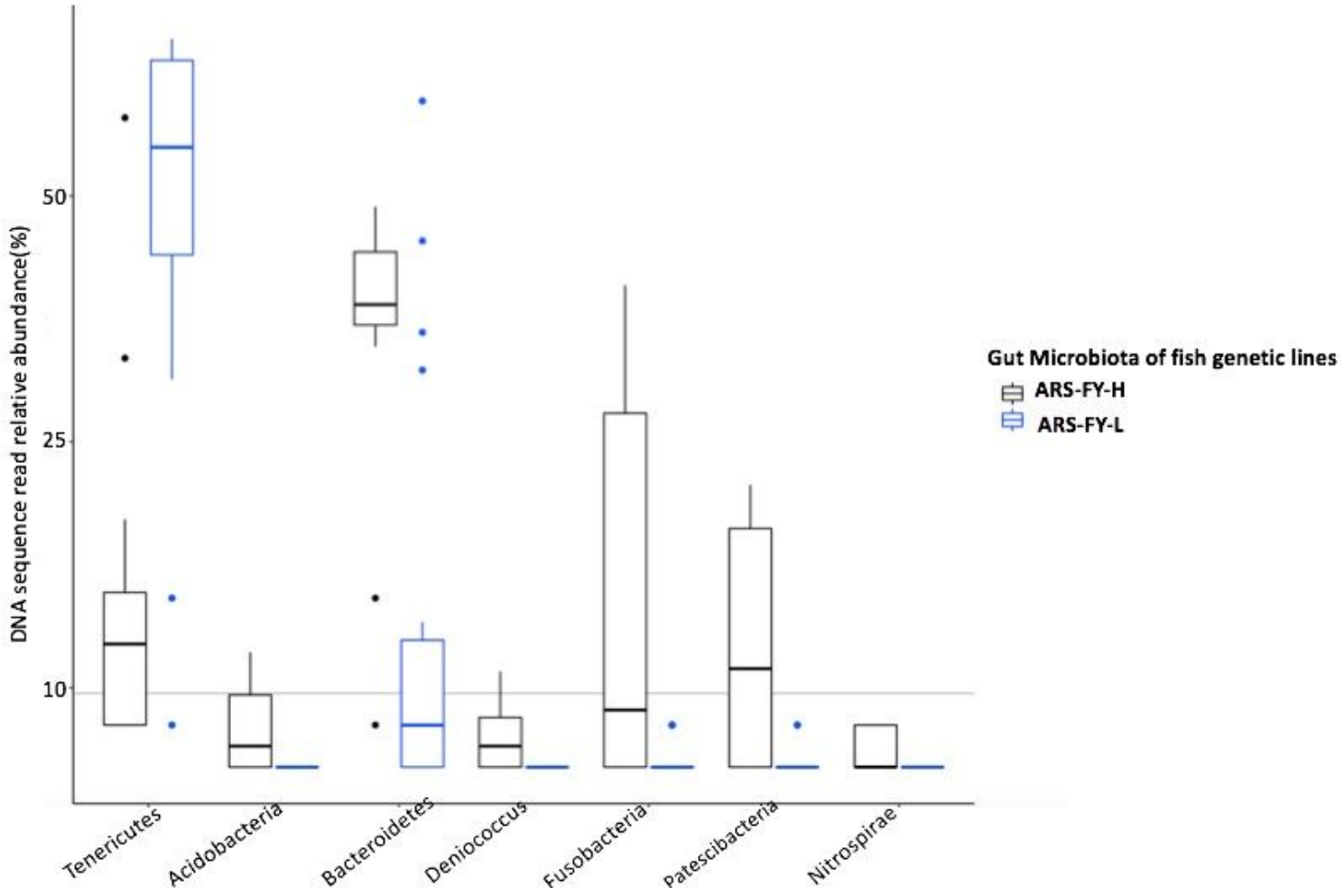

Figure 4

Significant difference in phyla diversity between ARS-FY-H and ARS-FY-L genetic lines ($p<0.05$). Boxplots show the median value as a bold black bar, the upper and lower limits of the box being the third and first quartile of the data, the whiskers extend up to 1.5 times the interquartile range, and the closed colored circles are outlier points.

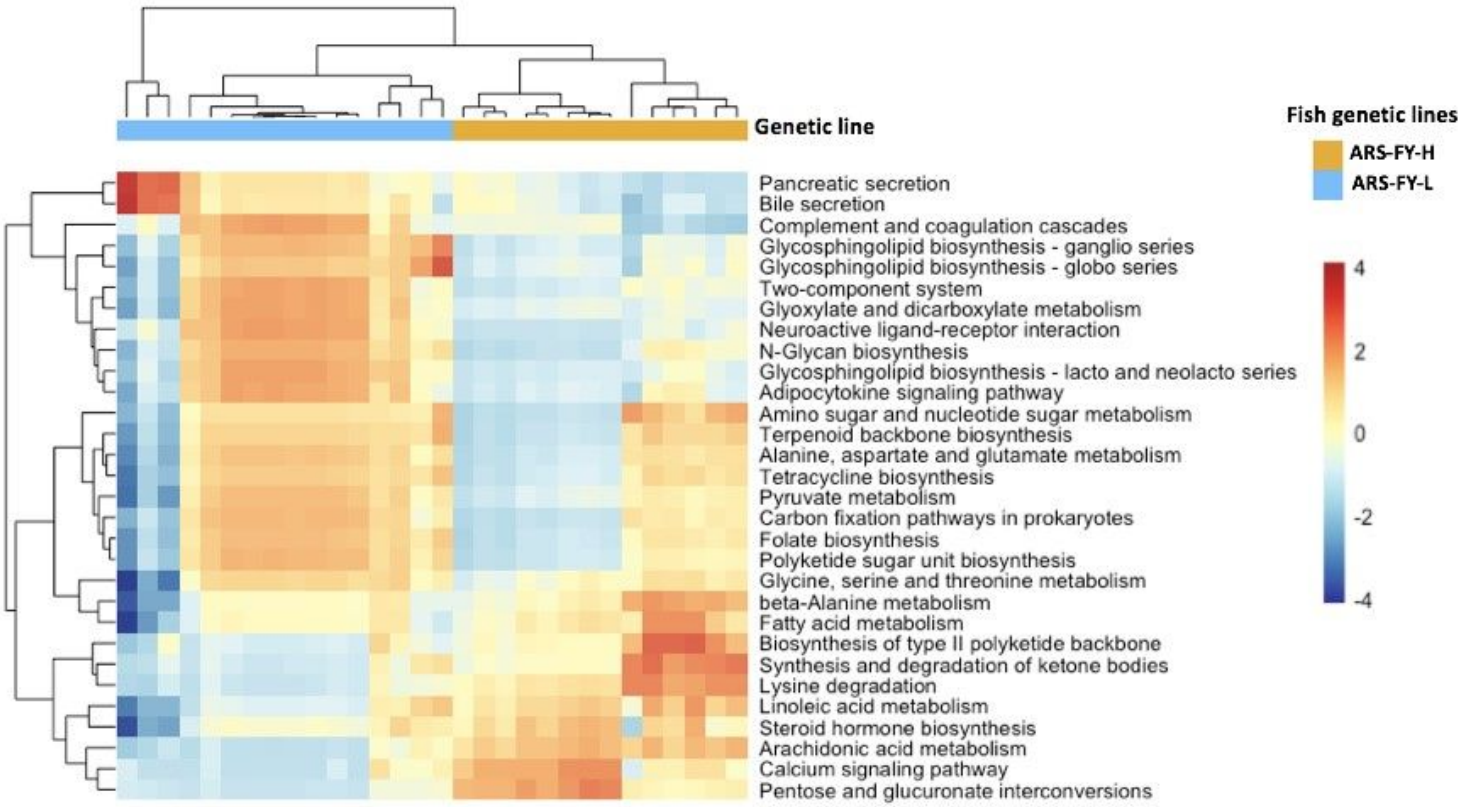

Figure 5

Heatmap showing metabolic pathways that differed significantly between the ARS-FY-H and ARS-FY-L genetic lines. Samples and pathways are clustered based on Euclidean distances. The abundance of each pathway was scaled to a range (-4, 4) with red and blue colors representing the relatively higher and lower abundance, respectively.

Supplementary Files

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

- Additionalfile1.xlsx
- Additionalfile4.xlsx
- NC3RsARRIVEGuidelinesChecklistfillable.pdf
- Additionalfile2.txt
- Additionalfile6.xlsx
- Additionalfile3.xlsx
- Additionalfile5.txt