Animal Microbiome

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Fecal microbiome analysis uncovers hidden stress effects of low stocking density on rainbow trout

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Abstract

Background Recirculating aquaculture systems can cause chronic stress in fish when stocking density is too high. However, this study tested whether low stocking density can cause fish stress. Adult rainbow trout, with an average weight of 1.517 kg (± 0.39), were subjected to low (12 kg/m3 ± 0.94) and moderate (43 kg/m3 ± 2.03) stocking densities for 24 days in a recirculating system maintained at 15 °C. At the end of the experiment, fecal microbiome analysis was carried out using a 16S rRNA amplicon sequencing. Additionally, an untargeted plasma metabolomics analysis was conducted.

Results The moderate stocking density group harboured greater numbers of commensals, such as *C. somerae*, *R. lituseburensis*, and *L. plantarum*. In contrast, detrimental species such as *S. putrifacens* and *P. putida* were abundant in the low-stocking density fish. Functional microbiome profiling revealed vitamin B12 salvage and synthesis in moderate stocking densities, which may support intestinal tight junction function. Additionally, vitamin B1 biosynthesis pathways were more abundant in the moderate stocking density group, which may function towards oxidative energy metabolism and protect against oxidative stress. A complementary plasma metabolomics study, although done at slightly different stocking densities and duration, confirmed the presence of blood metabolic stress markers. Elevated levels of L-lactic acid and L-Norvaline, L-Valine, and L-glutamine, indicate low stocking density fish were under stress. Furthermore, a *P4HA2* stress gene biomarker confirmed the occurrence of stress in low-density fish. This study suggests that low stocking density can induce stress in fish. Moreover, moderate stocking density leads to a distinct and beneficial fecal microbiome profile.

Conclusion Our study highlights the potential benefits of optimizing the stocking density of fish in recirculating aquaculture systems. This can improve fish health and welfare, promoting a more resilient fecal microbiome.

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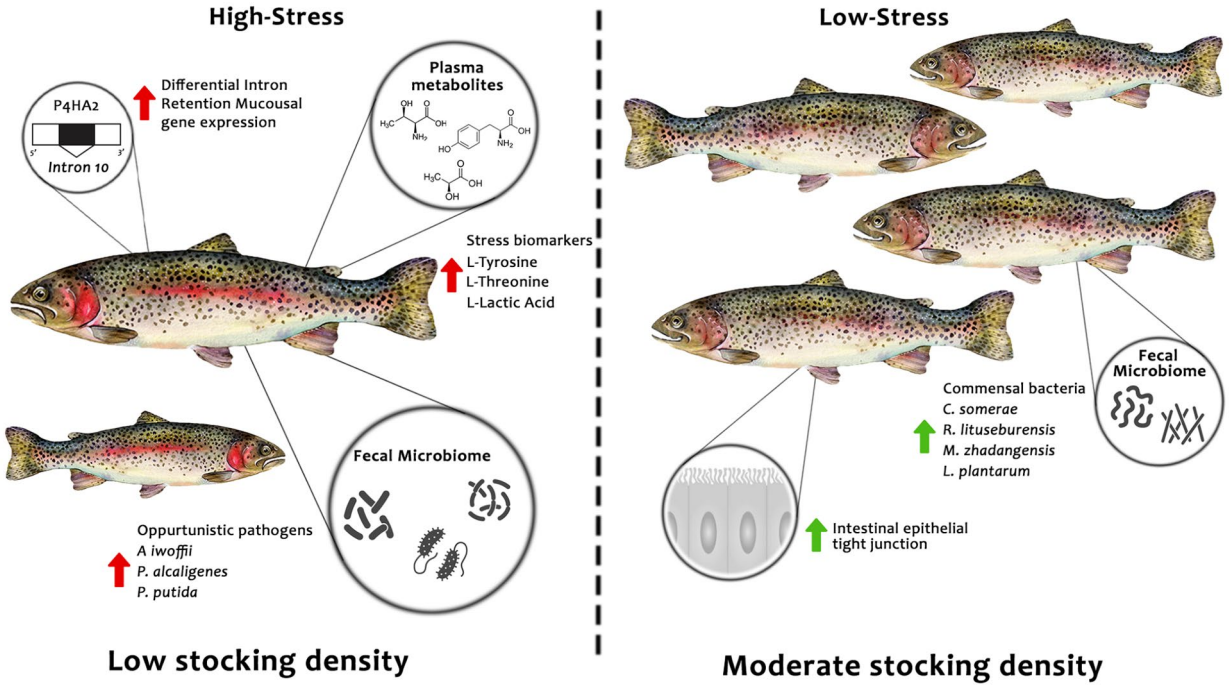
Graphical abstract



Keywords Recirculating aquaculture, Stress, Microbiome, Metabolomics, Rainbow trout, Oncorhynchus mykiss

Introduction

Intensive recirculating aquaculture exposes fish to chronic stressors such as crowding and fluctuating water quality. External stressors negatively affect fish growth and increase the risk of disease susceptibility via immune system impairment [1]. The combined effects of the nervous, endocrine, and immune systems regulate stress feedback, forcing the host to expend energy to maintain homeostasis [2]. The recurrent response to persistent stressors is maladaptive to the host and may hinder immunocompetence, feeding and growth performance, resulting in pronounced economic impacts for producers [3].

Due to the association between stress exposure and immune system impairment, adverse health effects and pathology may arise from common aquaculture practices, which jeopardize fish health and broader industry sustainability and profitability [1]. The native microbiome has evolved to participate in intricate connections between the gastrointestinal and immune systems [4]. The gut microbiome and downstream metabolites are noted to maintain host health via nutrient uptake, digestion, metabolism, and immune system functioning under homeostasis [5].

Chronic stress is known to disturb the gut microbiome and create long-term health defects in mammals. However, there is a lack of research focussed on stress-induced dysbiosis and illness in fish [6]. Increased blood plasma cortisol levels have been found to correlate with distinct gut bacterial profiles. Specifically, stress-perturbed microbiome profiles exhibit notable proliferation of opportunistic pathogens [7]. Studies have indicated that stress exposure can considerably change microbial communities in mice. In particular, the *Lactobacillaceae* family was found to be reduced in stress-challenged mice [8]. Due to the important effects of lactobacilli on preventing intestinal damage in the face of bacterial infections, reductions in their population may create significant leeway to pathology [9].

Few studies have investigated the effect of low stocking density on rainbow trout (*Oncorhynchus mykiss*). Bromage et al. reported an elevated stress response in juvenile rainbow trout stocked at 10 kg/m^3 compared to 80 kg/m^3 over 8 months [10]. Additionally, Marandel et al. similarly reported heightened plasma cortisol signatures in juvenile fish reared at 6.55 kg/m^3 compared to those reared at 37.87 kg/m^3, as well as 50% reduced survival at a low stocking density after 12 weeks [11]. Evaluation of stress response in juvenile Atlantic salmon revealed a diminished cortisol response after a 3-week paradigm consisting of unpredictable chronic stressors [12]. In addition, increased growth performance at high densities in

rainbow trout is suggested to be related to energy retention, as fish expend less energy in locomotion and can therefore maximize somatic growth [13].

Several studies outlining the relationship between stress and gut microbiome have revealed notable interactions in salmonids. Chasing stress was found to impart significant alterations to the gut microbiome of rainbow trout exposed over a period of 59 days, specifically resulting in increases in *Cetobacterium* and *Mycoplasma* abundances [14]. Juvenile Atlantic salmon subjected to a 2-week mild confinement stressor revealed a noteworthy flourishment of *Clostridia Gammaproteobacteria* in the gut macroenvironment concomitant with diminishment of lactic acid-producing *Carnobacteria*. Additionally, Uren et al. reported mucus-derived cortisol as lower than feces-derived, and reported no notable alterations to the skin microbiome [15]. Conversely, cold-shock stress during late embryogenesis resulted in pronounced epidermal microbiome perturbance in Atlantic salmon fry when interrogated 4 months post-hatch, supporting the notion of unique, tissue-specific microbiomes enhancing distinct environmental coping mechanisms [16].

Trout are known to establish social dominance hierarchies in low stocking density environments [11]. In such settings, some individuals may squander energy by engaging in aggressive behavior, which in turn leads to submissive individuals expending energy during chasing or wound healing. Therefore, low stocking densities may be harmful in rainbow trout aquaculture, due to increased aggression, which could negatively affect feed conversion and somatic growth. However, this issue has not received enough attention [17]. In mice, the variable presence of butyrate-producing taxa in the gut microbiome has been demonstrated to be a key regulator of social dominance hierarchies through the action of histone deacetylase 2 (*HDAC2*) activity localized in the medial prefrontal cortex (mPFC) [18]. Evidence in insect models further characterizes the link between *HDACis* and social caste crystallization [19]. The relationship between gut microbial taxa and social dominance hierarchies remains unexplored in fish and presents a unique opportunity for an in-depth categorization of the interplay between social structure, stress status, and bacterial abundance.

In this study, we tested the hypothesis that low stocking density can induce chronic stress in fish and that the fecal microbiome of rainbow trout reared at low stocking densities differs significantly from that of conspecifics reared at moderate stocking densities. Specifically, beneficial, immunostimulant taxa are predicted to be found in greater abundance in fish reared at optimal stocking densities. Conversely, we predict a heightened abundance of pathogenic bacteria in fish reared at low stocking densities wherein pronounced social dominance hierarchies can emerge. Additionally, in this study, we employed a plasma metabolomic profiling approach, confirming the presence of metabolic stress markers, including high levels of L-lactic acid and various amino acids [20, 21], indicating that the low-density fish were under chronic physiological stress. Furthermore, we validated a novel stress gene biomarker as an alternative to conventional cortisol measures, confirming the occurrence of stress in low-density fish.

The current body of research suggests that the gut microbiome has a strong heritable component [22]. Previous work in our lab has also indicated a noticeable difference in beta-dispersion influenced by host genetic lines [23]. Additionally, a recent study found that specific microbial taxa are involved in the interaction between stress and genetic lines in rainbow trout [14]. Studying the microbiome can contribute to improving the well-being, health, and productivity of fish by mitigating the negative effects of stress and developing stress-resistant genetic lines. As a secondary objective, this study aimed to assess the impact of genetic selection on the response of rainbow trout to low-density stress. We explored the relationship between fecal microbial composition under low versus moderate stocking density in two USDA-ARS genetic lines selected for muscle yield: High (ARS-FY-H) and Low (ARS-FY-L), as previously described [24].

Methodology

Stocking density experiment

Approximate 2-year-old all-female Rainbow trout, originally obtained from the US Department of Agriculture, National Center for Cool and Cold-Water Aquaculture (NCCCWA), were exposed to 24 days of density-induced stress. Six moderate-density group replicates were stocked in ~2.5 m^3 tanks at an average of 43 kg/m^3 ± 2.03, whereas six low-stocking-density group replicates were stocked at 12 kg/m^3 ± 0.94. All other parameters, including dissolved oxygen (DO ~ 10.0 mg/L), water temperature (15–15.5 °C), pH (~7.5), total ammonia (<0.5 mg/L), nitrate (<2.0 mg/L), and nitrate (<200 mg/L), remained constant both throughout the trial duration and between the experimental conditions. Additionally, moderate and low stocking density tanks were plumbed to the same recirculating aquaculture system, sharing the same water to avoid confounding effects due to variable water quality. A total of 259 fish representing 40 genetic families representing either high (ARS-FY-H) or low (ARS-FY-L) muscle yield genetic lines, as previously described [24], were distributed between experimental conditions, ensuring equal representation of families. Throughout the trial, fish received feed proportional to the tank stocking density via manual feeding at 48-hour intervals; the quantity of feed equated to 3% of the total biomass per diem based on initial biometrics. Fish were fed Zeigler broodstock

38−10 SS 6.5 MM pellets (Zeigler Feed, Gardners, PA). After the 24-day trial, the fish were anesthetized in a pH-buffered tricaine methanesulfonate (200 mg/L, pH=7.5) bath and fecal samples were collected from 46 fish (n=23 per group) by gently stripping the fish for 16S bacterial amplicon analysis. In addition, mucus was collected via gentle swabbing along the lateral aspect for *p4ha2* gene expression quantification (n=86), with 43 samples overlapped between assays. The samples were frozen in liquid nitrogen and stored at -80 °C until post-processing.

Microbiome sequencing

A total of 46 fecal samples were sequenced for 16S rRNA at Zymo Research in Irvine, CA. The specific sample pool was chosen for equal representation of the genetic family, muscle-yield genetic line, stocking experimental condition, and tank assignment (Additional File 1). DNA extraction was conducted with ZymoBIOMICS®-96 MagBead DNA Kit (Zymo Research, Irvine, CA). To amplify the 16S rRNA gene, a Quick-16STM Plus NGS Library Prep Kit was used. More specifically, the V3-V4 regions of the gene were targeted with the Zymo Quick-16STM Primer Set V3-V4 (Additional File 2). To construct the sequencing library, real-time PCR (95°C for 10 min 1 cycle, 95°C 30 s, 55°C 30 s, 72°C 3 min each for 42 cycles) was performed to better control replication cycles using Bio-Rad SYBR green polymerase (Hercules, CA, USA) and qPCR fluorescence readings were used to measure the final PCR product; the library was pooled according to molality. Select-a-size MagBeads DNA clean (Zymo Research, Irvine, CA, USA), and concentrator was used to clean the pooled library. Next, TapeStation and Qubit were used for library measurement (Agilent Technologies, Santa Clara, CA, USA, and Thermo Fisher Scientific, Waltham, MA, USA). Blank extraction and library preparation controls were used as negative controls to assess how much biological burden was created during the wet lab methods. As a positive control, the ZymoBIOMICS Microbial Community DNA Standard was used during the preparation of each library (Zymo Research, Irvine, CA, USA). The resulting library was sequenced via Illumina MiSeq, which was cycled 600 times; a v3 reagent kit was used, and sequencing was performed with 10% PhiX spike-in as a control.

Bioinformatic data processing

Raw 16S sequence reads were used to form amplicon sequences; the Dada2 pipeline was used to eliminate chimeric sequences [25]. Uclust by QIIME (v.1.9.1) was used to assign taxonomy; Zymo's internally curated taxonomy database was also used as a reference, and chloroplast and mitochondria were removed. QIIME was used to visualize beta-diversity results [26]. Linear discriminant analysis for effect size (LEfSe) was used to distinguish taxonomic groups with significantly variable abundance [27]. PICRUST2 (v2.5.2) was used first to generate metagenomic predictions based on 16S rRNA abundance data, and second, the gene content per amplicon sequence variant (ASV) was output to illustrate microbiome functional annotation pathways [28]. The predicted pathways were analysed for variable abundance between experimental conditions using the ggpicrust2 (v.1.7.3) package [29]. Pathways exhibiting a significant Benjamini−Hochberg corrected p-value (p<0.05) were visually displayed on a boxplot produced using ggplots2 [30].

Detection of stress via P4HA2 intron 10 retention

Mucus samples from the same fish used in the stress experiment were used for RNA isolation using Trizol manufacturer extraction protocol for total RNA (Molecular Research Center, Cincinnati, OH, USA). RNA was obtained from 43 of the 46 samples used for 16S sequencing, in addition to 43 extra samples totalling 86 samples, which were used in this analysis. Reverse transcription was conducted using Applied Biosystems High-Capacity cDNA Reverse Transcription Kit (Waltham, MA, USA). A quantitative polymerase chain reaction using Bio-Rad SYBR green polymerase (Hercules, CA, USA) was conducted (95 °C 3 min 1 cycle, 95 °C 10 s, 56 °C 30 s, 60 °C 30 s for 40 cycles). Expression of the *P4HA2* intron of interest was measured against an upstream exon expressed at a steady state. Primer sequences are provided in Additional File 2. The assay was run in duplicate, with the average Ct value generated for each sample using the following formula.

ΔΔCT=ΔCT *p4ha2* intron of interest − ΔCT *p4ha2* upstream exon.

Detection of stress via blood metabolomics

A confirmation experiment was conducted to compare the blood plasma metabolite levels of rainbow trout subjected to moderate versus low stocking density regimes; 147 fish were distributed among three tanks at moderate density (53.33 kg/m^3±2.87 kg), and three tanks stocked at low density (21.25 kg/m^3±3.06 kg) for 28 days. A total of 10 mL of blood originating from the caudal vein was collected from 20 fish in anti-coagulating tubes and centrifuged at 2200 rpm for 20 min for phase separation. Tandem mass spectrometry and high-performance liquid chromatography were conducted on plasma samples by MetWareBio (Woburn, MA) [31]. Samples stored at -80 °C were thawed on ice and vortexed for 10 s, and 50 µL of the sample and 300 µL of the extraction solution (ACN: methanol=1:4, V/V) containing internal standards were mixed in a 2 mL microcentrifuge tube. The sample was vortexed for 3 min and then centrifuged at 12,000 rpm for 10 min (4 °C). A total of 200 µL

of the supernatant was collected and placed at -20 °C for 30 min, followed by centrifugation at 12,000 rpm for 3 min (4 °C), and a 180 µL aliquot of the supernatant was used for LC–MS analysis.

The data were acquired via ultra-performance liquid chromatography (UPLC) (ExionLC 2.0) and tandem mass spectrometry (MS/MS) (QTRAP®6500) methods. The liquid chromatography conditions were as follows: chromatographic column, Waters ACQUITY UPLC HSS T3 C18 (1.8 µm). The mobile phase included the following phases: A, ultrapure water (0.1% formic acid added); B, acetonitrile (0.1% formic acid added); and G, 95:5 V/V for 0 min, 10:90 V/V for 10.0 min, 10:90 V/V for 11.0 min, 95:5 V/V for 11.1 min, and 95:5 V/V for 14.0 min. Multivariate analysis of variable importance in projection (VIP) from OPLS-DA modeling was used to select differentially abundant metabolites from different samples. Individual metabolites were screened for variable abundance between stocking density conditions using a t-test ($p<0.05$).

Results & discussion

Microbiome diversity uncovers stress effects of low stocking density

Bacterial 16S rRNA amplicon sequencing identified operational taxonomic units (OTUs) belonging to 21 phyla, 43 classes, 80 orders, 159 families, 482 genera, and 1554 species (Additional File 3). Notable abundance variation in 24 OTUs between moderate and low stocking density conditions was observed (Fig. 1, additional file 4). Specifically, 7 taxonomic units were found in greater abundance under low stocking density conditions, and 17 were higher in moderate stocking density conditions.

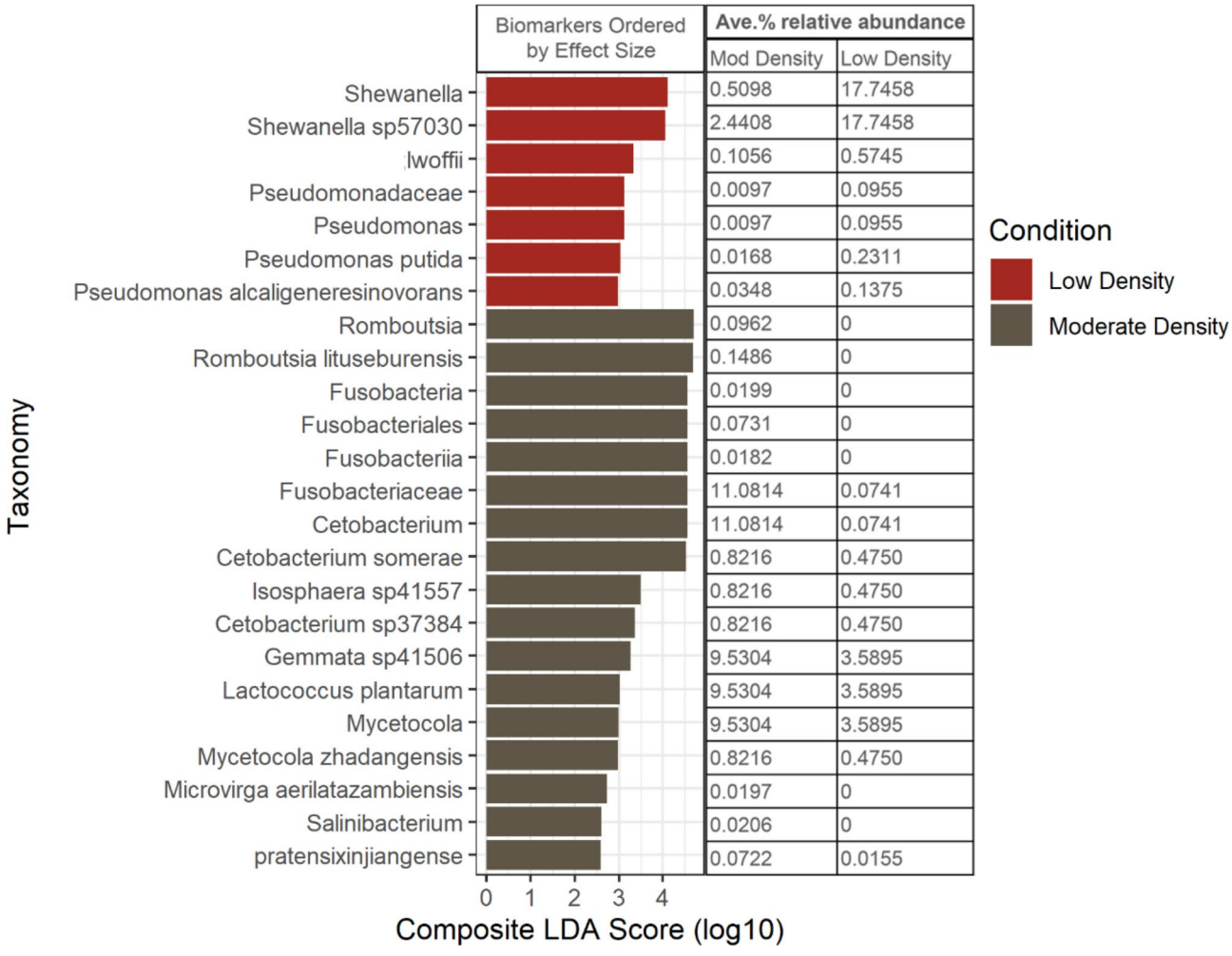


Fig. 1 Linear discriminant analysis effect size (LEfSe) analysis revealed 24 OTUs generated from fecal samples ($n=46$). Seven potential biomarker taxa were more abundant under low stocking density conditions, and 17 were more abundant under moderate stocking density conditions. The composite linear discriminant effect (LDA) scores displayed are greater than 2, and the biomarkers listed exceed a threshold for statistical significance ($p<0.05$). The average relative abundance percentage for each OTU in the moderate and low stocking density conditions is displayed on the right. The complete table containing relative abundances of differentially abundant OTU is found in Additional file 4

At low stocking densities, a significantly greater abundance of *Pseudomonas putida*, a known opportunistic pathogen in rainbow trout, was detected [32]. Nonspore-forming, gram-negative bacteria form biofilms in oxygenated aquatic environments and have been previously reported in *Plecoglossus altivelis* and *Seriola quinqueradiata* aquaculture [33]. Clinical signs reported in rainbow trout include ulcerations, dorsal lesions, necrosis, and bacterial septicaemia [32]. Notably, *P. putida* has been previously reported in epidermal and mucosal samples in fish, whereas we detected this microbe in fecal samples.

Another stress indicator found in greater quantities in the low stocking density group compared to the moderate stocking density, *Acinetobacter lwoffii*, is a recently characterized rainbow trout pathogen [34]. A strain of Acinetobacter lwoffii, capable of causing pathological damage to multiple organs, has also been isolated.

Additionally, *Pseudomonas alcaligenes* was identified in greater abundance under low stocking density conditions. *P. alcaligenes* has been previously classified as an opportunistic pathogen in several commercially relevant aquaculture species, namely Asian Swamp Eel *Monopterus albus* and the Chinese sturgeon *Acipenser sinensis* [35], as well as the ornamental Koi carp *Cyprinus carpio* [36]. *P. alcaligenes* infection induces notable mortality, preceded by clinical signs typical of bacterial infection, namely, epithelial haemorrhaging and lesions, as well as gill and submucosal inflammation in the intestines [36].

One biomarker found to be more abundant in the low stocking density group, the *Shewanella* genus, has been recorded as a pathogen in eye infections for humans, and conflicting reports both categorize *Shewanella* as potentially pathogenic bacteria or as a commensal bacteria in fish [37, 38]. Specifically, *Shewanella spp*. harbours 12 virulence genes that induce mortality in low-temperature aquaculture systems by forming biofilms on both biotic and abiotic surfaces [39]. Moreover, *S. putrefaciens* is responsible for spoilage in gadoid fish species, reducing trimethylamine oxide (TMAO) to trimethylamine (TMA) and emitting telltale spoilage and volatile off-flavour byproducts such as hydrogen sulfide [40]. Oral administration of a probiotic strain of *S. putrefaciens* isolated from gilthead seabream *to Vibrio harveyi* infected fish reduced mortality. Beneficial *S. putrefaciens* exhibits strong mucosal adhesion properties, persisting on the outer epidermis, gill, and intestinal mucous. Overall, 16S sequencing revealed a greater presence of *Shewanella* sp57030 and unclassified species with little known functional properties. Moreover, it is unclear whether the differential abundance of *Shewanella* at the genus level offers beneficial or detrimental qualities to the host.

We predicted a higher abundance of pathogenic bacteria in fish raised at low stocking densities. However, in this section, discussion of the pathogenicity of any taxa should be approached cautiously, as not all strains within a given species are pathogenic, and pathogenicity is influenced by a combination of host genetics and environmental factors.

On the other hand, *Lactococcus plantarum* was found to have greater richness in the fecal samples of fish at moderate stocking densities compared to the low stocking density. *L. plantarum* is known to improve fish welfare and is under investigation as a novel probiotic and antibiotic alternative in freshwater aquaculture due to its ability to colonize intestinal tracts and survive acidic and bile conditions common in the colonic environment [41]. Moreover, *L. plantarum* exhibits strong antimicrobial and antioxidant properties by secreting antimicrobial proteins known as bacteriocins ex-vivo. Antimicrobial proteins generate membrane pores in susceptible bacteria, leading to cell death and curtailing pathogenesis [42]. In vivo validation of probiotic and antimicrobial properties has been performed in Nile tilapia and common carp [43] as well as in rainbow trout [44].

Functional profiling of the microbiome reveals variable vitamin synthesis pathways

Differential functional capabilities of microbial communities at moderate and low stocking densities were examined using PICRUST2 (Fig. 2, Additional file 5). *Cetobacterium somerae*, a common anaerobe native to the digestive tract of freshwater fish, was reported to be more abundant under moderate stocking density conditions than the low stocking density (Fig. 1) [45]. Similarly, Suhr et al. reported a notable relationship between chasing stress, genetic line, and *C. somerae* abundance in rainbow trout [14]. In zebrafish, *C. somerae* has demonstrated immunomodulatory effects on the host attributable to innate vitamin B_{12} production [46]. An estimated 80% of the microbes inhabiting the intestinal tract of freshwater fish require vitamin B_{12} as a cofactor for steady-state metabolic activity; however, only 25% of gut microbes possess the ability to synthesize this vitamin in-situ [47]. Functional pathway analysis revealed enriched adenosylcobalamin (B_{12}) salvage from cobinamide I and II and adenosylcobalamin synthesis pathways from cobyrinate in moderate stocking density fish (Fig. 2) [48].

Exogenous B_{12} supplementation increases the expression of the crucial host gut epithelial tight barrier proteins *Claudin15*, *Occludin*, and *Zo-1* in *Danio rerio* [46]. However, the upregulation of these key proteins diminished substantially in the presence of vitamin B_{12} supplementation paired with antibiotics, supporting the link between the protective B_{12} microbiome-derived complex and host colonic epithelial tight junction activity. In the present study, we reported *Romboutsia litsurebensis* to be more abundant in the moderate stocking density group than the low stocking density group, and intestinal

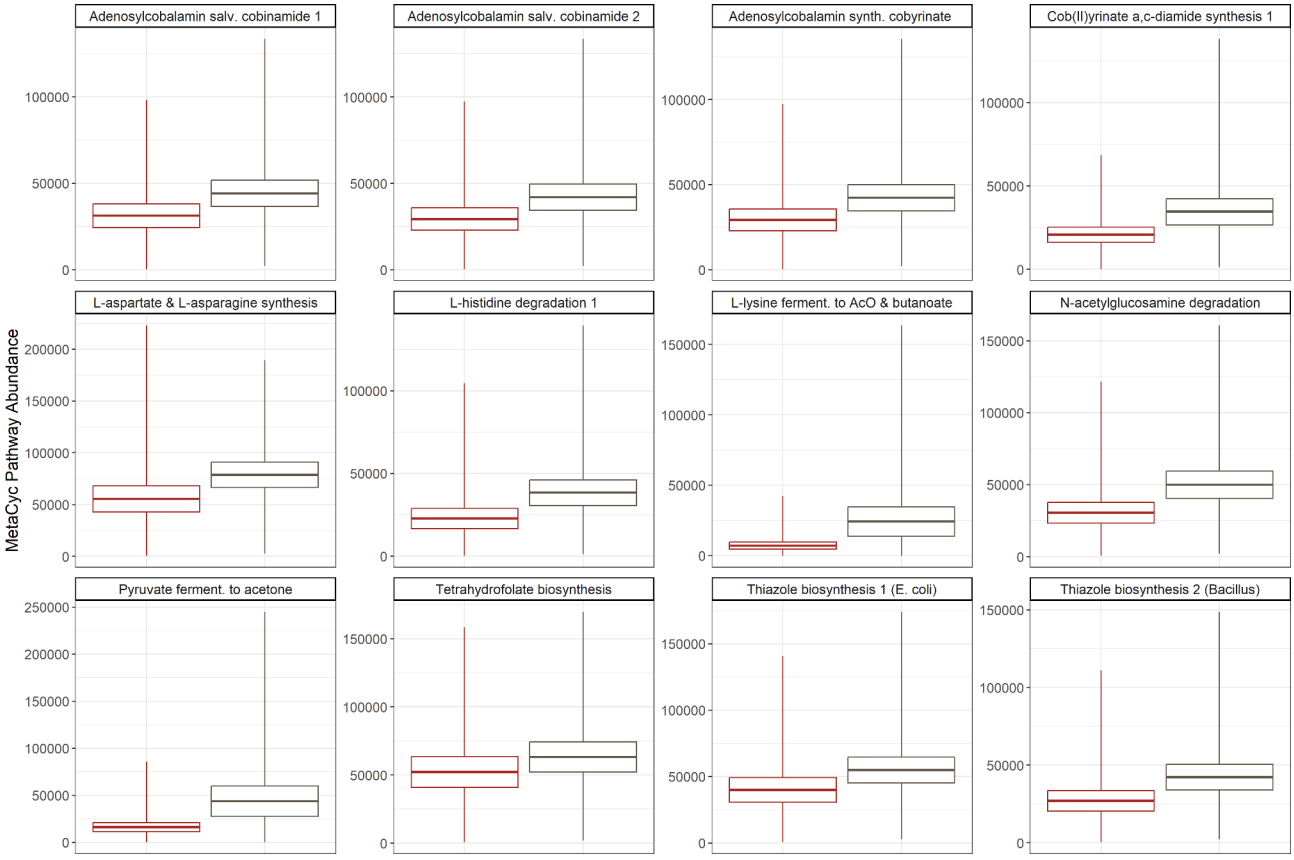


Fig. 2 Significantly differentially abundant ($P < 0.05$) MetaCyc pathways generated by PICRUST2, moderate stocking density is represented by the grey and low stocking density by red. A table containing significant EC pathways is found in Additional File 5

immunomodulatory effects appear to share commonalities with vitamin B_{12} tight junction enhancement. The glut of pathways responsible for the regeneration of adenosylcobalamin, the bioactive form of vitamin B_{12}, in the moderate stocking density group adheres to the hypothesis of B_{12}-mediated intestinal health. Increased vascular endothelial function and upregulation of the canonical intestinal tight junction proteins *ZO-1*, *Claudin5*, *Muc2*, and *Occludin* was also observed in murine models supplemented with *Romboutsia litsurebensis* [49]. Although the direct mechanism underlying the immunomodulatory action of adenosylcobalamin in the B_{12}-saturated gut environment remains unclear in freshwater teleost, we observed *C. somerae* and *R. litsurebensis* abundance as a key biomarker for a resilient microbiome primed for pathogenic insult.

The thiamine diphosphate biosynthesis super-pathway was more abundant in the moderate stocking density group than the low stocking density. Vitamin B_1 (thiamine) is indispensable for oxidative energy metabolism and is protective in reducing oxidative stress [50]. Similar to vitamin B_{12}, exogenous thiamine is absorbed through the intestine, and experimental data suggest that an estimated 81% of thiamine present in the posterior colon is derived from microbial production as opposed to dietary inclusion [51]. The thiamine deficiency complex (TDC) is linked to hepatocellular and brain cell necrosis with glycogen depletion in the liver and degeneration and glycogen depletion in muscle cells, leading to immobilization and abnormal swimming behaviour in adult rainbow trout [52]. Moreover, vitamin B1 deficiency has been shown to be vertically transmitted from mother to offspring, resulting in nearly 100% mortality in fry post-hatching [53].

Bacterial functional pathways enrichment of lysine fermentation in non-stress group

Bacterial functional pathways related to lysine fermentation to acetate and butanoate and pyruvate fermentation to acetone were found to be more abundant in the moderate stocking density group (Fig. 2, Additional File 5). Previous work categorizing the relationship between muscle yield genetic lines and fecal microbiome functional profile reported a notable enrichment of oligosaccharide fermentation to short-chain fatty acid pathways in the high muscle yield genetic lines [54]. *Planctomycetaceae isospharae* was more abundant in the moderate stocking density group. This genus has been previously

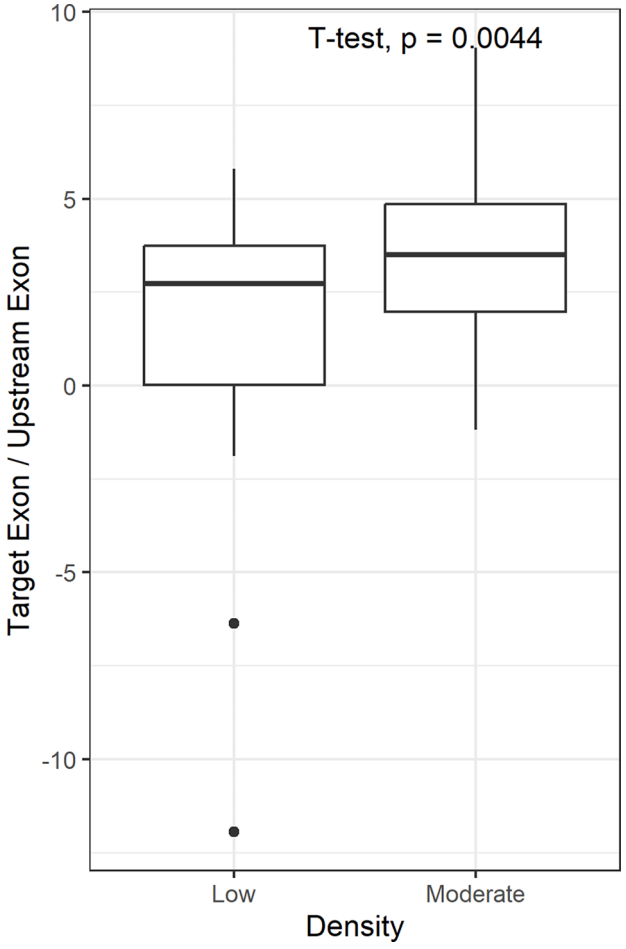


Fig. 3 Quantitative polymerase chain reaction of the *p4ha2* intron-10 retention marker compared to the upstream exon transcribed at steady state. The delta CT values obtained are inversely proportional to the intron-10 expression. Two-tailed t-tests revealed significant differences in the expression of the p4ha2 intron ($p = 0.0044$)

unreported in the intestines of freshwater fish and is commonly found in peatlands [55]. *P. isospharae* has an observed capacity for plant-derived organic matter decomposition and may contribute to the variable fermentation capacity observed between experimental groups, potentially resulting in increased somatic growth.

Stress confirmation via p4ha2 intron-10 retention marker

Plasma cortisol, blood glucose, and haematological measurements have been used as biomarkers for stress and have been well studied. However, conventional approaches are prone to circadian and endocrine-mediated fluctuations. In a yellow perch study, stress response was assessed using plasma cortisol concentrations, but the levels fluctuated within the stress conditions [56]. Differential intron retention belonging to the prolyl 4-hydroxylase subunit alpha-2 (*P4HA2*) gene was shown to serve as a generalized trout stress biomarker among several conditions [57]. Specifically, *P4HA2* catalyzes the hydroxylation of proline residues in newly synthesized collagen chains to form 4-hydroxyproline, stabilizing the collagen triple helices under nonnormal physiological conditions [58]. The retained *P4HA2* intron may lead to incomplete collagen hydroxylation, leading to protein instability and subsequent skin wounding or non-malleable and inflexible blood vessels [59].

This study used noninvasively collected mucosal samples to identify stressed fish, minimizing the need for highly invasive and onerous alternatives such as enzyme linked immunosorbent assay (ELISA). Compared with the moderate stocking density, low stocking density significantly increased prolyl 4-hydroxylase subunit alpha-2 intron-10 expression relative to an upstream exon (Fig. 3), suggesting that low-density conditions are undergoing generalized stress in the present study.

Stress confirmation via metabolomics

Blood metabolomic profiling revealed that L-lactic acid was elevated under low stocking density conditions (Table 1). This finding is notable because L-lactic acid is the terminal product of anaerobic glycolysis and has a noted regulatory function in stress-induced physiological and pathological conditions such as wound healing and ischemic tissue injury [60]. Proporato et al. demonstrated that endogenously produced lactic acid is implicated in reparative angiogenesis by recruiting endothelial progenitor cells, subsequently activating procollagen factors and leading to downstream extracellular matrix deposition in mice subjected to ischemic wounding [61]. Fish maintained at a low stocking density may exhibit increased capacity for wound healing in the form of L-lactic acid secretion as a response to repeated mechanical damage stemming from social hierarchy establishment and enforcement.

Under low stocking density conditions, several amino acids were also found in greater abundance, namely, L-Norvaline, L-Valine, and L-glutamine (Table 1). Evidence in carp indicates that supplemented dietary glutamine results in increased gene expression of intestinal tight junction proteins, specifically *ZO-1*, *occludin1*, *claudin2*, *claudin3*, and *claudin7*, in the intestinal mucosa [62]. Paradoxically, we reported a notable abundance of *R. litsurebnsis*, an immunomodulating bacterium important for maintaining intestinal tight barrier junctions partially through the action of *ZO*-1 and *Muc2* in murine models under moderate stocking conditions. Paired host transcriptome interrogation of the intestinal epithelium and fecal microbiome may elucidate the interactions between intestinal bacteria and the host metabolome under stress conditions. Notably, plasma cortisol level detected by metabolomics was not significantly different between the low and moderate stocking-density fish.

Table 1 Notable differentially abundant metabolites found in the blood plasma of rainbow trout exposed to variable stocking densities. FC denotes fold change when considering the Low/Moderate effect direction. A complete table of differentially abundant metabolites can be found in additional files 6

Compounds	Class	*P* value	FC	Low/Mod
Acrylamide	Alcohol & amines	0.0245	1.4033	up
Biotinamide	Alcohol & amines	0.0018	1.7857	up
N-acetyl-D-phenylalanine	Amino acid	0.0029	-1.3090	down
N-Acetyl-L-phenylalanine	Amino acid	0.0081	-1.2602	down
L-Threonine	Amino acid	0.0302	1.3767	up
L-Tyrosine	Amino acid	0.0443	1.4023	up
L-Norvaline	Amino acid	0.0075	1.5288	up
L-Valine	Amino acid	0.0075	1.5288	up
L-Glutamine	Amino acid	0.0087	2.3804	up
Glutathione Oxidized	Amino acid	0.0045	2.0483	up
Carnitine C5:1	Fatty Acid	0.0092	1.6642	up
Carnitine C4:0	Fatty Acid	0.0196	2.1004	up
Carnitine isoC4:0	Fatty Acid	0.0196	2.1004	up
L-Octanoylcarnitine	Fatty Acid	0.0433	2.5176	up
Carnitine C3:0	Fatty Acid	0.0245	2.59	up
Indole-3-lactic acid	Heterocycles	0.0001	-1.0726	down
1-Methyladenosine	Nucleotide	0.0138	3.3311	up
L-Lactic Acid	Organic acid	0.0121	1.4554	up

Relationship between genetic lines and stocking density stress

Host genetics impacts the gut microbiome [63] and the interaction between the host and the microbiome can influence the host's characteristics [64]. A recent study discovered that specific microbial groups are involved in the interplay between stress and genetic lines in rainbow trout [14]. A rainbow trout breeding program was established at the US Department of Agriculture, National Center for Cool and Cold-Water Aquaculture (NCCCWA). This program began as a growth-selected line in 2002 and underwent five generations of selection for improved growth performance before subsequently selecting for muscle yield, as outlined in a previous study [24]. The USDA-ARS muscle Yield – High (ARS-FY-H) and – Low (ARS-FY-L) genetic lines could be further studied to create stress-resistant genetic lines. Therefore, the current study examined the complex relationship between genetic lineage and the effects of stress due to stocking density on the fecal microbiome profile of rainbow trout.

We investigated the relationship between the fecal microbial composition and the rainbow trout High (ARS-FY-H) and – Low (ARS-FY-L) genetic lines. Bray–Curtis ordination beta dispersion measures indicated nonsignificant population-level clustering among genetic lines between the moderate and low stocking density cohorts (Fig. 4). However, a PERMANOVA test revealed a trend toward genetic line-specific clustering under moderate stocking density conditions but not low stocking density conditions. Research has highlighted the significant role of genetics in shaping the gut microbiome [65]. Previous studies have demonstrated an observable difference in beta-dispersion based on genetic lines associated with muscle yield [66]. There have been findings of a correlation between the microbial composition in fecal matter and the genetic lines of rainbow trout selected for muscle yield and growth [54, 66]. Further exploration using metagenomics approaches into the gut microbiome variation between the USDA-ARS muscle yield – High (ARS-FY-H) and – Low (ARS-FY-L) genetic lines could provide insights into the taxa linked to stress resistance.

Conclusion

We reported evidence for low stocking density-induced microbiome modulation in fish. The results highlight the importance of optimizing stocking density in recirculating aquaculture systems, which can improve fish health and welfare and promote a more resilient fecal microbiome. Additionally, the study shows that the fecal microbiome profile of fish at moderate stocking density is both distinct and beneficial, harbouring known immunostimulant species that may provide pathogenic resistance in rainbow trout. Functional profiling reveals enriched pathways for vitamin synthesis, potentially supporting intestinal tight barrier function and enhanced immunostimulant benefits to the host.

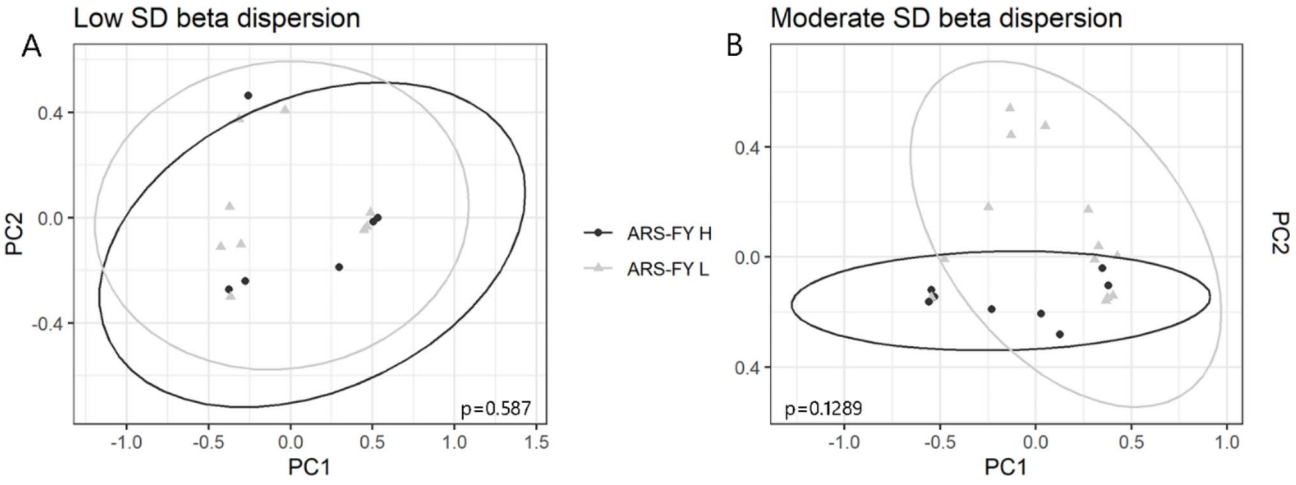


Fig. 4 Comparison of Bray–Curtis beta diversity between the ARS-FY-H and ARS-FY-L muscle yield genetic lines under various stocking density conditions using PERMANOVA. Black circles represent the high muscle yield genetic line, and grey triangles represent the low muscle yield genetic line. **(A)** Beta diversity clustering by genetic line within the low stocking density condition reflects slight dissimilarity ($p = 0.587$). (**B**) Beta diversity clustering by genetic line within the moderate stocking density condition reflects some clustering ($p = 0.1289$)

Abbreviations

ARS-FY-H	High muscle genetic line
ARS-FY-L	Low muscle genetic line
OTU	Operational Taxonomic Unit
KEGG	Kyoto encyclopedia of genes and genomes
SCFAs	Short chain fatty acids

Supplementary Information

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Supplementary Material 1

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Author contributions
G.R., F.J., R.T., and M.S. Conceived and designed the experiments. G.R., F.J., R.A., A.A., and M.S. Performed the experiments. G.R., F.J., and M.S. Analysed the data. G.R., F.J., and M.S. Drafted the paper. All authors read and approved the final manuscript.

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Data availability
The 16S rRNA sequence data supporting this study's findings have been deposited at NCBI (BioProject: PRJNA1102007).

Declarations

Ethics approval and consent to participate
The Animal Care and Use Committee of the University of Maryland reviewed and approved all practices used in this study (IACUC: 2064299).

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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