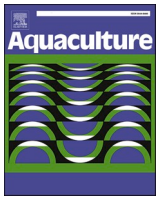
Aquaculture 594 (2025) 741448

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture



Effect of crowding stress on liver health, gut permeability and gut microbiota of genetically improved farmed tilapia (GIFT, *Oreochromis niloticus*)

Jian Zhang [a,1], Jie Chen [a,1], Hui Liang [a], Ming Li [a], Wenhao Zhou [a], Yalin Yang [b], Zhen Zhang [b], Qianwen Ding [c,*], Chao Ran [b,*], Zhigang Zhou [a,*]

[a] *Key Laboratory for Feed Biotechnology of the Ministry of Agriculture and Rural Affairs, Institute of Feed Research, Chinese Academy of Agricultural Sciences, Beijing 100081, China*

[b] *Sino-Norway Joint Lab on Fish Gut Microbiota, Institute of Feed Research, Chinese Academy of Agricultural Sciences, Beijing 100081, China*

[c] *National Collection of Livestock and Aquatic Microbes, Institute of Feed Research, Chinese Academy of Agricultural Sciences, Beijing 100081, China*

ARTICLE INFO

Keywords:
Crowing stress
Apoptosis
Gut permeability
Gut microbiota
GIFT

ABSTRACT

This study investigated the effects of crowding stress on liver and gut health in genetically improved farmed tilapia (GIFT, *Oreochromis niloticus*). Fish averaging 18.90 ± 0.01 g were reared at low (LD, 5 g/L) or high densities (HD, 100 g/L) for 14 days. The analysis revealed that the HD group significantly increased serum cortisol, glucose and adrenocorticotropic hormone (ACTH) levels in GIFT ($P < 0.05$). Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were elevated in the HD group ($P < 0.05$). TUNEL staining of liver tissues revealed that the fluorescence intensity indicating apoptosis was significantly higher in the HD group ($P < 0.05$). Additionally, the HD group exhibited increased expression of *p53* and *Caspase3* in the liver ($P < 0.05$). In comparison to the LD group, the HD group showed a significant increase in gut superoxide dismutase (SOD) and catalase (CAT) activities, as well as malondialdehyde (MDA) content ($P < 0.05$). The expressions of inflammatory-related genes (*tnfα*, *il-1β*, *il-8, and il-6*) and pro-apoptotic genes (*p53* and *Caspase7*) were significantly upregulated in the gut of the HD group ($P < 0.05$). Serum contents of D-lactate and diamine oxidase (DAO) were significantly elevated in HD group as comparison to LD group ($P < 0.05$), and significantly higher serum FITC-CM-dextran was observed in HD group versus LD group following oral gavage ($P < 0.05$). Furthermore, the HD group down-regulated the expressions of gut tight junction proteins (*tjp2a*, *hif-1a*, and *zo-1*) ($P < 0.05$), and significantly up-regulated the protein expression of phosphorylated myosin light chain 2 (P-MLC2) ($P < 0.05$). The 16S rDNA gene sequencing results indicated that the relative abundance of *Cetobacterium* was significantly lower ($P < 0.05$), and *Enterovibrio* and *Weissella* were significantly higher in the HD group ($P < 0.05$). Overall, crowding stress negatively affects GIFT liver and gut health, which is caused by inducing hepatic and intestinal apoptosis, impairing intestinal barrier function, and disrupting the gut microbiota.

1. Introduction

Aquaculture is one of the sources of food for human survival, and a sustained and stable supply of aquatic products is particularly important in times of extreme weather and regional conflicts (Galappaththi et al., 2020; Zhao et al., 2024). Due to the limitation of development and utilization of aquaculture areas, to increase the production and economic benefits, intensive aquaculture has become one of the mainstream aquaculture approaches, and the control of the production staff on the aquaculture density determines the direct production and benefits (Lin et al., 2018). In general, most fish have a clustering effect, and a density that is too low will not only be detrimental to their growth but also result in a waste of production resources (Santana et al., 2020). Conversely, a high-density environment can compress fish survival space, further triggering crowding stress (Li et al., 2023).

In the early phase of crowding stress, fish can adapt to a high-density environment and maintain their normal physiology by rebalancing their behavioral, physiological, biochemical, and molecular processes (Bai

* Corresponding authors.
E-mail addresses: dingqianwen@caas.cn (Q. Ding), ranchao@caas.cn (C. Ran), zhouzhigang03@caas.cn (Z. Zhou).
[1] These authors contributed equally to this work.

https://doi.org/10.1016/j.aquaculture.2024.741448
Received 6 June 2024; Received in revised form 18 July 2024; Accepted 2 August 2024
Available online 4 August 2024

et al., 2024). Fish regulate energy metabolism through hormone release during early adaptation to crowding stress and increase metabolism by increasing respiratory efficiency to adapt to crowding environments (Pederzoli and Mola, 2016). In addition, behaviourally, fish may alter their social interactions, e.g., by reducing activity range, to reduce somatic friction conflicts (Dara et al., 2023). Whereas, prolonged and intense high-density environment disrupts physiological homeostasis. Primarily, the activation of the hypothalamic-pituitary-interrenal (HPI) axis is crucial in responding to stress. This process starts with hypothalamus secretion of corticotropin-releasing hormone (CRH), stimulating pituitary adrenocorticotropic hormone (ACTH) release, which in turn prompts adrenal cortisol production (Amano et al., 2021; Shaughnessy et al., 2023). Cortisol is therefore a key bioindicator for assessing the degree of stress. Immediately thereafter, cortisol stimulates the metabolic organs of the body and mobilizes energy reserves to restore physiological homeostasis (Li et al., 2023; Bai et al., 2024). The liver and gut, as the major metabolic organs in fish, are essential for the catabolism of carbohydrates, lipids, and proteins to supply energy (Xie et al., 2021a). While a stable energy supply based on liver and gut health can mitigate the negative effects of stress to some extent, damage to metabolic organs leads to imbalances in energy metabolism, further threatening fish growth, reproduction, metabolism, immunity, and cellular function (Jia et al., 2022). Thus, there is an urgent need to add new insights into the effects of crowding stress on fish liver and gut health.

Genetically improved farmed tilapia (GIFT, *Oreochromis niloticus*) is known for its high growth rate, meat yield, and stable genetic characteristics, making it a significant economically farmed freshwater fish in China (Du et al., 2021). In 2023, China's tilapia aquaculture production has reached 1,816,800 t, making it one of the most productive freshwater economic fish (Wang et al., 2024). To fulfil the market demand, the intensive farming model has been widely used in GIFT culture. However, high-density stocking tends to lead to a variety of negative effects, including water quality deterioration, crowding stress, and individual differences (Sundh et al., 2019). Among them, crowding stress often leads to serious damage to GIFT health, which further leads to reduced production efficiency or even losses. Thus, the purpose of this study was to evaluate the effects of crowding stress on the liver and gut health of GIFT and to provide a rationale for finding strategies to alleviate crowding stress.

2. Materials and methods

2.1. Ethics statement

This research followed guidelines from the animal care committee at the Feed Research Institute (approval number 2021-AF-FRI-CAAS-001).

2.2. Fish and culture management

The tilapia (GIFT) used for the experiments were sourced from a freshwater economic fish breeding farm (Hainan, China) and transported to the aquaculture laboratory of the Chinese Academy of Agricultural Sciences in the Hebei International Agricultural Hi-Tech Industrial Park. Prior to the feeding trial, all fish were temporarily reared in recirculating aquaculture systems (RAS) within water exchange rate 90 L/h for 2 weeks to acclimatise to the culture environment. After 24 h of starvation, similarly sized and healthy fish (18.90 ± 0.71 g) were allocated at random to two 90-L tanks so that the culture densities of the two tanks were 5 g/L (LD) and 100 g/L (HD), respectively. The trial lasted for 14 days and basal diet (Table 1) was fed to all fish once a day on satiation. The fish were maintained under the below feeding conditions during this period: water exchange rate 90 L/h, water temperature 24 °C, ammonia nitrogen content <0.5 mg/L, nitrite concentration < 0.1 mg/L, dissolved oxygen ≥5.5 mg/L, and photoperiod of 12 h of darkness and 12 h of light.

Table 1
Formulation and proximate analysis of experimental diets (% dry matter).

Ingredients	Content (%)
Soybean meal	20.00
Rice bran	10.00
Rapeseed Cake	11.50
Fish meal	8.00
Chicken powder	8.00
Meat powder	4.00
DDGS	10.00
Flour	20.00
Soybean oil	3.00
Bentonite	2.00
Lysine	0.20
Methionine	0.05
Choline chloride	0.20
$Ca(H_2PO_4)_2$	2.00
VC phosphate	0.05
Premix[a]	1.00
Nutrient levels(%)	
Crude protein	35.25
Crude lipid	11.20
Ash	8.43
Moisture	7.38

[a] Premix was Provided by Beijing Sino-Norway Joint Aquaculture Technology Co., Ltd. The product meets NRC standard.

2.3. Sample collection

Samples were taken from GIFT at 1, 2, 3, and 14 days of culture phases. Fifteen fish from per group were randomly chosen for sampling after fasting for 24 h. Blood was harvested from the tail vein with a sterile syringe, centrifuged (850 *g*, 4 °C, 10 min) and serum stored at −80 °C for subsequent analysis. Then, samples of liver, hindgut and hindgut contents were taken, and the snap was frozen in liquid nitrogen, and stored at −80 °C. Additional hindgut and liver tissues were fixed in paraformaldehyde for the later histological investigations.

2.4. Analytical methods

2.4.1. Serum biochemical indices

Serum cortisol, ACTH, D-Lactate, and diamine oxidase (DAO) levels were quantified using ELISA kits from Jiangsu Meimian Industrial Company, by competitive methods. Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), and glucose were assessed using biochemical kits from Nanjing Jiancheng Biological Company, Nanjing, through enzymatic colorimetric methods. The determination of all indices was carried out according to the kit instructions.

2.4.2. Intestinal antioxidant parameters

Hindgut homogenization was conducted based on methods from our prior study (Hu et al., 2016). The quantitative quantification of proteins was performed using a total protein quantitative kit (Beyotime Biotechnology Industrial Co., Ltd., Shanghai). Intestinal superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), and malondialdehyde (MDA) were analyzed using ELISA kits (Jiangsu Meimian Industrial Co., Ltd., China) by enzymatic colourimetric methods according to the manufacturer's instructions.

2.4.3. Histology

Liver and intestinal tissue sections were prepped following our prior procedure (Zhang et al., 2023a, 2023b), processed for staining using the hematoxylin and eosin (H&E) procedure, observed under a digital slide scanner (ZEISS Axioscan 7, Germany), and analyzed morphologically using Image J software.

Liver sections were further stained with TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end labeling) for apoptosis. The sections were washed with phosphate buffer, soaked with proteinase K for 30 min and then washed with PBS. Then, a labeling solution consisting of terminal deoxynucleotidyl transferase, buffer and fluorescein dUTP was prepared, and the sections were incubated in the labeling solution for 2 h at 37 °C, followed by DAPI staining, PBS washing and neutral balsam sealing. Photographs were taken by a fully automated digital slide scanner (ZEISS Axioscan 7, Germany). The green positive mean fluorescence ratio was assessed using Image J software.

2.4.4. FITC-CM-dextran gavage test

FITC-CM-dextran was administered orally as per methods described in previous experiments (Ding et al., 2023). After four hours, blood was harvested from the caudal vein and centrifuged. Fluorescence intensity was measured using a SynergyMX Multi-Purpose MPP Detector (Biotek, USA).

2.4.5. Western blotting analysis

Hindgut tissue was lysed with ice-cold RIPA lysis buffer mixed with 1 mM PMSF and phosphatase inhibitor (Abcam, USA). Equal amounts of total protein were loaded onto 12% SDS-PAGE for electrophoresis and then transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, USA). After blocking non-specific binding with 5% skimmed milk in PBS, PVDF membranes were incubated with anti-GAPDH (Proteintech, 1E6D9, 1:1000) and anti-P-MLC2 antibodies (CST, 95777S, 1:1000). Blots were developed using horseradish peroxidase (HRP)-conjugated secondary antibody (GE Health, 1:3000) and the ECL-plus system. Colourimetric quantification of shaded portions was performed using Image Lab software.

2.4.6. Quantitative real-time PCR analysis

Total liver and intestinal RNA extraction followed previous methods (Chen et al., 2024), and RNA integrity was detected by 1.5% agarose gel electrophoresis. RNA was transformed into cDNA by using FastKing gDNA Dispelling RT SuperMix (TIANGEN, China). The qPCR assays were performed in 10 ul reactions containing 5 ul SYBR Green Master Mix buffer (TIANGEN, China). Sequences of primers are given in Table 2, with β-actin as the control gene. Data were analyzed using the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak, 2008).

2.4.7. Gut microbiota sequencing analyses

Four hours post-feeding, intestinal contents from each GIFT group were collected. Following protocols from a prior study (Zhang et al., 2022a), we conducted DNA extraction, amplified the V3-V4 region of 16S rRNA gene, and sequenced the DNA. Integrity checks of DNA samples were performed using agarose gel electrophoresis with quality were assessed by spectrophotometer. The V3-V4 region was amplified using PCR, and the products were high-throughput sequenced with primers 341F (CCTACGGNGGCWGCAG) and 806R (GGACTACHVGGGGTATCTAAT). Raw sequence data were refined using FastQC and NGS toolkit, and analyzed with Uparse 7.0.1090 to identify operational taxonomic units (OTUs). Taxonomic identifications for the OTUs were separately obtained, and the diversity among samples was evaluated based on these OTUs.

2.5. Statistical analysis

Data were analyzed using an unpaired samples *t*-test and Tukey's test for multiple comparisons. Results were presented as mean ± S.E. (standard error), with differences deemed significant at $P < 0.05$.

Table 2
Sequences of primers used for *q*RT-PCR in this study.

Target genes	Primer sequences (Forward/Reverse) (5′-3′)	Accession No.
β-actin	F: CAGCAAGCAGGAGTACGATGAGTC R: GTATGAGAAATGTGTGGTGTGTGGTTG	GBAZ01002549.1
P53	F: TTTTCTCCTCCCTGTTCGTGG R: CGGGAACCTCATGCTTCACT	GU594898.1
Caspase3	F: GGCTCTTCGTCTGCTTCTGT R: GGGAAATCGAGGCGGTATCT	GQ421464.1
Caspase7	F: GATTCCCTGTGTGGTCTCTATG R: GGTCAGTCTGTGGCATCATTA	XM_005471473
Bcl-xl	F: GCGCTTCAACGCAGTCATAG R: GCAGCTAGACCAAAGACCGT	NP_571882
tnfα	F: CTCAGAGTCTATGGGAAGCAG R: GCAAACACGCCAAAGAAGGT	NM_001279533.1
il-1β	F: ACAAGGATGACGACAAGCCAACC R: GGACAGACATGAGAGTGCTGATGC	XM_019365844.2
il-8	F: GCACTGCCGCTGCATTAAG R: GCAGTGGGAGTTGGGAAGAA	XP_001342606
il-6	F: ATAGCAAGCATCTACACGCATCTCC R: GGGCTGCCAGGGAATTGTAAGTC	XM_031742624.2
IFN-γ	F: GATCTTCATGGGTGGTGTTTTG R: GGTAGCGAGCCTGAGTTGTTG	XM_003448130.1
tgfβ	F: GCCCATCAGCTCACCTACAAATCC R: ATGACCGAAGAGGAGGAAGAGGAAG	XM_005457931.4
il-10	F: GCTTCCCCGTCAGGCTCAA R: CTGTCGGCAGAACCGTGTC	XM_013269188.3
Occludin	F: GGAGGAAAGCCGCAGTGTTCAG R: GTCGTAGGCATCGTCATTGTAGGAG	XM_025899615.1
Claudin1	F: GTCTGTTTCTGGGCGTGGTGTC R: ACTCCGACTGACTCCTCATCTTCC	XM_019367708.2
tjp2α	F: CACGGACACCATCGACTTGAACTC R: TCCTCCTCTGAACCATCCACCTTG	XM_025908597.1
hif-1α	F: AAGCAGACCGCAGATGTGAAGC R: TCCTCCTTCTCCAGTTCAGCCTTC	XM_005477039.4
zo1	F: CTATCCGTATCGGCAAGAACC R: CACCCTCTCATAGGCAGGGA	XM_019358158.1
zo2	F: GCTTTGGCATTGCTGTATCAG R: AACGAGTGGATGGCTCCATC	XM_019361305.1

Abbreviations: *P53*, tumor protein 53; *Caspase*, cysteinyl aspartate specific proteinase; *Bcl-xl*, B-cell lymphoma-extra large; *tnf*, tumor necrosis factor; *il*, interleukin; *IFN*, interferon; *tgf*, transforming growth factor; *tjp*, tight junction protein; *hif*, hypoxia-inducible factor; *zo*, zonula occludens.

3. Results

3.1. Crowding stress triggered stress response in GIFT

As shown in Fig. 1, crowding stress at 1, 2, and 3 days did not affect GIFT serum cortisol and ACTH contents ($P > 0.05$). After stressing for 14 days, serum ACTH, cortisol and glucose contents were significantly risen in the HD group ($P < 0.05$).

3.2. Crowding stress impairs liver health in GIFT

The liver morphology in GIFT is shown in Fig. 2A-B. The liver morphology of GIFT in the LD group was normal, while the liver cell nuclei in the HD group were gradually marginalized, vacuolization was aggravated, and the liver cord was not clear. Furthermore, serum ALT and AST contents were significantly risen in the HD group than in the LD group (Fig. 2C-D, $P < 0.05$).

3.3. Crowding stress induced apoptosis in the liver of GIFT

The TUNEL assay showed that there was a significant difference in the degree of apoptosis between the LD and HD groups (Fig. 3A). The fluorescence intensity ratio was significantly higher in HD group than in LD group, which indicated a significant rise in apoptosis in the HD group (Fig. 3B, $P < 0.05$). The expressions of *p53* and *Caspase3* were significantly risen in GIFT livers in the HD group (Fig. 3C, $P < 0.05$), with no significant difference in the expression of *Bcl-xl* ($P > 0.05$). Moreover,

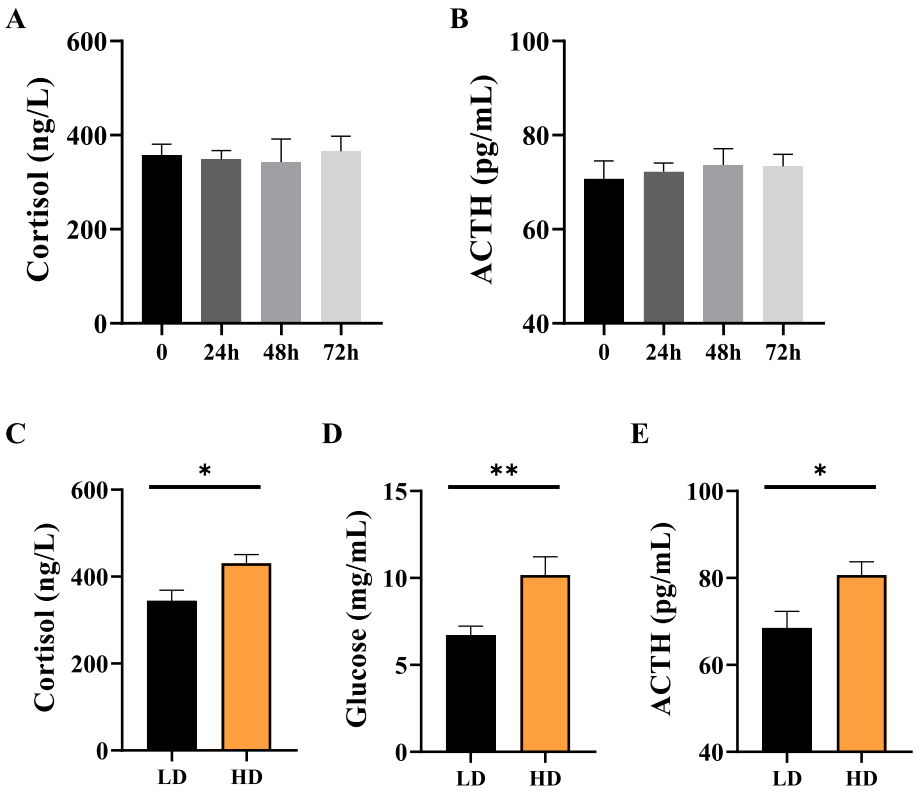


Fig. 1. (A-B) Effects of crowding stress on cortisol and ACTH in serum of GIFT within 3 days; (C-E) Effects of crowding stress on cortisol, glucose, and ACTH in serum of GIFT at 14 days ($n = 12$). Bars with asterisk indicate significant difference (unpaired samples *t*-test, *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$).

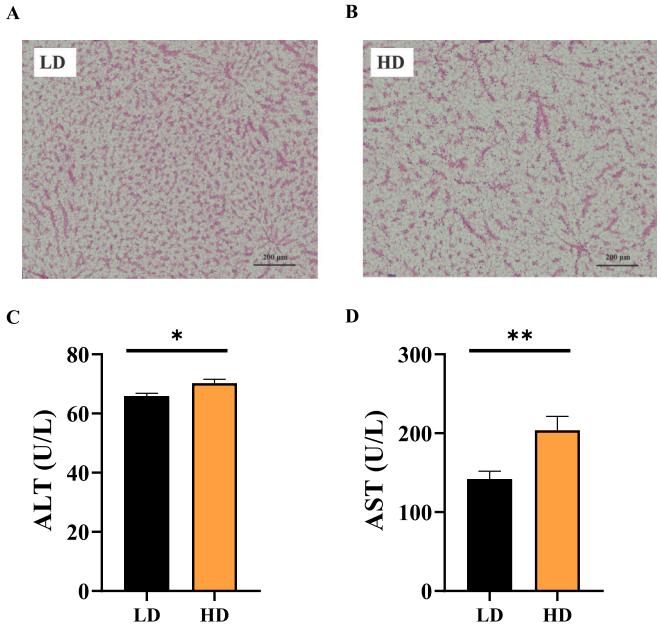


Fig. 2. (A-B) Observation of the liver morphology of GIFT by H and E staining (200×); Effects of crowding stress on (C) ALT and (D) AST in serum of GIFT (n = 12). Bars with asterisk indicate significant difference (unpaired samples *t*-test, *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$).

the expression of *Caspase7* in the HD group had a tendency to increase, but there was no significance ($P > 0.05$).

3.4. Crowding stress impaired gut morphology of GIFT

Intestinal villus of GIFT in the LD group kept fine integrity, while the intestinal villi in the HD group showed lysis and contraction, with a significant decrease in villus height (Fig. 4, $P < 0.01$) and a significant increase in villus width ($P < 0.001$). In comparison to the LD group, the intestinal muscle integrity was disrupted, and muscle thickness was significantly decreased in the HD group ($P < 0.001$).

3.5. Crowding stress reduced intestinal antioxidant capacity of GIFT

In the comparison with LD group, the HD group exhibited significantly higher intestinal SOD and CAT activities, as well as increased MDA content in GIFT ($P < 0.05$). Although GSH-Px activity also tended to increase, this change was not significant (Fig. 5, $P > 0.05$).

3.6. Crowding stress induced intestinal inflammation and apoptosis in GIFT

In the gut of GIFT, in comparison with the LD group, expressions of *tnfα*, *il-1β*, *il-8*, and *il-6* was significantly risen in the HD group (Fig. 6A, $P < 0.05$). Whilst anti-inflammatory gene (*tgfβ*) expression was significantly declined in the HD group ($P < 0.05$). The expressions of *IFN-γ* and *il-10* were not significantly different between groups ($P > 0.05$).

In the gut of GIFT, in comparison with the LD group, the HD group showed significantly increased expression of *P53* and *Caspase7* (Fig. 6B, $P < 0.05$). However, the expressions of *Caspase3* and *Bcl-xl* was not significantly different ($P > 0.05$).

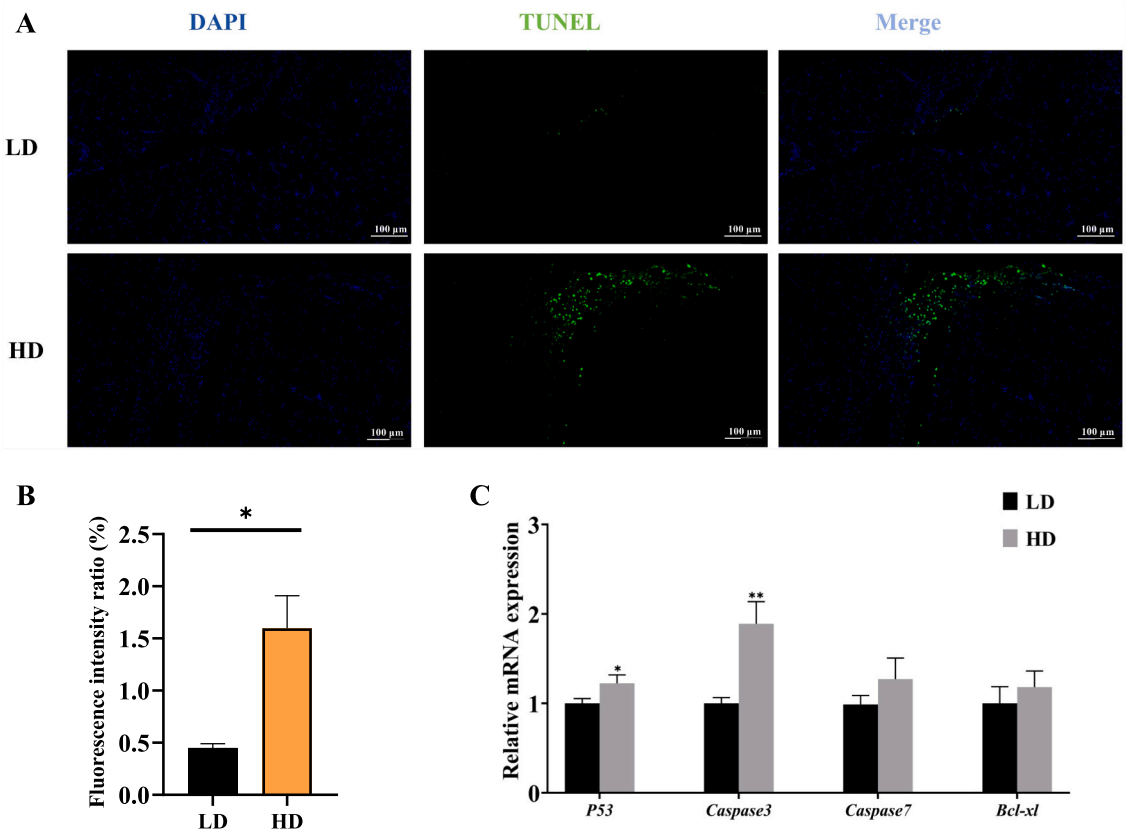


Fig. 3. Crowding stress induced apoptosis in the liver of GIFT. (A) TUNEL assays of liver tissues of GIFT, fluorescein-dUTP (green) to show apoptosis, and DAPI (blue) to show the nucleus; (B) Fluorescence intensity ratio of the two groups; (C) Effects of crowding stress on expression of liver apoptosis genes of GIFT (n = 12). Bars with asterisk indicate significant difference (unpaired samples *t*-test, *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$).

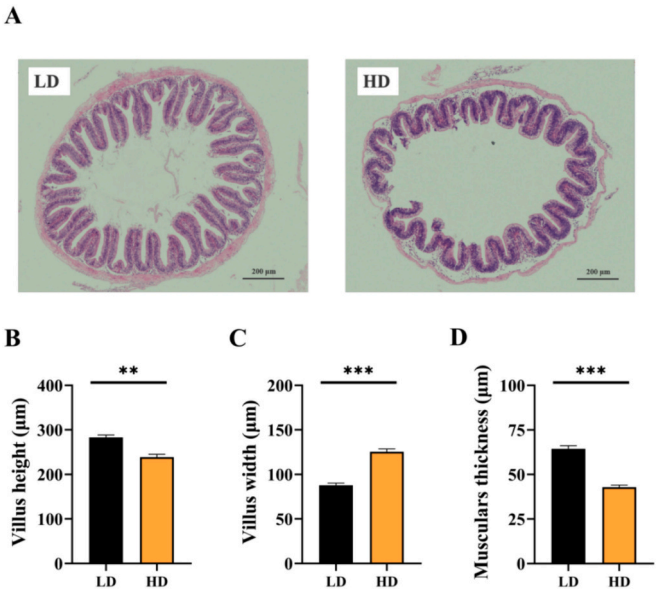


Fig. 4. (A) Observation of gut morphology of GIFT by H & E staining (200×). Quantification of gut histomorphology (n = 12): (B) Villus height, (C) Villus width and (D) Muscularis thickness. Bars with asterisk indicate significant difference (unpaired samples *t*-test, *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$).

3.7. Crowding stress impairs intestinal barrier and increases intestinal permeability of GIFT

Serum D-Lactate and DAO contents were significantly increased in GIFT in the HD group (Fig. 7A-B, $P < 0.05$). Consistently, significantly higher FITC-CM dextran was observed in HD fish versus LD group following oral gavage (Fig. 7C).

In comparison with the LD group, the HD group had significantly lower expressions of *tjp2a*, *hif-1a*, and *zo-1* (Fig. 7E, $P < 0.05$). Furthermore, there was a trend of decreased expression for *Occludin*, *Claudin1*, and *zo-2* in the HD group, though with no significant differences (Fig. 7E, $P > 0.05$). Additionally, the level of intestinal P-MLC2 was significantly higher in the HD group (Fig. 7D, $P < 0.05$).

3.8. Crowding stress altered the gut microbiota in tilapia

All Alpha diversity indexes tended to rise in the HD group (Fig. 8A-F), with Simpson, Shannon, and Pielou significantly higher in the HD group ($P < 0.05$), while the differences in Sob, ACE, and Chao1 were not significant ($P > 0.05$). The PCoA and PCA plots presented differences in distribution sites between groups (Fig. 8G-H), revealing differences in Beta diversity between the gut microbiota.

As shown in Fig. 9A, Fusobacteriota, Proteobacteria, and Bacteroidota are the predominant bacterial phyla in the intestines of GIFT. The HD group significantly decreased the relative abundance of Fusobacteriota and Bacteroidota, while boosting the relative abundance of Proteobacteria (Table 3). *Cetobacterium*, *Enterovibrio*, and *Plesiomonas* are the predominant bacterial genera in the gut (Fig. 9B). The HD group significantly decreased the relative abundance of *Cetobacterium* while increasing that of *Enterovibrio* (Table 4). The LEFSe analysis showed that

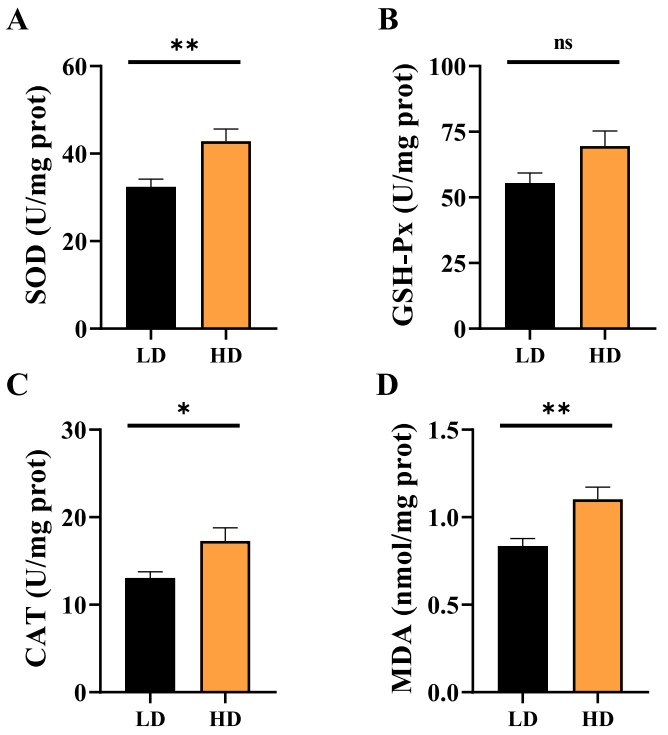


Fig. 5. Effects of crowding stress on gut antioxidant indexes of GIFT (n = 12). (A) SOD, (B) GSH-Px, (C) CAT and (D) MDA. Bars with asterisk indicate significant difference (unpaired samples *t*-test, *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$).

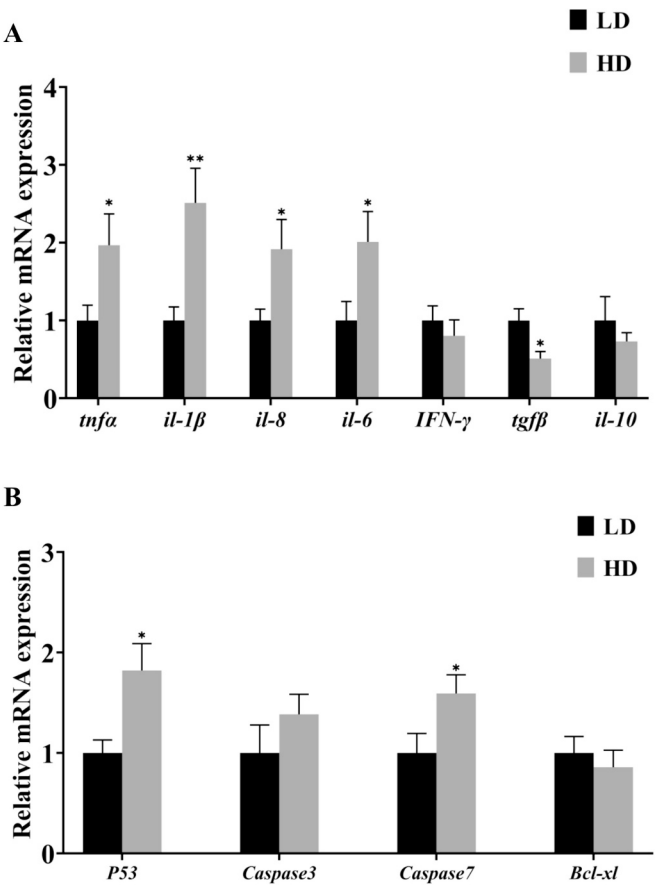


Fig. 6. Effects of crowding stress on expression of (A) gut inflammatory factors and (B) gut apoptosis genes of GIFT (n = 12). Bars with asterisk indicate significant difference (unpaired samples *t*-test, *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$).

the relative abundance of potentially pathogenic bacteria such as *Enterovibrio*, *Ralstonia*, *Aeromonas*, and *Staphylococcus* was increased in the HD group (Fig. 9C).

4. Discussion

Density is one of the key considerations in aquaculture production management, which can directly affect fish growth and survival, metabolism, organ function and immunity (Ran et al., 2016; Lin et al., 2018; Li et al., 2023). Numerous studies have confirmed that high-density aquaculture may jeopardize the viability of certain fish species by causing slow growth and increased growth dispersion (Lupatsch et al., 2010; Ran et al., 2016; Onxayvieng et al., 2021; Bi et al., 2023; Ghafarifarsani et al., 2023). This affects fish in two major ways. Firstly, when the culture density is above a threshold, it will lead to water quality deterioration due to excessive discharge of metabolic wastes, which in turn can have a cross-stressing effect on farmed individuals (Sundh et al., 2019). On the other hand, high-density aquaculture intensifies competition for water space among farmed individuals, resulting in mutual friction and aggressive behaviour, which can trigger a single crowding stress (Bi et al., 2023; Li et al., 2023). A research has shown that crowding stress can be triggered when the biomass of tilapia in the water column reaches 100 g/L (Qiang et al., 2014), which was referenced in the design of this study. In addition, we maintained similar water quality in two different density groups by maintaining a higher rate of aquaculture water circulating and increasing oxygen supply to reduce the effects of environmental variables and better facilitate the exploration of the mechanisms by which crowding stress affects liver and gut health of fish cultured at a high density.

Cortisol is an important stress-related marker secreted in the HPI axis of fish when they encounter stressors, and this marker is produced by ACTH released by the pituitary gland acting on the adrenal glands (Amano et al., 2021). Elevated plasma cortisol levels in fish are considered a typical signal of stress (Wu et al., 2016). In this study, 1, 2 and 3 days of crowding stress did not change the contents of cortisol and ACTH significantly in the serum of GIFT. In contrast, serum ACTH and cortisol contents were elevated in GIFT from the HD group after 14 days of crowding treatment, suggesting that 14 days of crowding causes a stress response in GIFT, which is analogous to the results of a study on the Egyptian Nile tilapia (Qiang et al., 2014), and it is hypothesised that the short-term crowding friction is of low number and frequency, which is not sufficient to trigger the release of HPI axis hormones in response to the stress response, whereas the accumulation of the number of long-term rubs and crowding and the increase in the frequency of the rubs have a direct inducing effect on the stress response, which needs further researches. Serum glucose content is also a vital indicator used to evaluate the stress response in fish. During the initial phase of stress, the blood glucose level of the organism decreases dramatically in response to the energy loss caused by stress (Sun et al., 2019). After this, the endocrine system is regulated, and blood glucose levels increase significantly, probably due to the fact that cortisol meets the energy requirements of fish in response to stress by promoting the glycolytic pathway (Zhu et al., 2021; Pan et al., 2022). In addition, we found that crowding stress resulted in a significant increase in blood glucose in GIFT, which is consistent with the study on striped catfish (*Pangasianodon hypophthalmus*) (Zaki et al., 2023) and hybrid sturgeon (♀*Acipenser baerii* × ♂*Acipenser schrenckii*) (Bi et al., 2023). To sum up, the present results of serum biochemistry indicated that 14 days of high-density treatment leads to crowding stress effects, and the establishment of the GIFT model is the basis for further research into the mechanisms of crowding stress effects on fish health.

ALT and AST are among the most active transaminases in animals,

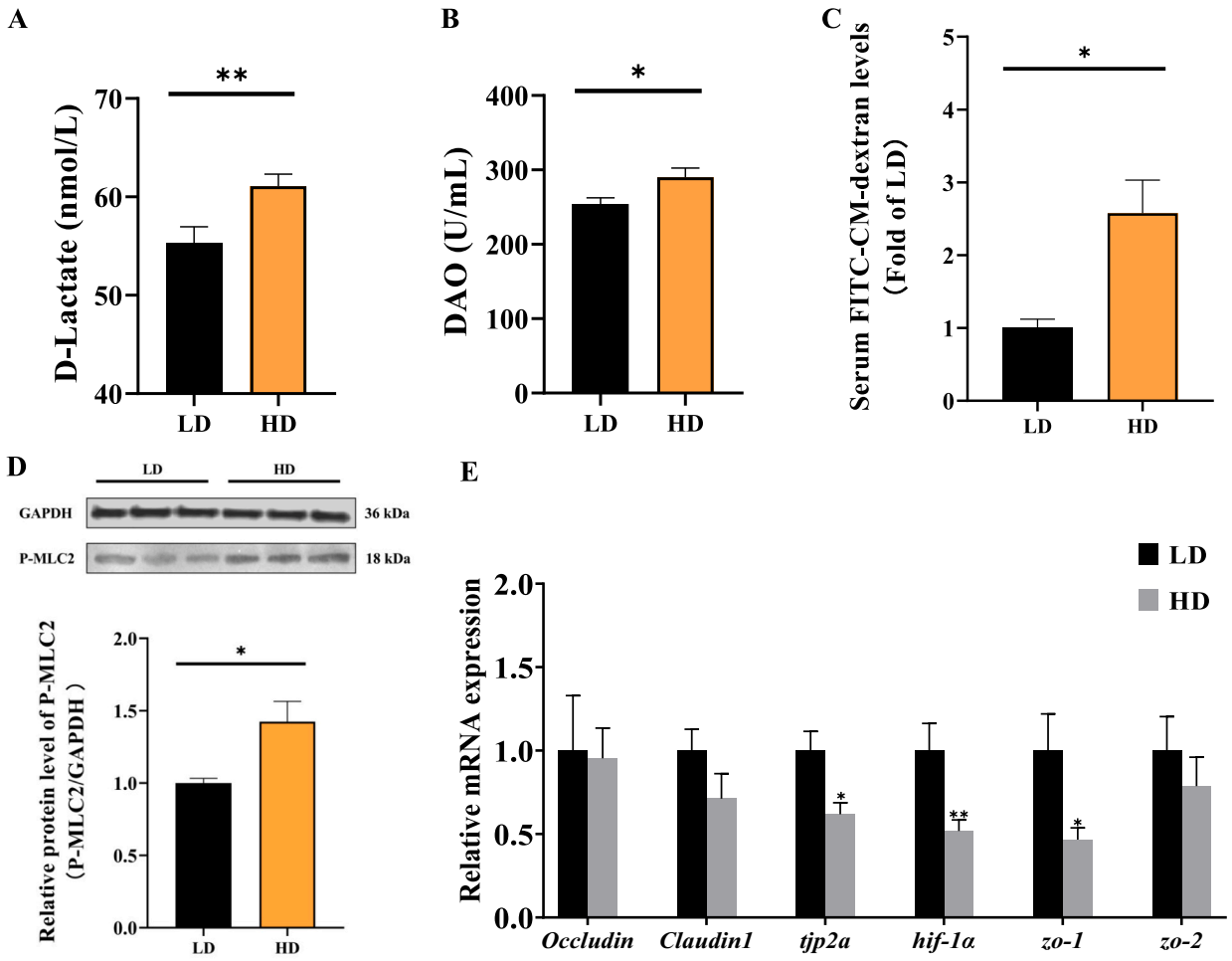


Fig. 7. Effects of crowding stress on (A) D-Lactate and (B) DAO levels in serum of GIFT (n = 12); Effects of crowding stress on (C) FITC-CM-dextran (fluorescein isothiocyanate-labeled carboxymethylated dextran) levels in serum of GIFT ($n = 6$); (D) Effects of crowding stress on protein expression of P-MLC2 (Phosphorylated myosin light chain 2) in gut of GIFT by western blot and quantification analysis ($n = 3$); (E) Effects of crowding stress on expression of gut tight junction protein of GIFT (n = 12). Bars with asterisk indicate significant difference (unpaired samples *t*-test, *: $P < 0.05$, **: $P < 0.01$, ***: $P < 0.001$).

playing a vital role in amino acid metabolism and the interconversion of proteins, lipids, and carbohydrates (Bonifacio et al., 2017; Xie et al., 2021a). ALT and AST are primarily active normally in hepatocytes, whereas they escape into the bloodstream when hepatocytes are damaged (Hu et al., 2022; Fauzi et al., 2024). Thus, the activities of these two aminotransferases in the serum are a visualized indicator of liver health. We found that ALT and AST were significantly risen in the HD group, suggesting that crowding stress caused some damage to liver health in GIFT. In addition, we found severe vacuolisation of the liver, indistinct hepatic cords and intracellular margination of the nuclei in the HD group in the tissue sections, which visually reflected the impairment of crowding stress on the morphology of the liver tissue, which the increased metabolic burden might cause (Wu et al., 2024). Metabolism is a chemical reaction that occurs within a cell. A metabolic burden can lead directly to impaired cellular function, damage to death, or potentially dangerous cells being removed in a process known as apoptosis (Nagata, 2018; Zhu et al., 2023). This study's TUNEL staining results confirmed that crowding stress induced apoptosis in GIFT hepatocytes. Apoptosis has been classified as exogenous (death receptor apoptosis pathway) and endogenous (mitochondrial apoptosis pathway) (Nagata, 2018). The exogenous pathway consists of extracellular death signals activating *Caspase* protease by binding to the corresponding receptor on the cell membrane surface, triggering apoptosis (Eimon et al., 2006). Another pathway, the endogenous pathway, is caused by overwhelming metabolic stress on mitochondria, where the transmembrane potential of damaged mitochondria is altered, increasing the permeability of the mitochondrial membrane and further activating *Caspase3*, *Caspase7*, which leads to apoptosis (Wang et al., 2021). Under the conditions of this study, expressions of *Caspase3* and *Caspase7* in the liver were up-regulated to a certain extent under crowding stress, suggesting that hepatocyte apoptosis in high-density-induced GIFT occurs through mobilisation of the apoptotic pathway in a caspase-dependent manner. Furthermore, it was shown that environmental stress activates the tumor suppressor protein *P53* in fish (Cheng et al., 2017; Cheng et al., 2018a). *P53* is a classic transducer of genotoxicity and plays a crucial function in modulating checkpoints of the cell cycle, stability of genes, and apoptosis (Fu et al., 2019). *P53* induces apoptosis by down-regulating the anti-apoptotic gene *Bcl-xl* and *Bax* transcription (Wang et al., 2018). In the present study, crowding stress increased the expression of *p53* in GIFT livers, suggesting that the high-density environment may trigger apoptosis in GIFT livers via the *P53*-activated apoptotic pathway.

The gut is a vital digestive, metabolic, and immune organ in fish, and its health is directly linked to the overall well-being of the fish (Zhang et al., 2022b). Therefore, the structural integrity of the gut is of paramount importance. Gut villus's length and width determine the digestive capacity of the gut, which will directly affect nutrient absorption and the rate of growth (Yang et al., 2019; Zhang et al., 2022a; Zhang et al., 2022b). In our findings, crowding stress reduced the length of villus, suggesting that intestinal digestibility was directly affected. Under stressful conditions, most of the steady-state energy supply comes

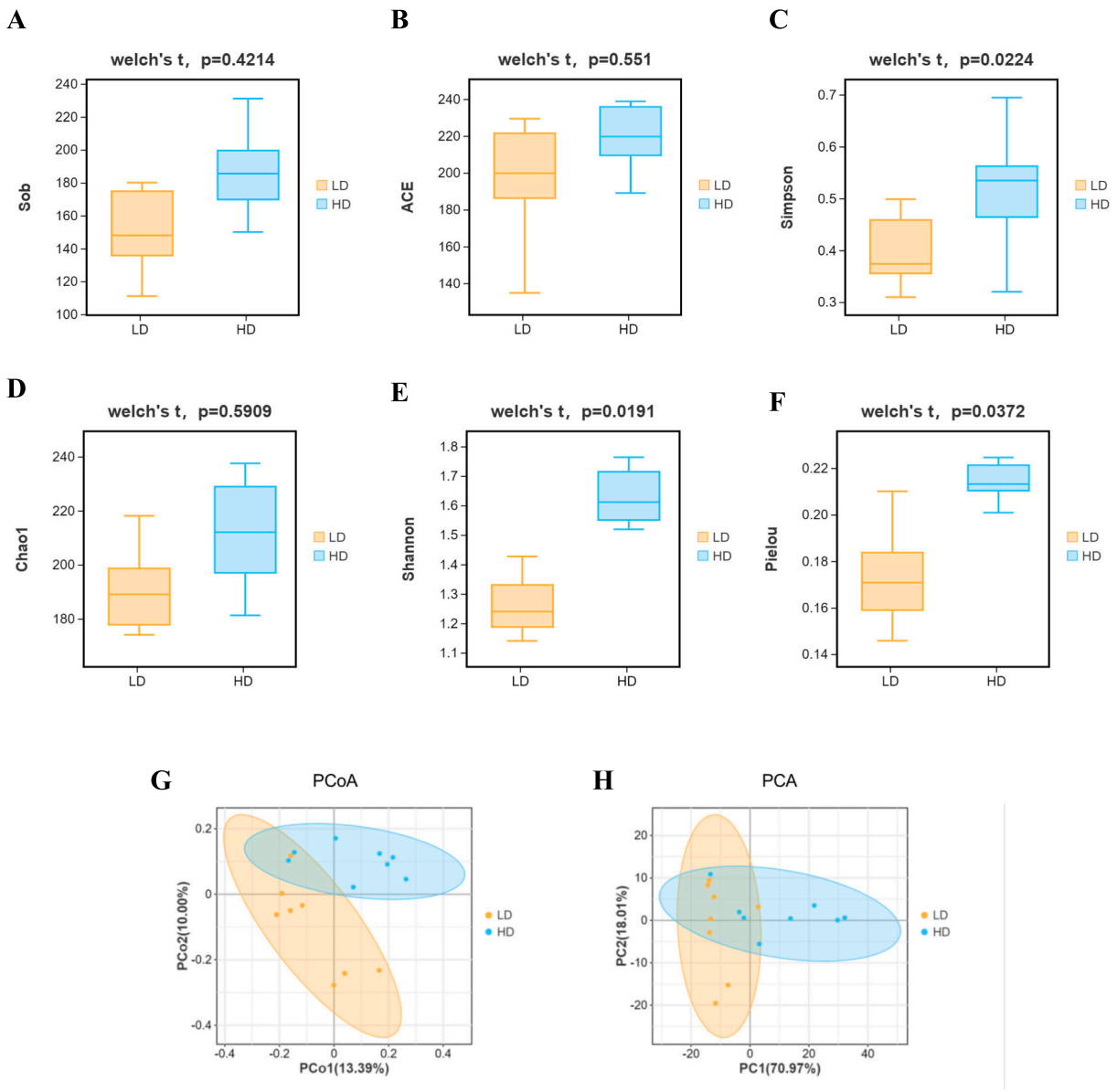


Fig. 8. Effects of crowding stress on gut microbial Alpha diversity indexes (A) sob, (B)ACE, (C)Simpson, (D)Chao1, (E)Shannon and (F) Pielou of GIFT (n = 8); (G) Principal coordinates analysis (PCoA) and (H) Principal component analysis (PCA) of the microbiota on phylum (n = 8).

from food, and reduced digestive capacity contributes to the damage caused by stress, as has been validated in several fish researches (Santos et al., 2012; Wen et al., 2017; Zhang et al., 2023a, 2023b). Furthermore, we found that crowding stress significantly increased the width of gut villus. The increase in villus width was thought to be positive, stemming from the increased contact area between the gut and the chyme (Zhang et al., 2023a, 2023b). However, it is often easy to ignore that the inflammatory response also causes an increase in villus width due to the swell of the intestinal villus caused by the infiltration of inflammatory factors (Niu et al., 2021). A study showed that ammonia-nitrogen stress led to oedema and haemorrhage in the gut of yellow catfish (*Pelteobagrus fulvidraco*) (Luo et al., 2023), which parallels the results of this study. Further, we did find significant changes in expressions of intestinal inflammatory factors in GIFT, encompassing elevated expression of pro-inflammatory factors (*tnfα*, *il-1β*, *il-8*, and *il-6*) and decreased expression of inflammation-suppressing factors (*tgfβ*), which once again confirms our results. Moreover, we similarly observed that crowding stress caused severe damage to the GIFT intestinal muscular, as evidenced by a significant reduction in muscular thickness. The intestinal muscularis is the most critical barrier for maintaining homeostasis in the intestinal lumen, and extensive muscular damage exacerbates the likelihood of intestinal inflammation and opportunistic bacterial invasion (Zhang et al., 2023a, 2023b). Numerous studies have confirmed that environmental stress causes a reduction in the muscular layer thickness of the fish gut, including, but not limited to, yellow catfish (*Pelteobagrus fulvidraco*) (Luo et al., 2023), largemouth Bass (*Micropterus salmoides*) (Wang et al., 2023), and cobia (*Rachycentron canadum*) (Yang et al., 2021a, 2021b, 2021c). In conjunction with these studies, oxidative stress is prevalent and may be the leading cause of damage to GIFT intestinal tissues caused by crowding stress. It has been shown that chronic stress disrupts the redox balance in fish organs such as the liver and gut, resulting in massive production of reactive oxygen species (ROS), resulting in oxidative stress (Gao et al., 2024). Overaccumulation of ROS in the process of oxidative stress can induce both apoptosis and tissue destruction (Eimon et al., 2006). SOD, GSH-Px and CAT are the major elements of the antioxidant enzyme system in fish, which decompose or convert ROS, thus preventing various intracellular structures or biomolecules from being damaged by the attack of ROS (Wang et al., 2023).

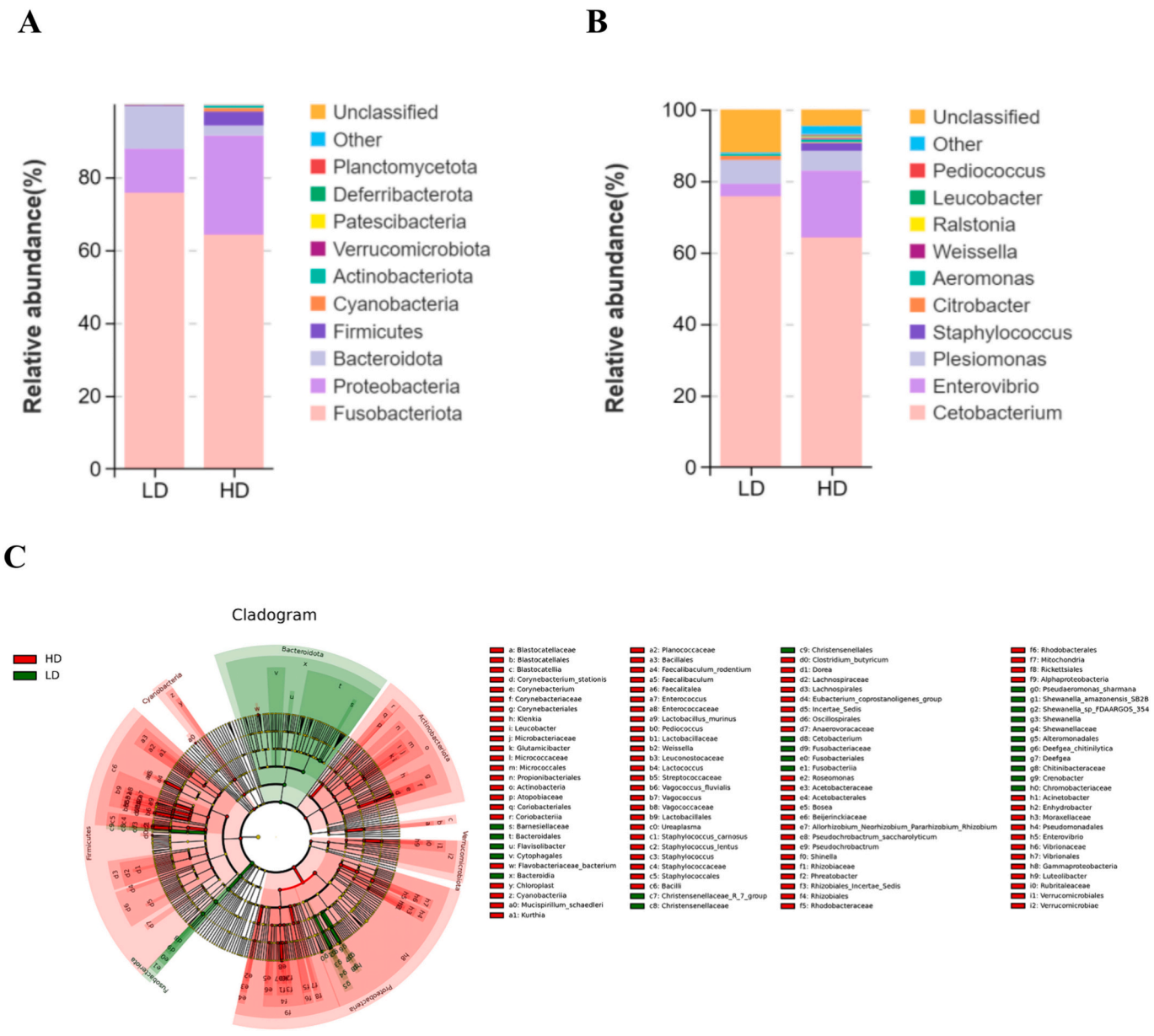


Fig. 9. The relative abundance of the major gut bacterial (A) phyla and (B) genera of GIFT (n = 8). (C) LEFSe (Linear Discriminant Analysis Effect Size) analysis identified bacterial species with significant differences in abundance between groups. These differences were indicated by colour: red signifies a significantly higher relative abundance in the HD group, while green signifies a significantly higher abundance in the LD group.

Table 3
The relative abundance of main phylum in the gut microbiota of GIFT.

Phylum name	Groups	
	LD	HD
Fusobacteriota	75.71 ± 6.35*	64.21 ± 10.66
Proteobacteria	12.08 ± 5.21	27.23 ± 12.42**
Bacteroidota	11.72 ± 9.28*	2.70 ± 3.05
Firmicutes	0.24 ± 0.15	3.87 ± 5.38
Cyanobacteria	0.09 ± 0.06	1.03 ± 1.82
Actinobacteriota	0.05 ± 0.08	0.60 ± 0.87
Verrucomicrobiota	0.03 ± 0.04	0.18 ± 0.12**
Patescibacteria	0.01 ± 0.01	0.06 ± 0.11
Other	0.02 ± 0.03	0.04 ± 0.03
Unclassified	0.03 ± 0.05	0.06 ± 0.04

Data represent the means (± SEM) (n = 8). Values in the same row having symbol (*) indicate significant difference ($P < 0.05$), and the symbol (**) indicate a highly significant difference ($P < 0.01$).

Table 4
The relative abundance of main genus in the gut microbiota of GIFT.

Genus name	Groups	
	LD	HD
Cetobacterium	75.71 ± 6.35*	64.20 ± 10.65
Enterovibrio	3.51 ± 5.06	18.68 ± 15.15*
Plesiomonas	6.61 ± 1.40	5.54 ± 1.94
Staphylococcus	0.05 ± 0.03	2.16 ± 4.08
Citrobacter	1.17 ± 1.63	0.26 ± 0.29
Aeromonas	0.45 ± 0.52	0.73 ± 0.94
Weissella	0.02 ± 0.02	0.47 ± 0.33**
Ralstonia	0.03 ± 0.02	0.31 ± 0.68
Other	0.43 ± 0.34	2.51 ± 3.23
Unclassified	12.00 ± 9.18*	4.56 ± 3.36

Data represent the means (± SEM) (n = 8). Values in the same row having symbol (*) indicate significant difference ($P < 0.05$), and the symbol (**) indicate a highly significant difference ($P < 0.01$).

Whereas MDA is generated by lipid peroxidation and its production can indicate the severity of oxidative stress (Taheri Mirghaed et al., 2020). We found that crowding stress increased the activities of SOD, GSH-Px and CAT, as well as MDA content in the gut of GIFT. Fish have been reported to preferentially use small molecule non-enzymatic antioxidant products under environmental stresses (e.g., vitamins C and E) to scavenge excess ROS (Cheng et al., 2018b; Liu et al., 2021; Yang et al., 2021a, 2021b, 2021c). When the non-enzymatic antioxidant products cannot scavenge excess ROS, fish begin synthesising antioxidant enzymes to moderate oxidative stress (Yang et al., 2021a, 2021b, 2021c). Thus the elevated intestinal SOD, GSH-Px, and CAT activities indicate that GIFT resists oxidative damage by synthesising antioxidant enzymes and increasing their viability under crowding stress, whereas the elevated MDA content is direct evidence of oxidative damage. Additionally, crowding stress induced elevated expression of the GIFT intestinal pro-apoptotic factors *P53* and *Caspase7*, suggesting apoptosis of the gut, most likely due to the excessive accumulation of H_2O_2 under oxidative stress (Lin et al., 2017).

Intestinal epithelial barrier permeability can be used directly to assess intestinal health, and the most critical determinant is tight junctions (Ding et al., 2023). Tight junctions are multi-protein complexes made up of transmembrane proteins (*Claudin*, *Occludin*) and peripheral membrane proteins (*zo*), which together form the main body of the tight junctions and are connected to the perijunctional actomyosin ring (PAMR) through the *zo* proteins (Bischoff et al., 2014). Intestinal epithelial permeability is controlled by tight junction proteins and regulated by their expression and distribution (Ulluwishewa et al., 2011). Hypoxia-inducible factor alpha subunit (*hif-1α*) is a hypoxia-induced oxygen-regulated subunit that modulates intestinal tight junctions (Shao et al., 2018). The tight junction protein alpha subunit (*tjp2α*) is essential for tight junction assembly and maintenance, and it is also an important part of the cellular junction involved in gene expression and cell behavioral signalling (Yang et al., 2021a, 2021b, 2021c). We found that crowding stress down-regulated the expression of GIFT intestinal *tjp2α*, *hif-1α*, and *zo-1* to a certain extent, suggesting that crowding stress can increase GIFT intestinal permeability. Besides, tight junctions are regulated by various signalling pathways, including MLCK, PKC, and MAPK (Ulluwishewa et al., 2011). Typically, myosin light chain kinase (MLCK)-mediated phosphorylation of myosin constricts the PAMR of junctions, which increases the tight junction gap and intestinal epithelial permeability (Jin and Blikslager, 2016). Our study found that crowding stress increased GIFT intestinal P-MLC2 protein levels, suggesting that the impairment of intestinal barrier function by crowding stress may be mediated through the MLCK/P-MLC2 pathway. Another piece of evidence is that serum D-Lactate and DAO contents were significantly increased in the high-density environment. It is worth mentioning that D-Lactate is a microbial metabolite in the gut, and DAO is a highly active intracellular enzyme in the cytoplasm of intestinal villous cells (Zhang et al., 2022a, 2022b). Only when there is structural impairment to the intestinal tract, the mucosal cells and microbial metabolites shed as a result of the damage enter the bloodstream through the damaged mucosa, resulting in elevated contents of D-Lactate and DAO in the blood (Zhang et al., 2022a, 2022b). Additionally, we used an exogenous marker validation method by performing FITC-CM-dextran gavage experiments. The higher fluorescence intensity in the serum of GIFT in the HD group is another illustration that crowding stress increases the intestinal permeability of GIFT. In another reported study, high stocking densities increased gut permeability in Atlantic salmon (*Salmo salar* L.) (Sundh et al., 2019). However, unlike our study, this report on Atlantic salmon focused on the stress caused by high density induced water quality deterioration rather than crowding stress. Hence, the mechanism of crowding stress on the gut permeability of fish still needs to be further explored.

The gut microbiota is vitally important for digestion, absorption, physiological metabolism, and immune resistance in the host. A balanced gut microbiota is beneficial to the animal's health (Zhang et al., 2023a, 2023b; Chen et al., 2024). In rats, removal of the gut microbiota by antibiotics significantly reduces the stress response and decreases the cortisol level in the blood, indicating that gut microbiota is a necessary component for the occurrence of the stress response (Ait-Belgnaoui et al., 2012; Rea et al., 2016). In addition, it has been testified that crowding stress disrupts the host's commensal gut flora in white shrimp (*Penaeus vannamei*) (Wang et al., 2020). Crowding stress altered the gut microbiota of GIFT in this study, as evidenced by changes in the relative abundance of the major bacteria. Crowding stress reduced the abundance of Fusobacteriota and Bacteroidota but increased the abundance of Verrucomicrobiota and Proteobacteria in the GIFT gut. In general, Proteobacteria is a conditionally pathogenic bacterium that is very sensitive to environmental factors and an increased abundance of Proteobacteria under stress conditions is inextricably linked to the development of enterocolitis was reconfirmed in this study (Shin et al., 2015). Besides, crowding stress reduced the abundance of *Cetobacterium* and increased the abundance of *Enterovibrio* and *Weissella* in the GIFT gut. *Cetobacterium*, as an indigenous probiotic in aquaculture, has been shown in our previous studies to have positive effects on growth, metabolism and immunity (Xie et al., 2021a; Xie et al., 2021b; Xie et al., 2022a; Xie et al., 2022b; Zhou et al., 2022). In contrast, *Enterovibrio* is a potential pathogen in aquaculture and is most commonly found in shrimp aquaculture, where significant increases in abundance directly threaten survival and growth (Alfiansah et al., 2020; Boopathi et al., 2023). These results could all suggest that crowding stress negatively alters the gut microbiota of GIFT and that the elevated alpha diversity index may be due to an increase in the number and type of pathogenic genera. Several researches have shown that oral probiotics improve the stress response of the host, mainly by repairing the intestinal barrier function (Ait-Belgnaoui et al., 2005; Anderson et al., 2010). However, whether probiotics directly modulate the host stress response needs to be further investigated.

Overall, the effects of crowding stress on GIFT liver and gut health were adverse. Our study demonstrated that crowding stress induced apoptosis in liver and gut cells, induced enteritis, and further impaired gut antioxidant and barrier functions, whereas increased intestinal permeability was a possibility for gut microbiota dysbiosis. In practice, proper density stocking is the basis for maintaining fish liver and gut health. If culture efficiency is to be improved by increasing density, regulatory strategies targeting the gut microbiota are potential therapeutic options and warrant continued in-depth investigation.

CRediT authorship contribution statement

Jian Zhang: Writing – review & editing, Writing – original draft, Formal analysis, Data curation. **Jie Chen:** Writing – review & editing, Visualization. **Hui Liang:** Validation, Investigation. **Ming Li:** Validation, Investigation. **Wenhao Zhou:** Validation, Investigation. **Yalin Yang:** Software, Resources. **Zhen Zhang:** Software, Resources. **Qianwen Ding:** Resources, Methodology, Investigation. **Chao Ran:** Supervision, Project administration, Funding acquisition, Data curation. **Zhigang Zhou:** Supervision, Project administration, Funding acquisition, Data curation.

Declaration of competing interest

The authors declare that they have no competing interests.

There is no conflict of interest in submitting the manuscript entitled "Effect of crowding stress on liver health, gut permeability and gut microbiota of genetically improved farmed tilapia (GIFT, *Oreochromis niloticus*)". This work represents an original research, the contents of which have not been previously published or considered for publication elsewhere. All listed authors have reviewed and approved the attached manuscript and agreed to its publication.

Data availability

Data will be made available on request.

Acknowledgments

This study was funded by National Natural Science Foundation of China (31972807, 32122088).

References

Ait-Belgnaoui, A., Han, W., Lamine, F., Eutamene, H., Fioramonti, J., Bueno, L., Theodorou, V., 2005. *Lactobacillus farciminis* treatment suppresses stress induced visceral hypersensitivity: a possible action through interaction with epithelial cell cytoskeleton contraction. Gut 55, 1090–1094. https://doi.org/10.1136/gut.2005.084194.

Ait-Belgnaoui, A., Durand, H., Cartier, C., Chaumaz, G., Eutamene, H., Ferrier, L., Houdeau, E., Fioramonti, J., Bueno, L., Theodorou, V., 2012. Prevention of gut leakiness by a probiotic treatment leads to attenuated HPA response to an acute psychological stress in rats. Psychoneuroendocrinology 37, 1885–1895. https://doi.org/10.1016/j.psyneuen.2012.03.024.

Alfiansah, Y.R., Peters, S., Harder, J., Hassenrück, C., Gärdes, A., 2020. Structure and co-occurrence patterns of bacterial communities associated with white faeces disease outbreaks in Pacific white-leg shrimp *Penaeus vannamei* aquaculture. Sci. Rep. 10, 11980. https://doi.org/10.1038/s41598-020-68891-6.

Amano, M., Amiya, N., Fukushima, K., Hagio, H., Yamamoto, N., Sakakura, Y., 2021. Effects of crowding stress on the hypothalamo-pituitary-interrenal axis of the self-fertilizing fish, *Kryptolebias marmoratus*. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 264, 111110 https://doi.org/10.1016/j.cbpa.2021.111110.

Anderson, R.C., Cookson, A.L., McNabb, W.C., Park, Z., McCann, M.J., Kelly, W.J., Roy, N.C., 2010. *Lactobacillus plantarum* MB452 enhances the function of the intestinal barrier by increasing the expression levels of genes involved in tight junction formation. BMC Microbiol. 10, 316. https://doi.org/10.1186/1471-2180-10-316.

Bai, C., Qi, X., Wang, Z., Wang, J., Qiu, L., Li, H., Zu, X., Li, H., Xiong, G., Liao, T., 2024. Effect of density stress on the physiological, biochemical, and immunological parameters of juvenile *Pelteobagrus fulvidraco* during simulated transportation. Aquacult. Rep. 34, 101911 https://doi.org/10.1016/j.aqrep.2023.101911.

Bi, B., Yuan, Y., Zhao, Y., He, M., Song, H., Kong, L., Gao, Y., 2023. Effect of crowding stress on growth performance, the antioxidant system and humoral immunity in hybrid sturgeon. Aquacult. Rep. 28, 101468 https://doi.org/10.1016/j.aqrep.2023.101468.

Bischoff, S.C., Barbara, G., Buurman, W., Ockhuizen, T., Schulzke, J., Serino, M., Tilg, H., Watson, A., Wells, J.M., 2014. Intestinal permeability–a new target for disease prevention and therapy. BMC Gastroenterol. 14, 189. https://doi.org/10.1186/s12876-014-0189-7.

Bonifacio, A.F., Ballesteros, M.L., Bonansea, R.I., Filippi, I., Amé, M.V., Hued, A.C., 2017. Environmental relevant concentrations of a chlorpyrifos commercial formulation affect two neotropical fish species, *Cheirodon interruptus* and *Cnesterodon decemmaculatus*. Chemosphere 188, 486–493. https://doi.org/10.1016/j.chemosphere.2017.08.156.

Boopathi, S., Ramu, M., Brindangnanam, P., Sudhakaran, G., Coumar, M.S., Arockiaraj, J., 2023. Microbiome analysis of *Litopenaeus vannamei* reveals *Vibrio* as main risk factor of white faeces syndrome. Aquaculture 576, 739829. https://doi.org/10.1016/j.aquaculture.2023.739829.

Chen, J., Zhang, J., Xie, M., Hao, Q., Liang, H., Li, M., Zhou, W., Zhang, Z., Ran, C., Zhou, Z., 2024. The effect of dietary supplementation with *lactobacillus rhamnosus* GCC-3 fermentation product on gut and liver health of common carp (*Cyprinus carpio*). Aquacult. Rep. 35, 101983 https://doi.org/10.1016/j.aqrep.2024.101983.

Cheng, C., Ye, C., Guo, Z., Wang, A., 2017. Immune and physiological responses of pufferfish (*Takifugu obscurus*) under cold stress. Fish Shellfish Immunol. 64, 137–145. https://doi.org/10.1016/j.fsi.2017.03.003.

Cheng, C., Guo, Z., Luo, S., Wang, A., 2018a. Effects of high temperature on biochemical parameters, oxidative stress, DNA damage and apoptosis of pufferfish (*Takifugu obscurus*). Ecotoxicol. Environ. Saf. 150, 190–198. https://doi.org/10.1016/j.ecoenv.2017.12.045.

Cheng, C., Guo, Z., Wang, A., 2018b. Growth performance and protective effect of vitamin E on oxidative stress pufferfish (*Takifugu obscurus*) following by ammonia stress. Fish Physiol. Biochem. 44, 735–745. https://doi.org/10.1007/s10695-018-0468-2.

Dara, M., Dioguardi, M., Vazzana, M., Vazzana, I., Carbonara, P., Alfonso, S., Cammarata, M., 2023. The role of spatial exploration and territoriality in establishing gilthead seabream (*Sparus aurata*) hierarchies, and their effects upon underlying stress physiology. Fishes 8, 132. https://doi.org/10.3390/fishes8030132.

Ding, Q., Hao, Q., Jin, Y., Zhang, Q., Xie, Y., Yang, Y., Olsen, R.E., Ringø, E., Ran, C., Zhang, Z., Zhou, Z., 2023. The effects of sodium propionate on intestinal barrier function of genetically improved farmed tilapia in a high-lipid formulation. Aquaculture 579, 740187. https://doi.org/10.1016/j.aquaculture.2023.740187.

Du, Z.Y., Nie, P., Liu, J., 2021. Genetic improvement for aquaculture species: a promising approach for aquaculture challenges and development. Rev. Aquac. 13, 1756–1757. https://doi.org/10.1111/raq.12600.

Eimon, P.M., Kratz, E., Varfolomeev, E., Hymowitz, S.G., Stern, H., Zha, J., Ashkenazi, A., 2006. Delineation of the cell-extrinsic apoptosis pathway in the zebrafish. Cell Death Differ. 13, 1619–1630. https://doi.org/10.1038/sj.cdd.4402015.

Fauzi, A., Kifli, N., Noor, M.H.M., Hamzah, H., Azlan, A., 2024. Bioactivity, phytochemistry studies and subacute *in vivo* toxicity of ethanolic leaf extract of white mulberry (*Morus alba* Linn.) in female mice. J. Ethnopharmacol. 325, 117914 https://doi.org/10.1016/j.jep.2024.117914.

Fu, X., Wu, S., Li, B., Xu, Y., Liu, J., 2019. Functions of p53 in pluripotent stem cells. Protein Cell 11, 71–78. https://doi.org/10.1007/s13238-019-00665-x.

Galappaththi, E.K., Ichien, S.T., Hyman, A.A., Aubrac, C.J., Ford, J.D., 2020. Climate change adaptation in aquaculture. Rev. Aquac. 12, 2160–2176. https://doi.org/10.1111/raq.12427.

Gao, F., Zhao, Y., Shi, X., Qiao, D., Pei, C., Kong, X., 2024. Signalling regulation of reactive oxygen species in fish inflammation. Rev. Aquac. https://doi.org/10.1111/raq.12895.

Ghafarifarsani, H., Hoseinifar, S.H., Adhami, B., Rohani, M.F., Van Doan, H., 2023. Dietary gallic acid influences serum enzymatic parameters and immunological responses in *Cyprinus carpio* exposed to crowding stress. Aquacult. Rep. 30, 101630 https://doi.org/10.1016/j.aqrep.2023.101630.

Hu, J., Ran, C., He, S., Cao, Y., Yao, B., Ye, Y., Zhang, X., Zhou, Z., 2016. Dietary microbial phytase exerts mixed effects on the gut health of tilapia: a possible reason for the null effect on growth promotion. Br. J. Nutr. 115, 1958–1966. https://doi.org/10.1017/S0007114516001240.

Hu, Z., Han, D., Zhang, T., Li, D., Tang, R., 2022. Ammonium induces oxidative stress, endoplasmic reticulum stress, and apoptosis of hepatocytes in the liver cell line of grass carp (*Ctenopharyngodon idella*). Environ. Sci. Pollut. Res. 30, 27092–27102. https://doi.org/10.1007/s11356-022-24072-8.

Jia, R., Hou, Y., Feng, W., Li, B., Zhu, J., 2022. Alterations at biochemical, proteomic and transcriptomic levels in liver of tilapia (*Oreochromis niloticus*) under chronic exposure to environmentally relevant level of glyphosate. Chemosphere 294, 133818. https://doi.org/10.1016/j.chemosphere.2022.133818.

Jin, Y., Blikslager, A.T., 2016. Myosin light chain kinase mediates intestinal barrier dysfunction via occludin endocytosis during anoxia/reoxygenation injury. Am. J. Phys. Cell Phys. 311, C996–C1004. https://doi.org/10.1152/ajpcell.00113.2016.

Li, W., Li, D., Yang, Q., Liu, L., Liu, J., Lu, J., Wang, Y., Tang, R., Li, L., Zhang, X., 2023. Long-term crowding stress induces chronic inflammatory response and declines the immunity of grass carp (*Ctenopharyngodon idella*). Aquaculture 577, 739976. https://doi.org/10.1016/j.aquaculture.2023.739976.

Lin, Y., Huang, J., Dahms, H., Zhen, J., Ying, X., 2017. Cell damage and apoptosis in the hepatopancreas of *Eriocheir sinensis* induced by cadmium. Aquat. Toxicol. 190, 190–198. https://doi.org/10.1016/j.aquatox.2017.07.008.

Lin, W., Li, L., Chen, J., Li, D., Hou, J., Guo, H., Shen, J., 2018. Long-term crowding stress causes compromised nonspecific immunity and increases apoptosis of spleen in grass carp (*Ctenopharyngodon idella*). Fish Shellfish Immunol. 80, 540–545. https://doi.org/10.1016/j.fsi.2018.06.050.

Liu, D., Gu, Y., Yu, H., 2021. Vitamin C regulates the production of reactive oxygen species through Wnt10b signaling in the gill of zebrafish. Fish Physiol. Biochem. 47, 1271–1282. https://doi.org/10.1007/s10695-021-00982-1.

Luo, L., Liu, S., Chen, B., Li, F., Deng, Y., Huang, X., Geng, Y., Ouyang, P., Chen, D., 2023. Chronic ammonia stress caused disorder of intestinal microbiota and damaged intestinal structure and function in yellow catfish (*Pelteobagrus fulvidraco*). Aquaculture 581, 740428. https://doi.org/10.1016/j.aquaculture.2023.740428.

Lupatsch, I., Santos, G.A., Schrama, J.W., Verreth, J.A.J., 2010. Effect of stocking density and feeding level on energy expenditure and stress responsiveness in European sea bass *Dicentrarchus labrax*. Aquaculture 298, 245–250. https://doi.org/10.1016/j.aquaculture.2009.11.007.

Nagata, S., 2018. Apoptosis and clearance of apoptotic cells. Annu. Rev. Immunol. 36, 489–517. https://doi.org/10.1146/annurev-immunol-042617-053010.

Niu, Y., Zhao, Y., He, J., Yun, Y., Shen, M., Gan, Z., Zhang, L., Wang, T., 2021. Dietary dihydroartemisinin supplementation alleviates intestinal inflammatory injury through TLR4/NOD/NF-κB signaling pathway in weaned piglets with intrauterine growth retardation. Anim. Nutrit. 7, 667–678. https://doi.org/10.1016/j.aninu.2020.12.009.

Onxayvieng, K., Piria, M., Fuka, M.M., Gavrilović, A., Liang, X., Liu, L., Tang, R., Li, L., Li, D., 2021. High stocking density alters growth performance, blood biochemical profiles, and hepatic antioxidative capacity in gibel carp (*Carassius gibelio*). Fish Physiol. Biochem. 47, 203–212. https://doi.org/10.1007/s10695-020-00905-6.

Pan, J., Chen, L., Ji, Y., Huang, Y., Bu, X., Zhu, J., Li, E., Qin, J., Wang, X., 2022. A crucial role in osmoregulation against hyperosmotic stress: carbohydrate and inositol metabolism in Nile tilapia (*Oreochromis niloticus*). Aquacult. Rep. 28, 101433 https://doi.org/10.1016/j.aqrep.2022.101433.

Pederzoli, A., Mola, L., 2016. The early stress responses in fish larvae. Acta Histochem. 118, 443–449. https://doi.org/10.1016/j.acthis.2016.03.001.

Qiang, J., Yang, H., He, J., Wang, H., Xu, P., Zhu, Z., 2014. Comparison on growth performance in three different strains of Nile tilapia and physiological responses after short-term high stocking density stress. J. Fishe. Sci. China. 21, 142–152. https://doi.org/10.3724/SP.J.1118.2014.00142.

Ran, C., Huang, L., Hu, J., Tacon, P., He, S., Li, Z., Wang, Y., Liu, Z., Xu, L., Yang, Y., Zhou, Z., 2016. Effects of dietary live and heat-inactive baker's yeast on growth, gut health, and disease resistance of Nile tilapia under high rearing density. Fish Shellfish Immunol. 56, 263–271. https://doi.org/10.1016/j.fsi.2016.07.001.

Rea, K., Dinan, T.G., Cryan, J.F., 2016. The microbiome: a key regulator of stress and neuroinflammation. Neurobiol. Stress. 4, 23–33. https://doi.org/10.1016/j.ynstr.2016.03.001.

Santana, T.M., Elias, A.H., Da Fonseca, F.A.L., Freitas, O.R., Kojima, J.T., Gonçalves, L. U., 2020. Stocking density for arapaima larviculture. Aquaculture 528, 735565. https://doi.org/10.1016/j.aquaculture.2020.735565.

Santos, G.A., Schrama, J.W., Capelle, J., Rombout, J.H.W.M., Verreth, J.A.J., 2012. Effects of dissolved carbon dioxide on energy metabolism and stress responses in European seabass (*Dicentrarchus labrax*). Aquac. Res. 44, 1370–1382. https://doi.org/10.1111/j.1365-2109.2012.03142.x.

Schmittgen, T.D., Livak, K.J., 2008. Analyzing real-time PCR data by the comparative C (T) method. Nat. Protoc. 3, 1101–1108. https://doi.org/10.1038/nprot.2008.73.

Shao, T., Zhao, C., Li, F., Gu, Z., Liu, L., Zhang, L., Wang, Y., He, L., Liu, Y., Liu, Q., Chen, Y., Donde, H., Wang, R., Jala, V.R., Barve, S., Chen, S., Zhang, X., Chen, Y., McClain, C.J., Feng, W., 2018. Intestinal HIF-1α deletion exacerbates alcoholic liver disease through inducing intestinal Dysbiosis and barrier dysfunction. J. Hepatol. 69, 886–895. https://doi.org/10.1016/j.jhep.2018.05.021.

Shaughnessy, C.A., Myhre, V.D., Hall, D.J., McCormick, S.D., Dores, R.M., 2023. Hypothalamus-pituitary-interrenal (HPI) axis signaling in Atlantic sturgeon (*Acipenser oxyrinchus*) and sterlet (*Acipenser ruthenus*). Gen. Comp. Endocrinol. 339, 114290 https://doi.org/10.1016/j.ygcen.2023.114290.

Shin, N., Whon, T.W., Bae, J., 2015. Proteobacteria: microbial signature of dysbiosis in gut microbiota. Trends Biotechnol. 33, 496–503. https://doi.org/10.1016/j.tibtech.2015.06.011.

Sun, J., Liu, Q., Zhao, L., Cui, C., Wu, H., Liao, L., Tang, G., Yang, S., Yang, S., 2019. Potential regulation by miRNAs on glucose metabolism in liver of common carp (*Cyprinus carpio*) at different temperatures. Comparati. Biochem. Physiol. D: Genom. & Proteom. 32, 100628 https://doi.org/10.1016/j.cbd.2019.100628.

Sundh, H., Finne-Fridell, F., Ellis, T., Taranger, G.L., Niklasson, L., Pettersen, E.F., Wergeland, H.I., Sundell, K., 2019. Reduced water quality associated with higher stocking density disturbs the intestinal barrier functions of Atlantic salmon (*Salmo salar* L.). Aquaculture 512, 734356. https://doi.org/10.1016/j.aquaculture.2019.734356.

Taheri Mirghaed, A., Paknejad, H., Mirzargar, S.S., 2020. Hepatoprotective effects of dietary Artemisia (*Artemisia annua*) leaf extract on common carp (*Cyprinus carpio*) exposed to ambient ammonia. Aquaculture 527, 735443. https://doi.org/10.1016/j.aquaculture.2020.735443.

Ulluwishewa, D., Anderson, R.C., McNabb, W.C., Moughan, P.J., Wells, J.M., Roy, N.C., 2011. Regulation of tight junction permeability by intestinal bacteria and dietary components. J. Nutr. 141, 769–776. https://doi.org/10.3945/jn.110.135657.

Wang, T., Wen, X., Hu, Y., Zhang, X., Wang, D., Yin, S., 2018. Copper nanoparticles induced oxidation stress, cell apoptosis and immune response in the liver of juvenile *Takifugu fasciatus*. Fish Shellfish Immunol. 84, 648–655. https://doi.org/10.1016/j.fsi.2018.10.053.

Wang, Y., Liu, M., Wang, B., Jiang, K., Wang, M., Wang, L., 2020. Response of the *Litopenaeus vananmei* intestinal bacteria and antioxidant system to rearing density and exposure to *Vibrio paraheamolyticus* E1. J. Invertebr. Pathol. 170, 107326 https://doi.org/10.1016/j.jip.2020.107326.

Wang, R., Li, W., Zhang, S., Song, Y., Dai, H., Tan, T., Hu, X., Xing, Y., 2021. The effects of intrinsic apoptosis on cystogenesis in *PKD1*-deficient ADPKD pig model. Gene 798, 145792. https://doi.org/10.1016/j.gene.2021.145792.

Wang, Q., Ye, W., Tao, Y., Li, Y., Lu, S., Xu, P., Qiang, J., 2023. Transport stress induces oxidative stress and immune response in juvenile largemouth bass (*Micropterus salmoides*): analysis of oxidative and immunological parameters and the gut microbiome. Antioxidants 12, 157. https://doi.org/10.3390/antiox12010157.

Wang, D., Gao, H., Song, D., Wu, F., 2024. China Fishery Statistics Yearbook. China Agricultural Publishing House.

Wen, B., Zhang, N., Jin, S., Chen, Z., Gao, J., Liu, Y., Liu, H., Xu, Z., 2017. Microplastics have a more profound impact than elevated temperatures on the predatory performance, digestion and energy metabolism of an Amazonian cichlid. Aquat. Toxicol. 195, 67–76. https://doi.org/10.1016/j.aquatox.2017.12.010.

Wu, H., Ohnuki, H., Ota, S., Murata, M., Yoshiura, Y., Endo, H., 2016. New approach for monitoring fish stress: a novel enzyme-functionalized label-free immunosensor system for detecting cortisol levels in fish. Biosens. Bioelectron. 93, 57–64. https://doi.org/10.1016/j.bios.2016.10.001.

Wu, Y., Xia, Y., Hu, A., Xiong, G., Wu, W., Shi, L., Chen, L., Guo, X., Qiao, Y., Liu, C., Yin, T., Wang, L., Chen, S., 2024. Difference in muscle metabolism caused by metabolism disorder of rainbow trout liver exposed to ammonia stress. Sci. Total Environ. 924, 171576 https://doi.org/10.1016/j.scitotenv.2024.171576.

Xie, M., Xie, Y., Li, Y., Zhou, W., Zhang, Z., Yang, Y., Olsen, R.E., Ringø, E., Ran, C., Zhou, Z., 2021a. Stabilized fermentation product of *Cetobacterium somerae* improves gut and liver health and antiviral immunity of zebrafish. Fish Shellfish Immunol. 120, 56–66. https://doi.org/10.1016/j.fsi.2021.11.017.

Xie, M., Zhou, W., Xie, Y., Li, Y., Zhang, Z., Yang, Y., Olsen, R.E., Ran, C., Zhou, Z., 2021b. Effects of *Cetobacterium somerae* fermentation product on gut and liver health of common carp (*Cyprinus carpio*) fed diet supplemented with ultra-micro ground mixed plant proteins. Aquaculture 543, 736943. https://doi.org/10.1016/j.aquaculture.2021.736943.

Xie, M., Hao, Q., Olsen, R.E., Ringø, E., Yang, Y., Zhang, Z., Ran, C., Zhou, Z., 2022a. Growth performance, hepatic enzymes, and gut health status of common carp (*Cyprinus carpio*) in response to dietary *Cetobacterium somerae* fermentation product. Aquacult. Rep. 23, 101046 https://doi.org/10.1016/j.aqrep.2022.101046.

Xie, M., Hao, Q., Xia, R., Olsen, R.E., Ringø, E., Yang, Y., Zhang, Z., Ran, C., Zhou, Z., 2022b. Nuclease-treated stabilized fermentation product of *Cetobacterium somerae* improves growth, non-specific immunity, and liver health of zebrafish (*Danio rerio*). Front. Nutr. 9, 918327 https://doi.org/10.3389/fnut.2022.918327.

Yang, M., Rahimnejad, S., Zhang, C., Song, K., Lu, K., Wang, L., 2019. Histomorphology of gastrointestinal tract in bullfrog *Rana* (*Lithobates*) *catesbeiana* and evaluation of the changes induced by a soybean meal-based diet. Aquac. Res. 51, 164–174. https://doi.org/10.1111/are.14361.

Yang, W., Guo, Y., Lu, J., Bao, Y., 2021a. Differential gene expression of tight-junction proteins and their correlation with PRSS8 and prognosis in colorectal cancer. J. Clin. Oncol. 39, 15. https://doi.org/10.1200/JCO.2021.39.15_suppl.e15548.

Yang, H.L., Liu, Z.Y., Jian, J.T., Ye, J.D., Sun, Y.Z., 2021b. Host-associated *Bacillus siamensis* and *Lactococcus petauri* improved growth performance, innate immunity, antioxidant activity and ammonia tolerance in juvenile Japanese seabass (*Lateolabrax japonicus*). Aquac. Nutr. 27, 2739–2748. https://doi.org/10.1111/anu.13399.

Yang, E.J., Zhang, J.D., Yang, L.T., Amenyogbe, E., Wang, W.Z., Huang, J.S., Chen, G., 2021c. Effects of hypoxia stress on digestive enzyme activities, intestinal structure and the expression of tight junction proteins coding genes in juvenile cobia (*Rachycentron canadum*). Aquac. Res. 52, 5630–5641. https://doi.org/10.1111/are.15438.

Zaki, M.A.A., Khalil, H.S., Allam, B.W., Khalil, R.H., Basuini, M.F.E., Nour, A.E.M., Labib, E.M.H., Elkholy, I.S.E., Verdegem, M., Abdel-Latif, H.M.R., 2023. Assessment of zootechnical parameters, intestinal digestive enzymes, haemato-immune responses, and hepatic antioxidant status of *Pangasianodon hypophthalmus* fingerlings reared under different stocking densities. Aquac. Int. 31, 2451–2474. https://doi.org/10.1007/s10499-023-01092-w.

Zhang, J., Dong, Y., Song, K., Wang, L., Li, X., Lu, K., Tan, B., Zhang, C., 2022a. Substituting fish meal with a Bacteria protein (*Clostridium autoethanogenum* protein) derived from industrial-scale gas fermentation: effects on growth and gut health of juvenile large yellow croakers (*Larimichthys crocea*). Fishes 7, 228. https://doi.org/10.3390/fishes7050228.

Zhang, J., Dong, Y., Song, K., Wang, L., Li, X., Tan, B., Lu, K., Zhang, C., 2022b. Effects of the replacement of dietary fish meal with defatted yellow mealworm (*Tenebrio molitor*) on juvenile large yellow croakers (*Larimichthys crocea*) growth and gut health. Animals 12, 2659. https://doi.org/10.3390/ani12192659.

Zhang, N., Yang, R., Fu, Z., Yu, G., Ma, Z., 2023a. Mechanisms of digestive enzyme response to acute salinity stress in juvenile yellowfin tuna (*Thunnus albacares*). Animals 13, 3454. https://doi.org/10.3390/ani13223454.

Zhang, J., Yu, M., Wang, J., Longshaw, M., Song, K., Wang, L., Li, X., Zhang, C., Lu, K., 2023b. Methanotroph (*Methylococcus capsulatus*, Bath) bacteria meal alleviates soybean meal-induced enteritis in spotted seabass (*Lateolabrax maculatus*) by modulating immune responses and the intestinal flora. Aquaculture 575, 739795. https://doi.org/10.1016/j.aquaculture.2023.739795.

Zhao, K., Gaines, S.D., García Molinos, J., Zhang, M., Xu, J., 2024. Effect of trade on global aquatic food consumption patterns. Nat. Commun. 15, 1412. https://doi.org/10.1038/s41467-024-45556-w.

Zhou, W., Xie, M., Xie, Y., Liang, H., Li, M., Ran, C., Zhou, Z., 2022. Effect of dietary supplementation of *Cetobacterium somerae* XMX-1 fermentation product on gut and liver health and resistance against bacterial infection of the genetically improved farmed tilapia (GIFT, *Oreochromis niloticus*). Fish Shellfish Immunol. 124, 332–342. https://doi.org/10.1016/j.fsi.2022.04.019.

Zhu, J., Wang, X., Bu, X., Wang, C., Pan, J., Li, E., Shi, Q., Zhang, M., Qin, J.G., Chen, L., 2021. Relationship between *myo*-inositol synthesis and carbohydrate metabolism changes in Mozambique tilapia (*Oreochromis mossambicus*) under acute hypersaline stress. Aquaculture 532, 736005. https://doi.org/10.1016/j.aquaculture.2020.736005.

Zhu, C., Zhou, W., Han, M., Yang, Y., Li, Y., Jiang, Q., Lv, W., 2023. Dietary polystyrene nanoplastics exposure alters hepatic glycolipid metabolism, triggering inflammatory responses and apoptosis in *Monopterus albus*. Sci. Total Environ. 891, 164460 https://doi.org/10.1016/j.scitotenv.2023.164460.