

Article

Effects of Different Stocking Densities on the Growth, Antioxidant Status, and Intestinal Bacterial Communities of Carp in the Rice–Fish Co-Culture System

Weixu Diao ^{1,†}, Rui Jia ^{1,2,3,†}, Yiran Hou ^{1,2}, Jianyou Gong ¹, Liqiang Zhang ^{1,2}, Bing Li ^{1,2,*} and Jian Zhu ^{1,2,*}

- ¹ Wuxi Fisheries College, Nanjing Agricultural University, Wuxi 214081, China; 2021113006@stu.njau.edu.cn (W.D.); jiar@ffrc.cn (R.J.); houyr@ffrc.cn (Y.H.); jianygo@foxmail.com (J.G.); zhangliqiang@ffrc.cn (L.Z.)
- ² Key Laboratory of Integrated Rice–Fish Farming Ecology, Ministry of Agriculture and Rural Affairs, Freshwater Fisheries Research Center, Chinese Academy of Fishery Sciences, Wuxi 214081, China
- ³ International Joint Research Laboratory for Fish Immunopharmacology, Freshwater Fisheries Research Center, Chinese Academy of Fishery Sciences, Wuxi 214081, China
- * Correspondence: lib@ffrc.cn (B.L.); zhuj@ffrc.cn (J.Z.); Tel.: +86-85550535 (B.L.); +86-85550414 (J.Z.)
- † These authors contributed equally to this work.

Abstract: Common carp (*Cyprinus carpio*) is a freshwater fish commonly farmed in rice fields, yet there were few studies on the intestinal functions and microbial community structure of common carp in the rice–carp co-culture system. Therefore, this study aimed to explore the effects of different stocking densities on the growth, antioxidant status, and intestinal bacterial composition of common carp in this system. This study was divided into three different stocking densities, including low density (LD, 10 fish, 52.9 g/m²), medium density (MD, 20 fish, 105.8 g/m²), and high density (HD, 30 fish, 158.7 g/m²), with a culturing period of 60 days. The results indicated that HD treatment inhibited the growth of common carp, as evidenced by the reduced final weight, WG, and SGR. In serum, the TG content in the HD group and the Cor content in the MD group were significantly increased. Meanwhile, HD treatment induced oxidative stress, manifesting specifically as increased SOD and CAT activities in the intestine or serum while reducing Gpx, GSH, and T-AOC in the serum. The 16S rDNA analysis indicated that the Simpson and Shannon indices of intestinal microbiota in the HD group were significantly higher than those in the LD group. At the phylum level, Proteobacteria, Cyanobacteria, and Firmicutes were dominant microbial communities in two groups. In addition, there was a significant difference between the two groups in the abundances of Actinobacterota and *Bifidobacterium*. Based on growth performances, biochemical indicators, and microbial diversity in rice–carp co-culture, low density (52.9 g/m²) may be more suitable in the rice–carp co-culture systems. In summary, this study contributes to a better understanding of common carp response to different stocking densities in the rice–carp co-culture system.

Keywords: common carp; integrated rice–carp co-culture system; oxidative stress; intestinal microbiota

Key Contribution: This study focused on the effects of different stocking densities on the structure of intestinal microbiota in the rice–carp co-culture system.



Citation: Diao, W.; Jia, R.; Hou, Y.; Gong, J.; Zhang, L.; Li, B.; Zhu, J. Effects of Different Stocking Densities on the Growth, Antioxidant Status, and Intestinal Bacterial Communities of Carp in the Rice–Fish Co-Culture System. *Fishes* **2024**, *9*, 244. <https://doi.org/10.3390/fishes9070244>

Academic Editor: David Sánchez Peñaranda

Received: 18 April 2024

Revised: 6 June 2024

Accepted: 18 June 2024

Published: 21 June 2024



Copyright: © 2024 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

1. Introduction

The rice–fish co-culture system represents an integrative ecological agriculture approach, utilizing paddy fields during the rice-growing season to simultaneously cultivate aquatic organisms [1]. This model fosters a symbiotic relationship between rice and aquatic animals, enhancing resource utilization and increasing production efficiency compared to traditional rice monoculture. The presence of aquatic animals aerates the soil and boosts dissolved oxygen levels, and their waste enhances soil fertility, thus reducing the application for pesticides

and chemical fertilizers [2]. Paddy are biodiverse environments rich in microorganisms and weeds, offering a rich food source for the aquatic animals. By harnessing the complementary benefits of both rice and fish, this co-culture system accomplishes the efficient reuse of water and maximizes land productivity, heralding a sustainable, high-quality, and efficient agricultural paradigm. It offers notable economic and social advantages, aligning with the trend toward green ecological agriculture [3]. In China, rice–fish co-culture has evolved into a significant aquaculture model. In 2022, this model spanned roughly 28,637 km² across China and yielded 3.87 million tons of aquatic products, making it the second most productive method in aquaculture, following pond culture [4].

In aquaculture practices, high stocking density is a prevalent approach to enhance yield. However, this method may precipitate stress due to intensified social interactions among the aquatic animals. This stress can lead to deleterious physiological responses since high stocking density often results in diminished growth rates and disrupted immune function in fish [5]. Existing research demonstrates that for yellow catfish (*Pelteobagrus fulvidraco*), when density becomes excessively high, there is a noticeable decrease in weight gain, body length, and condition factor, whereas the feed conversion ratio and coefficient of variation increase [6]. In juvenile grass carp (*Ctenopharyngodon idella*), elevated densities are associated with slowed growth, reduced immunity, and decreased antioxidant capacities [7]. Additionally, high density adversely impacts the growth and digestive enzyme activity of juvenile crayfish (*Procambarus clarkii*), eliciting notable alteration in the content of proteins, fats, glucose, and urea nitrogen in response to the escalated farming density [8].

The common carp (*Cyprinus carpio*), a significant freshwater aquaculture species in China, is renowned for its strong adaptability, rapid growth, and considerable cold resistance [9,10]. It is primarily cultivated in China in a variety of aquatic environments, including ponds, rice paddies, and lakes. Numerous studies have explored the effects of different stocking densities on the growth and physiological and biochemical parameters of carp in pond aquaculture [11,12]. However, few studies have focused on the impact of stocking density on the intestinal functions and microbial composition of carp, especially in integrated rice–fish farming systems. Therefore, this study aims to assess the effects of different stocking densities on the growth, intestinal antioxidant status, and microbial composition of carp in the integrated rice–carp co-culture system. These data will help to determine the optimal stocking density that enhances both economic and ecological outcomes in rice–carp farming.

2. Materials and Methods

2.1. Experiment Design

The common carp used in this experiment were supplied by the Jingjiang Experimental Base of the Freshwater Fisheries Research Center, Chinese Academy of Fishery Sciences. Healthy carp with uniform size (105.33 ± 2.52 g) were selected for the experiment. Three stocking densities were established: low density (LD, 10 fish, 52.9 g/m²), medium density (MD, 20 fish, 105.8 g/m²), and high density (HD, 30 fish, 158.7 g/m²), with each group having three replicates. Each rice–fish co-culture system covered an area of 20 m². On 20 June 2022, “Nangeng 5055” rice seedlings were transplanted into the experimental fields, and the rice was harvested in early November. The water level in the rice fields was maintained at 0.1–0.2 m. The experiment ran from September 1 to October 30. The fish were fed with feed (Cargill Changzhou, China) at 1% of fish body weight. The main nutritional components were crude protein 31.0%, crude fat 4.0%, and crude ash 18.0%. During the experiment, dissolved oxygen levels ranged from 3.2 to 5.8 mg/L, pH from 6.80 to 7.60, and water temperature from 18.5 to 30.5 °C.

2.2. Sample Collection

Fasting for 24 h, 20 fish were randomly selected from each group and anesthetized with MS-222. Blood was drawn from the caudal vein using a sterile 1 mL syringe. The collected blood was transferred into tubes (1.5 mL), resting for 4–5 h at 4 °C. Subsequently, serum was obtained by centrifugation at 3000 g for 10 min [13]. The serum was stored at −80 °C for further analysis. Following serum collection, the whole intestinal tissues and their contents were collected. After that, the separated intestinal tissues were quickly frozen in liquid nitrogen and then transferred and stored at −80 °C for further analysis. The blood and intestinal tissues from 4 fish were mixed to form one sample. The fish in this study received approval from the Freshwater Fisheries Research Centre (Wuxi, China), ensuring that all experimental practices adhered to animal welfare considerations.

2.3. Growth Parameters Analysis

Based on the recorded data of common carp weight, the growth rate and specific growth rate of common carp under different densities in the rice–carp co-culture system were calculated. The specific formulas are as follows:

$$\text{Weight gain rate, WG, \%} = (W_2 - W_1) / W_1 / 60 \times 100\%$$

$$\text{Specific growth rate of weight, SGR, \%} = (\ln W_2 - \ln W_1) / T \times 100\%$$

wherein (W_1) and (W_2) denote the initial weight and final weight (g), respectively, (L) represents the length of an individual (cm) at the end of the experiment, and (T) stands for the duration of the experiment (days).

2.4. Biochemical and Antioxidant Status Analysis

The biochemical parameters including cortisol (Cor), lactate (LA), glucose (Glu), (T-CHO), triglycerides (TG), acid phosphatase (ACP), alkaline phosphatase (AKP), albumin (Alb), and total protein (TP) were measured using commercial kits. Specifically, the reagent kits for measuring lactate (LA) and acid phosphatase (ACP) were sourced from Nanjing Jiancheng Bioengineering Institute (Nanjing, China) [14]; cortisol (Cor) was measured using kits provided by Shanghai Enzyme-linked Biotechnology Co., Ltd. (Shanghai, China) [15], and the analysis of total cholesterol (T-CHO), triglycerides (TG), glucose (Glu), alkaline phosphatase (AKP), albumin (Alb), and total protein (TP) was performed using the Mindray BS-400 fully automatic biochemistry analyzer [16].

We measured the levels of catalase (CAT), glutathione peroxidase (Gpx), glutathione (GSH), malondialdehyde (MDA), superoxide dismutase (SOD), and total antioxidant capacity (T-AOC) in serum and intestinal tissues. The reagent kits for Gpx were procured from Suzhou Grace Biotechnology Co., Ltd. (Suzhou, China); MDA and CAT were determined using kits from Shanghai Beyotime Biotechnology Co., Ltd. (Shanghai, China) [17], and GSH, SOD, and T-AOC measurements were carried out using kits supplied by Nanjing Jiancheng Bioengineering Institute [18].

2.5. Microbial Sequencing

The genomic DNA was extracted from the intestinal content using the QIAamp PowerFecal Pro DNA Kit (Qiagen, Shenzhen, China), following the manufacturer's instructions. The concentration and purity of DNA were assessed through electrophoresis on a 1% agarose gel. Amplification of the bacterial 16S rRNA gene V3–V4 regions was performed using PCR with the forward primer 341F (5'-CCTAYGGGRBGCASCAG-3') and the reverse primer 806R (5'-GGACTACNNGGGTATCTAAT-3'). Sequencing libraries were prepared according to the manufacturer's recommendations using the Illumina TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina Trading Co., Ltd., Shanghai, China), with the addition of index codes. The quality of these libraries was subsequently evaluated using the Qubit® 2.0 Fluorometer (Thermo Scientific, Shanghai, China). The sequencing process was completed using the Illumina NovaSeq platform.

Using the Uparse algorithm, effective tags from the reads were clustered, which were then classified into OTUs based on a 97% sequence identity criterion [19]. The OTUs were annotated and classified employing the Mothur method alongside the SILVA138.1 database [20]. Representative sequences for all OTUs underwent alignment via MUSCLE software to elucidate their phylogenetic relationships [21]. Alpha diversity indices, such as richness, Shannon, Simpson, and Evenness, were analyzed utilizing QIIME software (version 1.9.1). A principal coordinates analysis (PCoA) based on weighted Unifrac distances was conducted within the R project environment [22]. To determine the presence of statistically significant differences between groups, a *t*-test was conducted with a significance threshold established at $p < 0.05$.

2.6. Statistical Analysis

Data analysis was carried out using SPSS 26.0 software and Excel 2019 software. For the visualization of data, GraphPad Prism 8.0 software was chosen. The Shapiro–Wilk and Levene tests were used for normal distribution and homogeneity of variance, respectively. The differences in growth, physiological, biochemical, and antioxidant parameters among different groups were tested by one-way analysis of variance (ANOVA) followed by the least significant difference (LSD). Differences were deemed statistically significant at a *p*-value of less than 0.05. Results were expressed as the mean $\pm$ standard error (Mean $\pm$ SE).

3. Results

3.1. Change in Growth Performance

As indicated in Table 1, after 60 days of farming, the final body weight, WGR, and SGR of the HD group were significantly lower than those of the LD group ($p < 0.05$), whereas no significant differences were observed between the MD group and the other groups ($p > 0.05$).

Table 1. Growth parameters of common carp at different stocking densities after 60 days of farming.

Groups	Initial Weight (g)	Final Weight (g)	WG (%)	SGR (%/d)
LD	105.55 $\pm$ 0.33	178.3 $\pm$ 10.19 ^a	1.15 $\pm$ 0.096 ^a	0.84 $\pm$ 0.092 ^a
MD	105.72 $\pm$ 0.18	155.41 $\pm$ 8.15 ^{ab}	0.78 $\pm$ 0.054 ^b	0.62 $\pm$ 0.086 ^{ab}
HD	105.43 $\pm$ 0.24	136.36 $\pm$ 5.09 ^b	0.49 $\pm$ 0.047 ^c	0.41 $\pm$ 0.063 ^b

Note: After the same column of data, different letters indicate that the difference is significant ($p < 0.05$). Values are presented as means $\pm$ SE. LD, low stocking density; MD, medium stocking density; HD, high stocking density.

3.2. Change in Biochemical Indices in Serum

As shown in Figure 1, the levels of TG and Alb in the HD group were significantly higher than those in the LD and MD groups ($p < 0.05$). Moreover, the ACP and Cor levels in the MD group were markedly higher than those in the LD and HD groups ($p < 0.05$). The AKP, Glu, T-CHO, and LA have no significant changes among different groups ($p > 0.05$).

3.3. Change in Antioxidant Status in Serum and Intestine

In serum, compared to the LD group, the MD and HD groups exhibited a significant decrease in the levels of Gpx, T-AOC, and GSH ($p < 0.05$, Figure 2B,C,F). However, the SOD level in the HD group was significantly higher than in the LD group ($p < 0.05$, Figure 2E). Moreover, no significant differences were observed in MDA concentration and CAT activity among the different density groups ($p > 0.05$, Figure 2A,D).

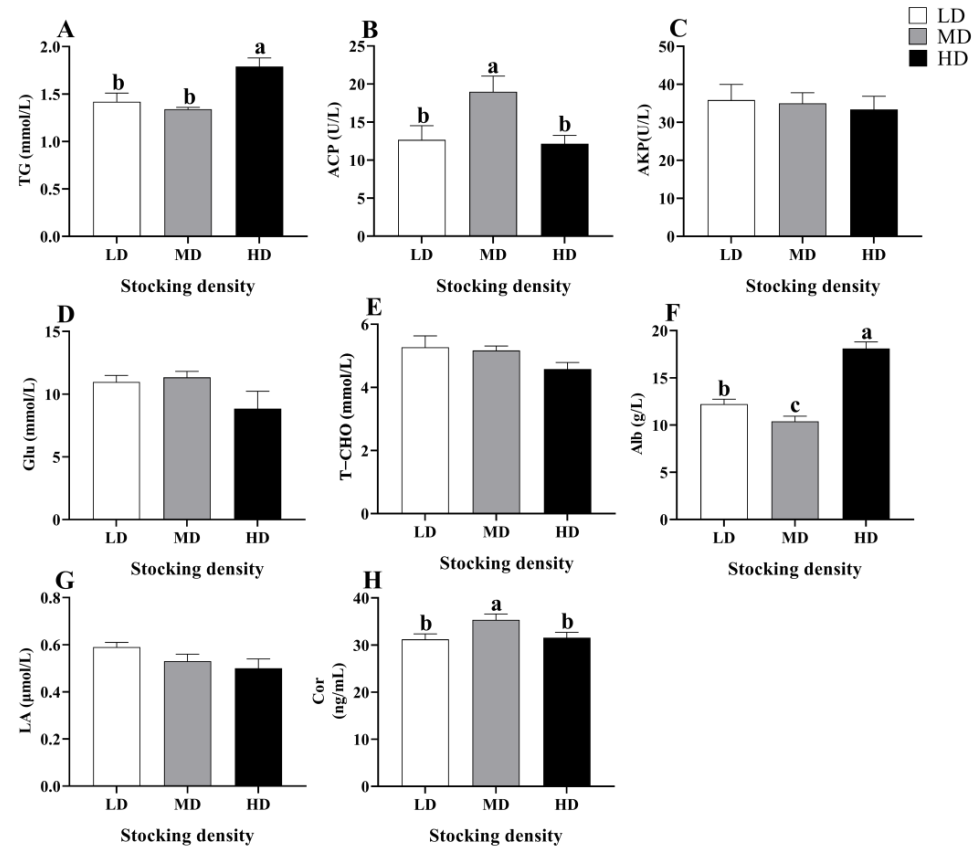


Figure 1. Physiological and biochemical indicators in the serum of common carp reared at different densities in an integrated rice–fish farming system. (A) TG, (B) ACP, (C) AKP, (D) Glu, (E) T-CHO, (F) Alb, (G) LA, and (H) Cor. Values are presented as means $\pm$ SE ($n = 5$). Different letters as superscripts indicate significant differences among the different groups ($p < 0.05$). LD, low stocking density; MD, medium stocking density; HD, high stocking density.

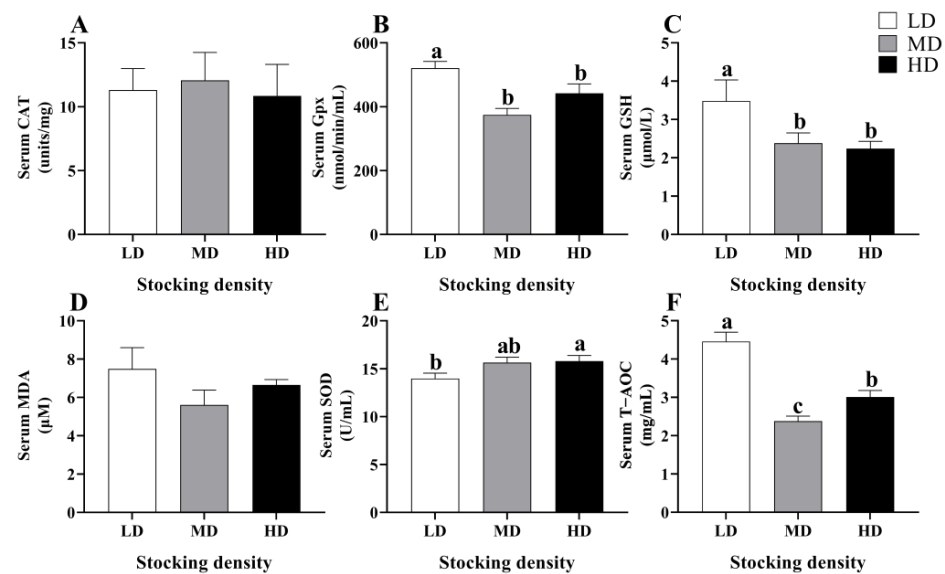


Figure 2. Antioxidative parameters in the serum of common carp reared at different densities in an integrated rice–fish farming system. (A) CAT; (B) Gpx; (C) GSH; (D) MDA; (E) SOD; (F) T-AOC. Values are presented as means $\pm$ SE ($n = 5$). Different letters as superscripts indicate significant differences among the different groups ($p < 0.05$). LD, low stocking density; MD, medium stocking density; HD, high stocking density.

In the intestines, compared to the LD group, MD and HD groups exhibited significantly decreased levels of CAT and GSH ($p < 0.05$, Figure 3A,C). However, the Gpx level in the HD group was significantly lower than that in both the LD and MD groups ($p < 0.05$, Figure 3B). Additionally, no significant differences were observed in the levels of SOD, T-AOC, or MDA in the intestines across the different density groups ($p > 0.05$, Figure 3D–F).

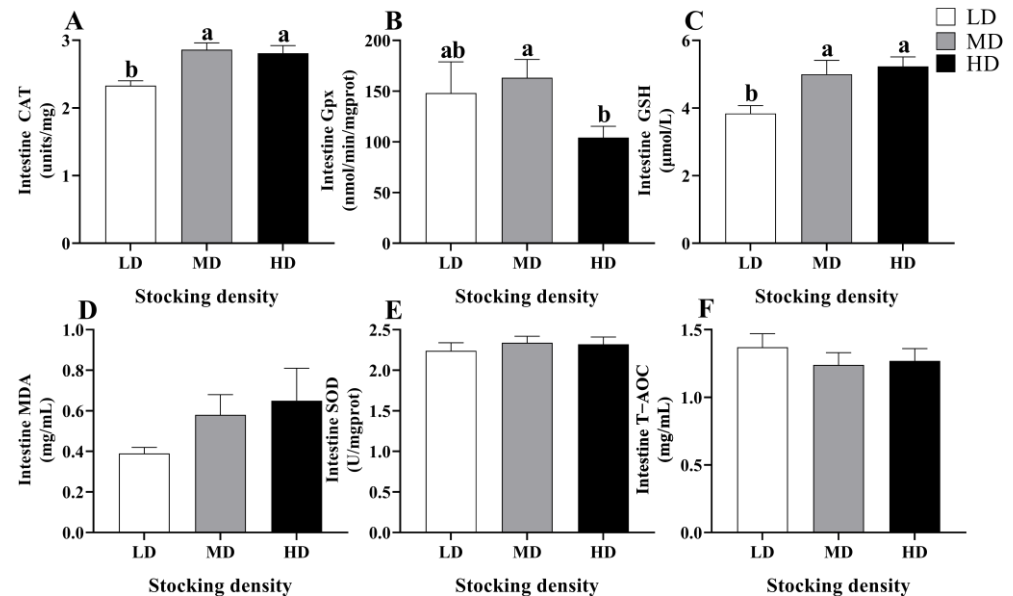


Figure 3. Antioxidative parameters in the intestine of common carp reared at different densities in an integrated rice–fish farming system. (A) CAT; (B) Gpx; (C) GSH; (D) MDA; (E) SOD; (F) T-AOC. Values are presented as means $\pm$ SE ($n = 5$). Different letters as superscripts indicate significant differences among the different groups ($p < 0.05$). LD, low stocking density; MD, medium stocking density; HD, high stocking density.

3.4. Alpha and Beta Diversities of Bacteria in Intestine

Alpha diversity analyses revealed that, compared to the LD group, the HD group exhibited a significant decrease in the Shannon and Simpson indices ($p < 0.05$; Figure 4B,D). However, no significant differences in Chao1 or ACE indices were observed between the two groups ($p > 0.05$; Figure 4A,C). Venn diagrams illustrated that a total of 1741 OTUs were identified across both groups, with 993 OTUs shared between them, accounting for approximately 57.04% of the total detected OTUs (Figure 4E). The PCoA results showed that there was no distinct separation in the clustering of gut bacterial communities between the LD and HD groups (Figure 4F).

3.5. Bacterial Composition in Intestine

The composition of gut bacteria demonstrated that at the phylum level, both the LD and HD groups showed a similar dominance of phyla, primarily Proteobacteria, Cyanobacteria, and Firmicutes (Figure 5A). Among these, Cyanobacteria are the most abundant in the LD group (45.74%), while Proteobacteria are most abundant in the HD group (33.85%).

At the genus level, the bacterial communities were mainly composed of *Aeromonas*, *Citrobacter*, and *Clostridium*, with *Aeromonas* being the genus with the highest abundance in both groups (LD: 25.53%, HD: 12.20%). Moreover, *t*-test analysis indicated that the HD group has a significantly higher relative abundance of Actinobacterota and *Bifidobacterium* compared to the LD group (Figure 6, $p < 0.05$).

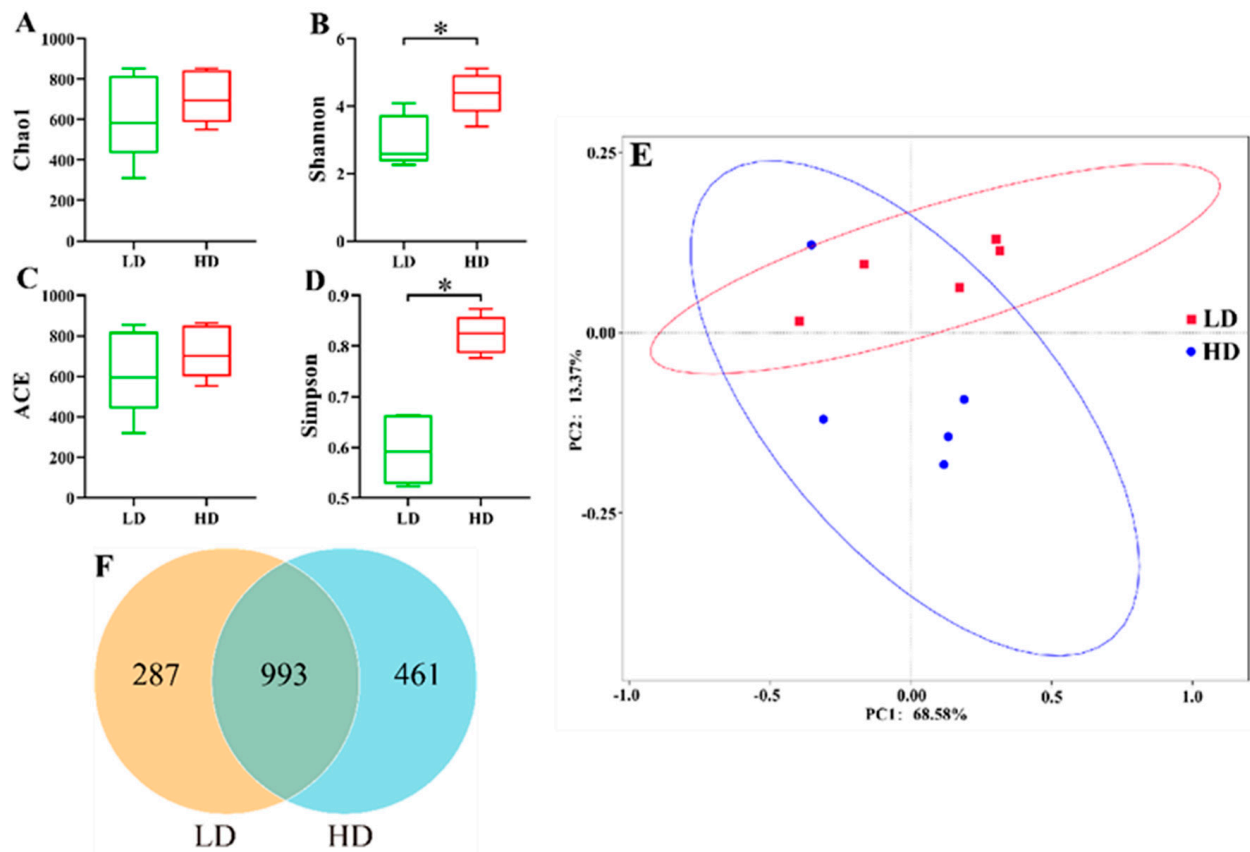


Figure 4. Differences in alpha and beta diversities of intestine bacterial communities of common carp between the LD and HD groups. (A–D) Alpha diversity index in different stocking densities; (E) Venn diagrams of OTUs; (F) principal coordinates analysis between different stocking densities. * $p < 0.05$. LD, low stocking density; HD, high stocking density.

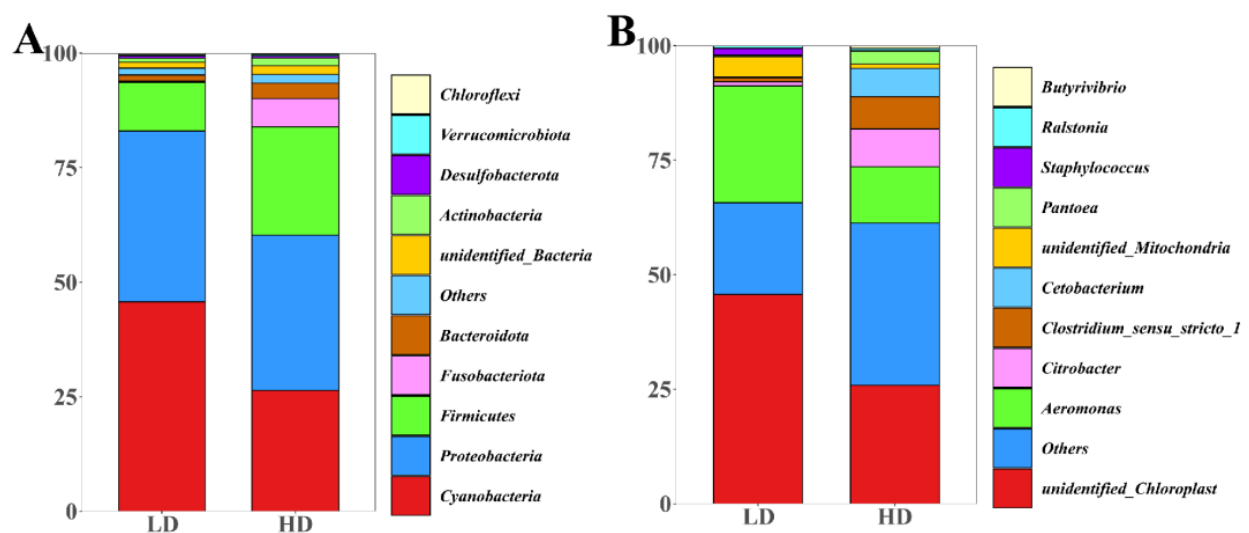


Figure 5. Intestinal bacterial composition at the level of phylum (A) and genus (B) in the LD and HD groups. LD, low stocking density; HD, high stocking density.

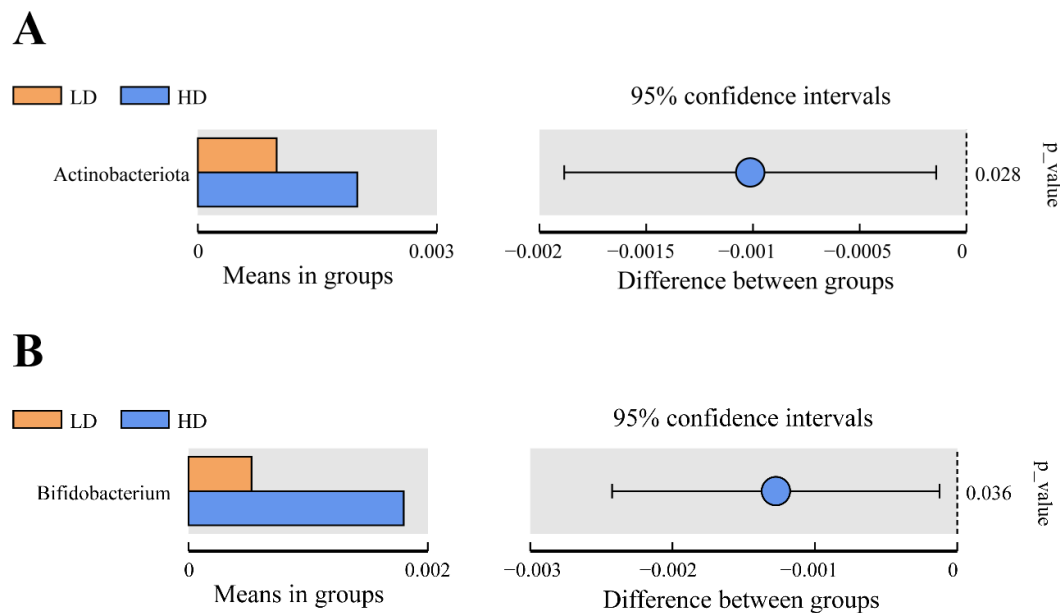


Figure 6. Analysis diagram of species differences at phylum level (A) and genus level (B) groups by *t*-test. LD, low stocking density; HD, high stocking density.

4. Discussion

The stocking density plays a critical role in determining the survival, growth, and overall health status of fish in aquaculture. Increasing stocking density in a balanced manner can both amplify fish yields and optimize water resource utilization. Nonetheless, excessive stocking densities may adversely affect farmed fish, such as inhibiting growth performance, deteriorating water quality, and increasing mortality and disease risk [23,24]. Karnatak et al. found a negative correlation between stocking density and growth performance in common carp, noting that higher densities resulted in decreased weight gain, specific growth rate, feed conversion ratio, and protein efficiency [25]. Similarly, research on barramundi (*Lates calcarifer*) indicated that increasing stocking densities from 350 to 1750 g/m³ significantly reduced both fish weight and feed utilization rates [26]. Wu et al. also reported that in yellow catfish, an increase in stocking density significantly hindered the final weight, growth rate, and specific growth rate [27]. Consistent with these observations, the current study revealed that common carp reared at HD exhibited significantly reduced final weight, weight gain rate, and specific growth rate compared to those reared at LD. This is likely attributed to intensified social interactions at higher densities, which increase energy expenditure and, consequently, inhibit growth.

The blood circulatory system is vital for the transportation of nutrients, delivery of oxygen, and metabolism of waste in fish [28]. It has been reported that high density, acting as a form of chronic stress, can modify the physiological and biochemical parameters in fish blood [29,30]. Blood glucose, cortisol, and lactate act as biomarkers for assessing stress levels in fish [31,32]. Blood glucose, a vital energy source, usually rises in response to unfavorable environmental conditions [33]. Cortisol assists in managing stress by regulating glucose metabolism, promoting the breakdown of proteins, and facilitating fat oxidation [34]. However, consistently high or uncontrolled levels of cortisol can detrimentally affect fish, inhibiting their growth [35]. Elevated lactate levels indicate a fish's struggle to maintain homeostasis, particularly in situations such as hypoxia [36]. In addition, lactate participates in the gluconeogenesis pathway to generate glucose, thereby providing energy to the body [37,38]. Previous research demonstrated that channel catfish (*Ictalurus punctatus*) displayed elevated blood glucose and cortisol levels following 60 days of cultivation at high densities, indicating an amplification of the stress response [39]. In this study, common carp in the MD group exhibited significantly elevated serum cortisol levels, whereas, in the HD group, the cortisol

level did not increase. We speculate that these results can be attributed to a dysregulation of the feedback mechanism under chronic stress induced by high-density cultivation conditions. The precise mechanisms behind these findings merit further exploration.

TG and T-CHO are the main lipids in blood, closely related to the organism's metabolism and physiological status, and can be used to assess the adaptability of fish to their environments [40]. Zhang et al. found that the TG levels in the HD group were significantly higher than those in the LD group, whereas the T-CHO levels showed no significant differences between groups in largemouth bass [41]. Mohamed et al. reported that both T-CHO and TG levels in the HD group were significantly higher than those in the LD group [39]. In this study, the TG content in the HD group was significantly higher than that in both the LD and MD groups. This elevation may be attributed to the increased demand for triglyceride reserves under high-density farming conditions, aiming to cope with the added energy expenditure, thereby leading to higher levels of TG.

Fish have evolved a comprehensive antioxidant defense system to ensure the maintenance of redox balance. This intricate system comprises both antioxidative enzymes (e.g., SOD, CAT, and GPX) and non-enzymatic antioxidants (e.g., GSH) [42]. However, chronic oxidative stress can deplete antioxidants and impair the antioxidant defense system, which may increase susceptibility to disease in fish [43]. Previous findings indicated that high stocking density, acting as a form of chronic stress, can induce oxidative stress in aquatic animals, accompanied by alterations in both enzymatic antioxidants (e.g., SOD and CAT) and non-enzymatic antioxidants (e.g., GSH). For instance, impaired antioxidant function was observed in rainbow trout (*Oncorhynchus mykiss*) and mandarin fish (*Siniperca chuatsi*) under high stocking densities, indicated by reduced levels of antioxidant parameters, such as SOD, GSH, and CAT [44,45]. Conversely, a positive response was observed in certain fish species, such as Japanese flounder (*Paralichthys olivaceus*) and large yellow croaker (*Larimichthys crocea*), where the levels of antioxidants (e.g., SOD, CAT, and GSH) tended to increase with heightened stocking density [46,47]. Furthermore, research has revealed that the T-AOC level in channel catfish (*Ictalurus punctatus*) and yellow catfish (*Pelteobagrus fulvidraco*) does not show significant differences under varying stocking density conditions [2,39]. In this study, HD led to an increase in SOD and CAT activities in the intestine or serum while simultaneously reducing the levels of Gpx, GSH, and T-AOC in the serum. This observation indicates a multifaceted response of aquatic organisms to stress conditions, emphasizing the intricate role of the antioxidant defense system in counteracting oxidative stress [48].

Numerous studies are highlighting the connection between intestinal microbiota and fish health, widely acknowledging that these microbial communities are essential in regulating intestinal immunity, nutrient absorption, and overall host health [49]. In this study, Alpha diversity analysis revealed that the Simpson and Shannon index of intestinal microbiota in the HD group of carp was significantly higher than that in the LD group, indicating an increased diversity of intestinal microbiota under high-density conditions. This observation aligns with the findings from the results of Wang et al. on the intestinal microbiota of banana prawn (*Fenneropenaeus merguensis*) [50]. Our study also found that Proteobacteria consistently occupied a substantial proportion of the intestinal microbiota. Similarly, Proteobacteria have been found to be the dominant bacterial phylum in the intestines of other aquatic animals.

In the LD and HD groups, Firmicutes and Clostridium were detected in the intestinal microbiota of carp, with Firmicutes serving as the dominant phylum, which aligned with the findings of Nie et al. [51]. At the genus level of intestinal bacteria, *Aeromonas* and *Citrobacter* serving as the dominant genus, this study's findings align with those reported by Tan et al. regarding the gut microbiome structure of wild Malaysian mahseer (*Tor tambroides*) [52]. It was observed that increased competition under higher densities prompts common carp to consume more phytoplankton and algae available in the environment. These dietary changes may be a critical factor in the significant increase observed in the relative abundance of the *Citrobacter* and *Mycobacterium* genera within the intestines in high-density environments. Additionally, there was a significant difference between

the two groups in the abundances of Actinobacterota and *Bifidobacterium*. *Bifidobacterium*, belonging to the Actinobacterota phylum, is among the most common bacterial species in the intestines of aquatic organisms and can directly reflect the health status of the intestine [53]. Wen et al. have demonstrated that bifidobacteria, as a probiotic, are beneficial for immune function [54]. Therefore, the increase in relative abundance of Actinomycetes and *Bifidobacterium* in high density may be an adaptive response, which contributed to maintaining intestinal health.

5. Conclusions

Our findings indicated that high stocking density reduced the growth performance, including the final weight, WG, and SGR in the rice–carp co-culture system. Our data also revealed that MD and/or HD treatments resulted in increased physiological stress, which was evidenced by alterations in lipid metabolism and antioxidant status, mainly including alterations in the levels of TG, Alb, SOD, GSH, and Gpx. Meanwhile, high stocking density increased the diversity of intestinal microbiota and the relative abundance of Actinobacterota and *Bifidobacterium*. In summary, this study provides a reference for the farming of carp in the rice–carp co-culture system. Based on growth performance, biochemical indicators, and microbial diversity, low density (52.9 g/m²) may be more suitable in the rice–carp co-culture systems.

Author Contributions: Conceptualization, writing—original draft preparation, software, W.D.; methodology, investigation, J.G.; validation, formal analysis, Y.H., R.J., and L.Z.; resources, data curation, writing—review and editing, B.L.; visualization, supervision, project administration, funding acquisition, J.Z. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the earmarked fund for CARS (CARS-45), Central Public-Interest Scientific Institution Basal Research Fund, CAFS (2023TD64), and National Key R&D Program of China (2019YFD0900305).

Institutional Review Board Statement: All animals in this study were approved by the Animal Care and Use Ethics Committee of the Freshwater Fisheries (2020TD60, 10-08-2022), and all procedures were performed according to Jiangsu Laboratory's Animal Management Guidelines (014000319/2008-00079).

Informed Consent Statement: Not applicable.

Data Availability Statement: The raw data of 16S rRNA used in this study have been submitted to the open database NCBI Sequence Read Archive (PRJNA1101697). All other data are contained within the main manuscript.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Lima, P.C.M.; Silva, A.E.M.; Silva, D.A.; Silva, S.M.B.C.; Brito, L.O.; Gálvez, A.O. Effect of Stocking Density of *Crassostrea* sp. in a Multitrophic Biofloc System with *Litopenaeus vannamei* in Nursery. *Aquaculture* **2021**, *530*, 735913. [\[CrossRef\]](#)
2. Diao, W.; Jia, R.; Hou, Y.; Dong, Y.; Li, B.; Zhu, J. Effects of Stocking Density on the Growth Performance, Physiological Parameters, Antioxidant Status and Lipid Metabolism of *Pelteobagrus fulvidraco* in the Integrated Rice-Fish Farming System. *Animals* **2023**, *13*, 1721. [\[CrossRef\]](#) [\[PubMed\]](#)
3. Nga, B.; Lüring, M.; Peeters, E.; Roijackers, R.; Scheffer, M.; Nghia, T. Chemical and Physical Effects of Crowding on Growth and Survival of *Penaeus monodon* Fabricius Post-Larvae. *Aquaculture* **2005**, *246*, 455–465. [\[CrossRef\]](#)
4. Yu, X.; Hao, X.; Dang, Z.; Yang, L. Report on the Development of China's Integrated Rice-Fish Farming (2023). *China Fish.* **2023**, 19–26.
5. Liu, J.; Zhao, Z.; Luo, L.; Wang, S.; Zhang, R.; Guo, K.; Bai, Q.; Li, H.; Li, M. Effects of Different Crab Stocking Density on Production Performance and Environmental Factors under Integrated Cultivation of Rice Crab in the Cold Areas. *Fresh Water Fish.* **2022**, *52*, 89–97.
6. Guan, W.; Liu, K.; Shi, W.; Xuan, F.; Wang, W. Scientific Paradigm of Integrated Farming of Rice and Fish. *Acta Ecol. Sin.* **2020**, *40*, 5451–5464.
7. Mabaya, G.; Unami, K.; Yoshioka, H.; Takeuchi, J.; Fujihara, M. Robust Optimal Diversion of Agricultural Drainage Water from Tea Plantations to Paddy Fields During Rice Growing Seasons and Non-Rice Growing Seasons. *Paddy Water Environ.* **2016**, *14*, 247–258. [\[CrossRef\]](#)

8. Nakasone, H. Runoff Water Quality Characteristics in a Small Agriculture Watershed. *Paddy Water Environ.* **2003**, *1*, 183–188. [\[CrossRef\]](#)
9. Huang, Z. In Vitro Bacteriostatic Test of Traditional Chinese Medicine against *Aeromonas hydrophila* of *Cyprinus carpio* Origin. *North. Chin. Fish.* **2023**, *42*, 437–440.
10. Dong, Z.; Luo, M. Main Methods, Genetic Analysis and Prospect of Common Carp (*Cyprinus carpio*) Breeding in China. *J. Fish. China* **2023**, *47*, 39–55.
11. Jui, R.A.; Haque, M.M.; Rahmatullah, S. Growth Performance of Silver Carp *Hypophthalmichthys molitrix* in Cage Stocked at Different Densities. *J. Bangladesh Agric. Univ.* **2018**, *16*, 322–327. [\[CrossRef\]](#)
12. Stanivuk, J.; Nagy, L.B.; Gyalog, G.; Ardó, L.; Vitál, Z.; Plavša, N.; Krstović, S.; Fazekas, G.L.; Horváth, Á.; Ljubobratović, U. The Rank of Intensification Factors Strength in Intensive Pond Production of Common Carp (*Cyprinus carpio* L.). *Aquaculture* **2024**, *583*, 740584. [\[CrossRef\]](#)
13. Ponpandy, N.; Kumar, V.A.; Subodh, G.; Annamalai, J.; Mallikarjun, H.C. Effects of Different Stocking Densities on Haematological, Non-Specific Immune, and Antioxidant Defence Parameters of Striped Catfish (*Pangasianodon hypophthalmus*) Fingerlings Reared in Finger Millet-Based Biofloc System. *Aquac. Int.* **2022**, *30*, 3229–3245.
14. Ray, G.W. Effects of Replacing Fishmeal with Alternative Plant Proteins Surce Including Spc, Wgm and Ddgs on Growth, Serum Biochemical Indices, and Antioxidative Functions, and Microbiota for Juvenile Shrimp *Litopenaeus vannamei*. Master's Thesis, Guangdong Ocean University, Zhanjiang, China, 2020.
15. Wang, J. Hypoxia Tolerance and Physiological Alterations in Turbot (*Scophthalmus maximus*). Master's Thesis, Shanghai Ocean University, Shanghai, China, 2021.
16. Hu, S.; Lin, Y.; Shi, X.; Chi, C. Effects of Dietary Supplementation of Silphium Perfoliatum on Growth Performance, Antioxidant Capacity and Lipid Metabolism of Juvenile *Megalobrama amblycephal*. *J. Fish. China* **2024**, *48*, 323–336.
17. Ren, Y.; Luo, J.; Wang, R.; Zhou, Z. Effects of Different Zinc Sources on the Intestinal Morphology, Digestive Enzymes, Antioxidant Capacity, and Mineral Element Deposition of Pearl Gentian Grouper. *Guangdong Feed J.* **2023**, *32*, 31–34.
18. Yong, S.; Yi, H.; Ziqin, W.; Jiancheng, Z.; Junzhi, Z.; Huan, Z.; Guihong, F.; Lei, Z. The Protective Effect of Taurine on Oxidized Fish-Oil-Induced Liver Oxidative Stress and Intestinal Barrier-Function Impairment in Juvenile *Ictalurus punctatus*. *Antioxidants* **2021**, *10*, 1690. [\[CrossRef\]](#) [\[PubMed\]](#)
19. Edgar, R.C. Uparse: Highly Accurate Otu Sequences from Microbial Amplicon Reads. *Nat. Methods* **2013**, *10*, 996–998. [\[CrossRef\]](#) [\[PubMed\]](#)
20. Wang, Q.; Garrity, G.M.; Tiedje, J.M.; Cole, J.R. Naive Bayesian Classifier for Rapid Assignment of Rrna Sequences into the New Bacterial Taxonomy. *Appl. Environ. Microbiol.* **2007**, *73*, 5261–5267. [\[CrossRef\]](#) [\[PubMed\]](#)
21. Edgar, R.C. Muscle: Multiple Sequence Alignment with High Accuracy and High Throughput. *Nucleic Acids Res.* **2004**, *32*, 1792–1797. [\[CrossRef\]](#) [\[PubMed\]](#)
22. Minchin, P.R. An Evaluation of the Relative Robustness of Techniques for Ecological Ordination. *Vegetatio* **2004**, *69*, 89–107. [\[CrossRef\]](#)
23. Ni, M.; Wen, H.; Li, J.; Chi, M.; Bu, Y.; Ren, Y.; Zhang, M.; Song, Z.; Ding, H. The Physiological Performance and Immune Responses of Juvenile Amur Sturgeon (*Acipenser schrenckii*) to Stocking Density and Hypoxia Stress. *Fish Shellfish Immunol.* **2014**, *36*, 325–335. [\[CrossRef\]](#) [\[PubMed\]](#)
24. De las Heras, V.; Martos-Sitcha, J.A.; Yúfera, M.; Mancera, J.M.; Martínez-Rodríguez, G. Influence of Stocking Density on Growth, Metabolism and Stress of Thick-Lipped Grey Mullet (*Chelon labrosus*) Juveniles. *Aquaculture* **2015**, *448*, 29–37. [\[CrossRef\]](#)
25. Gunjan, K.; Kumar, D.B.; Puthiyottil, M.; Tasso, T.; Suman, K.; Kumar, S.U.; Kanti, D.A.; Yusuf, A. Impact of Stocking Density on Growth, Feed Utilization and Survival of Cage Reared Minor Carp, *Labeo bata* (Hamilton, 1822) in Maithon Reservoir, India. *Aquaculture* **2021**, *532*, 736078.
26. Ezhilmathi, S.; Ahilan, B.; Uma, A.; Felix, N.; Cheryl, A.; Somu Sunder Lingam, R. Effect of Stocking Density on Growth Performance, Digestive Enzyme Activity, Body Composition and Gene Expression of Asian Seabass Reared in Recirculating Aquaculture System. *Aquac. Res.* **1963**, *53*, 1963–1972. [\[CrossRef\]](#)
27. Wu, Y.; Ma, H.; Yang, M.; Gan, H.; Lu, X.; Ruan, Z.; Lv, M. Effects of Stocking Density and Antimicrobial Peptides on Growth in Yellow Catfish *Pelteobagrus Fulvidraco* Cultured in Net Cages. *Chin. J. Fish.* **2020**, *33*, 66–71.
28. CristianAlin, B.; Mihaela, R.C.; Marian, B.; Lenuta, D.; LiviuDan, M.; Stefan, B.R.; Gabriela, D.; Elena, T. Comparative Study of Flesh Quality, Blood Profile, Antioxidant Status, and Intestinal Microbiota of European Catfish (*Silurus glanis*) Cultivated in a Recirculating Aquaculture System (Ras) and Earthen Pond System. *Life* **2023**, *13*, 1282. [\[CrossRef\]](#) [\[PubMed\]](#)
29. Qi, C.; Xie, C.; Tang, R.; Qin, X.; Wang, D.; Li, D. Effect of Stocking Density on Growth, Physiological Responses, and Body Composition of Juvenile Blunt Snout Bream, *Megalobrama amblycephala*. *J. World Aquac. Soc.* **2016**, *47*, 358–368. [\[CrossRef\]](#)
30. Guo, H.Y.; Dong, X.Y.; Zhang, X.M.; Zhang, P.D.; Li, W.T. Survival, Growth and Physiological Responses of Juvenile Japanese Flounder (*Paralichthys olivaceus*, Temminck & Schlegel, 1846) Exposed to Different Dissolved Oxygen Concentrations and Stocking Densities. *J. Appl. Ichthyol.* **2017**, *33*, 731–739. [\[CrossRef\]](#)
31. Tolussi, C.E.; Hilsdorf, A.W.S.; Caneppele, D.; Moreira, R.G. The Effects of Stocking Density in Physiological Parameters and Growth of the Endangered Teleost Species Piabanha, *Brycon insignis* (Steindachner, 1877). *Aquaculture* **2010**, *310*, 221–228. [\[CrossRef\]](#)

32. Marchi, A.; Bonaldo, A.; Di Biase, A.; Cerri, R.; Scicchitano, D.; Nanetti, E.; Candela, M.; Picone, G.; Capozzi, F.; Dondi, F.; et al. Towards a Free Wild-Caught Fishmeal, Fish Oil and Soy Protein in European Sea Bass Diet Using by-Products from Fishery and Aquaculture. *Aquaculture* **2023**, *573*, 739571. [\[CrossRef\]](#)
33. Shi, X. The Stress-Influences of Rearing Densities on Juvenile Amur Sturgeon, *Acipenser schrenckii*. Master's Thesis, Huazhong Agricultural University, Wuhan, China, 2006.
34. Wang, X.; Fang, X.; Peng, S.; Wang, Q.; Shi, Z. Impact of Abrupt Salinity Changes on Activity of Metabolic Enzymes, Antioxidant Enzymes and Cortisol Content in Serum and Liver of *Lateolabrax maculatus*. *Mar. Fish.* **2021**, *43*, 340–349. [\[CrossRef\]](#)
35. Ran, F.; Jin, W.; Huang, S.; Liu, C.; Li, Z.; Li, C. Research Progress on the Effects of Salinity Change on Fish. *Northwest A F Univ. (Nat. Sci. Ed.)* **2020**, *48*, 10–18.
36. Santos, E.L.R.; Rezende, F.P.; Moron, S.E. Stress-Related Physiological and Histological Responses of Tambaqui (*Colossoma macropomum*) to Transportation in Water with Tea Tree and Clove Essential Oil Anesthetics. *Aquaculture* **2020**, *523*, 735164. [\[CrossRef\]](#)
37. Lin, L.; Sun, X.; Xing, K.; Guo, Y. Effects of Stocking Density on Growth and Blood Biochemical Parameters in *Plectropomus leopardus*. *Fish. Sci.* **2017**, *1*, 1003–1111.
38. Zhu, M.; Kong, F.; Zhao, Q. Exercise Regulates Lactic Acid Metabolism. *Chin. J. Tissue Eng. Res.* **2023**, *27*, 322–328.
39. Refaey, M.M.; Li, D.; Tian, X.; Onxayvieng, K.; Tang, R. Physiological Responses of Channel Catfish (*Ictalurus punctatus*) Reared at Different Stocking Densities in a Recirculating Aquaculture System. *Aquaculture* **2022**, *557*, 738329. [\[CrossRef\]](#)
40. Lv, B.; Ye, Y.; Luo, H.; Ma, J.; Wang, Z.; Wu, D.; Tang, F.; Pu, Q. Effects of Dietary Sargassum Powder on Growth Performance, Tissue Enzyme Activity and Serum Biochemical Indices of Yellow Catfish (*Pelteobagrus fulvidraco*). *Feed Ind.* **2022**, *43*, 52–59.
41. Zhang, Q.; Hou, J.; Yang, J.; Liao, J.; Lu, J.; He, X. Captive Density on Growth Performance and Health Status of Largemouth Bass (*Micropterus salmoides*). *Acta Hydrobiol. Sin.* **2022**, *46*, 671–678.
42. Wojtczyk-Miaskowska, A.; Schlichtholz, B. DNA Damage and Oxidative Stress in Long-Lived Aquatic Organisms. *DNA Repair* **2018**, *69*, 14–23. [\[CrossRef\]](#) [\[PubMed\]](#)
43. Velázquez, J.S.; Herrejón, G.A.P.; Becerra, H.A. Fish Responses to Alternative Feeding Ingredients under Abiotic Chronic Stress. *Animals* **2024**, *14*, 765. [\[CrossRef\]](#) [\[PubMed\]](#)
44. Lu, K.; Zhang, Y.; Liang, X.; Tang, S. The Influence of Stocking Density on the Growth, Blood Biochemistry, and Antioxidant Capacity Indicators of Chinese Perch (*Siniperca chuatsi*). *Acta Hydrobiol. Sin.* **2023**, *47*, 473–478.
45. Hoseini, S.M.; Yousefi, M.; Mirghaed, A.T.; Paray, B.A.; Hoseinifar, S.H.; Van Doan, H. Effects of Rearing Density and Dietary Tryptophan Supplementation on Intestinal Immune and Antioxidant Responses in Rainbow Trout (*Oncorhynchus mykiss*). *Aquaculture* **2020**, *528*, 735537. [\[CrossRef\]](#)
46. Choi, C.Y.; Choi, J.Y.; Choi, Y.J.; Kim, B.-S.; Kim, J.-W. Effects of Green Wavelength Light on Antioxidant and Non-Specific Immune Responses of the Olive Flounder *Paralichthys olivaceus* Maintained at Different Stocking Densities. *Aquac. Eng.* **2019**, *84*, 23–28. [\[CrossRef\]](#)
47. Yu, J.; Wang, J.; Sun, P.; Tang, B. Physiological Response of *Larimichthys Crocea* under Different stocking Densities and Screening of Stress Sensitive Biomarkers. *Mar. Fish.* **2021**, *43*, 327–339. [\[CrossRef\]](#)
48. Zhang, Y. Effect of Air Exposure and Transportation Stress on Plasma Biochemical Profiles and *Hsp70* Mrna Expression of American Shad (*Alosa sapidissima*) Broodstock. Master's Thesis, Nanjing Agricultural University, Nanjing, China, 2016.
49. Ma, C.; Chen, C.X.; Jia, L.; He, X.X.; Zhang, B. Comparison of the Intestinal Microbiota Composition and Function in Healthy and Diseased Yunlong Grouper. *AMB Express* **2019**, *9*, 187. [\[CrossRef\]](#) [\[PubMed\]](#)
50. Wang, B.; Tao, H.; Liao, X.; Hu, S.; Zhao, J.; He, Z.; Han, X.; Chen, Z.; Sun, C. Effects of Stocking Density on Bacterial Community Characterization of Biofloc and Intestine of *Fenneropenaeus merguensis*. *J. Hunan Agric. Univ. (Nat. Sci.)* **2020**, *46*, 608–615.
51. Nie, Z.; Xu, G.; Shao, N.; Wang, B.; Gao, J.; Xu, P.; He, J. Comparison of Gut Microbiota in Carps from Fish Monoculture Ponds and the Rice-Fish Co-Culture System in Hani Terraces. *Acta Microbiol. Sin.* **2022**, *62*, 1473–1484.
52. Tan, C.K.; Natrah, I.; Suyub, I.B.; Edward, M.J.; Kaman, N.; Samsudin, A.A. Comparative Study of Gut Microbiota in Wild and Captive Malaysian Mahseer (*Tor tambroides*). *Microbiologyopen* **2019**, *8*, e00734. [\[CrossRef\]](#) [\[PubMed\]](#)
53. Pandiyan, P.; Balaraman, D.; Thirunavukkarasu, R.; George, E.G.J.; Subaramaniyan, K.; Manikkam, S.; Sadayappan, B. Probiotics in Aquaculture. *Drug Invent. Today* **2013**, *5*, 55–59. [\[CrossRef\]](#)
54. Wen, J.; Sun, X. Research Progress on Gut Microbiota Regulation in Aquatic Animals. *Feed Ind.* **2009**, 68–70. [\[CrossRef\]](#)

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.