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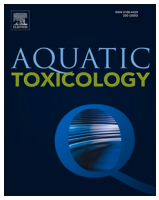

Effects of salinity on growth, physiology, biochemistry and gut microbiota of juvenile grass carp (*Ctenopharyngodon idella*)

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ABSTRACT

Grass carp (*Ctenopharyngodon idella*) is among the most important freshwater fish species in China. However, it remained unclear how salinity could affect grass carp. Two experiments were performed. The first experiment was a 4-day acute salt tolerance experiment with six salinities (0, 4, 8, 12, 16, and 20 ppt). The second experiment was an 8-week chronic salt stress experiment with three salinities (0, 2 and 6 ppt). To investigate the intestinal bacterial community of grass carp from three salinities (0, 2, and 6 ppt), the 16S rDNA sequencing was performed. The results showed that grass carp exhibited great adaptability to low salinity (2 ppt), with no significant difference in growth and maintained stable physiological and immune status. However, exposed to high salinity (6 ppt) caused significant deleterious effects on grass carp, including growth inhibition as well as physiological and immune-related changes. The gut microbiota in grass carp changed with salinity. With the increase of salinity, the proportion of beneficial bacteria in the gut of grass carp gradually decreased, while some harmful bacteria gradually occupied the dominant position. Changes in gut microbial composition ultimately affected the growth of grass carp. This study helps further clarify the effects of salinity on grass carp.

1. Introduction

Grass carp (*Ctenopharyngodon idella*) is among China's most important aquaculture species. Grass carp has been widely cultivated in China for more than 1700 years. According to the China Fishery Yearbook, in 2021, the total output of grass carp in China reached 5.75 million tons, an increase of 3.3% over 2020, and accounted for 21.80% of the output of freshwater fish. In addition to culturing grass carp, the industry has attempted to use several other culturing methods with the goal of improving the quality of fish meat, such as culturing crisp grass carp and brackish water grass carp. Grass carp cultured in brackish water exhibited better muscle quality and a special aroma (Zhang et al., 2021). However, using brackish water to culture grass carp remains in its infancy. The salinity suitable for grass carp growth, treatment time, and the impact of salinity on grass carp remain unknown.

Salinity is a vital factor that affects the survival, growth, physiological and immune function of fish (Steele et al., 1991). When the salinity of water changes, the corresponding organs in fish respond accordingly, resulting in a change in fish physiology. When exposed to salt stress, grass carp may undergo a series of physiological changes that eventually influence their growth. The intestine is an important immune organ that consists of a stable functional microbiota and intestinal mucosa (Lopez et al., 2020). Numerous gut microbiota are present in the gut of fish. Gut microbiota can affect the function of the intestine. Salinity has an effect on the composition of intestinal microorganisms in fish (Banerjee et al., 2017). Fewer beneficial bacteria are found in the gut microbiota of Tilapia (*Oreochromis mossambicus*) reared in fresh water than those reared in brackish water (Zhang et al., 2016). Additionally, when black molly (*Poecilia sphenops*) adapts to change in salinity, dominant bacteria in the host are completely replaced, which is driven by dominant bacteria at the corresponding salinity (Schmidt et al., 2015). Gut microbiota can also control gene expression associated with nonspecific immunity (Standen et al., 2013). The presence of beneficial and stable intestinal bacteria ensures the normal life activities of fish.

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Thus, to determine the effects of salinity on the growth, physiology, biochemistry and gut microbiota of grass carp (*Ctenopharyngodon idella*), the acute salt tolerance experiment and the chronic salt stress experiment were performed. The 16S rDNA amplicon sequencing was applied to investigate the effects of salinity on gut microbiota. Moreover, the effects of salinity on growth, physiological and immune status of grass carp were investigated. The findings of this study will provide a scientific basis for the development of grass carp aquaculture and the utilization of brackish water.

2. Materials and methods

2.1. Fish and rearing conditions

A total of 3000 grass carp with an initial body weight of 46.14±1.66 g and initial body length of 13.87 ± 0.86 cm were obtained from Chengyi Aquaculture Co., Ltd and fed in 24 round water tanks (500 L; diameter 0.8 m; depth 1 m). A multi-parameter water quality detector (PT-001B) was used to maintain the dissolved oxygen (DO) concentration> 6.0 mg/L, total ammonia nitrogen content< 0.1 mg/L, nitrite content< 0.05 mg/L, pH at 7.5- 8.5, and temperature at 29°C- 32°C. Water with different salinities was prepared by mixing fresh water with natural solar salt (Laizhou Zhongxiang Salt Industry Co., Ltd, China). A commercial pelleted diet (Chengyi Aquaculture Co., Ltd., China) was used to feed fish twice a day (at 07:00 a.m. and 18:00 p.m.).

2.2. Experiment on the acute salt tolerance of grass carp

To examine the acute salt tolerance of grass carp, a 4-day experiment was conducted. The treatment group was divided into five salinity gradients (4, 8, 12, 16 and 20 ppt) and the control group with 0 salinity was set. The experiment was repeated three times for each treatment and control group. Thirty grass carp were placed in each group, and all experimental fish were not fed during the whole experimental process to avoid any potential impact. During the experiment, two-thirds of the water was replaced daily by water with the same salinity to maintain the stability of water quality. The number of grass carp deaths was recorded every 24 hours. Grass carp were considered dead if they had no operculum movement or did not respond to stimulation (Xu et al., 2018).

2.3. Experiment on the chronic salt tolerance of grass carp

To test the chronic tolerance of grass carp, an 8-week experiment was conducted. The treatment group was divided into two salinity (2 and 6 ppt) and the control group with 0 salinity was set. The experiment was repeated three times for each treatment and control group. To prevent stress, we increased the salinity by 2 ppt daily until the corresponding experimental salinity gradient was reached. The water quality of each tank was monitored, and two-thirds of the water in the tank was replaced with water of the same salinity every day. All fish were fed with same commercial pelleted diet (Chengyi co., Ltd, Guangdong, China) twice a day (at 07:00 a.m. and 18:00 p.m.) until apparent satiation.

2.4. Sampling

After the chronic salt tolerance experiment, the fish were fasted for 12h and then anesthetized with MS-222 before sampling and measurement. Six fish were selected randomly from the treatment group (2 and 6 ppt) and control group for sampling. Blood was collected and maintained at 4°C for 3 h, followed by centrifuging at 4000 rpm for 10min. The supernatant of the serum thus obtained was stored in a freezer at −80°C. Visceral mass and liver were then obtained and weighted. Gill, kidney, liver, and gut samples were collected in liquid nitrogen and then transferred to -80°C ultra-low temperature freezer for storage. Guts from each group were dissected by sterile forceps to extrude gut contents. Gut contents were then frozen in liquid nitrogen and transferred to -80 °C ultra-low temperature freezer for storage for gut microbiota analysis.

2.5. Osmolarity, biochemical and physiological parameter analysis

Serum samples from 2.4 and water from the rearing pond were measured by an ice point osmometer (BS-100, China) to analyze the osmolarity. The concentrations of sodium (Na^+), potassium (K^+) and chloride (Cl^-), as well as the contents of glucose (Glu), creatinine (Cr) and triacylglycerol (TG) in serum were measured by Hiwell Diatek DR-200BS enzyme calibration (Wuxi, China), Na^+, K^+, Cl^-, glucose, creatinine and triacylglycerol test kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu, China).

2.6. Enzymatic activity analysis

The activities of superoxide dismutase (SOD), catalase (CAT), alkaline phosphatase (AKP), acid phosphatase (ACP) and the content of malondialdehyde (MDA) in gill, kidney, liver, and gut samples were measured by Hiwell Diatek DR-200BS enzyme calibration (Wuxi, China). The activity of T-ATPase was detected only in the gill. Special kits (Jiancheng Bioengineering Institute, Nanjing, China) were used to measure the activities of the above enzymes: SOD (Product code A001-1-2), CAT (Product code A007-1-2), MDA (Product code A003-1-2), AKP (Product code A059-2-2), ACP (Product code A060-1-1) and T-ATPase (Product code A070-2-2). All operational procedures were performed in accordance with the product instructions.

2.7. Intestinal bacterial community analysis

The genomic DNA of the samples was extracted by the CTAB method, and the purity and concentration of DNA were detected by agarose gel electrophoresis. Forward primer 314F (5′-CCTAYGGGRBGCASCAG-3′) and reverse primer 806R (5′-GGACTACNNGGGTATCTAAT-3′) were selected to amplify the V3-V4 region of the 16S DNA. TruSeq DNA PCR-free Sample Preparation Kit was used for library construction, and then quantified by Qubit. NovaSeq6000 was used for machine sequencing. Sample data was separated from the off-machine data. FLASH (v1.2.7, http://ccb.jhu.edu/software/FLASH/) was used to amputate the barcode and primer sequences (Magoč et al., 2011). The raw tags then went through strict filtering (Bokulich et al., 2013) to get high-quality clean tags.

Qiime (v1.9.1, http://qiime.org/scripts/split_libraries_fastq.html) (Caporaso et al., 2010) was used for quality control. The chimera sequences were then removed from obtained tags (Rognes et al., 2016). Uparse algorithm (Uparse v7.0.1001, http://www.drive5.com/uparse/) (Haas et al., 2011) was used to cluster all effective tags of all samples, and the sequences were clustered into OTUs (Operational Taxonomic Units) by default with 97% identity. Rarefaction curves were generated for the OTUs thus obtained to estimate bacterial diversity and richness (Caporaso et al., 2012).

2.8. Statistical analysis

Survival (%), specific growth rate (SGR, %), viscerosomatic index (VSI, %), hepatosomatic index (HSI, %), feed conversion ratio (FCR), mortality (%) and safe concentration (SC, ppt) (Krishnani et al., 2003; Mangang et al., 2021; Kang et al., 2022) were calculated using the following equations:

$$\textit{Specific growth rate}\ (SGR,\ \%) = [(lnW1 - lnW0)\ /\ t] \times 100$$

$$\textit{Viscerosomatic index}\ (VSI,\ \%) = (\textit{visceral weight}\ /\ W1) \times 100$$

$$\textit{Hepatosomatic index}\ (HSI,\ \%) = (\textit{liver weight}\ /\ W1) \times 100$$

$$\textit{Feed conversion ratio}\ (FCR) = \textit{feed intake}\ (g)\ /\ \textit{weight gain}\ (g)$$

$$Survival\ (\%) = (number\ of\ remaining\ fish\ /\ initial\ number\ of\ fish) \times 100$$

$$Mortality\ (\%) = (Number\ of\ dead\ fish\ /\ initial\ number\ of\ fish) \times 100$$

$$Safe\ concentration\ (SC,\ ppt) = LC50(96\ h) \times 0.1$$

W_0 and W_1 represent the average initial and final wet weights of each fish in each group, respectively; t represents the experimental period; LC50 (96 h) represents the 96 h median lethal concentration. Data were analyzed using SPSS version 22 (Chicago, USA). The LC50 was calculated by nonlinear regression after the normality and homogeneity of the data were confirmed by Kolmogorov Smirnov and Leven tests. Growth performance data were analyzed by one-way analysis of variance (ANOVA), and Tukey's multiple comparison test. With regard to the composition of the intestinal microbiota, species classification analysis of OTUs was carried out using the Kruskal Wallis test to obtain species abundance data at different classification levels (Nyman et al., 2017; Von Ahnen et al., 2019). $P<0.05$ was considered significant in all statistical tests. All data in the present study are presented as the means ± standard deviation (SD).

3. Results

3.1. Acute salt tolerance of grass carp

After the acute salt tolerance experiment, grass carp survived for 96h at salinities of 0, 4 and 8 ppt, while different degrees of death occurred at salinities of 12, 16, and 20 ppt. In the 12 ppt group, the mortality was 83.33% ± 5.44%. In the 16 ppt and 20 ppt groups, all grass carp died within 24 hours (Table 1). The results were analyzed by nonlinear regression and drawing (Fig. 1). The correlation coefficients R^2 of the nonlinear regression curves were all greater than 0.99. LC50-96 h and safe concentration of all groups were obtained (Table 2). The results of this experiment demonstrated that salinity should be controlled within the safe concentration of 1.173 ppt for the normal growth of grass carp in brackish water aquaculture.

3.2. Growth performance

After the chronic salt stress experiment, it was found that the final body weight (FBW) and the specific growth rate (SGR) of grass carp decreased, while the feed conversion ratio (FCR) increased with the increase of salinity. There was no significant difference in growth performance between the 0 ppt group and the 2 ppt group ($P>0.05$) (Table 3). However, there had been a significant decline in the growth performance of 6 ppt group. There were no significant differences in survival, hepatosomatic index (HIS) or viscerosomatic index (VSI) among different salinities ($P>0.05$).

3.3. Osmolarity, biochemical and physiological parameters

As shown in Fig. 2, the osmolarity of water increased as the salinity increased. There was no significant change in the serum osmotic pressure of grass carp under different salinities. The concentrations of Na^+ and Cl^- increased slowly with the change in salinity. On the other hand, a different trend was observed with K^+, which increased at 2 ppt and decreasing at 6 ppt. During salinity changes, the glucose in grass carp showed a similar trend to K^+, while the triglyceride decreased with the increasing salinity. The creatinine showed no significant differences among different salinities.

Table 1
Mortality of grass carp in different salinities at different time.

Salinity/ppt	Mortality (%)			
	24h	48h	72h	96h
0	0	0	0	0
4	0	0	0	0
8	0	0	0	0
12	13.33±7.20	25.56±3.14	70.00±9.81	83.33±5.44
16	100	100	100	100
20	100	100	100	100

Note: All data were presented as the mean ± standard deviation (SD).

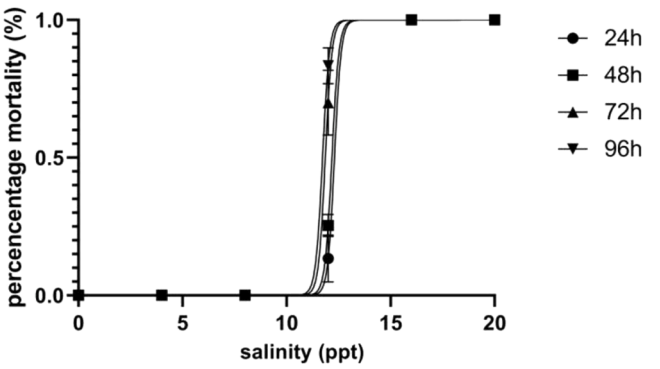

Fig. 1. 24h, 48h, 72h and 96h nonlinear regression analysis.

Table 2
Nonlinear regression parameters, median lethal concentration (LC50) and safe concentration (SC) at different time.

Time/h	Regression curve	correlation coefficient	LC50/ppt	SC/ppt
24	Fig. 1	R^2=0.9962	12.30	1.173
48		R^2=0.9991	12.18	
72		R^2=0.9928	11.86	
96		R^2=0.9979	11.73	

Table 3
The growth parameters of grass carp reared at different salinities during chronic salt tolerance experiment.

Salinity (ppt)	FBW (g)	survival (%)	HIS (%)	VSI (%)	SGR (%/d)	FCR
0	113.06 ±29.23^a	97.67 ±3.30^a	1.47 ±0.20^a	7.23 ±0.58^a	1.71 ±0.06^a	1.74 ±0.08^c
2	110.48 ±27.68^a	100.00 ±0.00^a	1.51 ±0.23^a	6.85 ±0.74^a	1.64 ±0.11^a	1.84 ±0.09^{bc}
6	62.54 ±18.37^c	100.00 ±0.00^a	1.37 ±0.30^a	7.08 ±0.83^a	0.56 ±0.11^c	2.45 ±0.08^a

Note: Survival, final body weight (FBW), specific growth rate (SGR) and feed conversion rate (FCR) were obtained from all fish. Hepatosomatic index (HIS) and viscerosomatic index (VSI) were obtained by measuring eighteen fish for each treatment. Different lowercase letters in the same column indicate significant differences ($p<0.05$).

3.4. Enzymatic activities

The enzyme activities in the gill, kidney, liver, and gut of grass carp reared at different salinity levels are shown in Fig. 3. SOD activity in the gill, kidney and liver was significantly different ($p<0.05$), and it increased as the salinity increased. CAT activity had significant differences in gill, liver and gut ($p<0.05$). CAT activity in the gut was significantly decreased at 2 ppt and 6 ppt ($p<0.05$). There was no significant difference in MDA concentration in the liver, but there was a significant difference in other tissues.

Among immune function-related enzymes, AKP activity increased with increasing salinity in the gill and liver, but decreased with increasing salinity in the gut, and significant differences were observed at 0 ppt and 6 ppt in these three tissues ($p<0.05$). The ACP activity rose and then decreased with increasing salinity in the gills, liver and

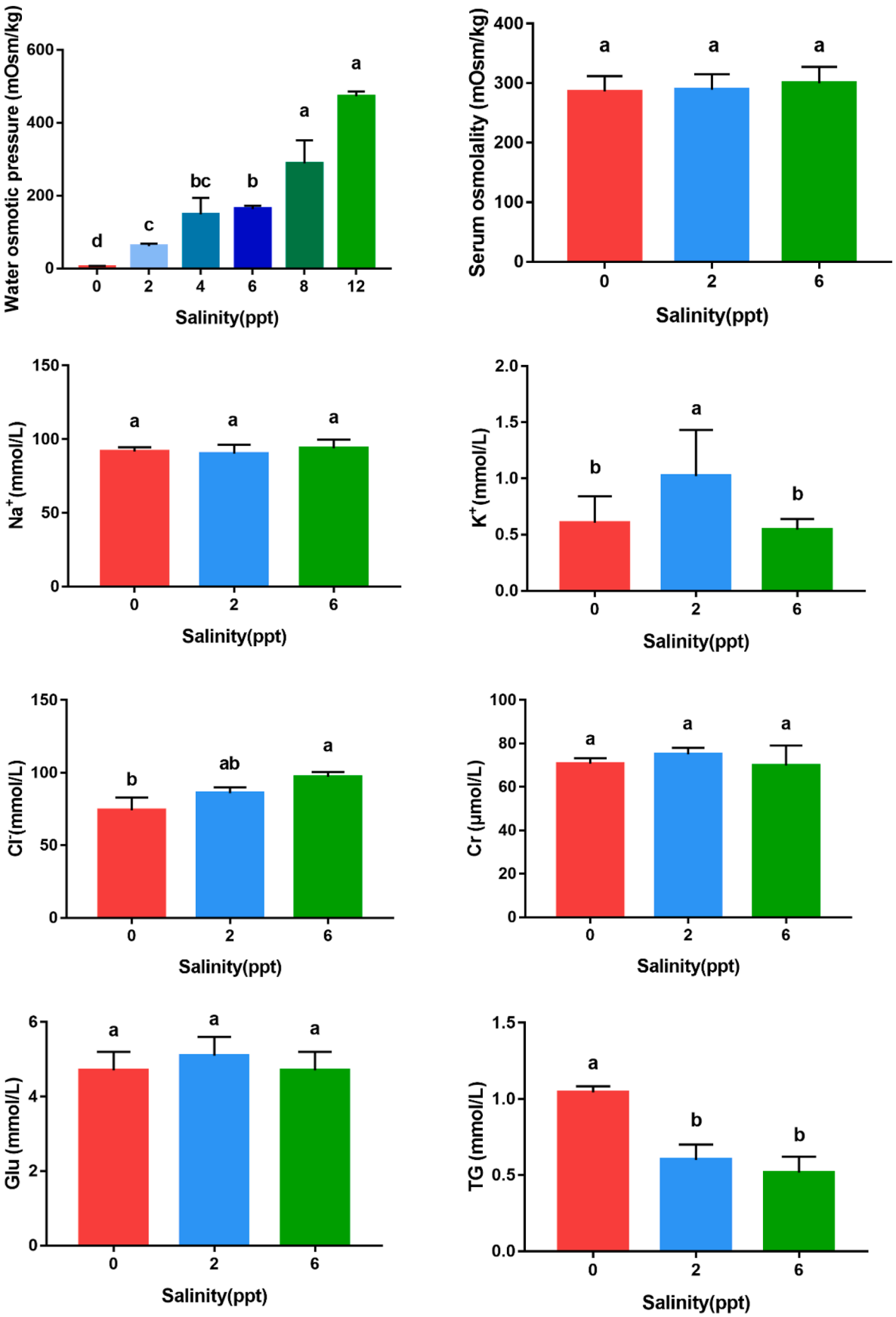

Fig. 2. Effects of salinity on osmolarity and serum biochemistry of grass carp. The biochemical indices of serum, including the concentration of glucose (Glu), creatinine (Cr), triacylglycerol (TG), Na^+, K^+ and Cl^- ions. Data were expressed as the means ± SD (n=18). Different superscript letters for each group indicate significant differences in the same tissue from fish exposed to different salinities.

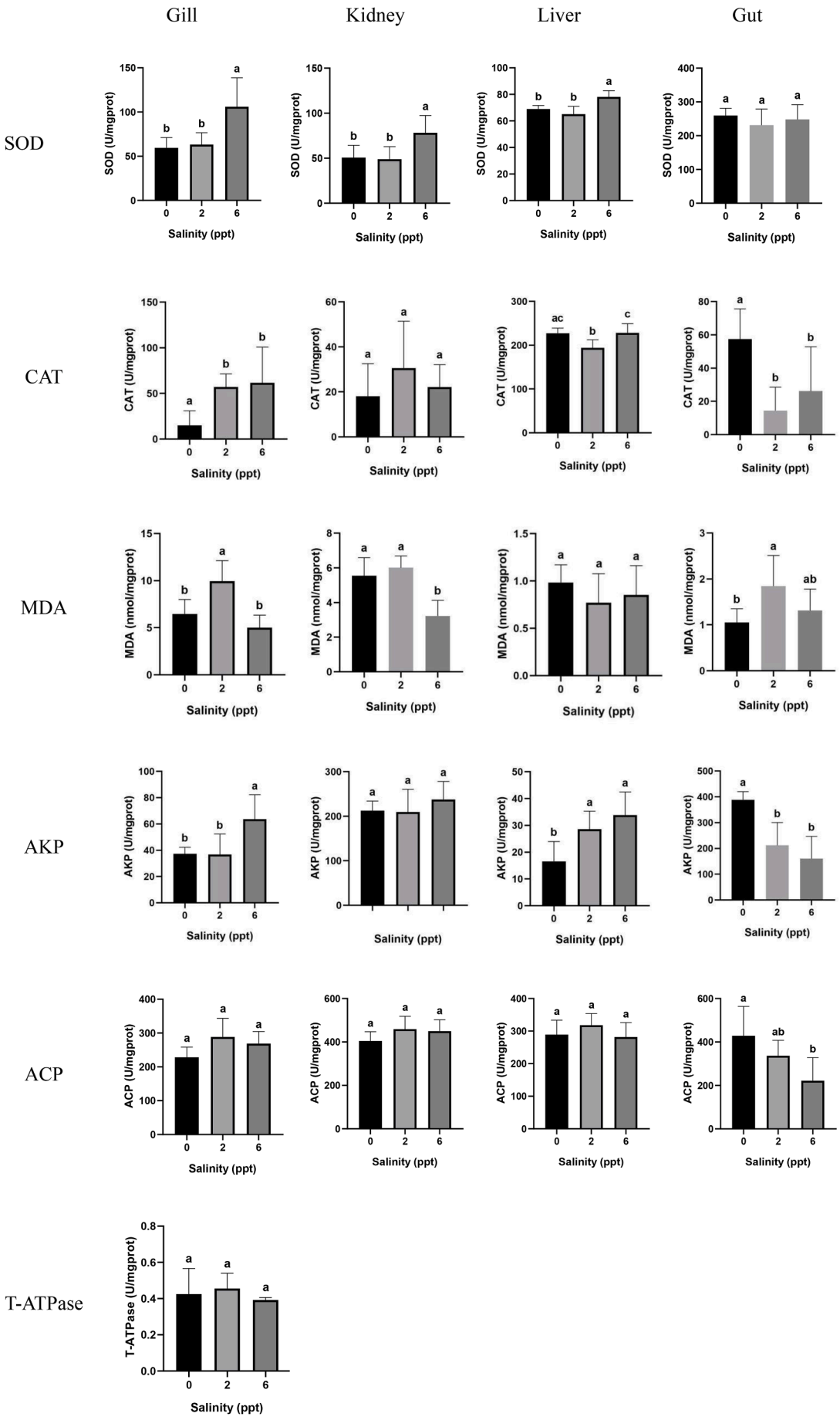

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Fig. 3. Biochemical and physiological parameters in the gill, kidney, liver, and gut of grass carp from different salinities. The activities of superoxide dismutase (SOD), catalase (CAT), alkaline phosphatase (AKP), acid phosphatase (ACP) and the content of malondialdehyde (MDA) were measured in the gill, kidney, liver, and gut. The activity of T-ATPase was measured only in gill. Data were expressed as the means± SD (n= 18). Different superscript letters for each group indicate significant differences in the same tissue from fish exposed to different salinity treatments.

kidneys, while the ACP activity decreased with increasing salinity in the gut. In addition, there was no significant difference in T-ATPase activity in the gill among different groups ($p > 0.05$).

3.5. Intestinal bacterial community analysis

3.5.1. Abundance and diversity of the intestinal bacterial community

A total of 801,292 raw reads were collected from intestinal microbial samples, with an average number of 89,033 reads per sample. After the removing chimeras, 3,748 OTUs were clustered from 572,995 effective tags. A venn graph showed that there were 879 common OTUs among the three groups, 133 unique OTUs at 0 ppt, 1088 unique OTUs at 2 ppt and 308 unique OTUs at 6 ppt (Fig. 4). Alpha diversity analysis of the samples showed that the rarefaction curve tended to be flat, and more data volumes resulted in only a few new OTUs. In the alpha diversity index, the Simpson and Chao1 have no significant difference, but the observed species and Shannon indices indicated that the species richness at 2 ppt was higher than that at 0 and 6 ppt (Table 4).

3.5.2. Composition of the intestinal bacterial community

Salinity changed the composition of the intestinal bacterial community of the grass carp from different salinities. The relative abundance of dominant bacteria in different salinity groups was altered. At the phylum level, Proteobacteria, Firmicutes and Fusobacteria were dominant in the intestinal microbial community of grass carp, and Proteobacteria was the most abundant phylum in all the groups (Fig. 5A). In 0 ppt group, Proteobacteria, Fusobacteria and Firmicutes were dominant in the intestinal bacterial community of grass carp. Compared with the 0ppt group, the proportion of Fusobacteria and Firmicutes in the 2 ppt group decreased significantly and was less than that in the 6 ppt group. The proportion of Actinobacteria, Verrucomicrobia and Acidobacteria in 2 ppt increased. Similar to 2 ppt, in 6 ppt, the proportion of Fusobacteria also decreased, while the proportions of Firmicutes, Actinobacteria, Verrucomicrobia and Acidobacteria increased.

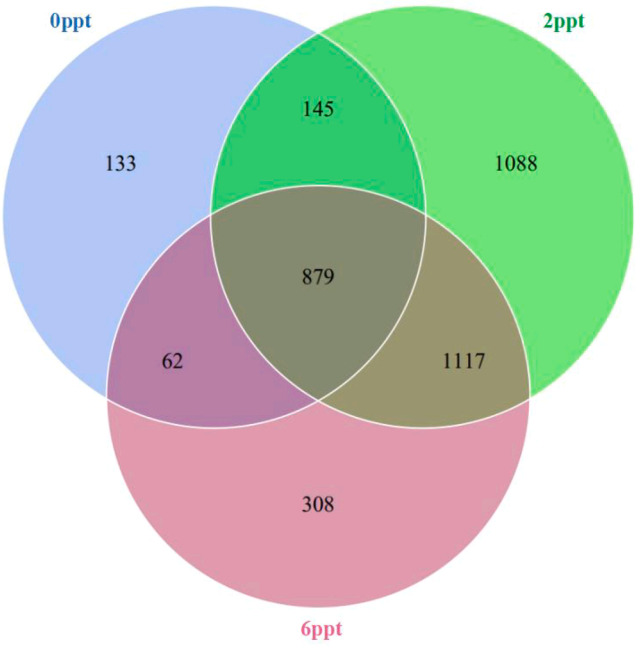

Fig. 4. Venn diagram. Venn diagram analysis depicts the numbers of shared and unique OTUs of gut microbiota from the 0, 2, and 6 ppt groups.

Table 4
Alpha diversity analysis of the gut microbiota of grass carp from different salinities (0, 2, and 6 ppt).

Salinity (ppt)	Observed species	Shannon	Simpson	Chao1
0	640.00±80.07^{b}	4.09±0.65^{b}	0.81 ±0.14^{a}	956.37 ±117.77^{a}
2	2152.67 ±150.88^{a}	6.81±1.00^{a}	0.89 ±0.09^{a}	2405.52 ±62.06^{a}
6	1218.33 ±537.77^{b}	6.13 ±1.06^{ab}	0.94 ±0.03^{a}	1765.37 ±937.05^{a}

Note: Different superscript letters in same column indicate significant differences ($p<0.05$).

At the genus level (Fig. 5B), *Cetobacterium* was dominant in the intestinal microbial community of Grass carp of control group. However, *Cetobacterium* and *Plesiomonas* showed a significant decline in the 2 ppt and 6 ppt groups. At 2 ppt, *Pseudomonas* and *Brevinema* became the dominant genera, while at 6ppt, *Vibrio, Exiguobacterium, Aeromonas* and *Pseudomonas* exceeded *Cetobacterium* and became the dominant genera.

3.5.3. Changes in the microbial community phylotypes

Beta diversity reflected the similarity of the bacterial community composition in different samples. Nonmetric Multidimensional Scaling (NMDS) is a non-linear model based on Bray-Curtis distance, which is among the Beta diversity evaluation methods. NMDS showed that they were distributed in different areas without intersection, and there was a separation between groups (Fig. 6A). To investigate the different abundances of bacterial taxa associated with salt exposure, a linear discriminant analysis (LDA) for the size effect between the 0 ppt, 2 ppt and 6 ppt groups was carried out. With an LDA score threshold of 4, *Cetobacterium, Actinobacteria* and *Alteromonadales* were enriched in the 0 ppt, 2 ppt, 6 ppt groups, respectively (Fig. 6B).

4. Discussion

Salinity is an important environmental factor that affects the survival, growth, and distribution of aquatic organisms. However, the effect of salinity on grass carp remained unknown. In this study, the effect of salinity on grass carp was studied by analyzing the survival, growth performance, physicochemical parameters and gut microbiota of grass carp at different salinities.

In the acute salt tolerance experiment, grass carp were cultured for 96h in different salinities. The results showed that all grass carp in 0, 4 and 8 ppt group survived for over 96 h, indicating that grass carp could adapt to acute low salinity tolerance. However, when salinity reached 12 ppt, grass carp began to die. The mortality rate increased with the increase of time. In the 16 ppt and 20 ppt groups, all grass carp died within 24 hours. The safe concentration of salt for grass carp was calculated by LC50-96h. The safe concentration is the concentration of a compound that does not produce adverse effects with long-term exposure (Allen et al., 2007). In fisheries, this value is the concentration of a toxic substance to which aquatic organisms can be permanently exposed at any developmental stage of their life cycle and their progeny without acute or chronic poisoning or potential harm. The results of this experiment indicate that salinity should be controlled within the safe concentration of 1.173 ppt for the normal growth of grass carp in briny aquaculture.

In the chronic salt stress experiment, the effect of salinity on the

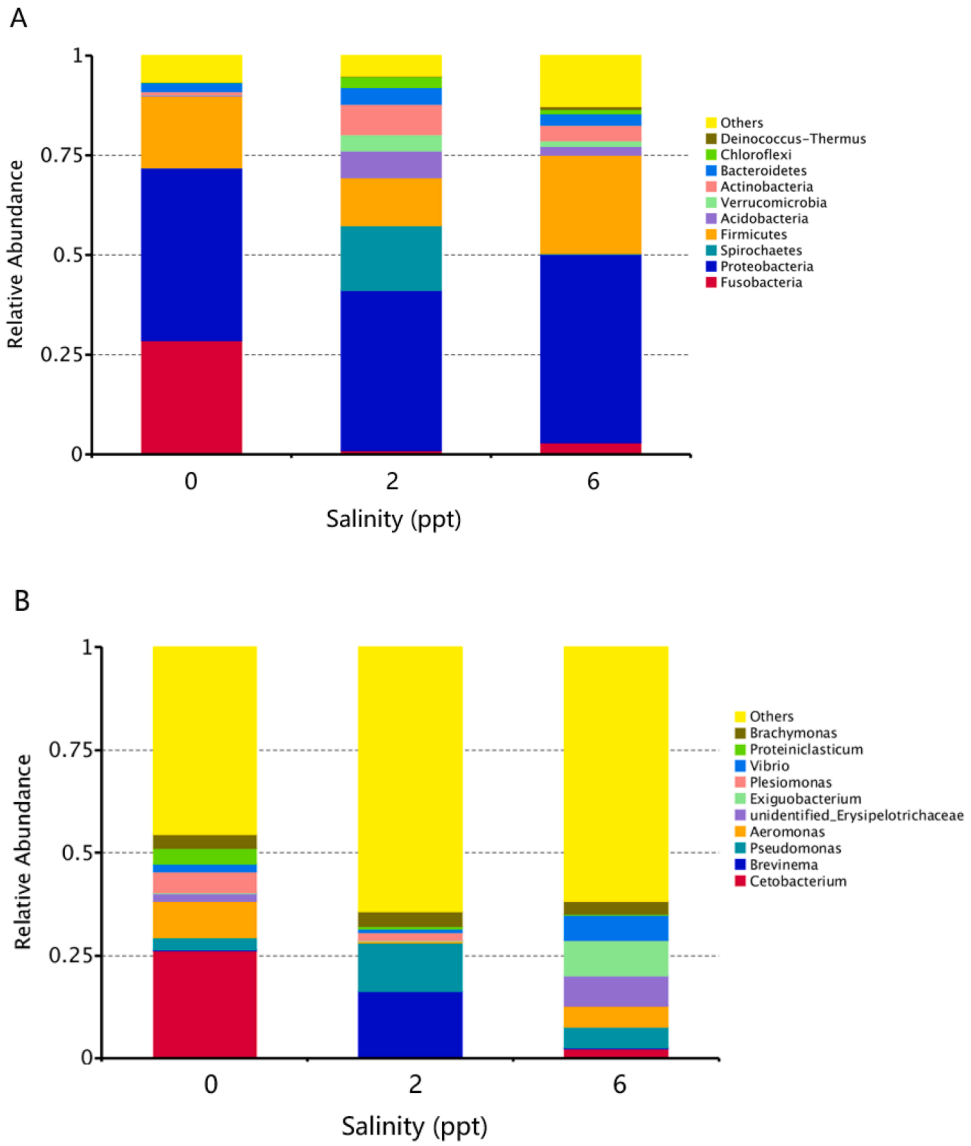

Fig. 5. Relative abundances of the dominant phyla (A) and genera (B). Taxa with relative abundances totaling <1% were combined into the minor member category.

growth of grass carp was studied. The results showed that the growth of grass carp was hindered with increasing salinity. Compared with the 0 ppt group, the final body weight of the 2 ppt group decreased slightly, but not significantly, while the growth of grass carp in the 6 ppt group was significantly inhibited. The variation trend of the specific growth rate was consistent with the result of the final body weight. Correspondingly, with increasing salinity, the feed conversion rate of grass carp also increased. The hepatosomatic index and viscerosomatic index did not change significantly. The results showed that low salinity such as 2ppt had no significant impediment on the growth of grass carp, while high salinity such as 6ppt slowed down the growth of grass carp. This may be due to the fact that freshwater fish grass carp need to consume extra energy to cope with changes in osmotic pressure and regulate osmotic pressure when facing increased salinity (Mirghaed et al., 2019), so that energy used for growth is expended to adapt to changes in salinity.

Subsequently, we studied the serum physiological and biochemical indices of grass carp, including serum ion concentration, osmotic pressure, blood glucose, creatinine, triacylglycerol content, etc. There was no significant change in the serum osmotic pressure of grass carp under different salinities, which indicates that grass carp can adjust and stabilize their serum osmotic pressure in the face of salinity changes. Under elevated salinity, fish usually compensate for water loss by inhaling large amounts of seawater through their body surface (He et al., 2017), removing the excess Na^+ and Cl^- in the serum by chlorine cells located on the gills, and the excreting excess Ca^{2+} and K^+ through the kidneys. The water-salt balance in osmotic pressure regulation of bony fishes mainly involves Na+, K^+ and Cl^- ion flows, and the trend is often the same as that for osmotic pressure. In this study, the concentrations of Na^+ and Cl^- increased slowly with the change in salinity. On the other hand, K^+ showed a different trend, increasing at 2 ppt and decreasing at 6 ppt. The results may indicate that passive diffusion of ambient water penetrating into the body in the face of low salinity (2 ppt) changes caused a rise in serum K^+ at 2 ppt in grass carp. However, in the face of higher salinity changes, grass carp regulated serum osmotic pressure balance through Na^+/K^+ -ATPase, pumped the sudden increase of Na^+ into the serum and K^+ into the cells, thus showing the rising then declining trend in the results. In the face of salinity changes, the blood sugar of grass carp showed a similar trend to K^+, while the triglyceride decreased with the increase of salinity. This result may indicate that grass carp need to consume triglyceride for fat mobilization in the face of increasing salinity to provide additional energy to maintain osmotic pressure homeostasis in grass carp.

Antioxidant enzymes are an important part of the antioxidant system

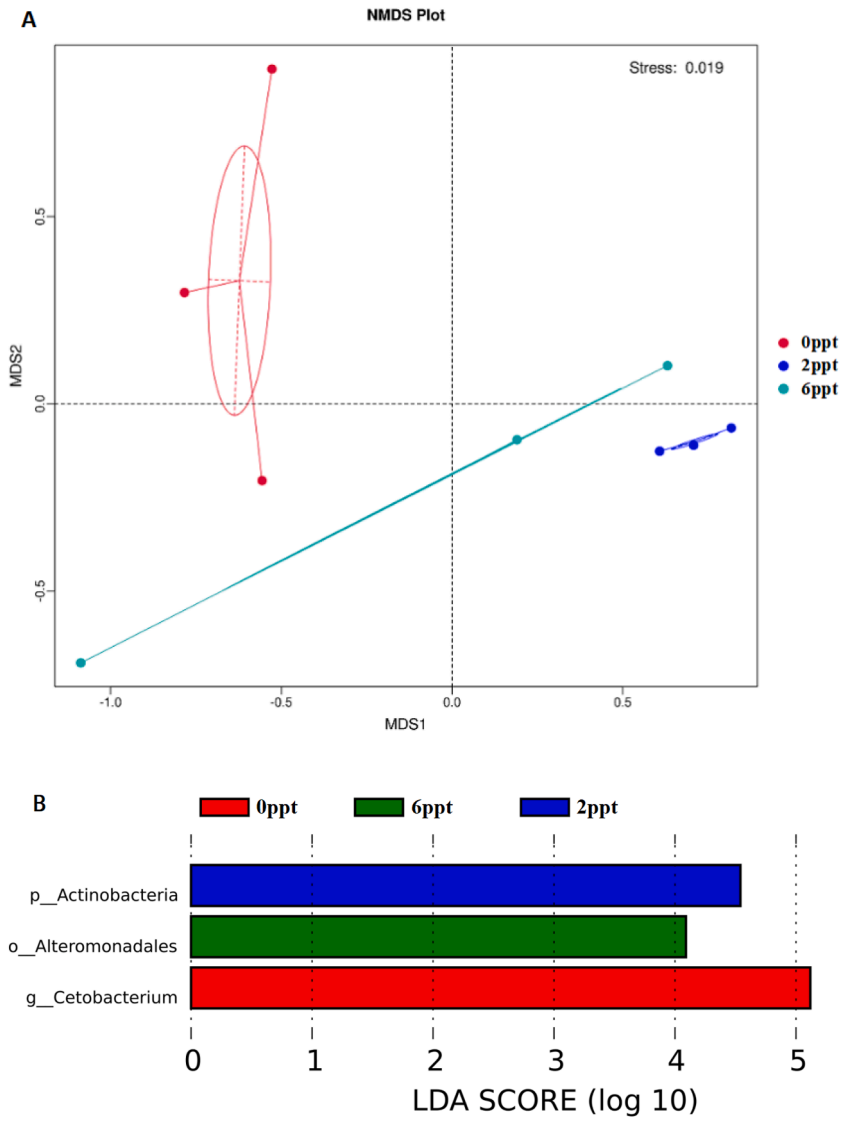

Fig. 6. Beta diversity of the gut microbiota of grass carp in the 0, 2, and 6 ppt groups. A. Non-Metric Multi-Dimensional Scaling (NMDS), each dot represents one sample and each color represents one group. B. Histogram of LDA value distribution, LDA score above 4.0 were selected, and the lowest taxonomic levels of OTUs are listed.

in organisms. CAT and SOD are the first line of defense against cell oxidative damage, while MDA is among the lipid peroxidation products and is an index to evaluate oxidative damage (Jia et al., 2019). ACP and AKP are important indices to measure immune function and health status. The results showed that salt stress led to increased SOD and CAT activities in the gill, kidney, and liver, indicating that high salinity (6 ppt) stimulated the stress response of grass carp. However, the CAT activity of 2 ppt and 6 ppt in gut was significantly lower than that of the 0 ppt, indicating that salinity inhibited gut CAT activity. Furthermore, the activities of ACP and AKP in gut also decreased significantly with increasing salinity. Therefore, the oxidative damage in gut led reduction of immune function (Reese et al., 2018). No differences were observed in the results for T-ATPase in the gills, which may be due to the fact that grass carp can adapt to lower salinities and the regulation of osmotic pressure is balanced in the fish. Combined with other studies (Mozanzadeh et al., 2021), it can be concluded that chronic salt stress had different effects on tissues and organs. The changes and damage due to immune stress in the liver and gut of fish affected the digestion and absorption of grass carp.

When facing salinity changes, the intestinal microbial composition of fish may also change correspondingly. Changes in the intestinal microbial composition of fish may affect the growth and physiological conditions of fish, and eventually lead to growth differences in fish (Dehler et al., 2017). In this study, the intestinal microorganisms of grass carp were studied at different salinities. At the phylum level, Proteobacteria and Firmicutes were abundant in each group. This is consistent with previous studies (Zhen et al., 2023). Proteobacteria is related to fish immunity (Liu et al., 2022). Firmicutes is associated with lipid metabolism, increasing the absorption of fatty acids (MD and Sanjay., 2022). In this study, the proportion of Proteobacteria and Firmicutes had little change under different salinities and remain dominant among different salinities, indicating that Proteobacteria and Firmicutes played an important role in maintaining lipid metabolism balance and immune homeostasis of grass carp facing salinity changes. In this study, the proportion of Fusobacteria was significantly reduced in the 2ppt group and the 6ppt group. Previous studies have shown that Fusobacteria are involved in complex organic compound metabolism and are associated with carbohydrate and lipid metabolism. In addition, Fusobacteria can participate in immune regulation of fish and help grass carp with anti-inflammatory and immunity by producing butyric acid (Terova et al., 2016). The significantly decreased proportion of Fusobacteria in the 2ppt and 6ppt groups may affect the anti-inflammatory and

immunity of grass carp.

At the genus level, *Cetobacterium* was dominant in 0ppt group, and significantly reduced in 2ppt group and 6ppt group. *Pseudomonas* and *Brevinema* were dominant in 2ppt group, while *Vibrio, Exiguobacterium, Aeromonas* and *Pseudomonas* were dominant evenly in 6ppt group. *Cetobacterium* was generally considered as beneficial bacteria (Schmidt et al., 2015). It was a common acetate producing bacteria in gut, which could stimulate insulin gene expression and regulate glucose utilization (Wang et al., 2021). Moreover, some species of *Cetobacterium* had the ability to produce vitamin (Zhou et al., 2018). Consistent with our result, Lin (2019) found that the dominant bacteria in gut of the eel (*Anguilla marmorata*) with faster growth rate were *Cetobacterium, Clostridium, Lactococcus, Bacteroides, Plesiomonas* and *Akkermansia*. The results showed that the abundance of *Cetobacterium* is positively correlated with the growth data of grass carp. *Cetobacterium* in the 6 ppt group reduced significantly and grass carp at 6 ppt showed a significant slowdown in growth.

In 6 ppt group, *Vibrio, Exiguobacterium, Aeromonas* and *Pseudomonas* exceeded *Cetobacterium* to become the dominant genus. *Vibrio* is generally considered as a harmful pathogen, associated with aquatic diseases (Santos et al., 2019). *Pseudomonas* is a common pathogen. It can produce a variety of virulence related substances, such as endotoxin, exotoxin A, elastase, collagenase and pancreatic peptidyase, etc. (Liu et al., 2022). *Exiguobacterium* can promote plant growth and is an important microbial inoculant. (Zhang et al., 2020). *Brevinema* may have negative effects on the growth of fish (Peng et al., 2021). In *Brachymystax lenok, Brevinema* was significantly decreased in slow growing group, compared to fast growing group (Hu et al., 2020). *Erysipelotrichaceae* existed in the gut of mammals, which was reported to be associated with metabolic disturbance and inflammatory diseases (Wu et al., 2021). *Aeromonas* was a potential pathogen in aquaculture. It would do harm to aquatic animals (Kitiyodom et al., 2019). The results showed that these harmful bacteria occupy a major position in the intestinal tract of grass carp in the 6ppt group, affecting the metabolism and immunity of grass carp and slowing the growth of grass carp.

The results showed that the changes in salinity changed the composition of intestinal microorganisms of grass carp at both phylum and genus levels. The results showed that with the increase of salinity, the proportion of beneficial bacteria in the gut of grass carp gradually decreased, no longer being the dominant bacteria. However, some harmful bacteria gradually occupied the dominant position. Changes in gut microbial composition ultimately affected the growth of grass carp.

5. Conclusion

In this study, we confirmed that grass carp could survive at the range of 0~8 ppt, and were most suitable for growth at a salinity lower than the SC (1.173). Grass carp showed good adaptability to low salinity (2 ppt), did not show a significant difference in growth and maintained a stable physiological and immune status. However, exposure to high salinity (6 ppt) produces significant deleterious effects, including growth inhibition as well as physiological and immune-related changes. The gut microbiota in grass carp changes with salinity. With the increase of salinity, the proportion of beneficial bacteria in the gut of grass carp gradually decreased, while some harmful bacteria gradually occupied the dominant position. Changed in gut microbial composition ultimately affected the growth of grass carp. The differences in growth rates may be related to the abundance of specific gut bacteria.

Author statement

The work has not been published elsewhere, and the manuscript has not been submitted to any other journal. All co-authors have seen a copy of the manuscript and agree to its submission.

CRediT authorship contribution statement

Dingrui Liu: Investigation, Visualization, Methodology, Writing – original draft, Data curation. **Zhuowei Zhang:** Visualization, Writing – review & editing. **Yikun Song:** Visualization, Methodology. **Jiayu Yang:** Visualization, Methodology. **Yuyou Lu:** Visualization, Methodology. **Wenjie Lai:** Visualization, Methodology. **Ziyi Wu:** Visualization, Methodology. **Dandan Zhao:** Supervision, Writing – review & editing. **Haoran Lin:** Supervision, Writing – review & editing. **Yong Zhang:** Supervision, Writing – review & editing. **Jin Zhang:** Conceptualization, Supervision, Writing – review & editing. **Shuisheng Li:** Conceptualization, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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