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# Impact of Different Diets on Adult Tri-Spine Horseshoe Crab, *Tachypleus tridentatus*

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**Abstract** Effective culture and management of adult tri-spine horseshoe crab, *Tachypleus tridentatus* can ensure that stock enhancement programs and aquaculture systems are maintained. To explore suitable feed for animals during the breeding season, Pacific oyster (*Ostrea gigas*) (oyster group; OG) and frozen sharpbelly fish (*Hemiculter leucisculus*) (frozen fish group; FG) were selected to feed 20 *T. tridentatus* male and female pairs, respectively. At the end of the experiment, intestinal samples were obtained to measure digestive enzymes activities. The intestinal flora were determined by 16S rDNA sequencing. No eggs were observed in the FG and one *T. tridentatus* adult died. No animals died in the OG, and $9.7 \times 10^4$ eggs were obtained. These results show that oysters are more suitable for the development and reproduction of adult *T. tridentatus* than frozen fish. Additionally, the digestive enzyme activity analysis revealed that animals in the OG exhibited higher protein digestibility than those in the FG, but no significant differences in lipid and carbohydrate uptake were observed between the groups. Furthermore, the intestinal flora analysis showed that operational taxonomic units (OTUs) and the Chao1 index were significantly higher in the OG than in the FG, but no significant difference was observed in the Shannon or Simpson indices between the groups. Our data indicate that the oyster diet improved the intestinal microbial diversity of *T. tridentatus*. We hypothesize that nutrients, such as oyster-based taurine, proteins, and highly unsaturated fatty acids, improve protease activity in the *T. tridentatus* digestive tract, alter the intestinal floral structure, and improve the reproductive performance of *T. tridentatus*.

**Key words** *Tachypleus tridentatus*; diet; reproductive performance; digestive enzyme activity; intestinal flora

## 1 Introduction

Horseshoe crabs are known as ancient 'living fossils' as they have survived for 400 million years on the Earth (Van Roy *et al*., 2010; Kwan *et al*., 2018). The hemolymph from *Tachypleus tridentatus* can be used to produce *Tachypleus* amebocyte lysate; therefore, *T. tridentatus* is of unique value for national public health security (Xie *et al*., 2021). Human disturbance and environmental pollution have caused a sharp decline of the *T. tridentatus* resource (Cai *et al*., 2021). In 2019, the International Union for Conservation of Nature Red List updated *T. tridentatus* to endangered status (Laurie *et al*., 2019), and the animal was listed as a Grade II protected species in the National Key Wildlife List of China in 2021. Due to the ongoing COVID-19 pandemic, the large-scale production of vaccines has led to increasing demand for *Tachypleus* amebocyte lysate and increased pressure on horseshoe crab conservation. Large-scale artificial breeding, larval culture, and field release measures are vital and reliable *ex-situ* conservation modalities for *T. tridentatus* resource management (Hong, 2011). Therefore, cultivation and management of adult horseshoe crab are of great importance to ensure enhanced release and culture of *T. tridentatus*. Food sources are crucial to the physiological activities of aquatic animals (Doxa *et al*., 2013). Mastering feeding demands and behaviors are key to successful artificial breeding programs, such as improving growth performance (Tacon *et al*., 2002), reducing mortality (Espinosa and Allam, 2006), enhancing adult fecundity (Pan *et al*., 2009), and replacing natural diets with artificial compound feed (Millamena, 2002).

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Horseshoe crabs have different dietary habits at different life stages. Mollusks, crustaceans, and polychaetes have been observed in gut samples from adult *Tachypleus gigas* in India (Chatterji *et al*., 1992) and adult *Carcinoscorpius rotundicauda* in Malaysia (John *et al*., 2012). However, studies on the diet of *T. tridentatus* have only been reported for juvenile and sub-adult animals. Hu *et al.* (2013) studied the diet of juvenile *T. tridentatus* at the third instar. Kwan *et al.* (2014) investigated the health status of juvenile *T. tridentatus* fed with different diets at the eighth instar, and Gao *et al.* (2003) conducted preliminary research on the diet of juvenile *T. tridentatus* with a prosomal width of 10−20 cm. There is a need for research on the diet of adult *T. tridentatus* during the breeding season, which has not been studied.

Different diets can lead to differences in intestinal microbial community structure, with intestinal microbiota affecting the host immune defense, digestion and absorption, nutritional metabolism, and other physiological functions (Chen *et al*., 2018). Little information is available on the intestinal microbiota diversity of *T. tridentatus*. Miao *et al.* (2020) analyzed the gut microbiota diversity of first and second instar *T. tridentatus*, and reported that initial molting rather than feeding has a significant effect on the intestinal flora of juvenile *T. tridentatus*. The effects of diet on intestinal microflora diversity in adult *T. tridentatus* remain unclear.

Based on the finding that major food sources for adult *T. tridentatus* and *T. gigas* are bivalves and fish (Guo *et al.*, 2021; Halim *et al.*, 2021), oysters and frozen fish were selected as diets for adult *T. tridentatus* in this study. Oysters have more moisture, crude protein, crude fat, and ash content than frozen fish (Li *et al.*, 2021). Moreover, oysters are rich in high-quality protein, glycogen, n-3 polyunsaturated fatty acids, essential amino acids, trace elements, and other nutrients (Li *et al.*, 2021). We then analyzed the dietary effects on reproductive performance and intestinal flora of adult *T. tridentatus* to provide a scientific and theoretical basis for selecting an artificial breeding diet for these animals.

## 2 Materials and Methods

### 2.1 Selection and Culture Management of Adult *T. tridentatus*

Horseshoe crabs (female, 4.21 kg±0.68 kg; male, 1.70 kg ±0.22 kg) were obtained from the South China Sea Fisheries Research Institute, Chinese Academy of Fisheries Sciences in March 2019. Twenty *T. tridentatus* male and female pairs were randomly selected for breeding studies (May−August). The average prosomal width of the females was 35.28 cm±4.62 cm, while that of males was 27.19 cm ±2.37 cm.

Culture studies were conducted in two indoor cement ponds (4 m×4 m×0.8 m) containing filter-disinfected seawater at 28−32℃, with 26%−30% salinity, pH of 7.4−7.8, and dissolved oxygen ≥4.0 mg $L^{-1}$. Ten animal pairs were randomly selected and placed in each pond, which was also equipped with a water circulating system, and a sand covered bottom (depth, 20−40 cm; grain size, 0.5−2.0 mm). The animals were fed once daily at 18:00 during culture. In one pond, animals were fed 20 mm (length)×8 mm (width)×5 mm (height) oysters (*Ostrea gigas*) (oyster group; OG), while the other pond was fed an equal portion of frozen fish (*Hemiculter leucisculus*) (frozen fish group; FG) with the same particle size, and both groups were fed at 3% of overall body mass. About 80% of the seawater in each pond was renewed daily. The sandy bottom and pond walls were cleaned once every 15 days. Health parameters, including final body weight, mortality, weight gain rate, and specific growth rate, were observed in all animals during the study. All horseshoe crabs were weighed at the beginning and the end of the experiment. Additionally, breeding activities were recorded in the groups, including egg number and hatching rate. Eggs were obtained by natural spawning. The total number of eggs spawned by the 10 pairs of horseshoe crabs was counted at the end of the experiment.

### 2.2 Sampling and Experimental Manipulation

After 120 days of feeding experiment, three *T. tridentatus* male and female pairs were randomly sampled from each pond. The digestive tract was dissected and stored at −80℃. Intestinal tissues (0.5 g per sample) were ground twice in an automatic freezing grinding instrument (JXFSTPRP-32L, Shanghai Jingxin Industrial Development Co., Ltd., Shanghai, China), and the supernatant was centrifuged at 4℃ (Thermo ST16, Shanghai, China) and stored to determine enzyme activities. Pepsin, trypsin, lipase, and α-amylase levels were determined using kits provided by Nanjing Jiancheng Bioengineering Institute (Shanghai, China).

Intestinal samples (0.5 g per sample) were also ground. Total intestinal bacterial DNA was extracted using the Tiangen DNA extraction kit (DP308, Tiangen Biotech Co., Ltd., Beijing, China), and the V3 and V4 regions of 16S rRNA were amplified. The polymerase chain reaction (PCR) was carried out in a 30 μL reaction system with 15 μL of Phusion® High-Fidelity PCR Master Mix (New England Biolabs, Ipswich, MA, USA), 0.2 μmol $L^{-1}$ of the forward and reverse primers, and about 10 ng of template DNA. The PCR conditions were initial denaturation at 98℃ for 1 min, followed by 30 cycles of denaturation at 98℃ for 10 s, annealing at 50℃ for 30 s, and elongation at 72℃ for 60 s, followed by a final elongation step at 72℃ for 5 min. Once successful amplification was indicated using 1% agarose gel electrophoresis, high throughput sequencing of the 16S rRNA gene was entrusted to Mingke Biotechnology Co., Ltd. (Hangzhou, China). The PCR primers were: 515F (5'-GTGCCAGCMGCCCGG-3') and 907R (5'-CCGTCAAT TCMTTTRAGTTT-3') DNA was amplified using TransStart Fastpfu DNA Polymerase (TransGen AP221-02, Beijing, China) with a PCR instrument (ABI GeneAmp® 9700, ABI, Foster City, CA, USA). Three replicates of each sample were mixed, and the PCR products from the same sample were recovered by 2% agarose gel electrophoresis. The PCR products were purified using the AxyPrepDNA Gel Recovery Kit (Axygen®, Tewkesbury, MA, USA) and

eluted in Tris-HCl (pH 7.4).

### 2.3 Intestinal Microbiome Analysis Determination of Taurine Content in Two Diets

Based on the Illumina PE250 sequencing tool, fast length adjustment of short reads and paired-end reads were filtered and spliced according to the overlapped relationships to generate good quality data. Operational taxonomic units (OTUs) were clustered to analyze differences in species abundance and the α- and β-diversity indices between the groups. Linear discriminant analysis effect size (LEfSe) was used to identify significant differences in the relative abundance of the bacterial taxa.

Muscle samples were collected randomly and locally from *Ostrea gigas* and *Hemiculter leucisculus* (six samples for each). Taurine concentrations in muscle samples were measured using taurine assay kit (Cell Biolabs, San Jose, CA, USA).

### 2.4 Data Analysis

Data were tested for normality and homogeneity of variance using the Shapiro-Wilk test, and the *t*-test was used to analyze differences between groups. The results are expressed as mean±standard deviation (SD). Duncan's multiple comparison test was used to analyze differences between groups, and $P<0.05$ was considered significant. Principal component analysis (PCA) was performed in R software (Version 4.0.5; The R Foundation for Statistical Computing, Vienna, Austria) to identify differences in the microbial structure between the groups.

## 3 Results

### 3.1 Effects of Different Diets on Adult *T. tridentatus* Growth and Reproductive Performance

No eggs were observed in the FG, and one adult *T. tridentatus* died. No horseshoe crabs died in the OG, and $9.7\times10^4$ eggs were obtained. These eggs were pale yellow spherical, with diameters of 2.98 mm±0.15 mm, and a hatching rate of 89%. The final body weight, weight gain rate, and specific growth rate of the OG group were higher than those of the FG group (Table 1).

Table 1 Influence of the different diets on growth of adult *Tachypleus tridentatus*

| Item | FG | OG |
|---|---|---|
| IBW (kg) | 3.05±0.32 | 2.98±0.21 |
| FBW (kg) | 3.59±0.56$^a$ | 4.03±0.75$^b$ |
| WGR (%) | 5.70±1.36$^a$ | 8.78±3.97$^b$ |
| SGR (%) | 0.14±0.76$^a$ | 0.40±0.89$^b$ |

Notes: IBW, initial body weight; FBW, final body weight; WGR, weight gain rate; SGR, specific growth rate. Different superscript letters indicate a significant difference between the treatment groups ($P<0.05$).

### 3.2 Effects of the Different Diets on Adult *T. tridentatus* Intestinal Enzyme Activity

As shown (Table 2), pepsin activity was significantly higher in the OG crabs than in the FG crabs ($P<0.01$, $t=-3.038$), while lipase, α-amylase, and trypsin activities were not significantly different ($P>0.05$).

Table 2 Influence of different diets on intestinal enzyme activities of adult *Tachypleus tridentatus*

| Item | FG (U (mg prot)$^{-1}$) | OG (U (mg prot)$^{-1}$) |
|---|---|---|
| Lipase (LPS) | 2.15±0.67 | 1.96±0.37 |
| α-amylase (AMS) | 0.34±0.09 | 0.27±0.08 |
| Pepsin | 0.67±0.56$^b$ | 5.89±5.13$^a$ |
| Trypsin | 182.37±105.67 | 313.20±347.65 |

Note: Different superscript letters indicate a significant difference between the treatment groups ($P<0.01$).

### 3.3 Effects of the Different Diets on the Adult *T. tridentatus* Intestinal Flora

#### 3.3.1 OTU cluster and species diversity analyses

In total, 237969 valid sequences were identified across the sample groups, including 114155 from the FG and 123814 from the OG. The OTUs belonged to 11 phyla, 17 classes, 32 orders, 50 families, 57 genera, and 55 species. OTU similarity and overlap between the groups were investigated using a Venn diagram (Fig.1A). In total, 1487 OTUs were identified in both groups, of which the numbers of OTUs in the FG and OG were 775 and 1272, re-

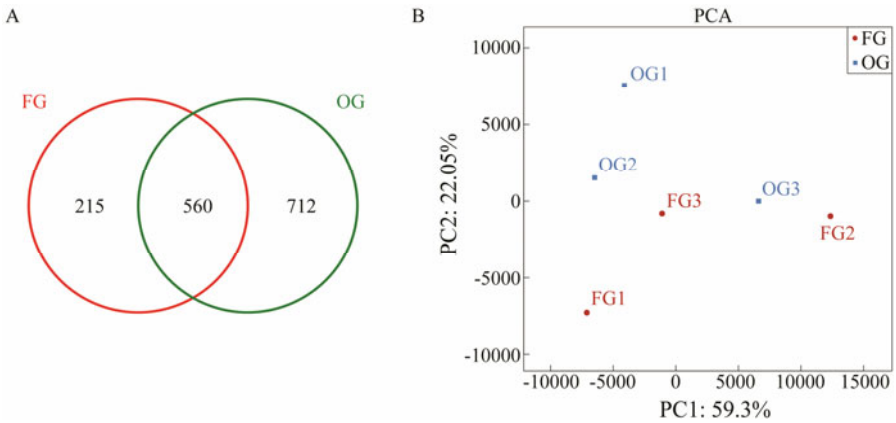


Fig.1 (A) Venn diagram used to count the number of shared and unique OTUs in different samples; the red circle represents the FG group, the green circle represents the OG group, and the overlap represents the number of shared OTUs between the two groups; (B) PCA of adult *Tachypleus tridentatus* intestinal flora. FG, frozen fish group; OG, oyster group.

spectively. A total of 560 OTUs were common between the groups, while there were 215 (FG) and 712 (OG) unique OTUs. These data indicate that oysters improved the intestinal microbial diversity of *T. tridentatus*.

The Chao1 index was used to evaluate the richness of the intestinal flora. The Shannon and Simpson indices have been commonly used to assess intestinal flora diversity; a higher Shannon index and a lower Simpson index indicate higher diversity in bacterial communities (Liu and Peng, 2021). Our results show that the coverage value of both groups was >0.99, suggesting that the results were reliable. The abundance (OTUs and Chao1 index) and diversity (Shannon index) of the intestinal flora were higher in the OG than those in the FG (Table 3). The OTUs and Chao1 indices were significantly different between the groups ($P<0.05$), while the Shannon and Simpson indices were not significantly different ($P>0.05$) (Fig.2).

PCA showed that diet (59.3%) was the main factor responsible for the difference of the intestinal content samples from *T. tridentatus* in the FG and OG groups (Fig.1B).

Table 3 Influence of the different diets on the intestinal microbial diversity index of *Tachypleus tridentatus*

| Intex | FG | OG |
|---|---|---|
| OTU | 273±35.00 | 693±216.40 |
| Chao1 | 287.33±24.38 | 719.67±211.89 |
| Coverage | 0.999±5.80E$^{-4}$ | 0.998±3.20E$^{-4}$ |
| Shannon | 2.59±0.67 | 4.10±0.80 |
| Simpson | 0.20±0.11 | 0.06±0.03 |

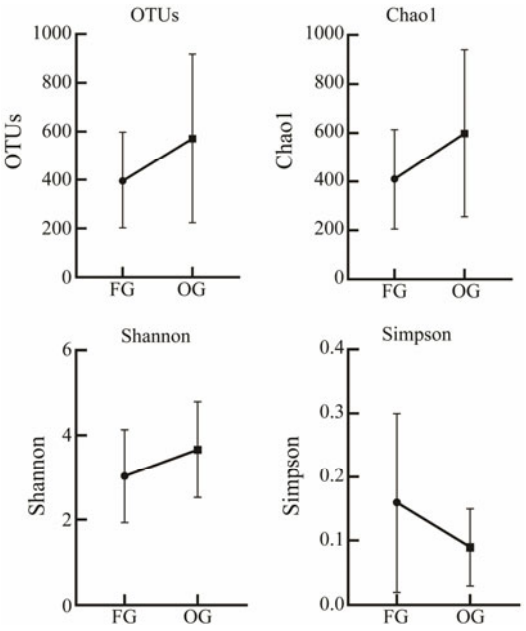


Fig.2 α-Diversity of the bacterial communities: The Chao1 index estimates richness; the Shannon and Simpson indices estimate diversity. FG, frozen fish group; OG, oyster group.

### 3.3.2 Community composition and intestinal flora abundance

The dominant phyla and genera in the *T. tridentatus* intestinal samples are shown in Fig.3. Proteobacteria (FG: 37.59% and OG: 20.59%), Tenericutes (FG: 34.89% and OG: 22.64%), Firmicutes (FG: 14.76% and OG: 21.62%), and Bacteroidetes (FG: 9.81% and OG: 16.85%) were the common dominant phyla. In addition, Fusobacteria (6.35%) was a unique dominant phylum in the OG, and the remaining abundances were<3% (Fig.3A). *Ralstonia* (14.02%), *Achromobacter* (5.73%), *Bacteroidales S24-7 group_norank* (5.49%), *Lactobacillus* (4.25%), *Delftia* (3.96%), and *Faecalibaculum* (3.93%) were the dominant genera in the FG, while *Ralstonia* (7.08%), *Achromobacter* (6.34%), *Bacteroidales S24-7 group_norank* (5.99%), and *Lactobacillus* (5.68%) were the dominant genera in the OG (Fig.3B).

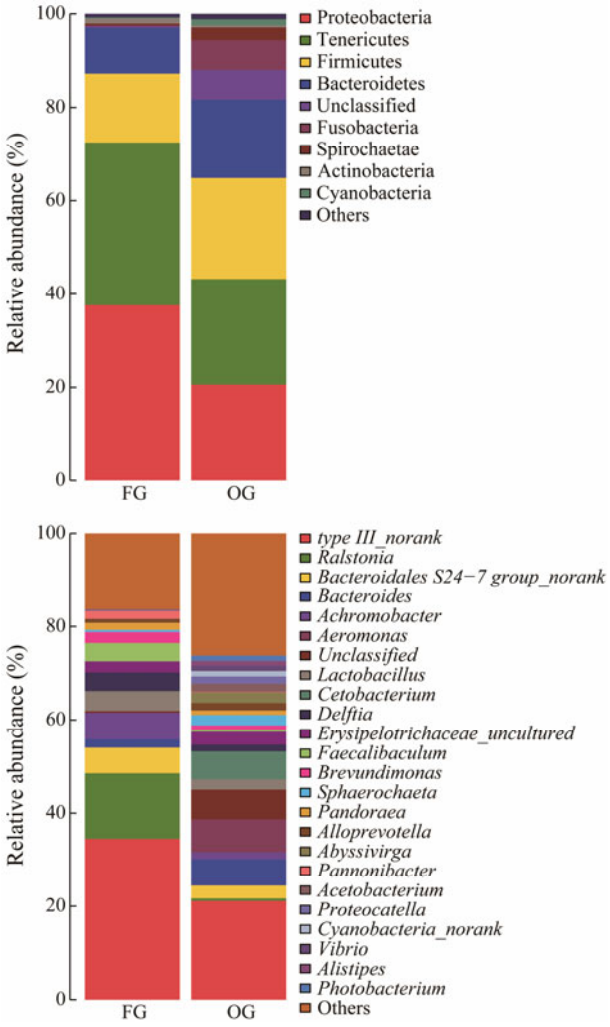


Fig.3 Dominant phyla (A) and genera (B) of intestinal flora in the different dietary groups. FG, frozen fish group; OG, oyster group.

### 3.3.3 Species differences among the intestinal flora groups and taurine content in two diets

The results of the LEfSe analysis showed that Fusobacteria was a phylum-level biomarker and *Roseomonas*, *Okibacterium*, *Epilithonimonas*, *Enterococcus*, and *Carboxylicivirga* were genus-level biomarkers between FG and OG (Fig.4).

The taurine content of two diets was significantly different ($P<0.05$). Taurine content in oysters was 362.19±13.46 mg(100 g)$^{-1}$, while *Hemiculter leucisculus* was 236.78±19.75 mg(100 g)$^{-1}$.

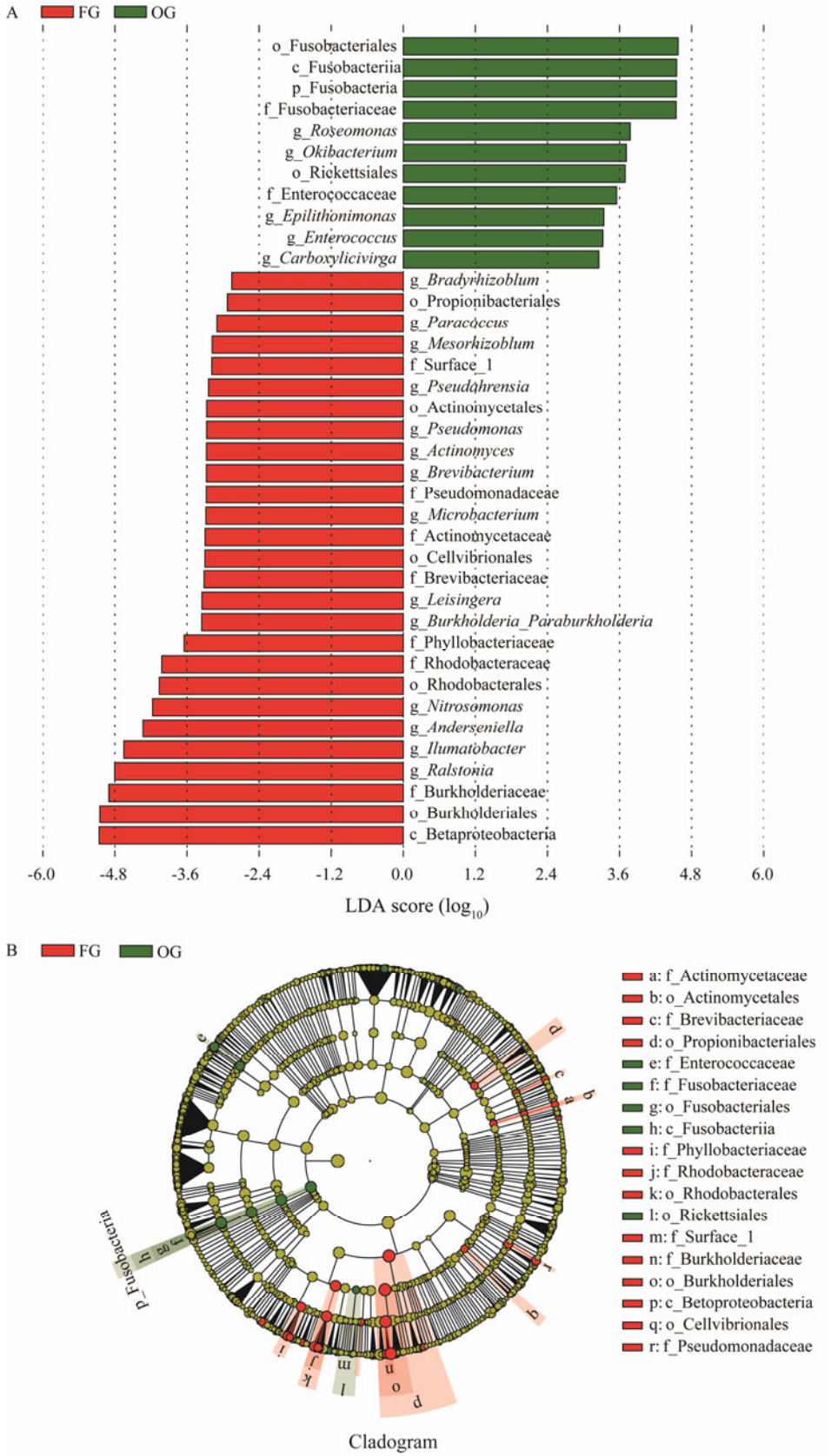


Fig.4 LEfSe results of intestinal microbial composition between the frozen fish group (FG) and oyster group (OG). A, LDA scores of the bacterial clades identified by the LEfSe analysis; B, Phylogenetic relationships of the bacterial clades revealed by LEfSe. The single characters before the underlines are abbreviations: p, phylum; c, class; o, order; f, family; g, genus.

## 4 Discussion

Our data show that adult *T. tridentatus* fed frozen fish (FG) did not spawn during the experiment (120 d), while animals fed oysters (OG) laid eggs, suggesting that the nutrients in oysters were more suitable for adult *T. triden-*

*tatus* growth and development than frozen fish. The frozen fish (*H. leucisculus*) had a high fat content (Zeng *et al.*, 2012). The different particle sizes and hardness of the diets may also be a reason for the results. Oysters are highly palatable with a soft meat quality, which are conducive to feeding horseshoe crabs. Frozen fish are difficult to chew and swallow. In addition, the width and thickness of the frozen fish pieces may have affected feeding of *T. tridentatus*. A previous study showed that the feed intake of juvenile *T. tridentatus* decreases with increasing particle size of the diet (Gao *et al.*, 2003). Considering these dietary factors, both diets were cut into small size pieces before feeding. Oyster soft tissues are rich in amino acids, with taurine levels accounting for almost half of all free amino acids (Fuentes *et al*., 2010). Taurine levels in oysters from the southwestern South China Sea are 16.81−19.83 mg g$^{-1}$ (Gao *et al*., 2013), which are 5−30 times higher than those of other marine fishes, such as *Dentex tumifrons*, *Chelidonichthys kumu*, and *Osmerus mordax* (Tan *et al*., 2000). Previous studies reported that taurine improves growth and reproduction (Xu *et al*., 2020; Yu *et al*., 2021). Oysters also contain highly unsaturated fatty acids (eicosapentaenoic acid and docosahexaenoic acid) that promote vitellogenesis and gonadal and embryonic development in aquatic animals and are key nutrients affecting the reproductive performance of adult fish (Bell and Sargent, 2003; Watanabe and Vassalloagius, 2003; Callan *et al*., 2014). Similarly, these molecules enhance the reproductive performance of aquatic animals, including *Cyprinus carpio* (Xu *et al*., 2016), *Pagrus maior* (Røjbek *et al*., 2014), and *Acipenser baeri* (Luo *et al*., 2015).

Pepsin activity was significantly higher in the OG than in the FG, but no significant difference was observed in intestinal α-amylase activity. This may be because oysters are rich in protein, glycogen, and taurine (Wang *et al*., 2011). Taurine increases protease activity and the feeding rate (Li *et al*., 2017; Wang *et al*., 2018). Similar to our data, Yu *et al.* (2021) reported that the protease activity of *Lateolabrax japonicus* was significantly higher in a taurine-treated group than that in a control group, whereas α-amylase activity was not significantly different. He *et al.* (2017) showed that feed containing 1.3% taurine enhances protease activity in the stomach, liver, and intestine of *Anguilla marmorata*. Other studies have reported that the intestinal protease activities of *Acrossocheilus hemispinus* (Liang *et al.*, 2018), *Salmo trutta fario* (Wang *et al*., 2017), and juvenile *Scylla paramamosain* (Dong *et al*., 2017) increase with dietary protein level. Thus, taurine could be supplemented in *T. tridentatus* feed to observe its specific effect on the digestive enzyme activity of *T. tridentatus*. Pepsin activity in the OG was significantly higher compared to the FG. Therefore, adult *T. tridentatus* of OG group had stronger ability to digest and absorb protein. Among the total fat of oyster, omega-3 highly unsaturated fatty acids (*n*-3 HUFAs) such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) accounted for 28% (Wang *et al*., 2003). Ma *et al*. (2005) found that turbot *Scophthalnus maximus* fed high-protein and high *n*-3 HUFAs diet had highest spawning-stock biomass. This finding is in agreement with the results found in our study.

Bacteria have a strong capacity to metabolize taurine, which can be directly broken down into carbon, nitrogen, and sulfur for growth (Cook *et al*., 2006). Ma *et al.* (2021) reported that taurine levels in feed affect the structure, richness, and diversity of the intestinal flora. Our data show that Proteobacteria, Tenericutes, Firmicutes, and Bacteroidetes were the common dominant phyla in the horseshoe crab groups, and Fusobacteria was the unique dominant phylum in the OG. Proteobacteria are Gram-positive bacilli that produce spores in harsh environments. Bacteroidetes are the largest Gram-negative bacilli group in the animal intestine and are involved in metabolic processes, such as digestion of nutrients and absorption (Francois *et al*., 2011). Fusobacteria are Gram-negative bacilli with a higher detection rate in human colorectal tumors than surrounding normal tissues (Kelly *et al*., 2018). Studies have shown that high-protein diets enrich Flavobacteria and Fusobacteria in male *Blattella germanica*, and a low-protein diet benefits the growth of Bacteroidetes, Rikenellaceae, and Tannerellaceae (Wang *et al*., 2021).

Oysters are high in protein and low in fat, and our results were consistent with previous studies. The quantity of *Bacteroidales S24-7 group_norank* was higher in the OG (5.99%) than in the FG (5.49%). These bacteria produce butyric acid, a short-chain fatty acid that can be produced by intestinal bacteria from cellulose fermentation (Tang *et al*., 2018). Butyrate-producing probiotics can reduce non-alcoholic fatty liver disease (NAFLD) progression in rats (Endo *et al*., 2013). Zhou *et al*. (2017) also confirmed that sodium butyrate attenuates high-fat diet (HFD)-induced steatohepatitis in mice by improving the gut microbiota and gastrointestinal battier. Short-chain fatty acids maintain homeostasis in the intestinal environment and regulate the immune response (Serino, 2019). *Roseomonas* is a pathogen that causes bacteriemia and infections in wounds, the urinary tract, and other body regions (Rihs *et al*., 1993). Pathogens were detected in the intestines of the OG, which may have been due to remnant, undigested oysters.

Horseshoe crabs have a wide variety of food sources in the natural environment, where nutrients are highly abundant, whereas a single diet is usually fed to animals under artificial culture conditions. The differences in intestinal flora between wild *T. tridentatus* and artificially cultured animals must be comprehensively characterized in terms of differences in nutrient digestion and absorption to develop compound feeds that promote *T. tridentatus* growth, development, and reproductive performance.

## 5 Conclusions

Adult *T. tridentatus* fed oysters had higher weight gain rate (WGR), specific growth rate (SGR), spawning-stock biomass. These results showed that oysters were more suitable for enhancing the growth and reproduction performance of adult *T. tridentatus* than frozen fish, which is of great significance for the recovery of the *T. tridentatus* resource. However, a high-protein diet can also provide nutrients for pathogens to breed while promoting animal

growth and reproduction. Thus, it is advisable to change the culture water frequently to remove the residual food and prevent replication of pathogens.

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