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Effects of Dietary Tannic Acid on Growth, Digestion, Immunity and Resistance to Ammonia Stress, and Intestinal Microbial Community in Pacific White Shrimp (*Litopenaeus vannamei*)

Han Gong ^{1,2}, Zhen Qin ^{1,2}, Zhao Chen ¹, Jitao Li ¹, Zhiqiang Chang ¹, Jian Li ¹ and Ping Chen ^{1,*}

¹ Key Laboratory for Development of Marine Fisheries, Ministry of Agriculture and Rural Affairs, Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Qingdao 266071, China

² Function Laboratory for Marine Fisheries Science and Food Production Processes, Qingdao National Laboratory for Marine Science and Technology, Qingdao 266237, China

* Correspondence: chenping@ysfri.ac.cn



Citation: Gong, H.; Qin, Z.; Chen, Z.; Li, J.; Chang, Z.; Li, J.; Chen, P. Effects of Dietary Tannic Acid on Growth, Digestion, Immunity and Resistance to Ammonia Stress, and Intestinal Microbial Community in Pacific White Shrimp (*Litopenaeus vannamei*). *Fishes* **2022**, *7*, 327. <https://doi.org/10.3390/fishes7060327>

Academic Editors: Daríel Tovar-Ramírez, Xiaolin Meng, Xulu Chang, Yafei Duan and Guokun Yang

Received: 9 September 2022

Accepted: 8 November 2022

Published: 10 November 2022

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Abstract: Tannic acid (TA) has great potential as a new feed additive. In this study, we investigated the effects of dietary TA on growth, digestion, nonspecific immunity, and resistance to ammonia stress and intestinal microbiota in *Litopenaeus vannamei*. The shrimp were fed diets containing different levels of TA: 0 mg/kg (TA-0), 200 mg/kg (TA-200), 400 mg/kg (TA-400), and 800 mg/kg (TA-800) for 56 days, followed by acute ammonia stress for 48 h. The results showed that dietary TA increased the survival rate of the TA-800 group. Dietary TA could improve the morphology of the hepatopancreas and intestinal tissues. After feeding different levels of TA for 56 days, the activities of amylase (AMS) and trypsin (Tryp) were increased, but the activity of lipase (LPS) was decreased. The activities of T-AOC, SOD, and PPO were higher in the hepatopancreas of the three TA treatment groups ($p < 0.05$). When shrimp were exposed to ammonia stress for 48 h, the activity of immune enzymes (LZM, T-AOC and SOD) and the expression levels of immune genes (*LZM*, *proPO* and *Cu/Zn-SOD*) were higher in the three TA treatment groups ($p < 0.05$). Furthermore, dietary TA also changed the composition of intestinal microflora by increasing the abundance of Planctomycetes, but decreasing the abundance of Bacteroides and Proteobacteria. The abundance of *Rhodopirellula*, *Ruegeria*, and *Rhodobacter* were higher, but that of *Paracoccus*, *Algoriphagus*, *Cellvibrio*, *Flavobacteriaceae*, and *Bacteroides* were lower in response to dietary TA. These results revealed that dietary TA had a positive effect on growth and intestinal microbial composition and enhanced the immune response to ammonia stress in shrimp. Therefore, TA can be a potential natural alternative antibiotic substitute for feed additives in shrimp, and the appropriate supplemental dosage is 400–800 mg/kg in the diet.

Keywords: *Litopenaeus vannamei*; tannic acid; immune; intestinal microbiota; ammonia stress

1. Introduction

The Pacific white shrimp *Litopenaeus vannamei* is one of the most economically important shrimp species, the production of which was 5.81 million tons in 2020, contributing to 51.7% of all mariculture shrimp in the world [1]. In order to produce large quantities in a short time, intensive aquaculture techniques and substances that stimulate growth are required [2]. However, dense growing environment is characterized by high density of individuals, which can easily lead to disease outbreaks, which can be stressful for organisms [3]. Shrimps spend all their growth stages in an aquatic environment and are inevitably affected by environmental changes [4]. Ammonia is an important environmental factor affecting the health of aquatic species, and its elevated concentration is due to the accumulation of organic sundries such as feces and unused feed in cultured water. Ammonia induces an anti-stress response in shrimp, thus alleviating oxidative stress, immune disorders, and susceptible disease, which leads to high mortality [5,6]. Therefore, the development

of ecofriendly disease preventative approaches is important for health management in shrimp aquaculture.

Under the background of “antibiotic prohibition” in feed, research on antibiotic substitutes has become one of the research hotspots in animal nutrition. Natural plant extracts have been widely studied as new feed additives for their positive effects on growth promotion and antioxidant and immune regulation of aquatic animals. Tannic acid (TA) is a polyphenolic compound that is ubiquitous in nature. Tannins represent a highly heterogeneous group of water-soluble polyphenolic compounds, divided into condensed tannins, which are C–C bound oligomers of catechin or leucoanthocyanidin, and hydrolyzable tannins, esters of monosaccharides with gallic acid or oligomers of gallic acid [7,8]. It has been reported that low-dose TA has beneficial effects on animal growth performance, immunity, intestinal health, and antioxidative stress ability [8,9]. Previous studies have indicated that the increased bacterial diversity in the intestines of organisms may play an important role in promoting nutrient digestion and absorption after a period of feeding condensed tannin-containing fish diets [10]. Similarly, sorghum TA up to 5.7 g/kg in diets has no adverse effects on Nile tilapia (*Oreochromis niloticus*) [11]. For example, TA in chestnut shells can improve the intestinal health of fish [12,13]. Zhu et al. found supplementation of a shrimp diet with 0.15% hydrolysable tannins to promote growth performance and digestive activity, improve antioxidant capacity, and optimally adjust intestinal bacterial structure [14]. Findings in livestock suggest that low concentrations of TA can improve the health status, nutrient digestion, and growth performance of chickens and pigs [15,16]. Therefore, TA has great potential as an alternative to feed nutrition addition.

The intestine is an important organ for digestion and absorption in aquatic animals, as well as an important barrier against infectious agents and toxins. The cultivation of a healthy digestive tract has a vital impact on the health and growth of aquatic animals [17]. In addition, intestinal microecology contains not only intestinal microbiota, but also a large number of intestinal microbiota metabolites [18]. Together, they affect host immune function and are important for the maintenance of intestinal homeostasis [19]. A multitude of agents can induce intestinal oxidative stress [20], barrier dysfunction [21], and morphological impairment [22,23], resulting in decreased absorptive function of nutrients and an increase in the incidence of disease [24]. Moreover, inflammation in the intestines of organisms further causes the proliferation of some potentially pathogenic bacteria, which may damage the barrier tissue and trigger intestinal inflammation [25–27]. Some studies have highlighted that chestnut shells rich in tannins may act as a prebiotic on fish intestinal microbiota, and chestnut shells can optimize intestinal microbiota health by regulating intestinal microbiota and immune levels to combat intestinal inflammation [10,12,28]. Tannins can regulate the abundance of beneficial or harmful bacteria in the intestine, thus altering the intestinal environment and its bacterial composition [13,29].

Therefore, the present study aimed to investigate the effects of dietary TA extracted from gallnut on (1) growth and feed utilization; (2) hepatopancreas and intestinal tissue structure; (3) intestinal digestive capacity and microbial community; and (4) hepatopancreas immune response of *L. vannamei* cultured under normal conditions and exposed to ammonia stress. These results will be essential for a better understanding of the roles of TA in the health regulation of *L. vannamei*.

2. Materials and Methods

2.1. Diet Preparation

Experimental diets were formulated according to the guidelines and nutritional requirements of the shrimp following NRC (2011). The basal diet (Hengxing Group Co., Ltd., Jiaxing, China) was used as the control diet. The TA used in this study belonged to the hydrolyzable tannins, which was extracted from gallnut and provided by Qingdao RBT Biotechnology Co., Ltd. (Qingdao, China). The gallnut was broken, screened, and then immersed in water. The impregnated water was clarified, pre-heated, and then spray-dried, and the resulting dry powder was sieved in order to obtain the final product. Four kinds of

experimental diets with different TA content were prepared: 0 mg/kg (TA-0), 200 mg/kg (TA-200), 400 mg/kg (TA-400), and 800 mg/kg (TA-800). TA was completely dissolved in water prior to being added to the diets. All ingredients were smashed to pass through a 320 µm sieve, mixed thoroughly, and then extruded into 2 mm pellets, dried at 55 °C for 12 h, and stored at −20 °C until use. The formulation and proximate composition of the experimental feed are shown in Table 1.

Table 1. The composition of the experimental diets.

Ingredient (g/100 g)	TA-0	TA-200	TA-400	TA-800
Ingredients				
Fish Meal	23	23	23	23
Solvent Extracted Soybean Meal	30	30	30	30
Whole Wheat	10	10	10	10
Corn Gluten (60% protein)	20.2	20.2	20.2	20.2
Dicalcium phosphate	5	5	5	5
Fish Oil	3.3	3.3	3.3	3.3
Bentonite	7.5	7.5	7.5	7.5
Lecithin	0.1	0.1	0.1	0.1
Vitamin Premix	0.1	0.1	0.1	0.1
Mineral Premix	0.1	0.1	0.1	0.1
Stable C	0.1	0.1	0.1	0.1
Lysine hydrochloride	0.6	0.6	0.6	0.6
Copper Sulfate	0.1	0.1	0.1	0.1
Total	100	100	100	100
Tannins acid	0	20	40	80
Proximate Composition				
Crude Protein	40	40	40	40
Crude Fiber	5	5	5	5
Ash	16	16	16	16
Crude Lipid	6	6	6	6
Total Phosphate	1.3	1.3	1.3	1.3
Lysine	1.8	1.8	1.8	1.8

2.2. Shrimp, Culture Conditions and Feeding Trial

Healthy *L. vannamei* with an average body weight of 0.3 ± 0.03 g were randomly selected from a local farming pond at Haifeng Aquaculture Co., Ltd. (Changyi, China). The shrimp were acclimatized for 7 days before the start of the feeding experiment and fed with control feed (Hengxing Group Co., Ltd., Jiaying, China). The water quality parameters were dissolved oxygen (DO) > 6 mg/L; salinity 32 ± 0.5 ; temperature 28 ± 0.5 °C and pH 7.8–8.2.

After acclimation, only shrimp in the molting stage were used for the experiments. A total of 6000 healthy shrimp were randomly distributed into four groups: TA-0, TA-200, TA-400, and TA-800. Each group had three replicate 5000 L plastic tanks, and each tank housed 500 shrimp. Each group was fed their corresponding experimental feed, and all experimental shrimp were fed to apparent satiation three times per day at 6:00, 12:00, and 18:00. The feeding trial lasted for 56 days, and the feeding quantity was adjusted according to the growth of the shrimp. About 30% of the water in each water tank was replaced every day, and the shrimp feces and uneaten feed were cleaned out each time. During the breeding period, the light–dark cycle was maintained in the natural environment.

At the end of the 56-day feeding trial, the hepatopancreas of the six shrimps from each tank was randomly sampled for biochemical and gene expression analyses, and the corresponding intestines were sampled for digestive enzyme activity and microbial community analysis. All experimental samples were frozen and stored in liquid nitrogen for further analysis.

2.3. Ammonia Stress Experiment

After the feeding trial, 50 shrimp were randomly selected from each tank for ammonia stress experiment. Based on the 48 h 50% lethal concentration (LC50) in *L. vannamei* under ammonia stress [30], the ammonia concentration in this study was set as 30.0 mg/L. The ammonia concentration of each tank was maintained at 30.0 mg/L by adding NH₄Cl solution to the fresh seawater, which was adjusted in time to ensure its stability. The concentration of ammonia in water was detected by indophenol blue spectrophotometry (GB 17378.4-2007). The ammonia stress lasted for 48 h, and the mortality of the shrimp in each group was recorded every 8 h. After ammonia stress for 48 h, the hepatopancreas of the six shrimp from each tank was randomly sampled for biochemical and gene expression analyses. All samples were frozen and stored in liquid nitrogen until analysis.

2.4. Growth Performance Analysis

At the end of the 56-day feeding trial, all shrimp were starved for 24 h and then counted and weighed in order to determine their growth performance. The average value of each 50 shrimp was considered as a valid value for each replicate ($n = 3$). The growth performance and feed utilization of the shrimp for all the groups were calculated using the following equations:

$$\text{WGR} = [(\text{Final body weight} - \text{Initial body weight}) / (\text{Initial body weight})] \times 100.$$

$$\text{SR} (\%) = (\text{Shrimp number final} / \text{Shrimp number initial}) \times 100.$$

$$\text{FCR} = \text{Feed consumed (dried)} / \text{WG}.$$

WGR = weight gain rate, SR = survival rate, and FCR = feed conversion ratio.

2.5. Histological Analysis

The hepatopancreas and intestines of three shrimp from each treatment group were randomly sampled after a feeding trial at 56 d and ammonia stress at 48 h, respectively. They were kept in 10% formalin for 24 h, and then dehydrated in a series of alcohol solutions (75–95%). After the samples were washed in toluene, they were embedded in paraffin to produce a dense wax block. Segments (4 μm) were cut with a rotary microtome and stained with hematoxylin and eosin (H&E). The tissue samples were further observed with a Nikon Eclipse E100 microscope (Tokyo, Japan) and analyzed with Image-Pro Plus 6.3 software (Media Cybernetics, Inc., Rockville, MD, USA) following the method of previous research [31].

2.6. Biochemical Analysis

Hepatopancreas and intestinal samples of the collected shrimp were homogenized and centrifuged to collect the supernatant for biochemical analysis. The hepatopancreas samples were used for the determination of superoxide dismutase (SOD), total antioxidant capacity (T-AOC), polyphenol oxidase (PPO), and lysozyme (LZM) activity. The intestinal samples were used to detect amylase (AMS), trypsin (Tryp), and lipase (LPS) activity. They were determined by the related commercial test kits (Jiancheng, Ltd., Nanjing, China).

2.7. Gene Expression Analysis

Total RNA from the hepatopancreas of six shrimp from each tank was extracted using TRIzol Reagent (Invitrogen, Waltham, MA, USA). The total RNA (1 μg) was reverse transcribed to cDNA using a PrimeScript™ RT Reagent Kit (Takara, Shiga, Japan), and the cDNA was diluted to 20 ng/ μL with diethylpyrocarbonate (DEPC) solution and then stored at $-20\text{ }^{\circ}\text{C}$ for further analysis. The β -actin gene of *L. vannamei* was chosen as an internal control. The primer sequences for β -actin, Cu/Zn superoxide dismutase (Cu/Zn-SOD), lysozyme (LZM), and prophenoloxidase (*proPO*) were obtained from previous studies [32], and are shown in Table S1. Real-time RT-qPCR was conducted in an ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA) using a SYBR® Premix Ex Taq™ II Kit (TaKaRa, Japan). The reactions were carried out in a total volume of 20 μL . The PCR program was 95 $^{\circ}\text{C}$ for 30 s, then 40 cycles of 95 $^{\circ}\text{C}$

for 5 s and 60 °C for 30 s, followed by 1 cycle of 95 °C for 5 s, 60 °C for 1 min and 95 °C for 15 s. DEPC-treated water for the replacement of template was used as the negative control. The relative gene expression level was shown as the fold change in expression relative to the TA-0 group, calculated by the $2^{-\Delta\Delta CT}$ comparative CT method.

2.8. Intestinal Microbiota Analysis

Total genomic DNA of intestinal microbes of six shrimp from each tank was extracted using the CTAB/SDS method, and then primers were used to amplify the V4 region of the bacterial *16S rDNA* gene (Table S1). PCR was carried out using Phusion® High-Fidelity PCR Master Mix (New England Biolabs, Ipswich, MA, USA). The PCR products were mixed in equidensity ratios and purified. Then, the sequencing libraries were constructed using the NEBNext® Ultra™ DNA Library Prep Kit for Illumina (NEB, Ipswich, MA, USA) and sequenced on an Illumina MiSeq platform.

Sequence analysis was performed by UPARSE software, and the operational taxonomic units (OTUs) were defined with $\geq 97\%$ similarity [33]. Chimeric sequences were determined by UCHIME [34]. Alpha diversity was calculated using three metrics, including the Chao1, Simpson, and Shannon indices, using Mothur software (version v.1.30.1) [35]. A Venn diagram was used to count the number of unique and shared OTUs in multiple samples. Beta diversity was evaluated by principal coordinates analysis (PCoA) plots based on weighted and unweighted UniFrac metrics, using the package Ape. Microbial composition was analyzed at the phylum, class, and genus levels. Linear discriminant analysis (LDA) and effect size (LEfSe) analysis was performed using the Python LEfSe package to identify differential bacterial taxa within different groups [36]. Random forest analyses were performed based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) level 3 using the R random forest package. A Cytoscape network was constructed to analyze the correlations and differences between microbial communities based on OTU abundance using Cytoscape software.

2.9. Statistical Analysis

All the data were presented as the mean values \pm standard deviation (SD). The one-way analysis of variance (ANOVA; SPSS v22.0) was used to determine the significance of differences between the control and treatment groups, if the values were distributed normally and the variances were homogeneous. If there was only an overall difference, multiple comparisons were made by the Duncan test. A value of $p < 0.05$ was regarded as statistically significant. Percentage data of the weight gain and survival were transformed before the ANOVA.

3. Results

3.1. Growth Performance and Survival of the Shrimp

The shrimp fed the TA diet had a lower FCR and higher weight gain than those fed the TA-0 diet (Table 2). The TA-400 group had the highest growth performance and feed utilization. After a 56-day feeding trial, the survival rate of the shrimp in the three TA groups was significantly higher than that in the TA-0 group ($p < 0.05$), and the TN-800 group's survival rate was significantly higher than that of the TN-200 group ($p < 0.05$) (Figure 1A). Additionally, after ammonia stress for 48 h, the survival rate of the TA-0 group was approximately 15.67%, and that of the TA-800 group was approximately 30.67%. The TA-800 group's survival rate was significantly higher than that of the TA-0 group ($p < 0.05$) (Figure 1B).

Table 2. Growth performance and feed utilization of *L. vannamei* that were fed the control and TA diets for 56 days.

Index	TA-0	TA-200	TA-400	TA-800
IW (g)	0.30 ± 0.03 ^a			
FW (g)	4.47 ± 0.76 ^{ab}	4.68 ± 0.98 ^{ab}	5.16 ± 0.92 ^a	5.07 ± 1.16 ^a
WGR (%)	14.32 ± 1.83 ^b	14.60 ± 1.56 ^b	16.11 ± 1.36 ^a	15.75 ± 2.20 ^{ab}
FCR	1.09 ± 0.02 ^a	0.98 ± 0.02 ^b	0.80 ± 0.01 ^c	0.78 ± 0.01 ^c

Mean values ± SD ($n = 3$). Identical values in same row with a similar superscript are no longer substantially exclusive ($p > 0.05$) by the Duncan test. Where IW = initial weight, FW = final weight, WGR = weight gain rate, FCR = feed conversion ratio.

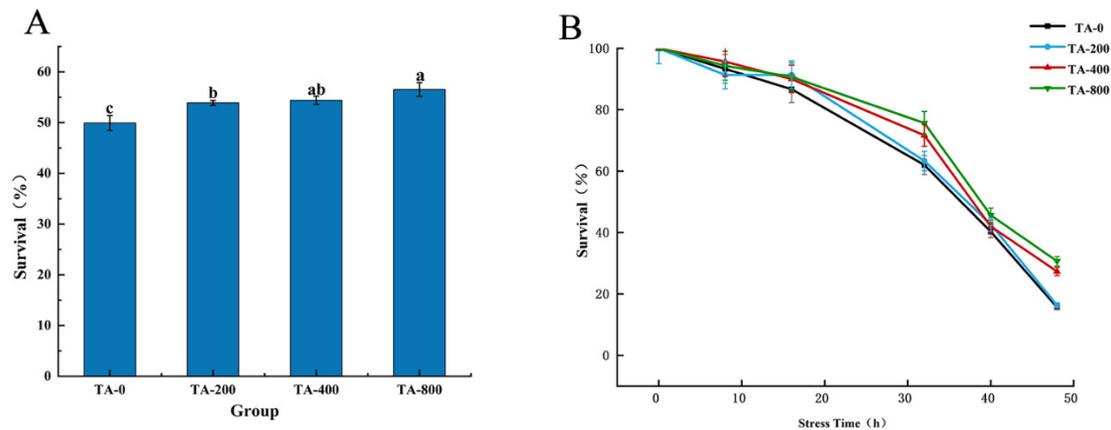


Figure 1. Effects of dietary TA on the survival of *L. vannamei* at the feeding trial 56 days and ammonia stress for 48 h. (A) The survival of shrimp fed the four experimental diets 56 days; (B) the survival of shrimp after ammonia stress for 48 h. Vertical bars represented the mean values ± SD ($n = 3$). Data marked with different letters were significantly different ($p < 0.05$) among groups.

3.2. Histological Structure of the Hepatopancreas and Intestines

After the shrimp were fed TA-supplemented diets for 56 days, the hepatopancreas of four group individuals presented a normal functional structure, with the differentiation of the storage cells (R-cell), secretory cells (B-cell). The control group showed a gap between the bile canaliculus, cell lysis, and cell necrosis with dissolution of some areas of the basement membrane. However, in the TA-800 treatment group, the structure of hepatopancreatic tubules was relatively normal, the structure of hepatocytes was clear, and a few vacuoles appeared in hepatopancreatic tubules. After ammonia stress, compared with the TA-800 group, the hepatopancreas in the TA-0, TA-200, and TA-400 groups exhibited obvious histological damage under ammonia stress for 48 h. The nucleus fell off, and the gap between hepatic tubules was obvious. There was obvious cell lysis and cell necrosis, and the cell morphology was damaged. However, the damage gradually weakened with increasing TA concentration (Figure 2).

After the shrimp were fed TA-supplemented diets for 56 days, TA could make the intestinal epithelial cells tightly connected, and the microvilli on the free surface of the cells were arranged neatly and densely. TA could promote the benign growth of intestinal tissue. After ammonia stress for 48 h, varying degrees of damage were observed inside the hepatopancreatic tissue of the TA-0, TA-200, and TA-400 groups. The intestinal epithelial cells of the TA-0 group fell off, and the intestinal wall was damaged. However, the intestinal epithelial cells were relatively intact in the TA-800 group. TA could effectively prevent the intestinal tract from being damaged by ammonia stress (Figure 3).

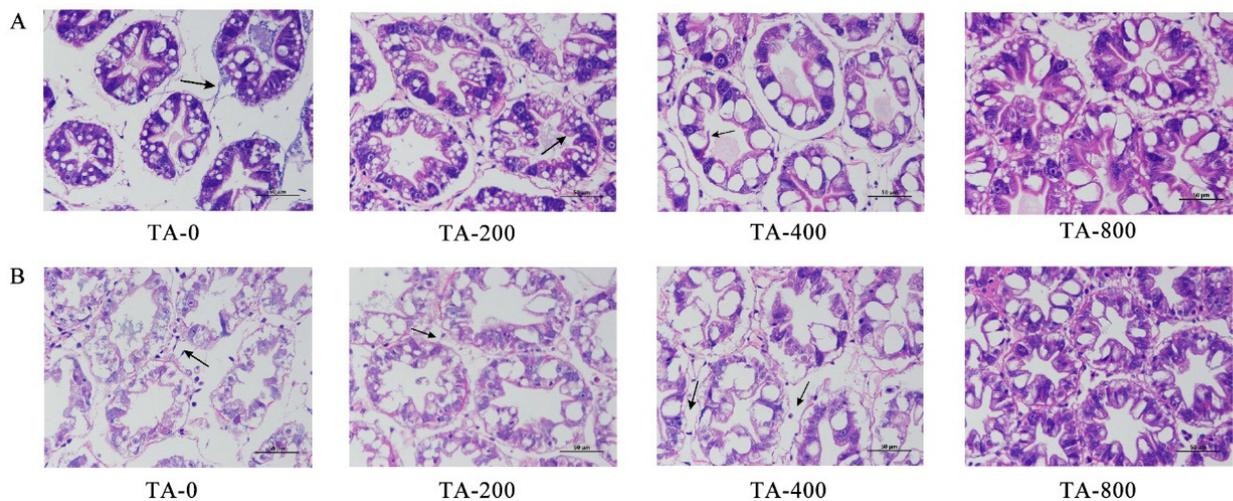


Figure 2. Hepatopancreas histology of *L. vannamei* in the feeding trial after 56 days and ammonia stress after 48 h. (A) The feeding trial lasted 56 days; (B) ammonia stress lasted for 48 h. Whole segments were marked with H&E. Scale bar: 50 μm. The arrows in the figure indicate the differences between the treatment groups.

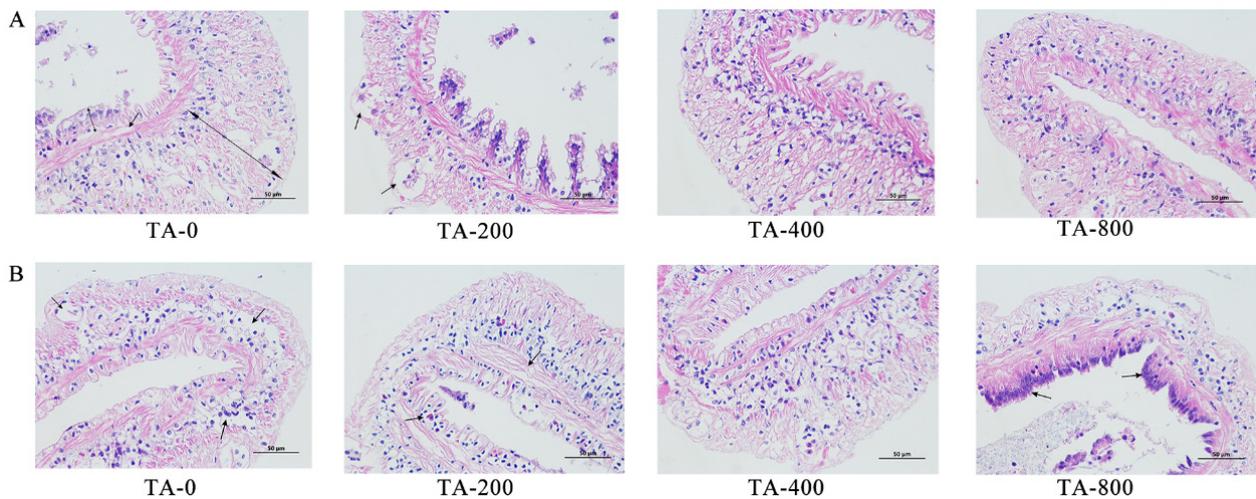


Figure 3. Intestine histology of *L. vannamei* at the feeding trial after 56 days and ammonia stress after 48 h. (A) The feeding trial lasted 56 days; (B) ammonia stress lasted for 48 h. Whole segments were marked with H&E. Scale bar: 50 μm. The arrows in the figure indicate the differences between the treatment groups.

3.3. Intestinal Digestive Enzyme Activity

After shrimp were fed TA-supplemented diets for 56 days, compared with the TA-0 group, the AMS and TryP activities were highest in the TA-800 group ($p < 0.05$). The TryP activity increased with the addition of TA to the diet. The LPS activity was lower in the TA-400 and TA-800 groups ($p < 0.05$), and higher in the TA-200 group (Figure 4).

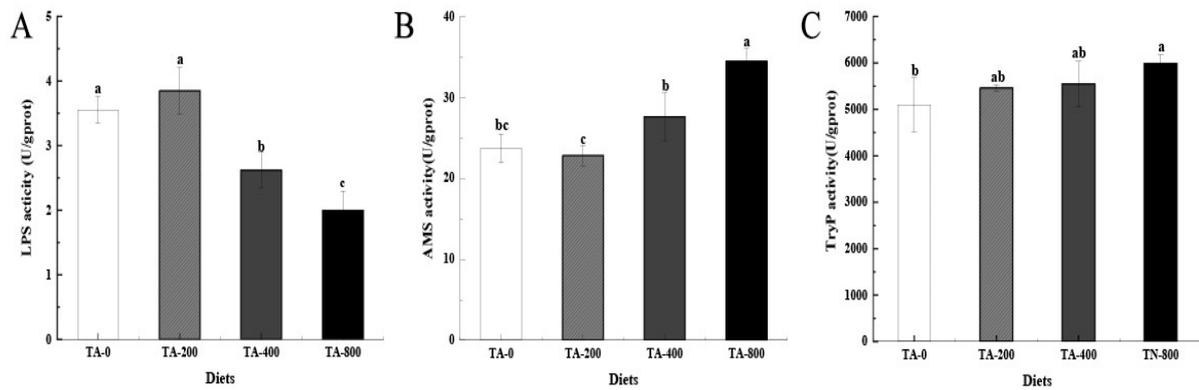


Figure 4. Effects of dietary TA on digestive enzyme activities in the intestines of *L. vannamei*. (A) LPS activity; (B) AMS activity; (C) Tryp activity. Vertical bars represented the mean values \pm SD ($n = 3$). Data marked with different letters were significantly different ($p < 0.05$) among groups.

3.4. Hepatopancreas Immune Enzyme Activity

After shrimp were fed TA-supplemented diets for 56 days, compared with the TA-0 group, the activities of T-AOC and PPO were higher in the three TA treatment groups ($p < 0.05$). LZM activity was higher in the TA-800 group ($p < 0.05$) but lower in the TA-200 and TA-400 groups ($p < 0.05$). SOD activity was higher in the three TA treatment groups, while there was no significant difference with the TA-0 group (Figure 5).

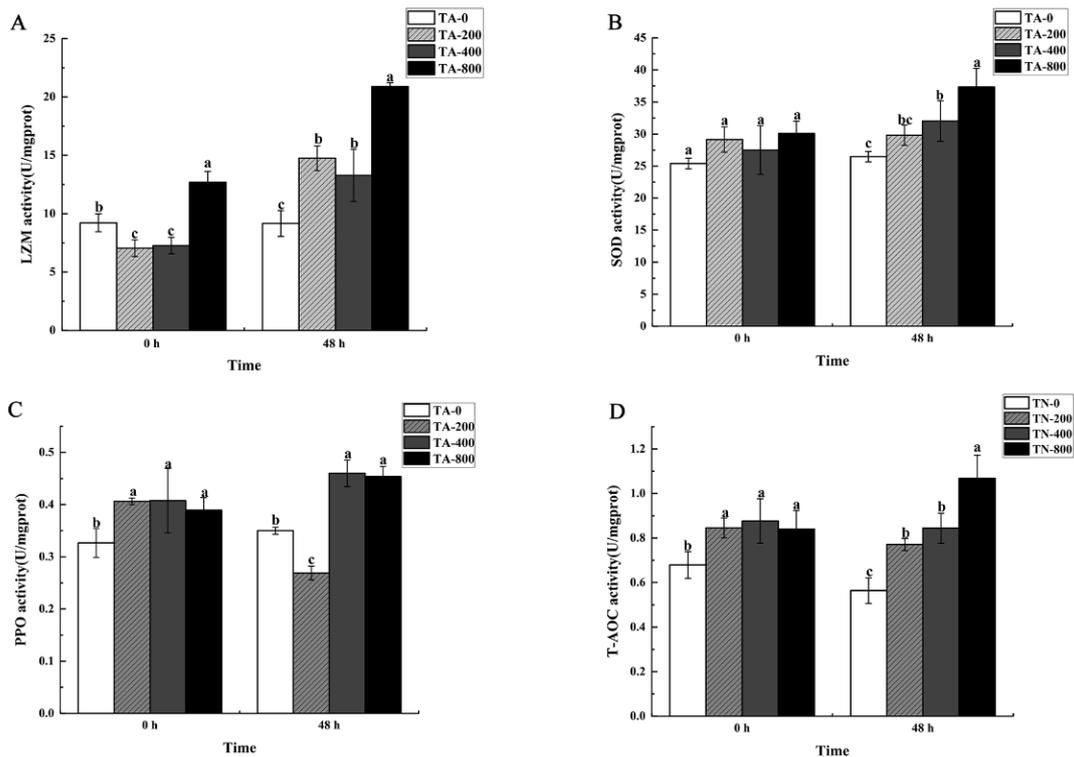


Figure 5. Immune enzymes activity in the hepatopancreas of *L. vannamei* that were fed the control and TA diets for 56 days (0 h) before being exposed to ammonia stress for 48 h. (A) LZM activity; (B) SOD activity; (C) PPO activity; (D) T-AOC activity. Vertical bars represented the mean values \pm SD ($n = 3$). Data marked with different letters were significantly different ($p < 0.05$) among groups.

When the shrimp were exposed to ammonia stress for 48 h, PPO activity was higher in the TA-400 and TA-800 groups compared with the TA-0 group ($p < 0.05$), but lower in the

TA-200 group ($p < 0.05$). LZM and T-AOC activities were higher in the three TA treatment groups ($p < 0.05$). SOD activity was significantly higher in the TA-400 and TA-800 groups ($p < 0.05$) (Figure 5).

3.5. Hepatopancreas Immune Gene Expression Levels

After shrimp were fed TA-supplemented diets for 56 days, compared with the TA-0 group, the relative expression of the LZM and *Cu/Zn-SOD* genes all increased in the TA groups after shrimp were fed TA-supplemented diets for 56 days ($p < 0.05$, Figure 6). The relative expression of *proPO* was higher in the TA-400 and TA-800 groups (Figure 6).

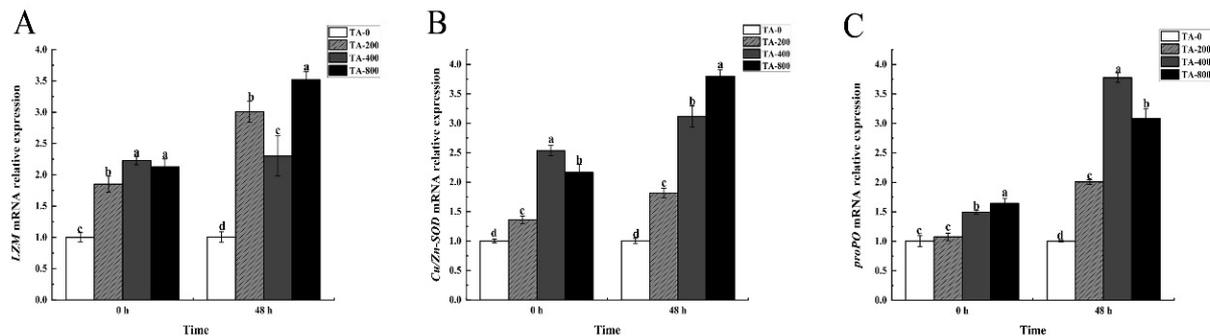


Figure 6. Immune gene expression levels in the hepatopancreas of *L. vannamei* that were fed the control and TA diets for 56 days (0 h) before being exposed to ammonia stress for 48 h. (A) LZM mRNA expression; (B) *Cu/Zn-SOD* mRNA expression; (C) *proPO* mRNA expression. Vertical bars represent the mean values \pm SD ($n = 3$). Data marked with different letters were significantly different ($p < 0.05$) among groups.

When the shrimp were exposed to ammonia stress for 48 h, the variation in the relative expression of the LZM, *Cu/Zn-SOD*, and *proPO* genes was obvious compared with that in the TA-0 group, and they were all higher than that in the TA-0 group ($p < 0.05$). The relative expression of the *Cu/Zn-SOD* gene increased with the addition of TA ($p < 0.05$) (Figure 6).

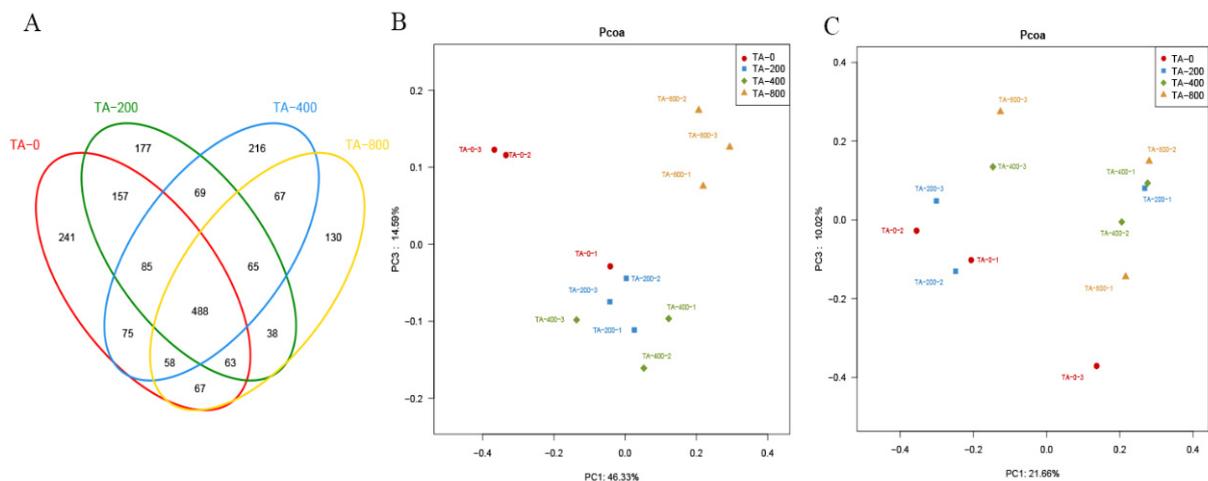
3.6. Intestinal Microbial Richness and Diversity

A total of 249,037 high-quality clean reads were obtained from all intestinal microbial samples, and the average number of clean reads per sample was 62,259. Rarefaction curves of the observed species per sample were sufficient (Figure S1). The alpha richness and diversity of the intestinal microbiota were assessed using the Chao1, Shannon, and Simpson indices (Table 3). Compared with the TA-0 group, the Simpson index was lower in the TA-400 group ($p < 0.05$), while there was no significant change in the TA-200 and TA-800 groups. The Shannon index in the TA-800 groups was higher than that in the TA-0 and TA-200 groups ($p < 0.05$). The Chao1 index was higher in the three TA treatment groups, and the highest value was in the TA-800 group ($p < 0.05$). A total of 488 OTUs coexisted in the four groups; the number of unique OTUs was lower in the three TA treatment groups, and the lowest number was in the TA-800 group (Figure 7A). According to PCoA, based on weighted and unweighted UniFrac distances, the intestinal microbial samples of the TA-400 and TA-800 groups were clearly separated from the TA-0 group, while those of the TA-200 group could not be distinguished from the TA-0 group (Figure 7B,C).

Table 3. Alpha diversity of intestinal microbial of *L. vannamei* that were fed the control and TA diets for 56 days.

Index	TA-0	TA-200	TA-400	TA-800
Reads				
Mean sequences	63,881	65,123	58,035	61,998
OTUS	745 ± 18 ^a	635 ± 12 ^b	668 ± 18 ^b	768 ± 38 ^a
Community diversity				
Simpson index	0.06 ± 0.005 ^a	0.05 ± 0.007 ^{ab}	0.03 ± 0.006 ^b	0.06 ± 0.023 ^a
Shannon index	3.47 ± 0.36 ^b	3.48 ± 0.38 ^b	4.12 ± 0.16 ^a	4.24 ± 0.32 ^a
Community richness				
Chao1	712.67 ± 24.01 ^b	714.00 ± 17.35 ^b	748.33 ± 58.15 ^{ab}	816.00 ± 24.25 ^a

Values are mean values ± SD ($n = 3$). Different letters indicate significant differences ($p < 0.05$).

**Figure 7.** Intestinal microbial diversity and composition of *L. vannamei* that were fed the control and TA diets for 56 days. (A) The number of unique and shared OTUs between different treatments indicated by the Venn diagram. (B) PCoA plots based on weighted UniFrac metrics. (C) PCoA plots based on unweighted UniFrac metrics.

3.7. Intestinal Bacterial Composition

The dominant flora species of the four treatment groups were basically the same, but the abundance changed. At the phylum level, the dominant bacteria were Proteobacteria, Bacteroidetes, Planctomycetes, and Actinobacteria. Compared with the TA-0 group, the relative abundances of Proteobacteria and Bacteroidetes were lower in the three TA treatment groups, while the relative abundance of Planctomycetes was higher. The relative abundance of Actinobacteria was lower in the TA-200 group but higher in the TA-400 and TA-800 groups (Figure 8A).

At the class level, the relative abundance of Cytophagia, Gammaproteobacteria, Flavobacteria, and Betaproteobacteria decreased, but the abundance of Alphaproteobacteria, Planctomycetacia, and Mollicutes increased with the addition of TA to the diet (Figure 8B). Differences in microbiota abundance were also observed at the genus level. Certain genera, such as *Rhodopirellula*, *Ruegeria*, and *Rhodobacter*, were higher in the three TA experimental groups, but *Paracoccus*, *Algoriphagus*, *Cellvibrio*, *Flavobacteriaceae*, and *Bacteroides* were lower. In addition, *Vibrio* was lower in the TA-400 and TA-800 groups (Figure 8C).

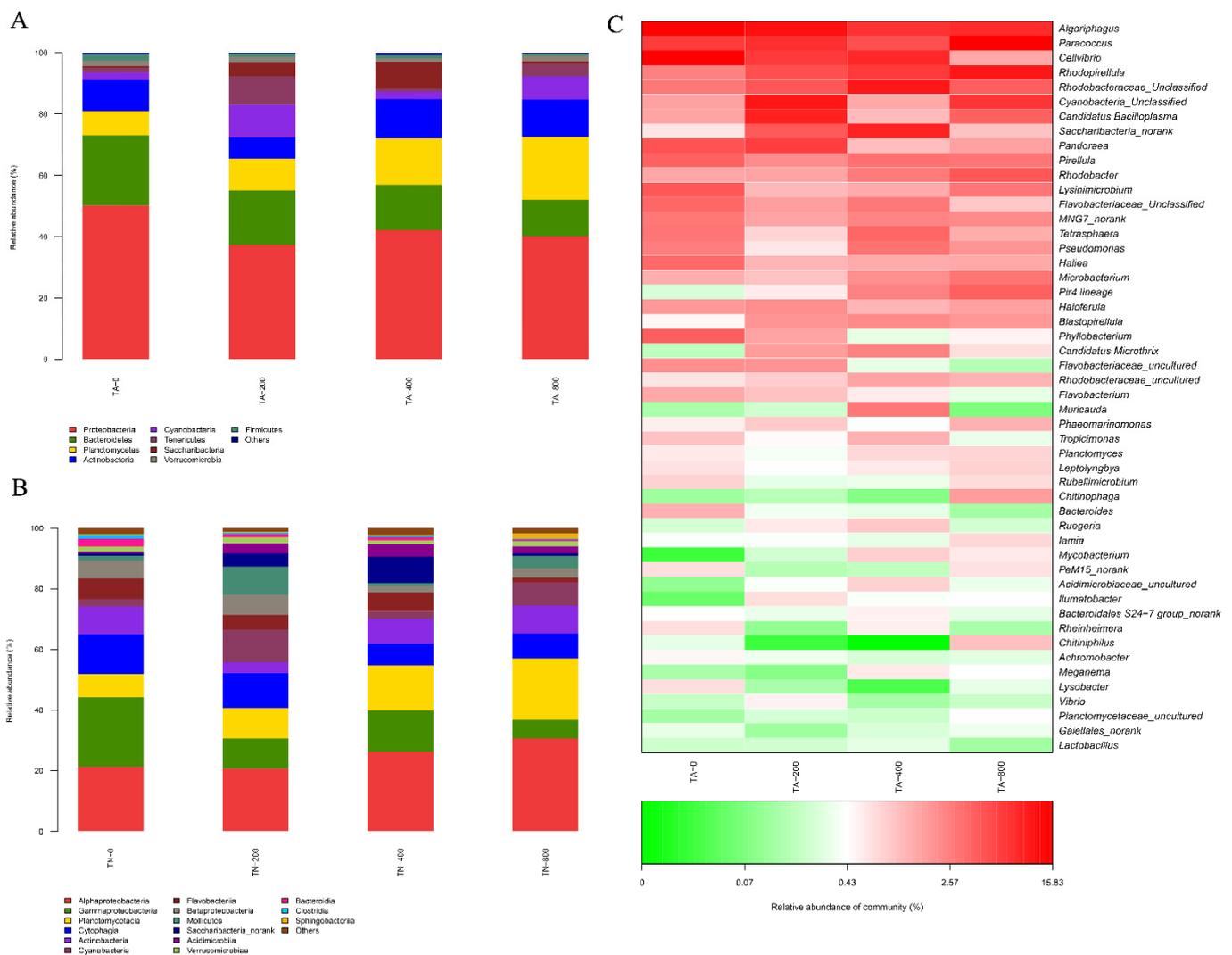


Figure 8. Average relative abundances of dominant bacterial in the intestines of shrimp under different treatments. (A) Phyla level, (B) classes level, and (C) genera level.

3.8. Differential Analysis of Intestinal Bacterial Community

The specific bacterial taxa were identified from the four groups based on LefSe analysis. According to the LefSe cladogram, nine families were enriched in the TA-0 group, including *Cryptosporangiaceae*, *Demequinaceae*, *Rikenellaceae*, *Flavobacteriaceae*, *Comamonadaceae*, *Aeromonadaceae*, *Cellovibrionaceae*, *Chromatiaceae*, and *Xanthomonadaceae*; three families were enriched in the TA-200 group, including *Burkholderiaceae*, *Legionellaceae*, and *T9d*; seven families were enriched in the TA-400 group, including *Intrasporangiaceae*, *Desulfomicrobiaceae*, *Mycobacteriaceae*, *Gemmatimonadaceae*, *Marinilabiaceae*, *Elev_16S_1332*, and *Caldilineaceae*; and four families were enriched in the TA-800 group, including *Rhodobacteraceae*, *Microbacteriaceae*, *Neisseriaceae*, and *TM146* (Figure 9A). Among the bacteria with an LDA score >4.0 were, *Gammaproteobacteria*, *Cellovibrionales* (from order to genus), *Flavobacteriia* (from class to family), *Demequinaceae*, and *Lysinimicrobium* were in the TN-0 group; *Cyanobacteria* (from phylum to family), *Burkholderiaceae* (from family to class), *Pandora*, *Collinsella*, and *Oceanirhabdus* were in the TN-200 group; *Saccharibacteria*, *Actinobacteria*, *Muricauda*, *Tetrasphaera*, and *Intrasporangiaceae* were in the TN-400 group; and *Rhodobacteraceae* (from order to family), *Alphaproteobacteria*, *Pir4_lineage*, and *Rhodobacter* were in the TN-800 group (Figure 9B).

3.9. Network and Functional Analyses of Intestinal Bacterial Community

Network analyses further confirmed the correlation and differences of the microbiota. Based on the correlation network analysis of the bacterial phylum, the top abundance OTU2 and OTU3 belonged to Proteobacteria, and the OTUs of Bacteroidetes had the highest correlation with those of Actinobacteria (Figure 10A). In the network branching diagrams of the microbes among different groups, the genera *Cellvibrio*, *Lysinimicrobium*, *Rubellimicrobium*, and *Flavobacterium* were dominant in the TA-0 group; the class Cyanobacteria and genus *Pandoraea* were dominant in the TA-200 group; the genera *Muricauda*, *Candidatus Microthrix*, *Tetrasphaera*, *Tropicimonas*, and *Ruegeria* were dominant in the TA-400 group; and the genera *Phaeoamarinomonas*, *Rhodobacter*, *Pir4 lineage*, and *Microbacterium* were significant in the TA-800 group (Figure 10B).

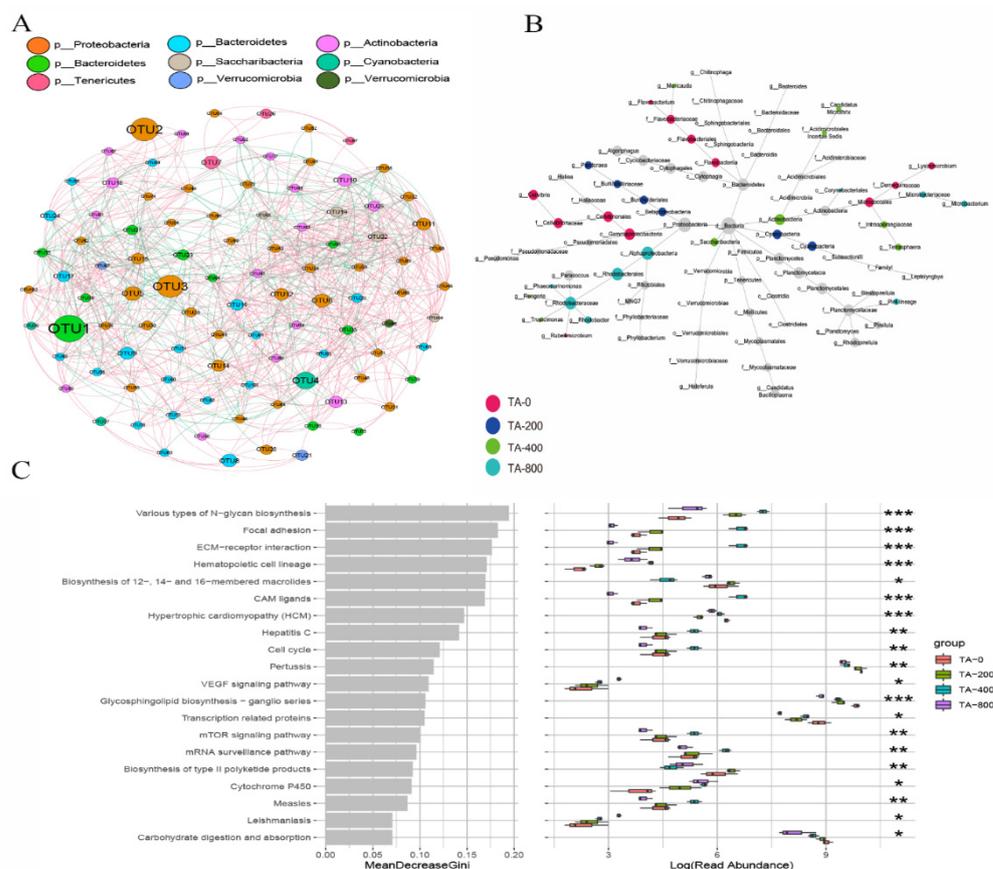


Figure 10. Network and metabolism analyses of the intestinal microbes of *L. vannamei* in different treatments. (A) Relevance network analyses based on the bacterial phylum level. The nodes represent the phylum classification of the OTUs, and the node size indicates the relative abundance of each taxon. The nodes representing the same phylum are shown in the same colour. Lines between two OTUs represent the correlation. (B) Differential network analyses: the nodes represent the species classification, and the node size indicates the relative abundance. The node colour provides information about the difference; black indicates no significant difference, and the remaining colours indicate significant differences in the corresponding group. The names of taxa with an abundance above 1% are provided. (C) Microbial metabolism prediction based on KEGG pathway analysis. * Indicates a significant difference ($p < 0.05$) among groups. ** Indicates a highly significant difference ($p < 0.01$) among groups. *** Indicates a extremely significant difference ($p < 0.001$) among groups.

The predicted function of the intestinal microbiota was analyzed. Based on KEGG classification, compared with the TA-0 group, “VEGF signaling pathway”, “various types of N-glycan biosynthesis”, “cytochrome P450”, and “hematopoietic cell lineage” were

higher in the three TA treatment groups, but “carbohydrate digestion and absorption” and “glycosphingolipid biosynthesis-ganglio series” were lower (Figure 10C).

4. Discussion

4.1. Effects of Dietary TA on the Growth Performance and Histological Structure of the Shrimp

TA is widely present in most plant species used for nutrition addition, and has been applied to aquaculture activity with positive effects on aquatic animal nutrition absorption and health [37–40]. For example, adding hydrolysable tannins to feed can improve the feed conversion ratio of shrimp [14]. Adding 0.05–0.15% hydrolyzed tannins to feed can improve the growth performance of pearl gentian grouper [41]. Adding TA to the diet can increase the intestinal surface area, promote the absorption of nutrients, and make better use of them [42]. In the present study, B and R cells were observed in the hepatopancreas of all experimental groups. They can help with nutrient absorption and the synthesis of digestive enzymes, and the increase in R cells in the dietary TA supplement group may be due to tannins, which give them easier access to nutrients and better liver protein status, thus improving the growth of shrimp [43–45]. In this study, supplementation with 400–800 mg/kg TA in diets promoted growth, reduced the feed conversion ratio, and improved the survival rate of *L. vannamei* after ammonia stress.

4.2. Effects of Dietary TA on the Digestive Enzyme Activity of the Shrimp

The activity of digestive enzymes is directly related to the digestion and absorption of nutritional material and to the growth of the animals [46]. It has been reported that a low dosage of TA has positive effects on the digestion and absorption function of organisms [47]. The addition of 1.0% chestnut tannin to the diet of meat rabbits could significantly increase the activities of amylase and trypsin [48]. Dietary supplementation with 0.15% hydrolyzable tannic acid could promote the growth performance and digestive activity of shrimp [14]. Previous studies have shown that dietary tannins stimulate the organism’s neural pathways as well as epinephrine, which promotes the expression of amylase [49]. In this study, dietary TA increased the activity of AMS in the intestine of *L. vannamei*, indicating that TA could enhance nutrient digestibility and contribute to improving growth performance. Tannins inhibit the absorption of amino acids in vitro mainly by combining with Tryp and dietary protein [50]. However, in the present study, the activity of Tryp was higher in the TA groups than in the other treatments. Previous research has shown that tannins affect protein digestion primarily by forming less digestible complexes with dietary protein, rather than inhibiting digestive enzymes [51]. LPS activity was lower in the TA-400 and TA-800 groups. In a study, hydrolysable tannin decreased the activity of lipase, and the central reason was the high binding strength between tannin and lipase [52]. Therefore, this suggests that TA can reduce lipid deposition by inhibiting the uptake of dietary lipids by the organism. The differences in the activities of digestive enzymes indicate the complexity of the digestion and degradation of different nutrients by tannins, which may be related to the structure of TA, types of digestive enzymes, experimental subjects, and complex and changeable environments in organisms.

4.3. Effects of Dietary TA on the Immune Enzyme Activity and Gene Expression of the Shrimp

Environmental stress can accelerate oxygen uptake and produce excessive reactive oxygen species in shrimp, and lead to oxidative stress in crustaceans. Suboptimal environmental conditions can induce the production of excessive ROS, which are harmful to organisms [53]. Organisms rely on antioxidant enzyme systems, including antioxidant enzymes and proteins, to resist oxidative stress and maintain physiological homeostasis. Aquatic animals adapt to their environment by using antioxidant systems to remove excess ROS. T-AOC activity reflects the total status of antioxidant enzymes, while SOD provides the first line of ROS elimination from cells [54]. It has been reported that tannins have a positive effect by stimulating antioxidant defense functions [55–58]. In this study, T-AOC and SOD activity were higher in the hepatopancreas of *L. vannamei*, as indicated by

higher transcript levels of *Cu/Zn-SOD*. The *Cu/Zn-SOD* multigene family is known to elicit oxidant defense and cytokine network through the action of phagocytes (macrophages) and humoral factors. This reveals that TA might enhance the antioxidant capabilities and non-specific immunity of shrimp to defend against ammonia stress.

Nonspecific immunity plays an important role in shrimp when the organism suffers environmental stress [59]. The main role of *LZM* is to hydrolyze the cell wall of bacteria, while *PPO* is the product of the *proPO* activation pathway, which is closely related to the activation of cellular defense and the emergence of phagocytosis factors [60]. The *LZM* and *proPO* systems have important effects on immune defense in crustaceans [61,62]. The addition of tannic acid produces immune protection or positive effects in aquaculture [63]. In this study, the activities of *PPO* were higher in the three TA treatment groups, and the activities of *LZM* were highest in the TA-800 group. Additionally, the expression levels of the *LZM* and *proPO* genes were upregulated in the three TA treatment groups. This indicates that exposure to ammonia stress and TA can better activate the immune system of shrimp and stimulate the release of *LZM* from lysosomes to resist immune stress.

4.4. Effects of Dietary TA on the Intestinal Microbiota of the Shrimp

The intestinal microbiota is closely associated with host health [64,65]. In this study, after shrimp were fed the TA diets for 56 days, TA supplementation increased the diversity and altered the composition of the intestinal microbiota in *L. vannamei*. It has been reported that Proteobacteria, Bacteroidetes, and Planctomycetes are the dominant phyla in the intestine of *L. vannamei* [66]. Proteobacteria participate in carbon complexes and nitrogen degradation [67,68]. Bacteroidetes can promote energy metabolism by increasing carbohydrate metabolism [69]. Planctomycetes contains anaerobic ammonia-oxidizing bacteria, which play important roles in reducing ammonia nitrogen in aquatic systems and contributing to the global nitrogen cycle [70]. In this study, the increased abundance of Planctomycetes and the decreased abundance of Bacteroidetes and Proteobacteria indicated that dietary TA might affect the ability of *L. vannamei* intestinal bacteria to metabolize nutrients.

In our experimental results, several major bacterial genera also showed obvious differences among the experimental groups. Among them, we focus on bacteria that are relevant to host health. Rhodobacteraceae can contribute to the global carbon and nitrogen cycle by breaking down large amounts of organic material, such as “marine snow”. According to previous study [71], it plays an important role in maintaining the health and promoting the growth of aquatic animals [72,73]. The present study shows that the highest relative abundance of Rhodobacteraceae was observed in the TA-800 group, which was consistent with the best growth performance of *L. vannamei*. These results suggest that dietary TA can promote the growth of shrimp by regulating the intestinal flora abundance. *Paracoccus*, belonging to Proteobacteria, is a denitrifying bacterium that can help to reduce nitrate to molecular nitrogen [74]. In our study, the abundance of *Paracoccus* increased in some TA groups, and TA increased the abundance of beneficial bacteria in the intestine.

Various opportunistic pathogens exist in the intestine of shrimp, such as Flavobacteriaceae [75]. In this study, the decreased abundance of Flavobacteriaceae indicated that dietary TA might reduce the pathogen abundance in the intestine of shrimp. *Vibrio* is a common pathogen in aquatic animals [76], and Scalbert et al. found that TA achieved antibacterial activity by reducing the content of iron ions [77]. In this study, the reduced levels of this bacterium indicated that TA reduced the risk of putative opportunistic pathogens in the *L. vannamei* intestine. *Algoriphagus* was used as a model strain for classification and identification. There have been few studies on *Candidatus Bacilloplasma*, which is classified as Tenericutes, in the intestine, and its role in the intestine is not clear [78], but it is often enriched in the intestines of diseased shrimp [79]. *Pandora* has the ability to degrade lignin [80]. *Cellvibrio* can utilize glucose in an oxidative manner and hydrolyze cellulose and other polysaccharide complexes [81]. In this study, the change in the abundances of *Algoriphagus*, *Cellvibrio*, *Candidatus Bacilloplasma*, and *Pandora* indicated that TA could potentially affect the abundance of harmful or beneficial bacteria in the intestine and the metabolic

capacity of shrimp. Furthermore, a large number of KEGG metabolic pathways fluctuated, such as “carbohydrate digestion and absorption”, “glycosphingolipid biosynthesis-ganglio series”, “cytochrome P450”, “hematopoietic cell lineage” and “various types of N-glycan biosynthesis”. This phenomenon revealed that dietary TA could regulate the intestinal microbiota metabolic function of *L. vannamei*. Therefore, dietary TA supplementation could cause intestinal microbiotic variations in *L. vannamei*, and the increased microbial diversity might contribute to counteracting the adverse effects of ammonia exposure.

5. Conclusions

The present study revealed that dietary TA improved growth, digestion, nonspecific immunity, resistance to ammonia stress, and the intestinal microbial community. TA did not have any negative effects or severe changes in the intestine and hepatopancreas morphological structure in *L. vannamei*. Furthermore, TA not only enhanced the relative abundances of beneficial bacteria, but also lowered the relative abundance of opportunistic pathogenic bacteria in shrimp. The results showed that 400–800 mg/kg TA showed the most application value in shrimp aquaculture. The interactions among growth performance, antioxidant capability, and the composition of intestinal microbiota in *L. vannamei* deserve further exploration.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/fishes7060327/s1>, Table S1. Primer sequences used for quantitative real-time PCR in this study. Figure S1. Rarefaction curves of intestine microbial samples with OTUs of *L. vannamei*.

Author Contributions: Conceptualization, P.C.; methodology, P.C. and J.L. (Jitao Li); formal analysis, H.G. and Z.Q.; investigation, Z.C. (Zhao Chen) and Z.C. (Zhiqiang Chang); data curation, H.G. and P.C.; writing—original draft preparation, H.G.; writing—review and editing, H.G., P.C. and J.L. (Jitao Li); Supervision, J.L. (Jian Li); funding acquisition, J.L. (Jitao Li), P.C. and J.L. (Jian Li). All authors have read and agreed to the published version of the manuscript.

Funding: This project was financially supported by the National Key R & D Program of China (2019YFD0900505), the earmarked fund for CARS-48, and the Central Public-interest Scientific Institution Basal Research Fund of CAFS (2020TD46, 2021XT06).

Institutional Review Board Statement: No human subjects were included in this study. The collection and handling of all shrimps used in this study were approved by the Experimental Animal Ethics Committee, Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences (Approval code: YSFRI-2022031, and date: 29 October 2022).

Data Availability Statement: The datasets generated and analyzed during the current study are available from the corresponding author on reasonable request.

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

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