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Effect of dietary L-tryptophan on the survival, immune response and gut microbiota of the Chinese mitten crab, *Eriocheir sinensis*

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ABSTRACT

This study investigated the influence of L-tryptophan (L-trp) on the survival, immune response and gut microbiota of the Chinese mitten crab, *Eriocheir sinensis* (with an average weight of 16.58 ± 2.20 g). After 30 days of feeding with diets supplemented with L-trp at 0.36%, 0.47%, 0.73% and 1.05% (groups 1, 2, 3 and 4, respectively), the survival rate and bacterial challenge (*Aeromonas hydrophila*) were evaluated, the activities of antioxidant and phosphatase enzymes in the serum were assessed, and the gut microbiota were measured via high-throughput Illumina sequencing. The results showed that the supplementation of L-trp significantly improved the survival rate of crabs ($P < 0.05$). After feeding for 7 days, it was observed that a high L-trp diet significantly increase the survival rate relative to a basal diet after a 96-h post-challenge with *A. hydrophila* ($P < 0.05$). The activity of CAT and AKP in the serum were increased by the addition of L-trp. The activity of CAT and AKP in the serum in group 4 were higher than those in group 1 ($P < 0.05$). Furthermore, we observed that adjunction of the L-trp can significantly increase the richness and diversity of the gut microbiota. The dominant phylum in the intestine of the Chinese mitten crab were *Tenericutes*, *Proteobacteria*, *Firmicutes*, *Chloroflexi* and *Actinobacteria*. The L-trp in the diets increased the richness of *Proteobacteria*, *Firmicutes* and *Actinobacteria* in the intestine significantly. These bacteria were all dominant bacteria and had a specific role in promoting the immunity of *E. sinensis*. Therefore, it could be inferred that L-trp supplementation is beneficial in the diet of *E. sinensis*. Based in these results, the dietary 0.47% or 0.73%L-trp supplemented is found to be optimum to improve *E. sinensis* survival.

1. Introduction

The animal gut is a complex micro-ecosystem that functions in digestion and nutrient absorption and defense against disease. The gut-associated microbiota has increasingly been determined to play a unique role in the host's gut development, immune responses, disease resistance and homeostasis [1–3]. Previous studies have shown that the manipulation of the microbial composition of the gut of farmed fish and crustaceans can have a marked effect on animal health, growth and survival [4]. Many factors, such as internal structure, host conditions, water quality, season, infection with bacteria or virus and diet composition, can affect the gut microbiota of aquatic animals [5–7]. Considerable efforts have also been made to find dietary ingredients that can modulate the dominant bacterial community in the intestinal system [8,9]. By far, many studies have proved that nutritional regulation is an important technique for the maintenance of gut immune function, suah as E. sinensis by dietary astaxanthin [10], Asian seabass *Lates calcarifer* dietary prebiotic inulin [11], and Atlantic salmon *Salmo salar* by different dietary levels of soybean meal [12].

Tryptophan (Trp) is an essential amino acid in human nutrition and plays an important role in physical functions, such as promoting protein synthesis, exerting antioxidant capacity and alleviating the stress response [13]. Trp is also used as a forage additive for the aquaculture industry. It has been reported that dietary L-trp reduces cannibalism and aggressiveness and enhances growth in Atlantic cod *Gadus morhua* [14] and mud crab *Scylla serrata* [15]. Qiu et al. have documented that

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L-trp supplemented in the flow protein diets can increase the activity of Phenoloxidase (PO), Catalase (CAT) and Superoxide dismutase (SOD) in the serum of *Litopenaeus vannamei* [16]. In addition, it has been found that the activity of Alkaline Phosphatase (AKP) in the serum of *L. vannamei* increased significantly when tryptophan was added to the diets [17]. Our previous studies have shown that dietary supplementation of L-trp could improve the food intake and survival rate of the freshwater Chinese mitten crab *E. sinensis* [18]. Increasing evidence has indicated that the microbial influence on Trp metabolism and the serotonergic system may play an important role in intestinal immunity, appetite and digestive regulation in mammals [19]. A report from Cell showed that indigenous bacteria from the human gut microbiota regulated host serotonin biosynthesis; a later report from Nature demonstrated that gut microbiota produced dozens of metabolites including tryptophan, phenylalanine and tyrosine, affecting intestinal permeability and systemic immunity. These studies revealed that Trp or 5-HT were closely related to microbiota and intestinal immunity [20,21]. Although Wen (2014) and Jiang et al. (2015) have verified that dietary L-trp modulates the intestinal immune response and barrier function in the young grass crap *Ctenopharyngodon idella* [22,23], there are no clinical or experiment studies that evaluated the effects of L-trp on the gut microbiota in crustaceans, especially *E. sinensis*.

The Chinese mitten crab is an economically important crustacean in freshwater aquaculture production in China. With the rapid increase of the *E. sinensis* aquaculture industry, numerous diseases have recently evolved, thereby leading to huge economic losses [24]. For example, hepatopancreatic necrosis disease (HPND or “shuibiezi” in Chinese), which affects *E. sinensis*, has been a major problem in the crab-cultivated area with an increased prevalence and incidence in recent years [25]. Studies showed that there were statistically significant changes in the gut microbiota of HPND-infected crabs [26]. The interaction between gut bacteria and their hosts has been recognized as a crucial factor affecting the wellness of aquatic animals [27–29]. Therefore, the study of gut-host interactions has become a hot topic aiming to improve immune function. The gut microbiota of *E. sinensis* have gained particular attention with emphasis on changing the microbiota by changing the composition of the feed. To promote healthy cultivation, we investigated the effects of different dietary different levels of L-trp on the survival, immune response and gut microbiota. The data may provide valuable insights for clarifying the role of L-trp in the gut microbiota of *E. sinensis* and provide the basis for its application in *E. sinensis* production in the future.

2. Materials and methods

2.1. Experimental crabs and ethics statement

All animals were handled in accordance with permits that were established by the Animal Experiments Ethics Committee of Shanghai Ocean University for the care and use of laboratory animals.

Intact intermolt juvenile crabs, with a body weight of (16.58 ± 2.20) g were obtained from a commercial farm in Chongming Island (Shanghai, China). The crabs were acclimated in a circulating system containing thoroughly aerated freshwater and a UV-treated PVC tube as a shelter. They were fed the basal diet for one week prior to the start of the experiment. During the experiment, the water quality parameters were maintained at a temperature of 19–22 °C, a dissolved oxygen level > 6.0 mg/L, a pH 7.6–7.8 and a total nitrogen < 0.05 mg/L.

2.2. Experimental diets and sampling

Intermolt juvenile Chinese mitten crabs were fed four purified diets containing L-trp at four levels: 0.36% (basal diet, namely, control group), 0.47%, 0.73% and 1.05% (group 1, 2, 3 and 4, respectively) for 30 days [15,30]. The crabs were divided into four groups based on the

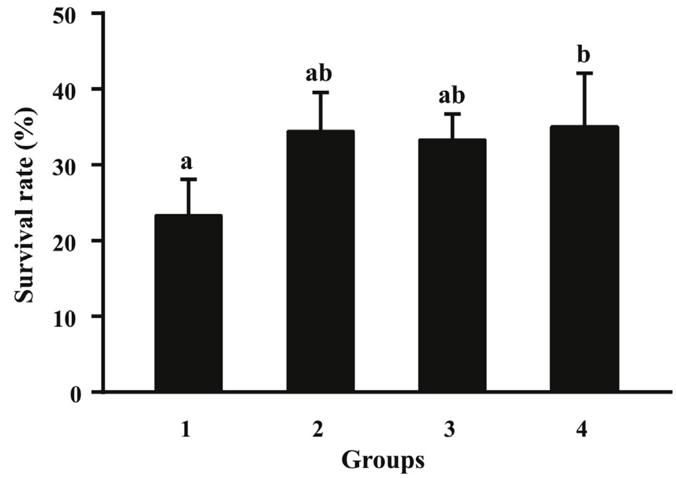

Fig. 1. Survival of crabs (n = 20) fed with different dietary levels of L-trp. Means not sharing the same superscript are significantly different ($P < 0.05$).

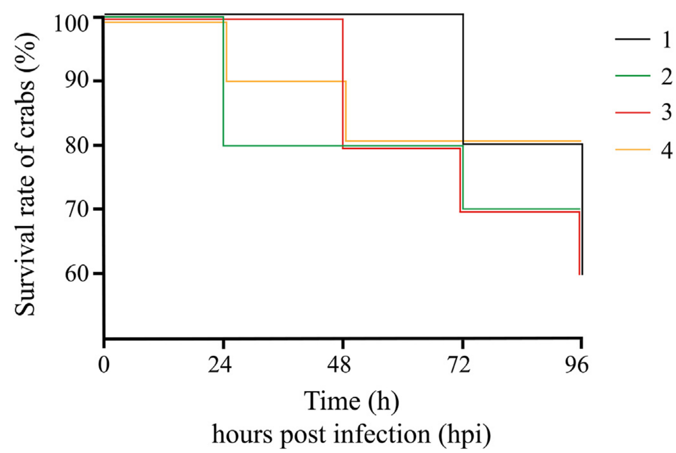

Fig. 2. Survival of crabs (n = 10) within 96 h among the different dietary L-trp feeding levels during artificial infestation with *Aeromoas hydrophila.*

L-trp concentrations with three replicates each and 30 crabs in each tank (830 L). Each tank was filled with 500 L water, and 2/3 of the water was replaced once every three days with aerated water. The crabs were fed once daily with corresponding diets at 5% of their body weight at 6 p.m. The dead crabs were removed in time. After being fed the diets for 7 days, ten crabs randomly selected from each group were equally transferred to another set of identical tanks and later used for the bacterial challenge test. After feeding with the experimental diets for 30 days, the total number and survival rate were counted and calculated using the following equations: Survival (%) = 100 × (final no. of crab/initial no. of crab). At the end of the trail, crabs were hungry for 24 h before sampling. Then, ten crabs from each tank were taken, and hemolymph was drawn with a sterile 1-mL syringe from the arthrodial membrane of the third pereiopod. The hemolymph was kept at 4 °C and centrifuged at 5000 r/min for 15 min to collect supernatant for the analysis of immune (AKP and acid phosphatase) and antioxidant enzyme (total antioxidant capacity, SOD and CAT) activities [31]. The contents of the hindgut from each crab were squeezed out into to a sterile centrifuge tube, pooled, and stored at −80 °C for gut microbial analysis. All efforts were made to reduce animal suffering.

2.3. Bacterial challenge and serum immune parameters analysis

The bacterial strain of *Aeromonas hydrophila* was obtained from the pathogenic library of Shanghai Ocean University. The 96-h bacterial

Table 1
The serum immune response of crabs (n = 10) fed with different levels of dietary L-trp.

Parameter	groups			
	1	2	3	4
T-AOC (U/mL)	7.12 ± 0.46	7.46 ± 1.15	7.31 ± 2.48	10.46 ± 1.47
T-SOD (U/mL)	475.17 ± 16.28	454.94 ± 15.65	459.14 ± 17.02	444.29 ± 14.16
CAT (U/mL)	2.28 ± 0.69[a]	6.03 ± 1.30[b]	5.12 ± 0.24[ab]	16.95 ± 1.33[c]
ACP (U/100 mL)	1.46 ± 0.28	1.72 ± 0.35	2.01 ± 0.23	1.70 ± 0.28
AKP (U/100 mL)	1.56 ± 0.22[a]	2.39 ± 0.25[ab]	2.61 ± 0.16[b]	3.42 ± 0.58[c]

Notes: The different lowercase letters in the same row denote significant difference ($P < 0.05$).

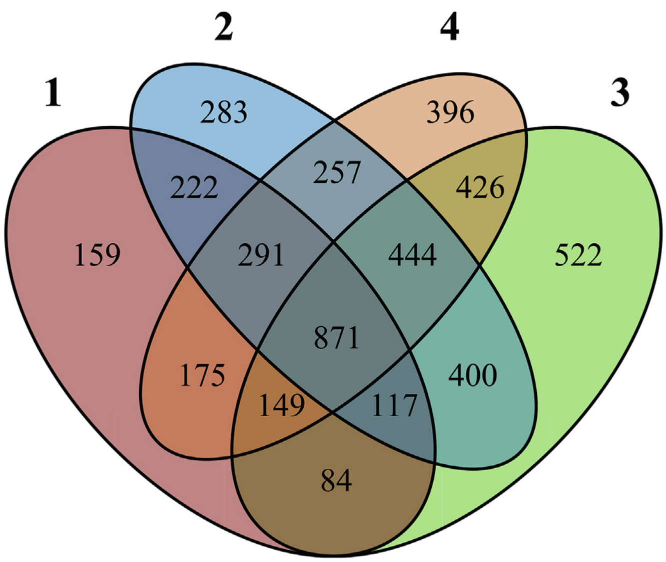

Fig. 3. Venn diagrams demonstrating the distribution of OTUs shared by crabs (n = 5) fed with different dietary levels of L-trp. The numbers indicate the correlated OTUs in the total sequences of each group.

median lethal dose (LD50) was determined on crabs fed with basal diet. Fifty crabs were randomly sampled for each dose test by pereiopod injection using five graded dosed of *A. hydrophila* (10^4, 10^5, 10^6, 10^7 and 10^8 CFU per crab). The results showed that the 96-h LD50 was 7.0×10^5 CFU per crab. At the end of the feeding trial, crabs were injected on the pereiopod with 1.0×10^6 live *A. hydrophila*. Mortalities were recorded daily for 96 h, and dead crabs were immediately removed.

The total antioxidant capacity (T-AOC) was determined by colorimetry [32]. SOD activity was measured according to the method of Beauchamp and Fridovich [33]. The superoxide anion radical (O_2^-) was produced by the reaction of xanthine and xanthine oxidase, which was later oxidized to nitrite. The color was developed under the reaction of a color reagent, and then the absorbance was measured. CAT activity was determined by measuring the decrease in H_2O_2 concentrations [34]. After 20 μL sample was added to the reagent, the mixture was incubated for 60 s at 37 °C. Acid phosphatase (ACP) and AKP levels were measured following the methods of Sanders [35]. Under the acidic and alkaline conditions, ACP and AKP could decompose disodium phosphate to produce free phenol and phosphoric acid. Phenol reacted with 4-aminoantipyrine (AAP) in an alkaline solution, and then was oxidized by potassium ferricyanide to form a red pyrene derivative. The above indicators were all detected following the instructions of the kits in the Nanjing Jiancheng Bioengineering Institute.

2.4. Gut DNA extraction, PCR amplification of the 16S rRNA gene, amplicon sequence and sequence data processing

The total DNA was extracted using Fast DNA SPIN extraction kits (MP Biomedicals, Santa Ana, CA, USA) according to the manufacturer's instructions. Harvested DNA samples were measured on a NanoDrop spectrophotometer (Thermo Scientific, NC2000, Waltham, MA, USA) and agarose gel electrophoresis to estimate DNA quantity and quality, respectively. The 16S rRNA gene comprising V3-V4 regions was amplified by PCR using barcoded fusion primers (forward primers: 5′ ACTCCTACGGGAGGCAGCA 3′, reverse primers: 5′GGACTACHVGGGTWTCTAAT 3′). PCR products were purified with Agencourt AMPure Beads (Beckman Coulter, Indianapolis, IN) and quantified using the PicoGreen dsDNA Assay Kit (Invitrogen, Carlsbad, CA, USA). Only PCR products without primer dimers and contaminant bands were used for sequencing by synthesis. In this experiment, each purified PCR product was subjected to Illumina-based high-throughput sequencing (Shanghai Personal Biotechnology Co., Ltd., Shanghai, China).

The Quantitative Insights Into Microbial Ecology (QIIME, v1.8.0) pipeline was employed to process the sequencing data, as previously described [36]. The raw paired-end reads from the original DNA fragments were merged using FLASH [37] and assigned to respective original samples according to the unique barcodes. High-quality sequences for bioinformatics analysis were clustered into operational taxonomic units (OTUs) based on a 97% sequence similarity according to UCLUST [38]. A respective sequence was selected from each OTU using default parameters. OTUs containing less than 0.001% of total sequences across all samples were discarded. The species richness for each community was estimated based on the OTU abundance matrix. A Venn diagram was generated to represent the number of shared and unique species among the groups and percentages (%) of OTUs.

2.5. Biodiversity analysis

For alpha diversity analysis, we rarified the OTU to several metrics, including Chao1 richness estimator, ACE metric (Abundance-based Coverage Estimator) and the indexes of Shannon, Chao 1, Simpson and ACE. Beta diversity analysis were performed to investigate the structural variation of microbial communities across samples using UniFrac distance metrics [39,40] and visualized via heatmap of RDA-identified key OTUs, principal coordinate analysis (PCoA), nonmetric multidimensional scaling (NMDS), and the unweighted pair group method with arithmetic mean (UPGMA). Differences in the Unifrac distances for pairwise comparisons among groups were determined using Student's t-test and the Monte Carlo permutation test with 1000 permutations. The results were visualized through the box-and-whiskers plots. Taxa abundances at the phylum, class, order, family, genus and species levels were statistically compared among groups using the Metastats Program [41].

2.6. Microbial function prediction

Microbial function was predicted using PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States). The OTU abundance was normalized automatically using 16S rRNA gene copy numbers from known bacterial genomes in Integrated Microbial Genomes (IMG). The predicted genes and their functions

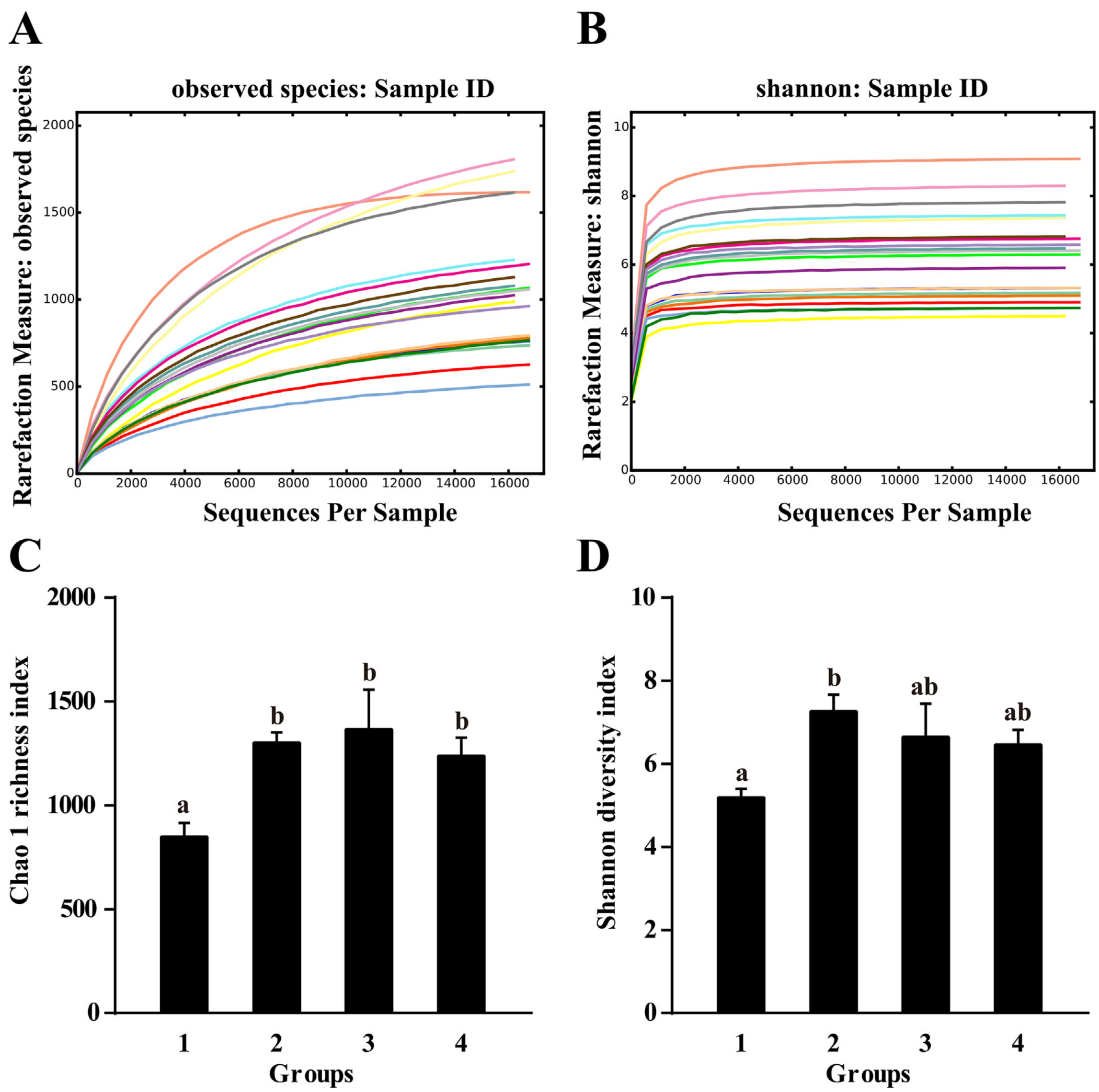

Fig. 4. Richness and diversity of the gut microbiota in crabs (n = 5) fed with different dietary L-trp.
(A) Rarefaction curves; (B) Shannon curves; (C, D) Chao 1 and Shannon richness and diversity index of each group. Means not sharing the same superscript are significantly different ($P < 0.05$).

were aligned to the KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway database.

2.7. Statistical analysis

All data were presented as the mean ± standard error (mean ± S.E.) and subjected to a one-way analysis of variance (ANOVA) using SPSS (V22.0, IBM Corporation, NY, USA) followed by Tukey's comparison tests. Data were considered to be statistically significant when $P < 0.05$.

3. Results

3.1. Effect of dietary L-trp on survival

Compared to the control group, crabs fed with L-trp showed a gradual increase in their survival rates (Fig. 1). Additionally, crabs fed the 1.03% L-trp showed a significantly higher survival rate than those in the control group ($P < 0.05$).

3.2. Effect of dietary L-trp on the bacterial challenge and serum immune parameters

The challenge test showed that crabs fed high dietary L-trp had significantly higher survival rates than those fed a basal diet (Fig. 2).

Crabs fed high L-trp diets had significantly higher CAT and AKP activities in the serum than group 1 (control group) ($P < 0.05$) (Table 1). Regardless of the degree of L-trp in the diets, the serum T-AOC, SOD and ACP activities of crabs showed no significantly difference ($P > 0.05$).

3.3. DNA sequence data and operational taxonomic unit

An Illumina HiSeq 2000 sequencing system was used to generate 715, 129 high-quality reads from the E. sinensis gut microbiota with an average of 35, 756 sequences per sample (range from 30, 380 to 41, 231). The obtained sequences were regarded as bacteria. Based on a 97% similarity level, all effective reads were clustered into 33, 106 OTUs. There were 4826 OTUs after streamlining, and these were characterized into different taxonomic levels, including the phylum,

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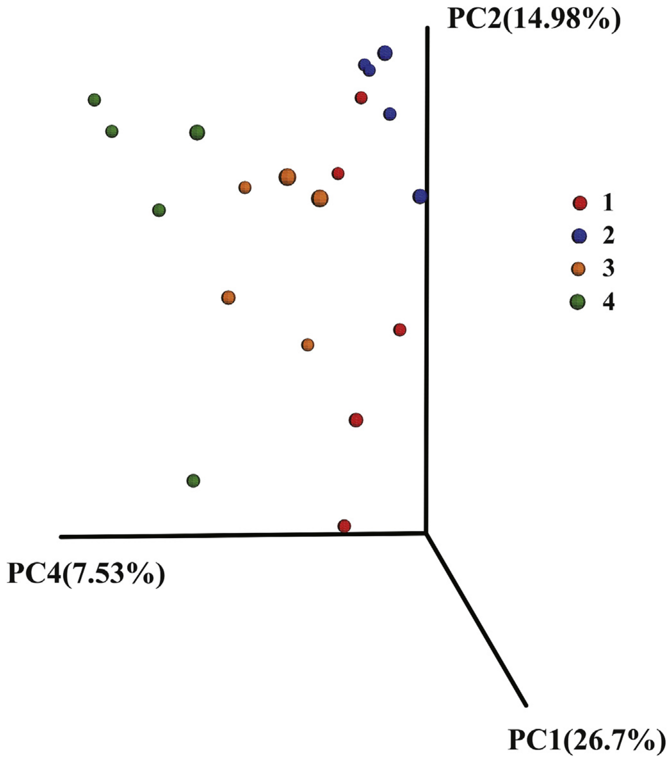

Fig. 5. Principal coordinate analysis (PCoA) of bacterial OTUs between the different dietary L-trp groups.

class, order, family, genus and species based on Greengene database through QIIME and on a 97% species similarity. As shown in Fig. 3, a total of 871 OTUs coincided in all the crab treatments. Additionally, 159, 283, 522 and 396 OTUs were uniquely identified in crabs from groups 1, 2, 3 and 4, respectively.

3.4. Alpha richness and diversity analysis

Rarefaction and Shannon analyses showed that most of the gut microbial diversity in each sample was sufficiently captured with the current sequencing depth (Fig. 4A and B). The sequencing depth among all of the groups was calculated using bootstrap, Chao 1 richness and Shannon diversity index analyses. The gut microbiome in the experimental groups demonstrated significant increases in the Chao 1 richness when compared with the control ($P < 0.05$) (Fig. 4C). Moreover, the Shannon diversity index showed an upward trend, with an increase of L-trp in the dietary levels. However, only group 2 was higher than the control significantly ($P < 0.05$), and the others exhibited no significant differences ($P > 0.05$).

3.5. Beta diversity analysis

Beta diversity analysis based on the UniFrac metrics revealed a clear distinction in the microbiota through PCoA (Fig. 5). The results showed that the distance between group 1 and group 2 was the closest, indicating that the composition of gut microbiota was the most similar, while that of group 4 was the least similar, indicating significant differences in the microbiota composition.

3.6. Taxonomic composition analysis

At the phylum level, the dominant bacteria in the gut of *E. sinensis* were *Tenericutes* (48.7%), *Proteobacteria* (22.0%), *Firmicutes* (8.1%), *Actinobacteria* (5.9%), *Bacteroidetes* (5.1%) and *Chloroflexi* (4.9%) (Fig. 6). The results showed that there was no significant effect of adding different levels of L-trp on the abundance of *Tenericutes* ($P > 0.05$). However, relative to the control crab, 1.05% L-trp significantly increased the abundance of *Proteobacteria*, *Firmicutes*, *Actinobacteria* and *Bacteroidetes* ($P < 0.05$). Moreover, crabs fed with 0.47% L-trp had more *Chloroflexi* than control crabs ($P < 0.05$).

The heatmap analysis of *E. sinensis* gut microbiota abundance at the genus level showed that *Ralstonia* and *Achromobacter* were more abundant in the control group than in the other experimental groups ($P < 0.05$). However, the abundances of *Thermomonas*, *Novosphingobium*, *Shewanella* and *Pseudomonas* in the experimental groups with L-trp were greater than in the control ($P < 0.05$). *Flavobacterium*, *Streptococcus*, *Leptonema*, *Rhodoplanes*, *Clostridium* and *Luteolibacter* were more abundant in group 2 than in the total groups. Group 3 had a comparative advantage in the abundance of *Pseudoxanthomonas*, *Candidatus-Hepatoplasma*, *Anaeromyxobacter*, *Thiobacillus* and *Allobaculum*. Moreover, the abundance of *Sporosarcina*, *Chitinilyticum*, *Bacillus*, *Legionella*, *Paracoccus, Phycicoccus*, *Cupriavidus* and *Agromyces* were more advantageous in group 4 (Fig. 7).

The top 20 most abundant OTUs as inferred by GraPhlAn are shown in the cladogram of gut microbiota, with *Actinobacteria* being the largest phylum among the top four OTUs (Fig. 8). *Dysgonomonas* was the most abundant genus.

Comparing predicted microbial function of feces, we detected a significant enrichment in the metabolism of energy, carbohydrates and amino acids (Fig. 9).

4. Discussion

This report is the first to describe the effects of L-trp on the survival, immune response and gut microbiota of *E. sinensis*. A growing body of data implicates the gut microbiome in the regulation of gastrointestinal tract function in general with a specific emphasis on its impact on Trp and serotonin (5-HT) in mammals. However, to date, there are no reports available on *E. sinensis*. Many studies have also published that Trp, a precursor of serotonin, is beneficial in improving the growth performance of many important aquaculture crustaceans, including juvenile mud crab (*S. serrata*), *L. vannamei* and freshwater crayfish (*Astacus leptodactylus*) [15,30,42]. The effective mechanism of Trp or 5-HT in this process is still unclear. In this study, we found that the supplementation of L-trp also significantly improved the survival of *E. sinensis*. All of the results suggest that the addition of L-trp in diets may improve the survival of animals. Moreover, studies of *Astacus leptodactylus*, *L. vannamei*, *S.serrata* found the survival of these animals can be improved by respective supplementation 1%, 0.36 and 0.5–1% [15,30,42]. These results are consistent with our results.

In view of the low survival rate of mud crab *S. serrata* and *E. sinensis* in the indoor aquaculture system reported by *Laranja* (2010) and us, the disease resistance of *E. sinensis* against *A. hydrophila* after dietary L-trp for 7days was evaluated [18]. Our study suggested that L-trp application significantly improved the *E. sinensis* bacterial resistance. A similar observation was reported by Azeredo et al. for the Senegalese sole, *Solea senegalensis* [43].

Based on the above results, we wanted to know whether the high survival and antibacterial ability is related to antioxidant and phosphatase enzyme levels. Therefore, we evaluated the activities of these enzymes after supplementation with different dietary levels of L-trp. Antioxidant enzymes, such as T-AOC, CAT and SOD are all essential cellular components used to eliminate excessive reactive oxygen species such as hydrogen peroxide (H_2O_2). They are also important indexes used to evaluate the stress response and immune level of the organism. SOD can catalyze the disproportionation reaction of superoxide anion radicals and eliminate excess superoxide radicals in the body [44]. The present study found that with the increase in L-trp, there were no significant changes in the activity of SOD of the serum between the four groups. Similar results have been reported by Zhang et al. on the sea cucumber, *Apostichopus japonicus Selenka* [45]. Although melatonin is

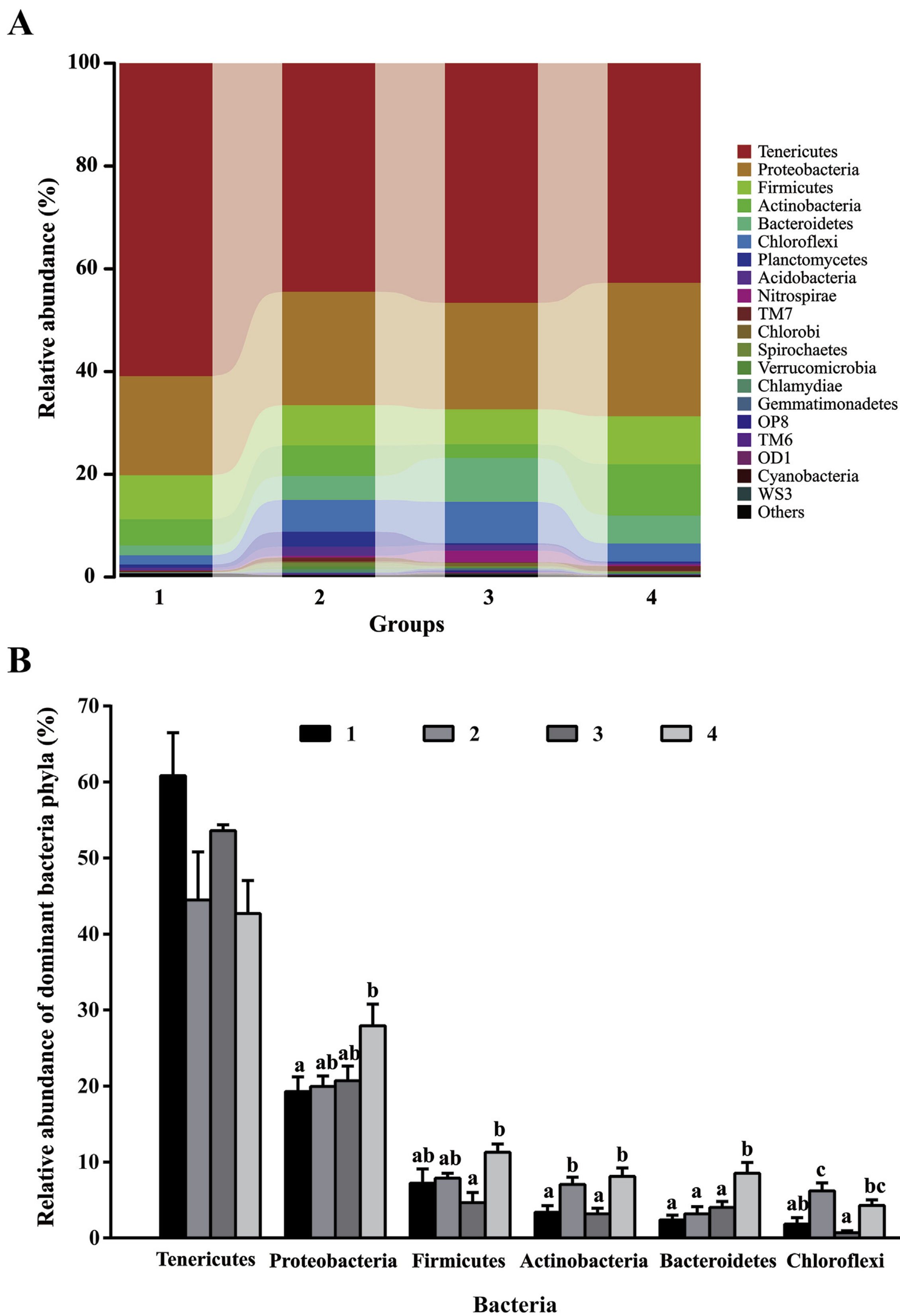

Fig. 6. Structure and composition of the gut bacterial communities of crabs (n = 5) fed with different dietary L-trp levels on the phylum levels of taxonomy. (A) community taxonomy composition and abundance map; (B) changes in abundance of dominant bacterial phyla at different TRP dietary levels. Each value represents the mean ± SEM (n = 5). Means not sharing the same superscript are significantly different ($P < 0.05$).

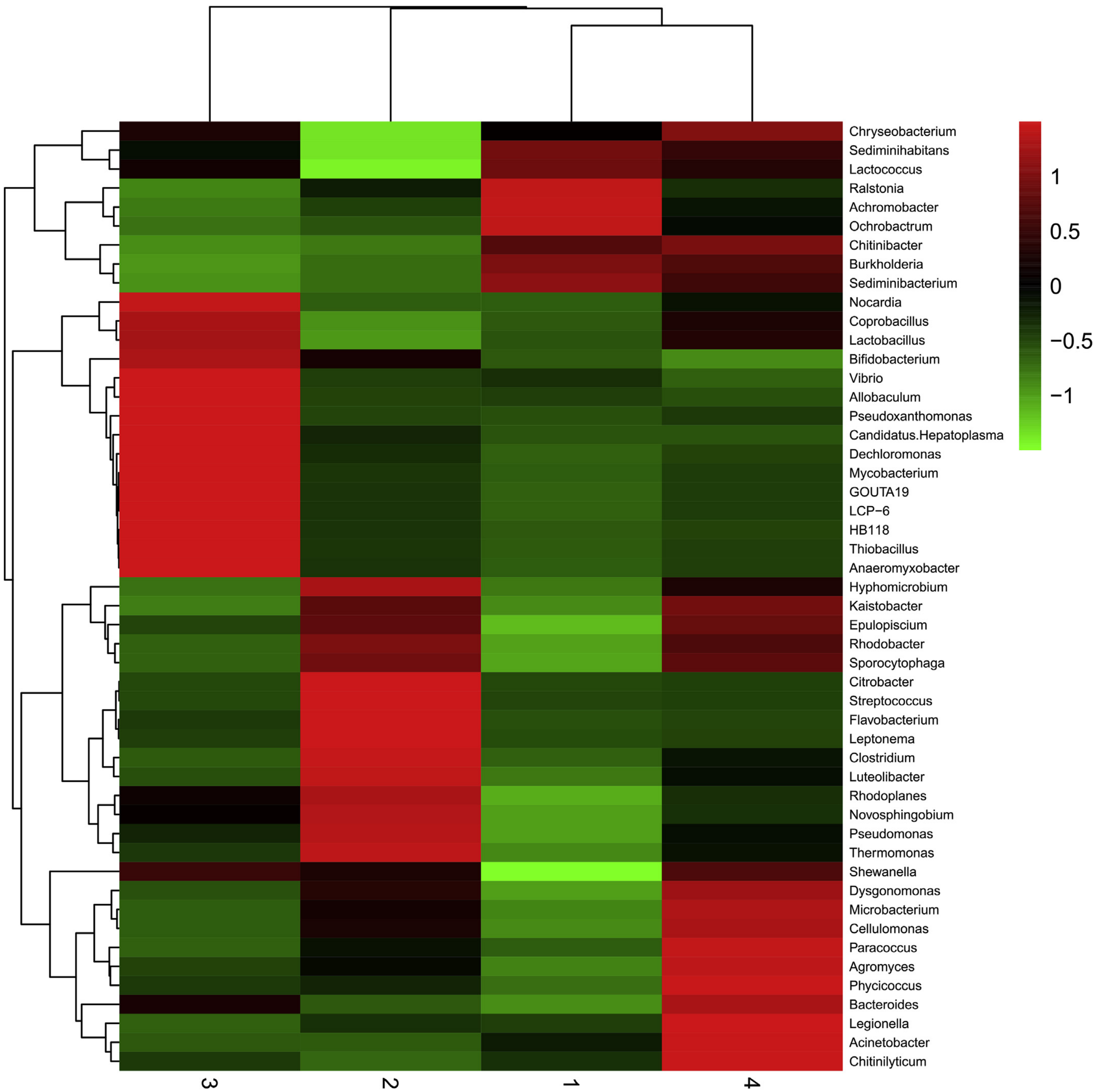

Fig. 7. Heatmap of the abundance of the *E. sinensis* gut microbiota at the genus level at different L-trp dietary levels. Red represents the more abundant genus in the corresponding sample, and green represents the less abundant genus. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

an endogenous metabolite of tryptophan, it plays an important role in the antioxidant function and immune regulation of the organism [46,47]. Interestingly, tryptophan's antioxidant effect was possibly different from that of melatonin. However, the activity of CAT in the serum increased significantly with increases in the level of Trp in the diet. This result was consistent with the observation of Ma et al. in *L. vannamei* [48]. CAT is one of the "scavenger enzymes", that is, it removes free radicals. It also promotes the decomposition of H_2O_2 into molecular oxygen and water and protects cells from H_2O_2 poisoning. CAT is one of the key enzymes in biological defense systems [49]. ACP and AKP are two important non-specific phosphohydrolases considered to be important non-specific indicators of crustaceans and are the evaluation indexes to reflect the health status of aquatic animals [50,51]. The results of the present study demonstrated that the activity of AKP in serum of crabs fed diet with L-trp increased significantly. It was consistent with the reports that adding tryptophan to feed aquatic animals such as *L. vannamei* and *C. carpiovar Jian* can increase the activity of AKP in the serum [17,52]. Therefore, the phosphatase activity could also be considered as a sensitive indicator of different diets supplementation, except for in stress [45,53].

In recent years, the concept of health has gradually started to be included in ideas about the ideal comprehensive and healthy life. Many farmers are committed to using eco-culture and immune enhancers to reduce the risk of breeding Chinese mitten crabs, and feed additives

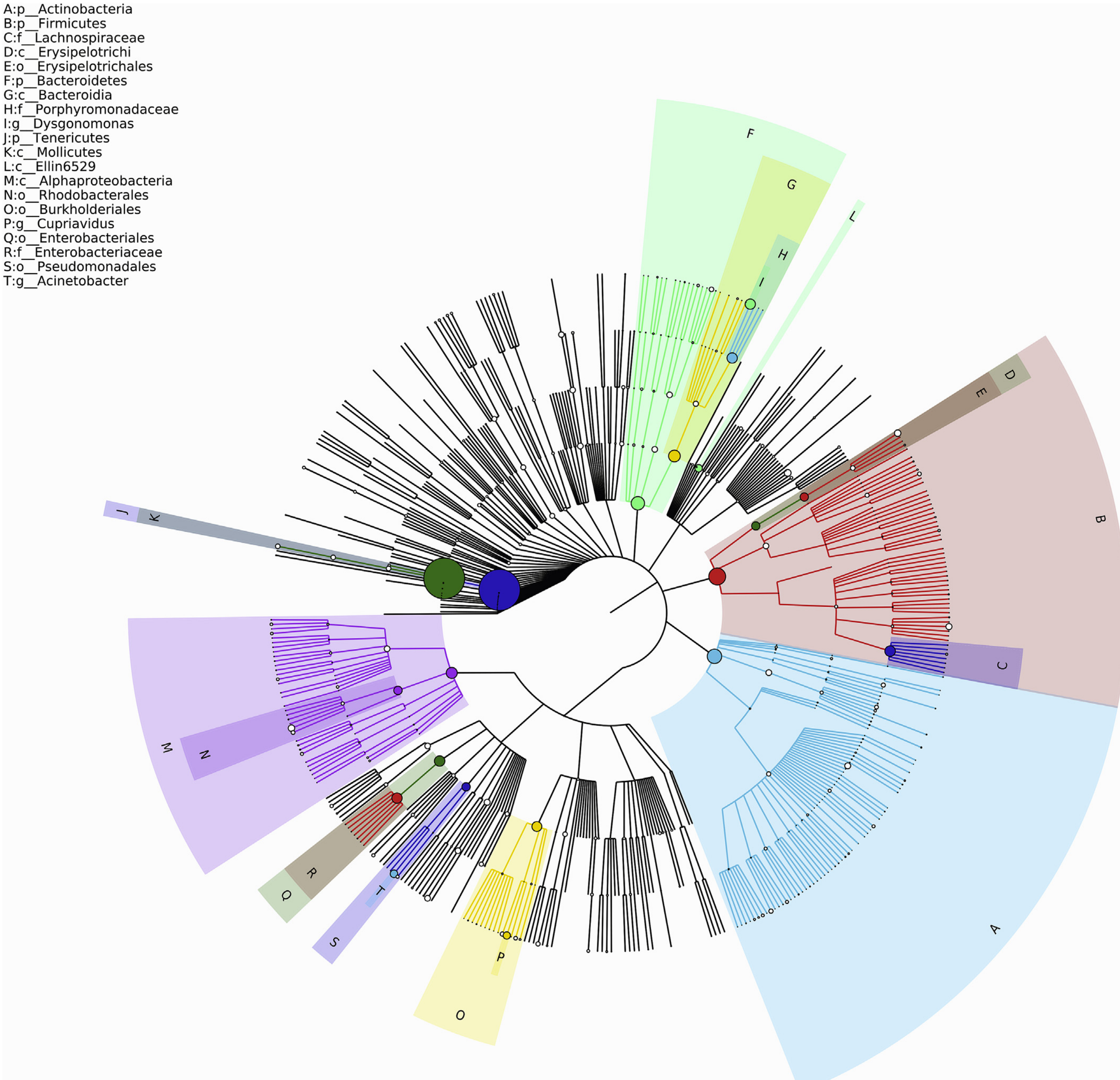

Fig. 8. Cladogram of the gut microbiota that are the top 20 most abundant as inferred by GraPhlAn. Node size is proportional to the advantage abundance; the color indicates the relative concentration of the clusters. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

have become a research hotspot. The balance of gut microbiota is one of the important factors affecting the health of aquatic animals. Many studies have shown that essential amino acids contain numerous anti-nutritional factors and antigens that can affect gut microbiota and the innate immune system in several fish species and crustaceans [9]. As a functional amino acid, tryptophan can participate in the regulation of various physiological functions, such as nutrient metabolism, digestion and absorption, immune function and anti-stress response [54–56]. In medicine, tryptophan appears to be an important amino acid in inflammatory bowel disease (IBD) patients since they have lower levels of serum and fecal tryptophan compared to healthy subjects [57]. The tryptophan supplemented diet reduced inflammation in mice or piglets have been proved [58,59]. However, no such reports have been made for *E. sinensis*. In this study, the 16S rRNA V3-V4 region was amplified in the gut microbiota of the *E. sinensis* after feeding with four L-trp diets by high-throughput sequencing technology. The dominant phyla were identified in the crabs, including *Tenericutes*, *Proteobacteria*, *Firmicutes*, *Chloroflexi* and *Actinobacteria*. In recent years, several studies have found that the *Tenericutes*, *Proteobacteria* and *Firmicutes* are the native bacteria in *E. sinensis*, which further confirms the reliability of the experimental results [44,60,61]. Crabs fed L-trp diets had higher intestinal bacterial richness and diversity. Although, Liang [62]found that a crosstalk between dietary Trp and intestine in nutrition, microbial metabolism, and mucosal immunity in Weaned piglets, in crustacean the relationship between Trp or 5-HT and microbiota or intestinal immunity were still superficial. Further studies in this aspects need be

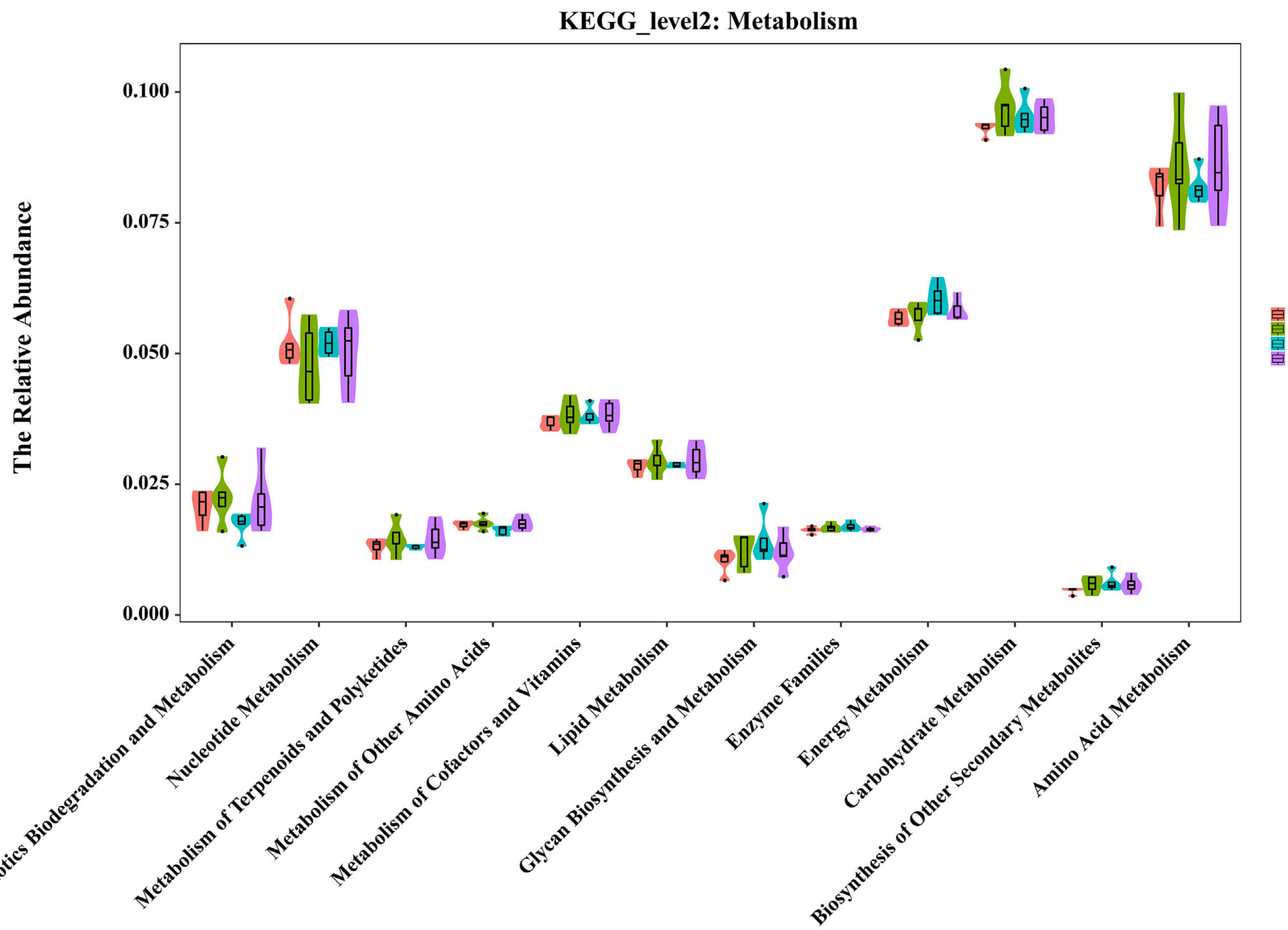

Fig. 9. KEGG for the prediction of bacterial metabolism.

done in the future.

In the present study, we found that the relative abundance of *Proteobacteria*, *Firmicutes* and *Actinobacteria* increased with increases in the dietary L-trp levels. Interestingly, similar findings were reported for fish, crabs and humans. Ingerslev et al. found that a plant diet gave rise to a prebiotic effect in rainbow trout (*Oncorhynchus mykiss*) by increasing their intestinal *Firmicutes* [63]. He also found that an organic acid and essential oils mixture beneficially affected the intestinal microflora of Pacific white shrimp (*L. vannamei*) and improved the immune response and disease resistance of *L. vannamei* by increasing the abundance of *Firmicutes* [64]. Fitzstevens suggested that *Firmicutes* in Human milk would boost infant and this group is closely related to the genera *Weissella*, *Leuconostoc*, *Staphylococcus*, *Streptococcus* and *Lactococcus* [65]. Furthermore, in this study, the genera *Candidatus Hepatoplasm* and *Bacillus* significantly increased with the addition of L-trp. A previous study has shown that *Candidatus Hepatoplasma* was beneficial to its isopod host under low-nutrient conditions [66]. *Bacillus spp.* have been used as dietary supplements to promote tilapia health performance and protect these fish from various infections [67,68]. These changes may be important reasons for the enhancement of immunity in *E. sinensis.*

Moreover, we found that the four groups of gut microbiotas were more active in three metabolic pathways (energy metabolism, carbohydrate metabolism and amino acid metabolism) based on PICRUST prediction of metabolic function of KEGG microbiota. Further studies are needed to confirm that the dietary addition of L-trp is beneficial to the physiological metabolism of *E. sinensis* and improving the health of gut microbiota and immune responses.

5. Conclusion

To date, little is known regarding the intestinal bacterial community of the crustacean in the commercial aquaculture settings. In this study, the addition of an appropriate amount of L-trp can enhance the survival and the disease resistance of *E. sinensis* against *A. hydrophila*. In addition, L-trp increased the antioxidant and immune capacity of *E. sinensis*, but its mechanism needs further study. Because *E. sinensis* belongs to the benthos group and lives in a complex water environment, microorganisms in the aquatic environment may also change the structure and type of the gut microbiota that interacts with food. Therefore, whether changes in the gut microbiota of the Chinese mitten crab are only mediated via L-trp remains to be further discussed, and its mechanism of action warrants further investigation.

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