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Effects of dietary *Gelsemium elegans* alkaloids on intestinal morphology, antioxidant status, immune responses and microbiota of *Megalobrama amblycephala*

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ABSTRACT

Numerous plant extracts used as feed additives in aquaculture have been shown to stimulate appetite, promote growth and enhance immunostimulatory and disease resistance in cultured fish. However, there are few studies on the famous Chinese herbal medicine *Gelsemium elegans*, which attracts our attention. In this study, we used the *Megalobrama amblycephala* to investigate the effects of *G. elegans* alkaloids on fish intestinal health after diet supplementation with 0, 5, 10, 20 and 40 mg/kg G. *elegans* alkaloids for 12 weeks. We found that dietary *G. elegans* alkaloids at 40 mg/kg improved intestinal morphology by increasing villus length, muscle thickness and villus number in the foregut and midgut and muscle thickness in the hindgut ($P < 0.05$). These alkaloids also significantly improved intestinal antioxidant capabilities by increasing superoxide dismutase, catalase, total antioxidant capacity and malondialdehyde levels and up-regulated intestinal *Cu/Zn-SOD* and *Mn-SOD* ($P < 0.05$) at 20 and 40 mg/kg. Dietary *G. elegans* alkaloids improved intestinal immunity *via* up-regulating the pro-inflammatory cytokines *IL-1β*, *IL-8*, *TNF-α* and *IFN-α* and down-regulating expression of the anti-inflammatory cytokines *IL-10* and *TGF-β* ($P < 0.05$) at 20 and 40 mg/kg. The expression of Toll-like receptors *TRL1*, *3*, *4* and *7* were also up-regulated in intestine of *M. amblycephala* ($P < 0.05$). In intestinal microbiota, the abundance of *Proteobacteria* was increased while the *Firmicutes* abundance was decreased at phylum level after feeding the alkaloids ($P < 0.05$). The alkaloids also increased the abundance of the probiotic *Rhodobacter* and decreased the abundance of the pathogenic *Staphylococcus* at genus level ($P < 0.05$). In conclusion, dietary *G. elegans* alkaloid supplementation promoted intestine health by improving intestine morphology, immunity, antioxidant abilities and intestinal microbiota in *M. amblycephala*.

1. Introduction

Intestinal status and intestinal microbiota are essential for fish, they play an important role in nutrient absorption of fish, also as a barrier to invading pathogens and to provide a niche for the indigenous microbiota [1]. Unlike terrestrial animals, the aquatic animals are directly exposed to water and the relationships between the intestinal microbiota and nutrient absorption or disease resistance are more complex [2]. Despite the composition of intestinal microbiota in fish is related to the species and culture environment, the diversity and composition of bacteria were also plasticity in different feeding conditions [3]. Therefore, we can control the feeding conditions to improve the intestinal status of fish.

For instance, the current practices of high-density aquaculture result in high fish yield at the expense of deteriorated water quality due to organic matter accumulation [4]. This state has a negative impact on the stability of the microbiota by increasing pathogens and decreasing probiotic species. This has a direct effect of the intestinal microbiota in fish and is also reflected in intestinal immune system status [5]. Feed additives including plant extracts [6,7], amino acids such as valine [8] and prebiotics including oligosaccharides [9] have been shown to be beneficial for fish intestinal health. The plant extracts attract our

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attention because they are inexpensive, locally available and environment friendly [10]. However, current practices most often utilize chemical drugs, such as antibiotics, which have negative impacts on the environment and leads to drug resistance and significant amounts of unmetabolized drug remaining in the environment [11].

In the present study, we examined the effects of *Gelsemium elegans* alkaloids as a plant-derived feed additive to assess its maintaining intestinal health qualities. *G. elegans* belongs to a small genus of the family Loganiaceae that are widely distributed in China and Southeast Asia. This plant has been used as a Chinese herbal medicine for treatment of neuropathic pain, inflammation, rheumatic arthritis and anxiety [12–14]. *G. elegans* is a medicinal plant that contains numerous bioactive compounds including koumine, gelsemine and gelsenicine [15,16]. In our previous study, there have been applications of *G. elegans* alkaloids in fish. Its main concern is the effects of *G. elegans* alkaloids on growth and immunity, but does not involve the effects of intestinal status and intestinal microbiota [17]. In this study, We used extracts from *G. elegans* and examined its effects on *Megalobrama amblycephala* intestine, a commercially important herbivorous, freshwater fish [18]. Fish intestinal status and the intestinal microbiota are of great importance to the growth and health of fish, which have become the hotspot in the field of immunity and disease control of aquatic animals in the world [19,20].

The health status of fish is reflected in their morphology, intestinal immunity, antioxidant status and microbiota and these are important indicators for evaluating the health and welfare of fish [21]. To evaluate the effects of our treatment procedures, we compared these intestinal health parameters after treatment with *G. elegans* alkaloids as a dietary addition. In addition, these experiments evaluated the intestinal microbiota of *M. amblycephala* to generate an overall understanding of the endogenous bacterial populations of fish.

2. Materials and methods

2.1. Diet preparation

The diets of *G. elegans* alkaloids levels were formulated in Table S1 and the basal diet composition is based on previous studies [22]. The basal diet was used as control diet (NC), and 5, 10, 20, 40 mg/kg G. *elegans* alkaloids were supplemented to formulate as experimental diets. Basal diet proximate analysis was performed according to the method outlined by the Association of Official Analytical Chemists [23,24]. All the ingredients were thoroughly mixed with fish oil, *G. elegans* alkaloids and water, and then were made sinking pellet feed through the pelletizer. Lastly, the pellets were air-dried to below 10% moisture. After drying, all diets were sealed in bags and stored at −20 °C for further use. The *G. elegans* alkaloids were provided by Manst Biotechnology Co., Ltd (Chendu, China).

2.2. Experimental fish

Experimental fish were obtained from the Lihong Fishery Company (Guangzhou, China). The fish were fed a basal diet for 2 weeks prior to the start of the experiments to acclimate them to the experimental diet. Following this, similar sized *M. amblycephala* (3.73 ± 0.03 g) were randomly distributed into 15 tanks at 30 fish per tank and fed twice daily at 8:00 and 17:00 until apparent satiation for 12 weeks. The water temperature (25–28 °C) pH (7.2–7.8) and dissolved oxygen (5.5–6.0 mg/L) were maintained at all times. The fish were reared under 12-h light 12-h dark photoperiods and each diet treatment was tested in triplicate.

2.3. Sample collection

After the feeding trial, fish were fasted for 24 h before sampling and randomly selected fish from each tank and were euthanized using 100 mg/L tricaine methanesulfonate (MS-222, Sigma, USA). The foregut, midgut and hindgut from 3 fish per tank were collected in 1.5 mL sterile centrifuge tubes containing 4% paraformaldehyde for morphological analysis. Intestinal samples were taken from 3 fish per tank for antioxidant enzyme activity assay and gene expression analysis and 12 parallel intestine samples were collected from each treatment concentration, 4 fish per tank for Miseq sequencing. Intestines were quickly frozen in liquid nitrogen and stored at −80 °C after removal from the fish.

2.4. Morphological analysis

The foregut region encompassed 1 cm after the stomach pyloric portion and the hindgut region was the region within 1 cm before the anus [25]. All samples were washed in sterile PBS (pH 7.4) and fixed in 4% paraformaldehyde for 48 h and washed in 70% ethanol. Intestinal regions were embedded in paraffin and 5 μm sections were stained with haematoxylin-eosin. All slides were evaluated in a Pannoramic MIDI scanner (3D Histech, Budapest, Hungary). Images were analyzed using Image-pro plus 6.0 software (Media Cybernetics, Rockville, MD, USA) software.

2.5. Antioxidant activity assays

Superoxide dismutase (SOD), catalase (CAT), total antioxidant activity (T-AOC) and malondialdehyde (MDA) contents were measured using commercial test kits (Nanjing Jiancheng Bioengineering, China) following the manufacturer's protocols. Briefly, intestinal samples in cold phosphate buffered saline (PBS) pH 7.4 were homogenized and then centrifuged for 15 min at 3500 rpm and the supernatant was used for biochemical assays using spectrophotometry.

2.6. Gene expression analysis

RNA from intestine samples was extracted using TransZol Up Plus RNA kits (TransGen Biotech, Beijing, China) according to the manufacturer's instructions. The quantity of total RNA was determined by UV spectroscopy using a NanoDrop 2000 instrument (Thermo Scientific, Pittsburg, PA, USA) and RNA integrity was evaluated by electrophoresis in 2% agarose gels. RNA was reverse transcribed using a PrimeScript RT reagent kit with gDNA Eraser (Takara, Dalian, China) according to the manufacturer's instructions. The cDNA were stored at −80 °C for gene expression analysis. Real time PCR was carried out in a Biorad CFX96 Real-Time instrument using a SYBR Green Supermix (Biorad, Hercules, CA, USA). Thermal cycling conditions were 95 °C for 2 min, followed by 39 cycles of 95 °C for 15 s, 60 °C for 30 s, 72 °C for 30 s and a final cycle of 72 °C for 7 min. Melting curves were performed from 60 to 90 °C. Gene specific primers have been previously described (Table S2). Relative gene expression levels were normalized against the expression level of β-actin. All samples were run in triplicate and each assay was repeated three times.

2.7. DNA extraction, amplicon library preparation and Miseq sequencing

The intestinal microbiota genomic DNA was extracted using Takara MiniBEST Genomic DNA Extraction kit according to manufacturer's protocols. DNA integrity was determined by electrophoresis in 2% agarose gels and UV spectrometry as described above. PCR reactions were used to amplify the V3–V4 region of the 16S rRNA genes using primers 338F: 5′- ACTCCTACGGGAGGCAGCA-3′ and 806R: 5′-GGACTACHVGGGTWTCTAAT-3′ as previously described [26]. The PCR reactions were performed on the ABI GeneAmp PCR System 9700 (Applied Biosystems, Waltham, MA, USA) as follows: 98 °C for 2 min, followed by 30 cycles of 98 °C for 30 s, 50 °C for 30 s, 72 °C for 40 s and a final step of 72 °C for 5 min. The PCR products were visualized after electrophoresis in 2% agarose gels and DNA bands were gel purified

Table 1
Effects of dietary *G. elegans* alkaloids on gut morphology of *M. amblycephala*.

	0 mg/kg	5 mg/kg	10 mg/kg	20 mg/kg	40 mg/kg
	Foregut				
Villus length (μm)	564.18 ± 15.91^{a}	569.05 ± 22.87^{a}	585.83 ± 27.63^{ab}	601.10 ± 25.84^{b}	611.19 ± 30.26^{b}
Muscle thickness (μm)	95.05 ± 3.54^{a}	94.00 ± 7.92^{a}	100.21 ± 9.31^{ab}	105.46 ± 5.80^{ab}	110.98 ± 7.49^{b}
Goblet cells (per villus)	30.93 ± 4.57^{ab}	28.67 ± 1.40^{a}	33.07 ± 8.86^{ab}	39.67 ± 6.69^{b}	37.20 ± 3.65^{ab}
Villus number	20.33 ± 1.53^{a}	20.00 ± 2.65^{a}	22.67 ± 4.04^{ab}	21.67 ± 1.15^{ab}	26.33 ± 2.89^{b}
	Midgut				
Villus length (μm)	323.22 ± 23.37^{a}	342.33 ± 32.18^{ab}	349.74 ± 20.41^{ab}	334.32 ± 38.24^{ab}	388.03 ± 54.89^{b}
Muscle thickness (μm)	78.72 ± 10.12^{a}	75.87 ± 2.46^{a}	89.23 ± 8.85^{ab}	83.41 ± 11.39^{a}	102.66 ± 12.18^{b}
Goblet cells (per villus)	38.60 ± 7.16^{a}	37.13 ± 5.69^{a}	36.53 ± 7.19^{a}	34.60 ± 4.13^{a}	36.67 ± 2.19^{a}
Villus number	14.00 ± 2.00^{a}	14.67 ± 1.53^{a}	14.67 ± 1.53^{a}	16.00 ± 2.00^{a}	20.67 ± 4.51^{b}
	Hindgut				
Villus length (μm)	411.67 ± 24.34^{a}	518.86 ± 79.34^{b}	419.05 ± 19.62^{a}	420.33 ± 25.85^{a}	439.95 ± 39.73^{ab}
Muscle thickness (μm)	67.21 ± 3.52^{a}	69.31 ± 6.28^{a}	68.17 ± 9.59^{a}	72.03 ± 3.76^{ab}	82.47 ± 8.17^{b}
Goblet cells (per villus)	35.07 ± 10.02^{a}	35.13 ± 6.97^{a}	27.40 ± 2.55^{a}	35.87 ± 2.58^{a}	36.73 ± 4.72^{a}
Villus number	12.67 ± 2.89^{a}	13.00 ± 2.00^{a}	13.67 ± 1.15^{a}	14.00 ± 2.00^{a}	13.33 ± 0.58^{a}

Data are presented as mean ± SD. Values within the same row with different superscripts were significantly different ($P < 0.05$).

using the AxyPrep DNA Gel Extraction kit (Axygen Biosciences, Union City, CA, USA) and then quantified by flourimetry in a QuantiFluor ST instrument (Promega, Madison, WI, USA) [27]. DNA pools were generated from the purified products in equimolar ratios and paired-end sequenced (2 × 300) using an Illumina MiSeq platform (Illumina, San Diego, CA, USA) following standard protocols [28].

2.8. Bioinformatics analysis

Before analysis the raw fastq data were processed using a Trimmomatic (http://www.usadellab.org/cms/?page=trimmomatic) and FLASH (https://ccb.jhu.edu/software/FLASH/) to remove low quality sequences. Operational taxonomic units (OTUs) were assigned using QIIME 1.91 [29]. UPARSE 7.1 (http://drive5.com/uparse) was applied to cluster the OTUs at an identity threshold of 97% and then UCHIME 4.2 was utilized to remove chimeric sequences, and got the Effective Tags [30,31]. The OTUs that reached a 97% nucleotide similarity level were used for alpha diversity analyses using the observed species, Chao1, Shannon, Good's coverage estimator, Simpson and phylogenetic diversity indices using Mothur 1.30.1 software [32] and R-package ggplot2. For beta diversity analysis, principal coordinate analysis (PCoA) and nonmetric multidimensional scaling (NMDS) analysis based on the Bray Curtis algorithm were applied using ggplot2 and Vegan R-package. Microbiota composition analysis was performed to identify the specific microbiota taxa (microbiological markers) associated with *G. elegans* alkaloids. The microbiota were compared using linear discriminant analysis effect size (LEfSe) method (http://huttenhower.sph.harvard.edu/lefse) [33] using a Kruskal-Wallis test alpha value of $P < 0.05$. The PICRUSt algorithm was used to predict metabolic activities of bacterial communities [34] and the intestinal microbiota of different diet groups were compared using the Kruskal-Wallis test at different taxon levels. The tests for significance were two-sided and $P < 0.05$ was considered statistically significant. Functional inferences were made from the Kyoto Encyclopedia of Gene and Genomes (KEGG) catalog. The pathway functions were categorized at level 3. BugBase (https://bugbase.cs.umn.edu/) was used to predict bacterial phenotypes. The results of other analyses were visualized using R package software and Excel (Microsoft, Seattle, WA, USA).

2.9. Statistical analysis

Miseq sequence data were analyzed using R package software. Differences were analyzed using one-way analysis of variance (ANOVA) in SPSS 16.0 software (IBM, Armonk, NY, USA). Real-time PCR data were analyzed using the $2^{-\Delta\Delta Ct}$ method. Results with $P < 0.05$ between groups were declared statistically significant. All results were presented as means ± SD and all experiments were carried out at least in triplicate.

3. Results

3.1. Effect of dietary G. elegans alkaloids on intestinal morphology

We examined the intestinal morphology and villus length increased as a function of increasing dietary *G. elegans* alkaloid levels. Significant increases were observed in the 20 and 40 mg/kg groups for the foregut and in 40 mg/kg for the midgut ($P < 0.05$). However, the villus length values of hindgut were maximal in 5 mg/kg group ($P < 0.05$) and other treatment groups did not reach significant levels. The muscle thickness also increased with the increasing dietary *G. elegans* alkaloids levels and this was significant in the 40 mg/kg group for all three sample types ($P < 0.05$). Goblet cells increased significantly in the 20 mg/kg group in the foregut ($P < 0.05$) but levels in the midgut and hindgut were not significant. Moreover, the villus number in the foregut and midgut in 40 mg/kg groups increased significantly ($P < 0.05$) but we found no significant changes in the hindgut between control and treatment groups (Table 1 and Fig. 1).

3.2. Effect of dietary G. elegans alkaloids on intestinal antioxidant ability

We next measured intestinal antioxidant abilities after fish exposure to the *G. elegans* alkaloids. The SOD activities of all 4 experimental groups were significantly greater than controls ($P < 0.05$) with a maximal peak in the 40 mg/kg group (Fig. 2a). The CAT activities in the 5, 20 and 40 mg/kg groups were also significantly higher than controls ($P < 0.05$) but the 10 mg/kg group was not significantly different compared to controls (Fig. 2b). The intestinal T-AOC was also significantly increased in the 10, 20 and 40 mg/kg groups ($P < 0.05$) compared to controls and this was positively correlated with dose (Fig. 2c). MDA levels in the 10, 20 and 40 mg/kg groups were also significantly greater compared to controls ($P < 0.05$). In contrast, there was a significant decrease in the 5 mg/kg group (Fig. 2d).

3.3. Effect of dietary G. elegans alkaloids on intestinal immune-related gene expression

Immune-related gene expression was also upregulated when compared with controls. The interleukin genes *IL-1β* and *IL-8* were significantly up-regulated in the fish fed *G. elegans* alkaloids ($P < 0.05$) and a maximal level was reached in the 40 mg/kg group. In contrast, the *IL*-10 mRNA levels were significantly down-regulated in all experimental groups compared with controls ($P < 0.05$) (Fig. 3a). The

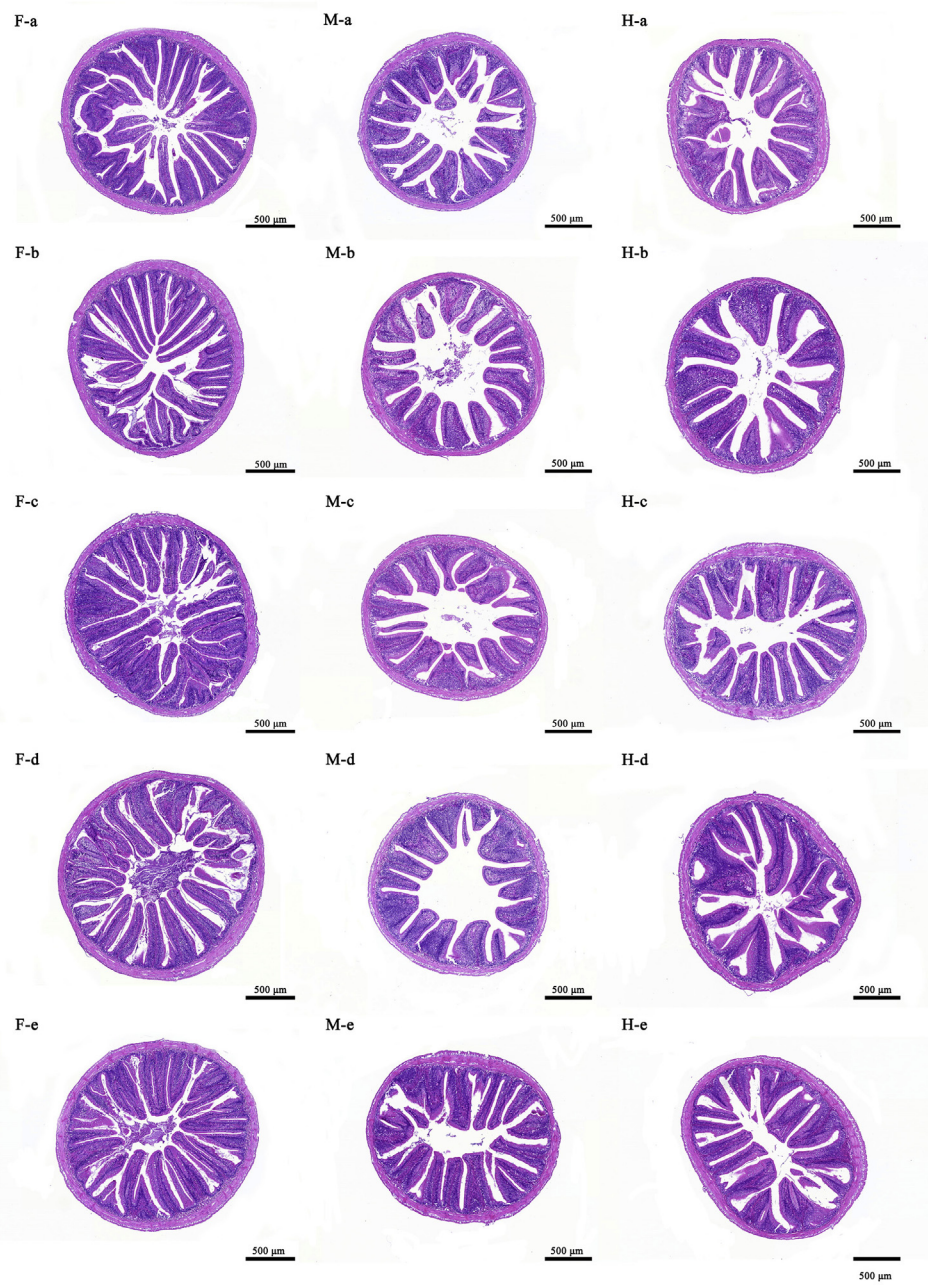

Fig. 1. Morphological analysis of foregut, midgut and hindgut in *M. amblycephala* fed with various *G. elegans* alkaloids levels. F, M and H represent foregut, midgut and hindgut samples, respectively. Samples taken from fish cultured in the presence of *G. elegans* alkaloids at 0, 5, 10, 20 and 40 mg/kg are represented by a, b, c, d and e, respectively.

cytokine genes *TNF-α* and *IFN-α* displayed significant increases in the 20 and 40 mg/kg groups ($P < 0.05$). However, *TGF-β* levels were significantly down-regulated for all experimental groups compared with controls ($P < 0.05$). In particular, the *TNF-α* gene was significantly up-regulated in the 10 mg/kg group ($P < 0.05$) (Fig. 3b). The Toll-like receptor genes *TLR1*, *3*, *4* and *7* were also significantly up-regulated in the experimental groups ($P < 0.05$) with the exception of *TLR4* in the 10 mg/kg group. The other TLR genes reached their peak values in the 20 and 40 mg/kg groups ($P < 0.05$) and TLR7 was the most highly expressed (Fig. 3c). The mRNA levels of the oxidative stress regulator *Cu/Zn-SOD* was significantly decreased in the 10 mg/kg group and thereafter was significantly increased ($P < 0.05$). *Mn-SOD* mRNA levels were significantly increased in the 10, 20 and 40 mg/kg groups ($P < 0.05$) (Fig. 3d).

3.4. Characteristics of sequencing data

The alpha diversity analysis we performed indicated that our observed species index represented the number of OTUs that we actually observed. The observed species index for the 10, 20 and 40 mg/kg groups were significantly higher than controls ($P < 0.05$) (Fig. 4a). Sample richness was calculated using the Chao1 index and these same groups were again significantly higher than controls ($P < 0.05$) (Fig. 4b). To quantify diversity we also used the Shannon and Simpson indices and both these indices in the 10 mg/kg group displayed significant increases compared with controls ($P < 0.05$) (Fig. 4c and e). We also utilized the Good's coverage of each sample to estimate sequencing completeness. The Good's coverage indices for the 10, 20 and 40 mg/kg groups were all significantly lower than the control group ($P < 0.05$) but most of them were > 97% (Fig. 4d). This indicated that

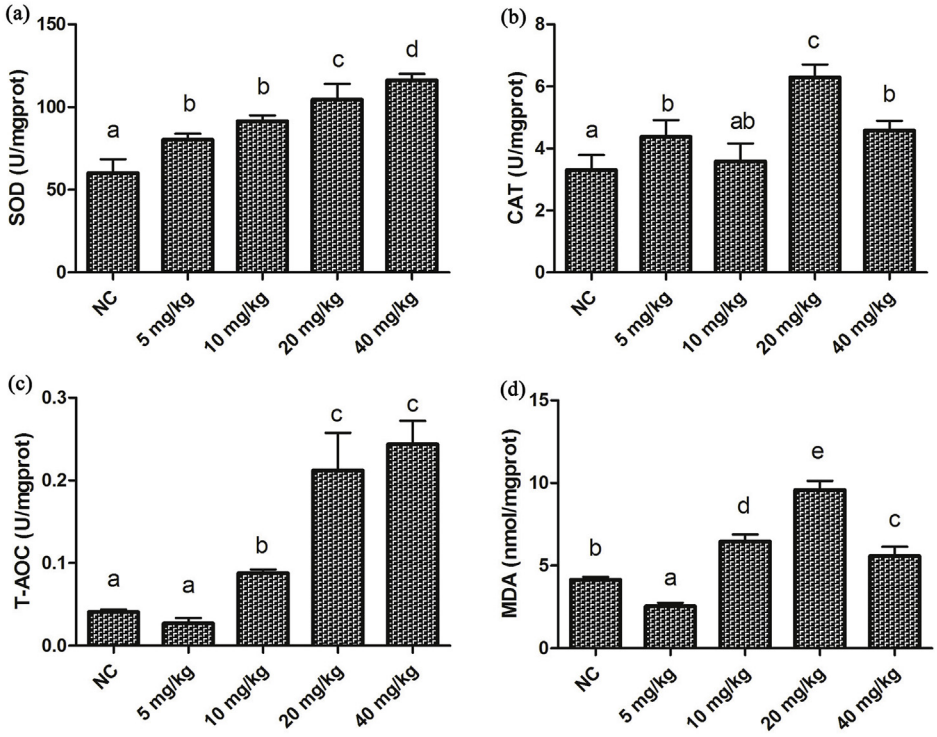

Fig. 2. Effect of dietary *G. elegans* alkaloids on intestinal antioxidant abilities in *M. amblycephala*. (a) Superoxide dismutase (SOD); (b) Catalase (CAT); (c) Total antioxidant capacity (T-AOC); (d) Malondialdehyde (MDA). NC, negative control. Data were expressed as mean ± SD (n = 3). Values with no common superscript differed significantly ($P < 0.05$).

the sequences identified were representative of the majority of the bacteria in this study.

A phylogenetic diversity index calculates the evolutionary distance relationships between OTUs based on a random sampling of OTUs. However, only the phylogenetic diversity index in the 40 mg/kg group displayed significant differences compared with controls ($P < 0.05$) (Fig. 4f). This indicated that the evolutionary distances between OTUs in all the experimental groups except for 40 mg/kg were close and

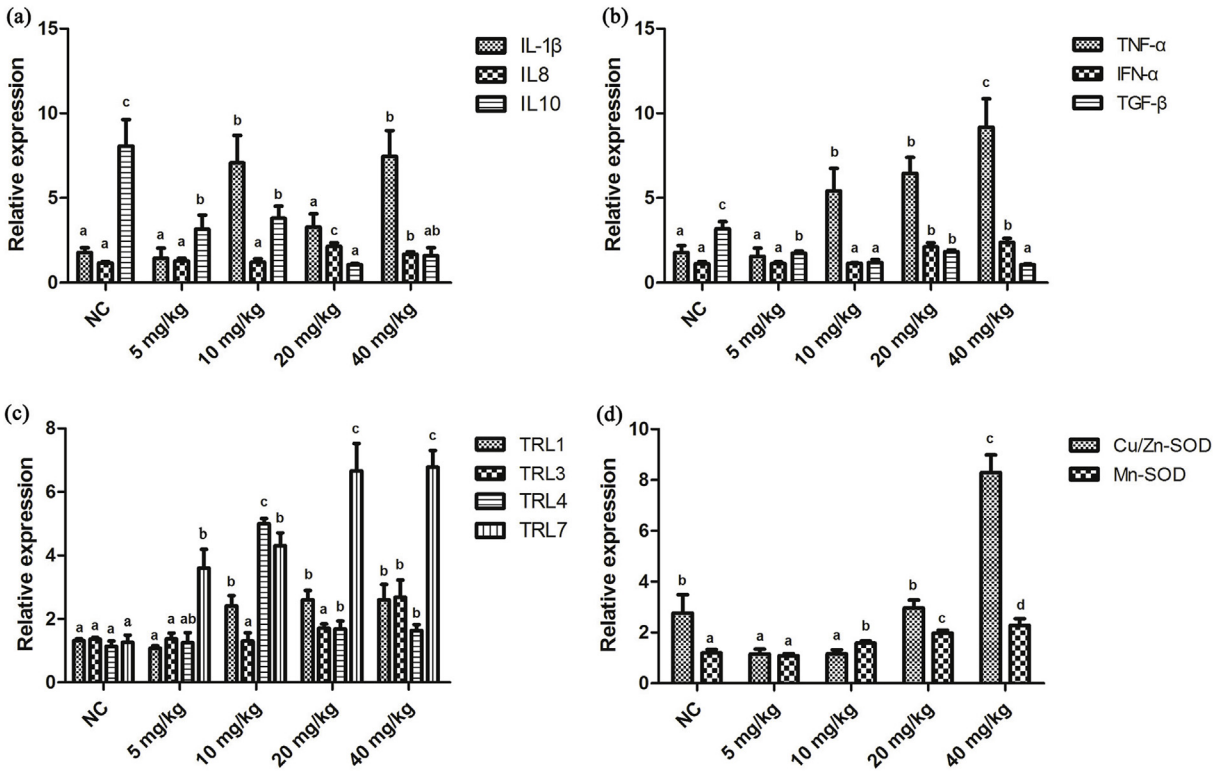

Fig. 3. Relative expression of immune related genes in the intestine of *M. amblycephala* fed G. *elegans* alkaloids. (a) Interleukin 1β (*IL-1β*), interleukin 8 (*IL-8*); and interleukin 10 (*IL-10*); (b) Tumor necrosis factor α (*TNF-α*), interferon α (*IFN-α*) and transforming growth factor β (*TGF-β*); (c) Toll-like receptors 1, 3, 4 and 7 (TLR1, 3, 4 and 7); (d) Cu/Zn superoxide dismutase (*Cu/Zn-SOD*) and Mn superoxide dismutase (*Mn-SOD*). NC, negative control. Values were expressed as mean ± SD (n = 3) and values with no common superscript differed significantly ($P < 0.05$).

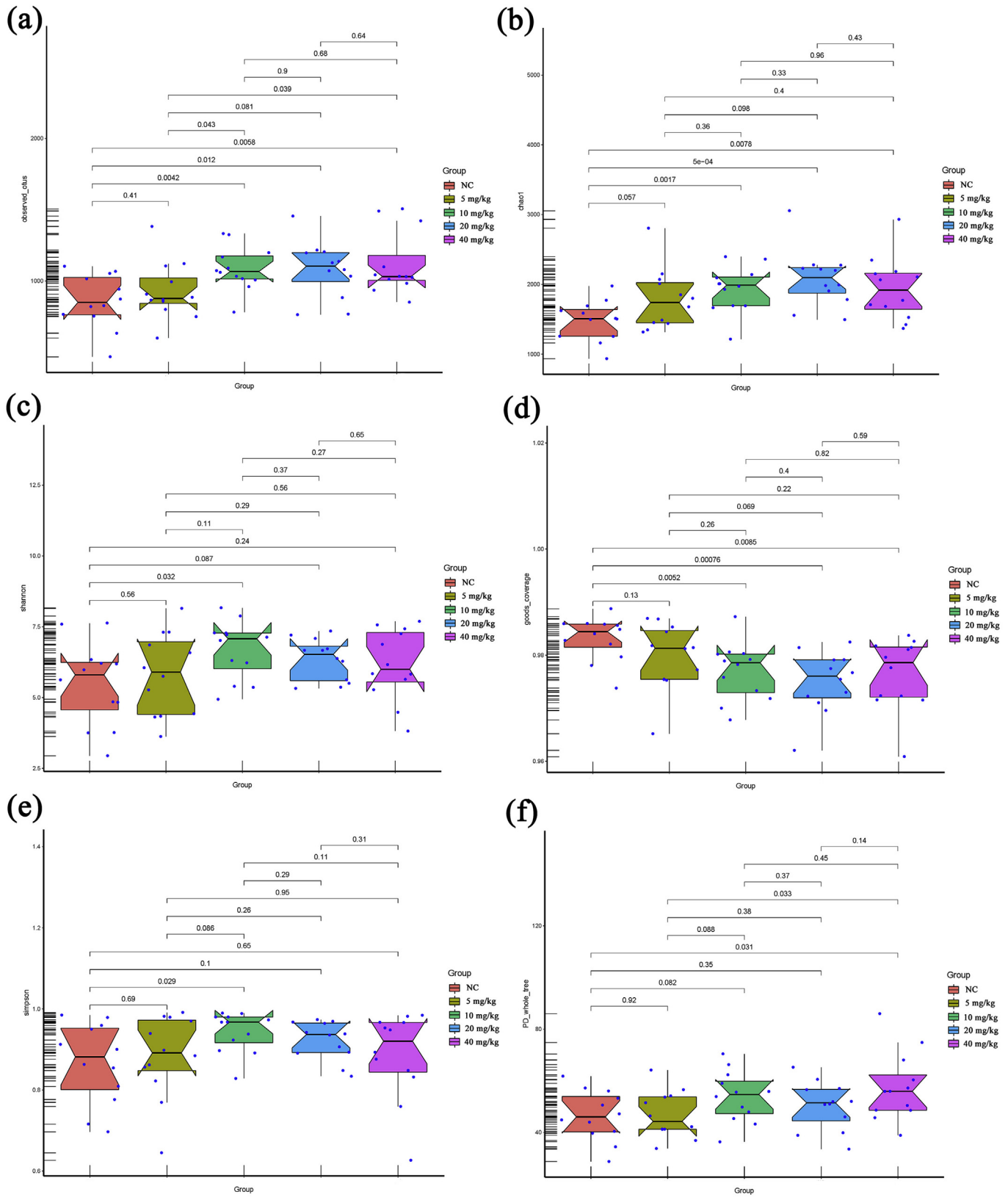

Fig. 4. The alpha diversity index of gut microbiota in *M. amblycephala* fed *G. elegans* alkaloids. (a) Observed species index; (b) Chao1 richness estimator; (c) Shannon-Weiner index; (d) Good's coverage estimator; (e) Simpson diversity index; (f) Phylogenetic diversity index. $P < 0.05$ indicates a significant statistical difference.

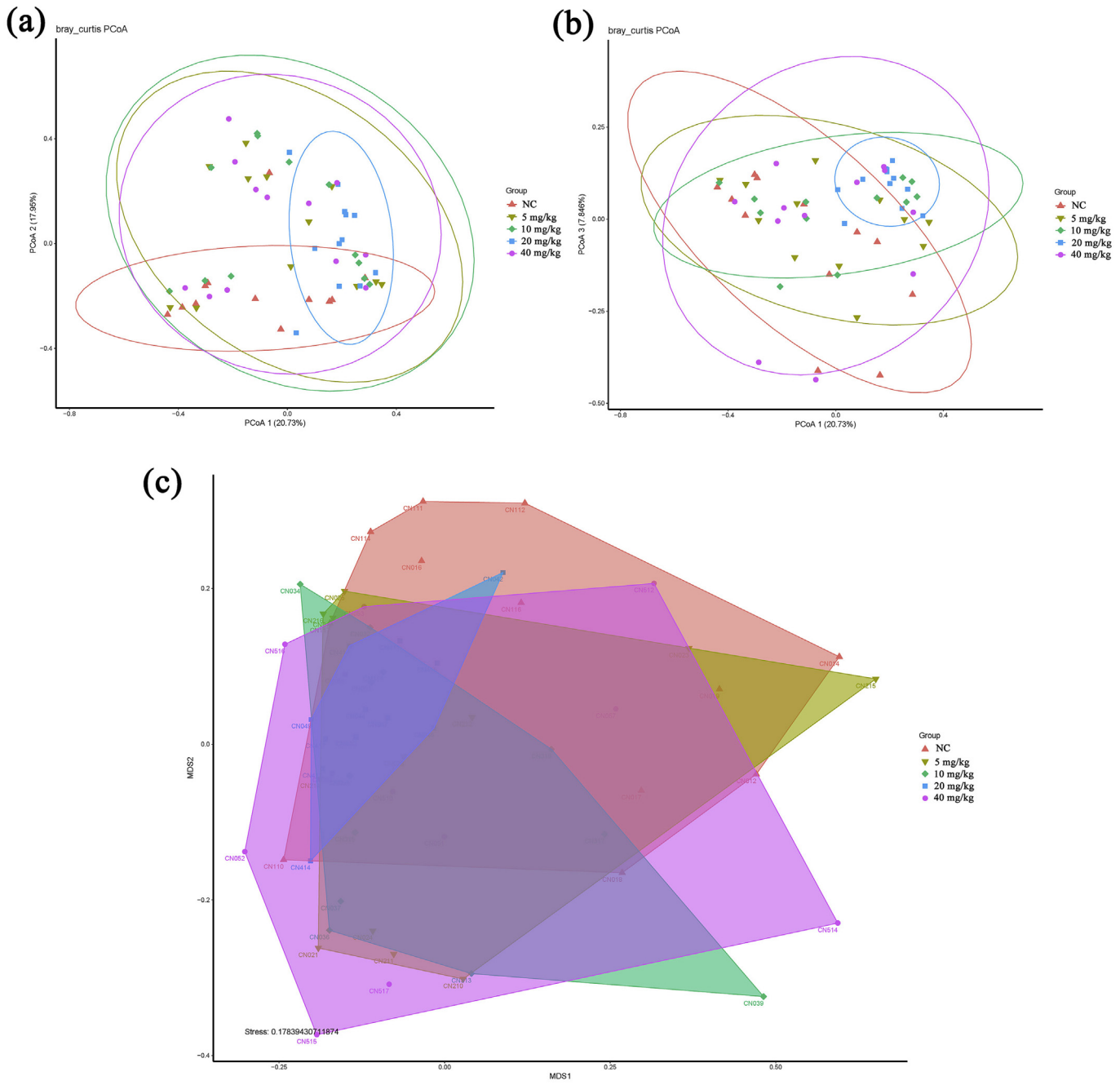

Fig. 5. Beta diversity analysis of intestinal bacterial communities in *M. amblycephala* (a) PC1 versus PC2 (b) PC1 versus PC3 (c) Nonmetric multidimensional scaling (NMDS) analysis. PCoA and NMDS analysis were based on Bray Curtis algorithm.

possessed similar compositions. Additionally, beta diversity analysis clustered with PC1 = 20.73%, PC2 = 17.95% and PC3 = 7.846% by PcoA analysis. Interestingly, the 20 mg/kg group was quite different from other groups using PCoA and NMDS analysis (Fig. 5a, b and 5c).

3.5. The dominant bacterial taxa of intestinal microbiota among five diet groups

We used a taxonomic analysis to view the intestinal community structures at different taxonomic levels. We identified OTUs in 30–40 phyla between the control and the 4 experimental groups. We also identified from 598 to 688 genera in our 5 groups as well as a range of members in other taxonomic groups (Fig. 6a). When we determined the intersection of these sets of taxonomic classifiers, we identified 25, 66, 124, 235, 371 and 401 common classifiers at the phylum, class, order, family, genus and species levels, respectively (Fig. 6b). At the phylum level, the control group was dominated by phyla *Proteobacteria* (44.57%), *Firmicutes* (18.92%), *Fusobacteria* (13.65%) and *Bacteroidetes* (6.53%) and these represented 77.14% of the total bacterial sequences (Fig. 7b). The 5 mg/kg group was dominated by the *Proteobacteria* (65.34%), *Firmicutes* (6.06%), *Fusobacteria* (6.22%) and *Bacteroidetes* (5.4%), representing 83.02% of the total reads (Fig. 7c). The 10 mg/kg group was dominated by *Proteobacteria* (61.4%), *Bacteroidetes* (10.06%), *Firmicutes* (6.06%) and *Fusobacteria* (4.97%), accounting for 82.49% of the reads (Fig. 7d). The 20 mg/kg group was dominated by *Proteobacteria* (69.08%), *Bacteroidetes* (12.76%), *Actinobacteria* (3.6%), *Firmicutes* (3.35%) and *Fusobacteria* (2.25%), representing 91.04% of the reads (Fig. 7e). The highest dosage group 40 mg/kg was dominated

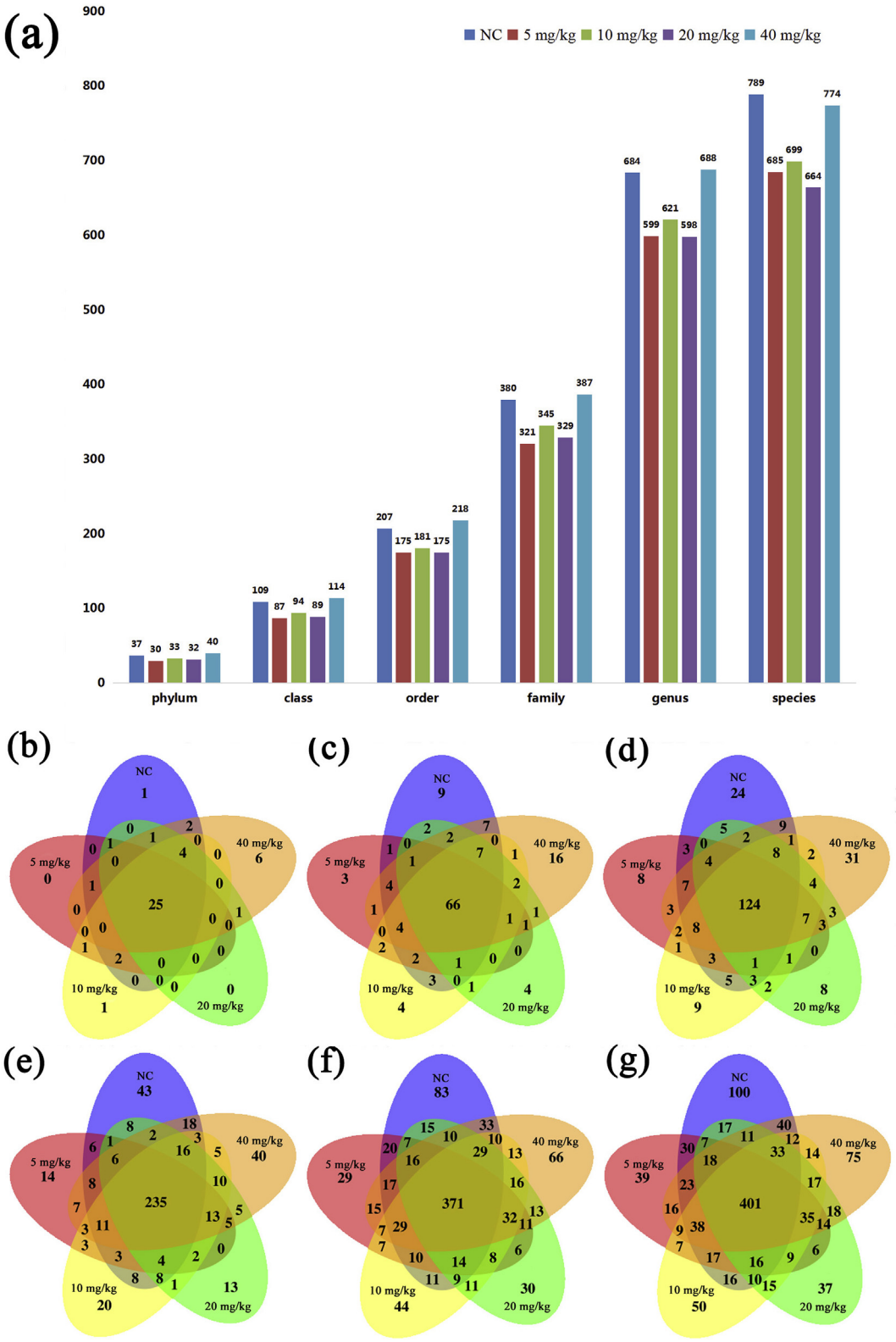

Fig. 6. Overall intestinal microbiota structures in the intestines of *M. amblycephala*. (a) Total OTUs from phylum to species for each group; (b–g) Representation of the above groupings illustrating OTU overlaps from phylum to species, respectively.

by *Proteobacteria* (58.67%), *Fusobacteria* (10.68%), *Bacteroidetes* (6.59%) and *Actinobacteria* (4.06%) accounting for 80% of the reads (Fig. 7f).

3.6. Differences in the bacterial community compositions among five diet groups

To identify whether there were specific bacterial taxa associated with *G. elegans* alkaloid treatments, the gut microbiota were compared

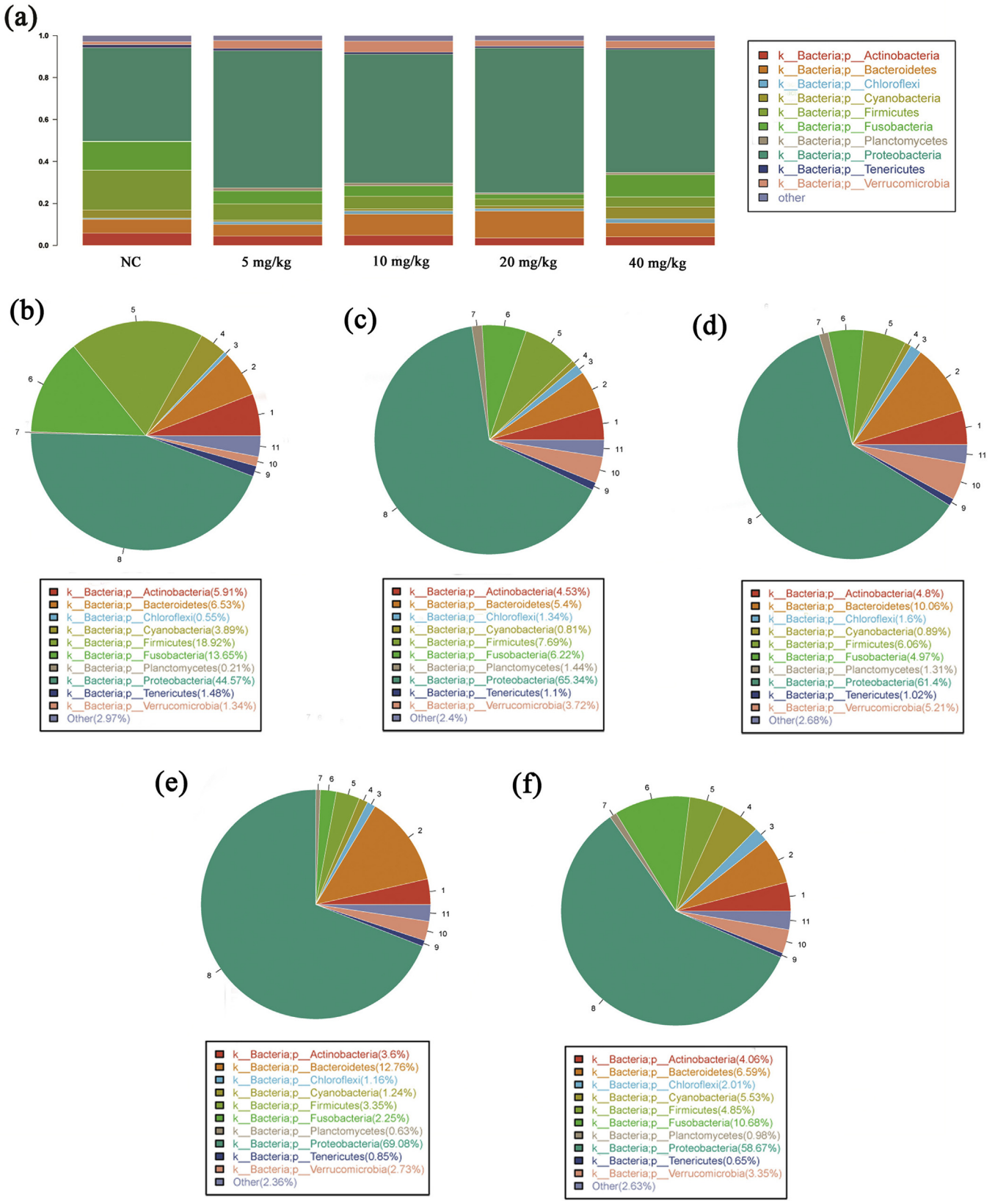

Fig. 7. The intestinal microbiota composition at the phylum level. Microbiota composition of bacterial taxa at phylum level in the five groups (a) and respectively in control group (b), 5 mg/kg group (c), 10 mg/kg group (d), 20 mg/kg group (e) and 40 mg/kg group (f).

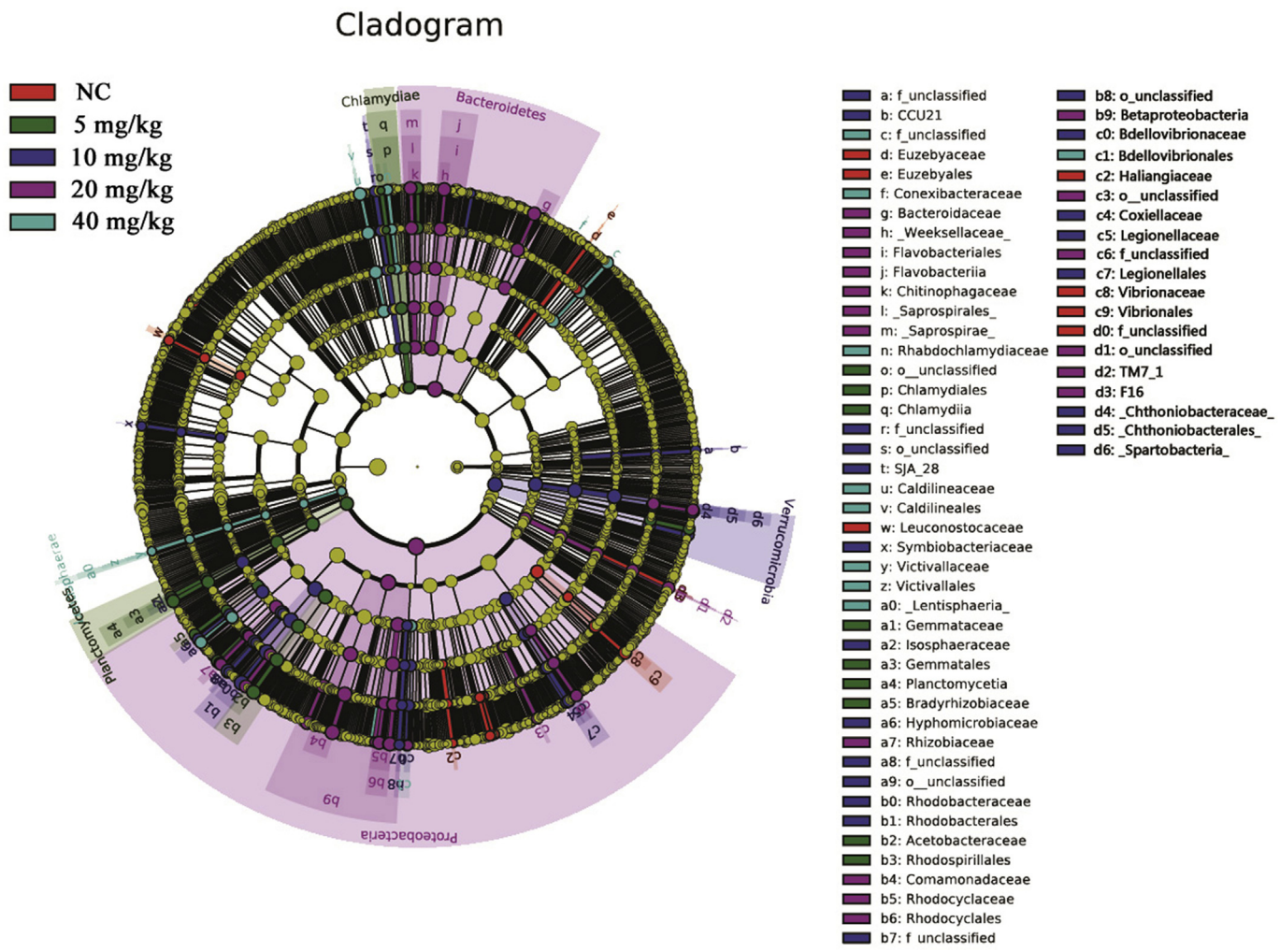

Fig. 8. LEfSe identification of the most differentially abundant microbiota taxons at all classification levels. The circle radii from inside to outside in the figure represent the classification level from phylum to genus, respectively, and the diameter of the small node represents the relative abundance. Significantly discriminant taxon nodes are colored according to grouping and differential nodes not statistically significant are colored yellow. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

using linear discriminant analysis effect size (LEfSe) analysis. We found that the primary differences were in the *Proteobacteria*, *Firmicutes*, *Bacteroidetes*, *Verrucomicrobia*, *Planctomycetes* and *Chlamydiae* at the phylum level (Fig. 8) and the specific relative abundances of these species were displayed in Fig. 9a ($P < 0.05$). At the genus level the primary differences were identified for *Flavihumibacter* and *Cloacibacterium* (*Bacteroidetes*), *Staphylococcus*, *Weissella* and *Veillonella* (*Firmicutes*). *Hyphomicrobium*, *Rhodoplanes, Agrobacterium*, *Rhodobacter, Azospira*, *Dechloromonas*, *Bdellovibrio* and *Aquicella* (*Proteobacteria*) and *Chthoniobacter* and *Luteolibacter* (*Verrucomicrobia*). The genera *Rhodobacter* and *Staphylococcus* were the most abundant bacteria overall (Fig. 9b). In addition, we found the relative abundances of different bacteria in these experimental groups were higher than that in the control group, except for those in *Firmicutes*. These results indicated that dietary supplementation with *G. elegans* alkaloids significantly increased the relative abundance of *Proteobacteria*, *Bacteroidetes* and *Verrucomicrobia* and significantly reduced that of the *Firmicutes*. Furthermore, these significantly different bacterial groups could be used as microbiological markers to differentiate between *G. elegans* alkaloid treatment and controls.

3.7. Functional prediction of the microbiota

We used the PICRUSt algorithm to estimate metabolic functions in the populations. Most functions were related to amino acid metabolism including valine, leucine and isoleucine degradation, tryptophan metabolism, lysine degradation, β-alanine metabolism, fatty acid metabolism, cytochrome P450 including xenobiotic and drug metabolism, glutathione metabolism and cell division (Fig. 10). By comparing the predicted results of PICRUSt gene functions with the KEGG database we identified 79 differential KEGG pathways. Among these we found 10 pathways related to amino acid metabolism including amino and nucleotide sugar metabolism, cysteine and methionine metabolism, D-glutamine and D-glutamate metabolism, lysine biosynthesis, alanine, aspartate and glutamate metabolism, valine, leucine and isoleucine degradation, lysine degradation, tryptophan metabolism, β-alanine and phenylalanine metabolism. We also identified 6 pathways related to fatty acid metabolism including glycerolipid and glycerophospholipid metabolism, fatty acid elongation in mitochondria, biosynthesis of unsaturated fatty acids, arachidonic acid and fatty acid metabolism. There were also 6 pathways related to glycometabolism including starch and sucrose metabolism, fructose and mannose metabolism, glycolysis, gluconeogenesis, pentose phosphate pathway, galactose metabolism and glycosphingolipid biosynthesis. In addition, we identified 10 pathways related to DNA replication including RNA transport and degradation, cell cycle, aminoacyl tRNA biosynthesis, DNA replication, homologous recombination, purine and pyrimidine metabolism, nucleotide excision repair and non-homologous end joining. Three of the

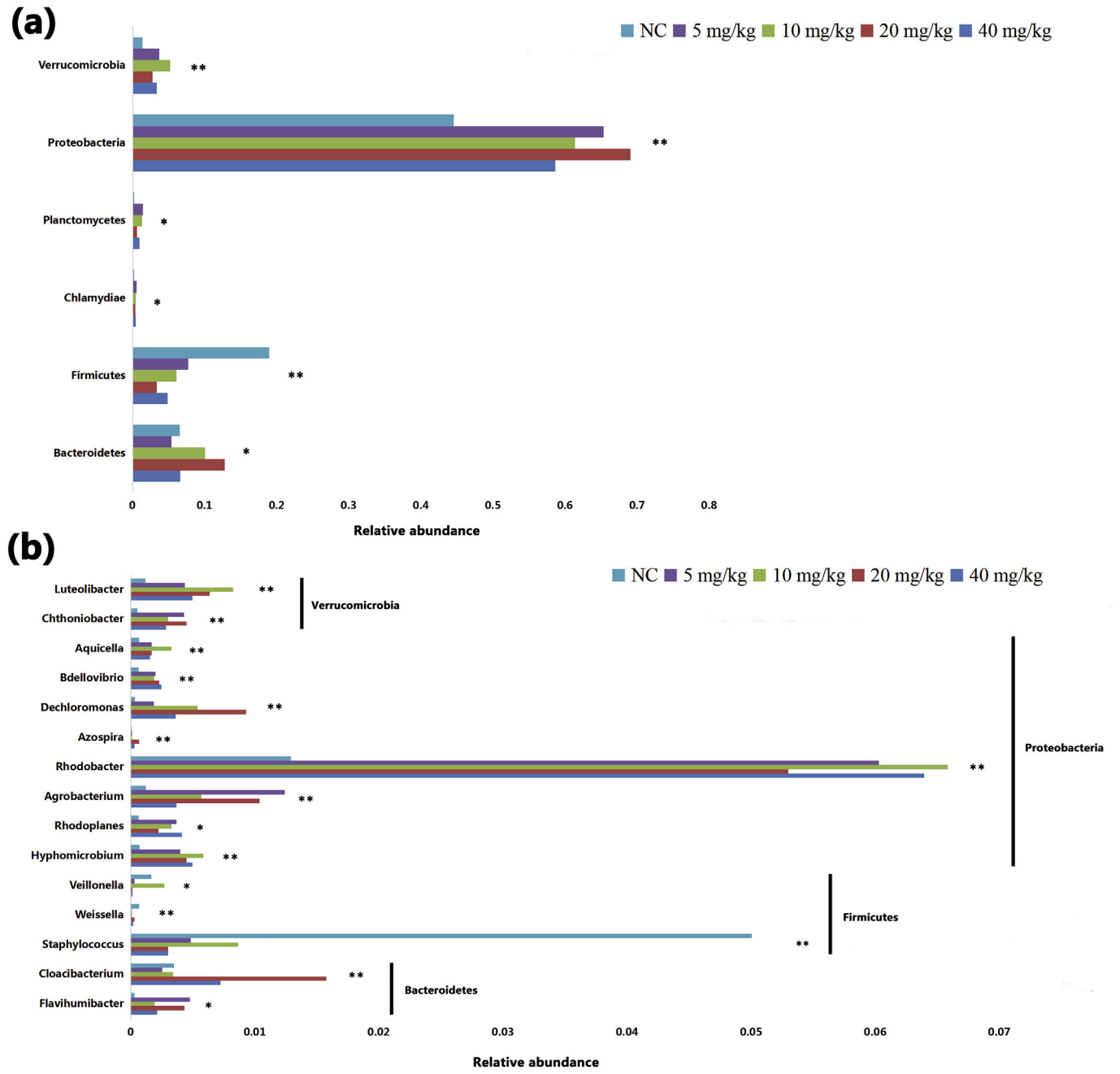

Fig. 9. Comparison of differentially abundant microbiota taxons in the intestine at the phylum and genus levels. (a) The most abundant taxons at the phylum level; (b) The most abundant taxons at the genus level. *, $P \leq 0.05$; **, $P \leq 0.01$.

pathways were related to oxidative stress including P450 xenobiotic metabolism, glutathione metabolism and the peroxisome. Finally, we found 2 pathways related to the tricarboxylic acid cycle including pantothenate and CoA biosynthesis and the synthesis and degradation of ketone bodies (Fig. 11). These functional predictions assisted in the understanding of the mechanism of action of the *G. elegans* alkaloids on *M. amblycephala*. In addition, we also used BugBase to predict bacterial phenotypes and we found the relative abundance of the 20 mg/kg group was significantly higher than control group in oxidative stress tolerance ($P < 0.01$) (Fig. 12).

4. Discussion

The intestine and intestinal microbiota play important roles in fish health by stimulating the immune system, aiding in nutrient absorption and decreasing disease susceptibility [35]. Previous studies have demonstrated that extracts from dandelions, *Panax notoginseng* and *Echinacea purpurea* possess positive effects in the fish intestine [36–38]. In our study, we found that 20 and 40 mg/kg G. *elegans* alkaloids significantly increased villus length and number, muscle thickness and goblet cell numbers in the *M. amblycephala* intestine. This indicated that *G. elegans* alkaloids are stimulatory to intestinal development in these fish. In addition, we found that the *G. elegans* extracts elevated the antioxidant indices SOD, T-AOC, CAT and indicated that the *G. elegans* alkaloids improved the intestinal anti-oxidative status of *M. amblycephala.* At the same time, we also found that the expression of MDA increased after feeding the alkaloids, and the MDA is not a good indicator for fish intestinal health. The increased MDA may be related to oxidative stress in fish induced by total alkaloids [39]. In fish intestine, fish have developed antioxidant systems to prevent oxidative damage,

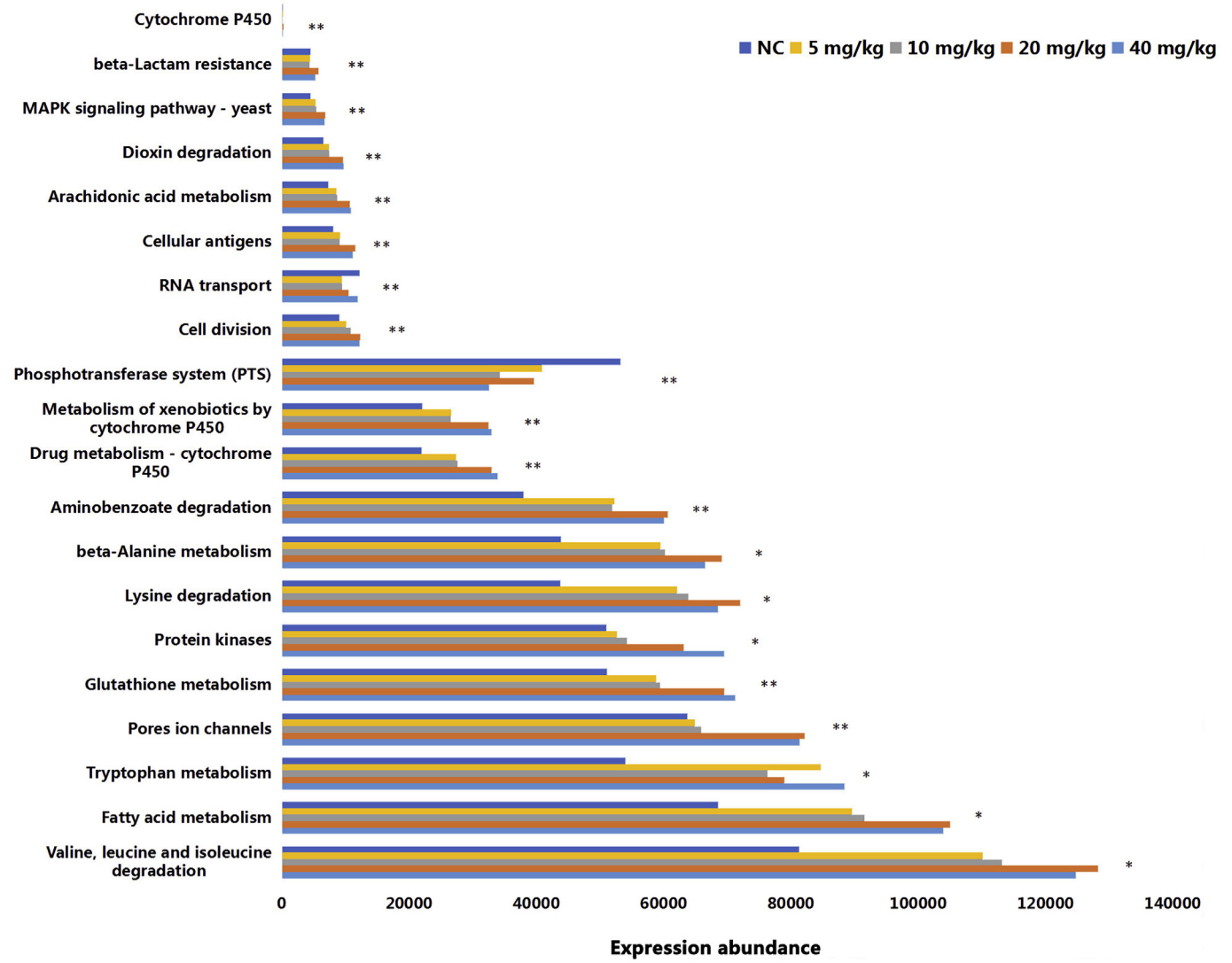

Fig. 10. A comparison of bacterial functions in the five diet groups. PICRUSt software was used to analyze the functional differences between different groups and differences were analyzed by the Kruskal-Wallis test. *, $P \leq 0.05$; **,$P \leq 0.01$.

which including superoxide dismutase (SOD) and catalase (CAT) [40]. The SOD and CAT worked together to reducing oxidative damage. Firstly, the SOD converted harmful superoxide free radicals into hydrogen peroxide, then the CAT immediately decomposed it into completely harmless compounds [41]. The T-AOC levels directly reflected antioxidant capacity [42]. These antioxidant enzymes are stress- and immune-response biomarkers that can be used to evaluate impacts on fish health [43]. Previous studies have shown that some plant extracts exhibit antioxidant stress properties in fish [1,44,45]. Furthermore, studies have shown that the monomer compounds of *G. elegans* elevate the levels of antioxidant indexes in eukaryotic microorganisms [46,47]. We also found that two commonly studied SOD genes *Cu/Zn-SOD* and *Mn-SOD* were up-regulated in the 40 mg/kg group and the results are similar to our previous study [17]. Therefore, this study indicated that the *G. elegans* alkaloids can improve intestinal antioxidative status of *M. amblycephala*.

Many studies reported that the plant extracts can also act as fish immunostimulants that enhance defense mechanisms or immune responses resulting in enhanced disease resistance [48]. Cytokines, such as nterleukins, interferons, and tumor necrosis factors plays a significant role in fish immune response [49]. Previous studies have shown that plant immunostimulants up-regulated pro-inflammatory cytokines and down-regulated anti-inflammatory cytokines in carp fed *Spirulina platensis* and *Rehmannia glutinosa* [50,51]. We found a similar same trend and the *G. elegans* alkaloids up-regulated pro-inflammatory cytokines *IL-1β*, *IL-8*, *TNF-α* and *IFN-α* and down-regulated anti-inflammatory cytokines *IL-10* and *TGF-β*. We also found that these alkaloids up-regulated *TRL1*, *3*, *4* and *7* and in particular, *TRL7* levels were increased significantly using the lower concentration levels of the alkaloids. TLRs activate transcription factors and mitogen-activated protein kinases to promote a pro-inflammatory response [52]. Recent studies have identified plant extracts as potential modulators of TLRs and hold promise for use as treatments for controlling inflammation and stimulating the immune response in fish [53].

In the aquatic environment, fish are directly exposed to numerous microbial pathogens so that the fish intestinal microbiota is more complex than for terrestrial animals. The fish intestinal microbiota plays a key role in gastrointestinal physiology and function as well as the immune response [54]. The microbial community in the fish intestine may be similar to that in aquaculture water and sediment, but aquaculture feed also has a significant impact on intestinal microbiota [55,56]. Plant extracts have been applied in aquaculture to improve fish health but the interaction with the fish gut microbiota is still poorly understood. In our study, the diversity and richness of gut microbiota increased after *G. elegans* alkaloid feeding and this most likely contributed to the homeostasis of the gut microbiota. PCoA and NMDS

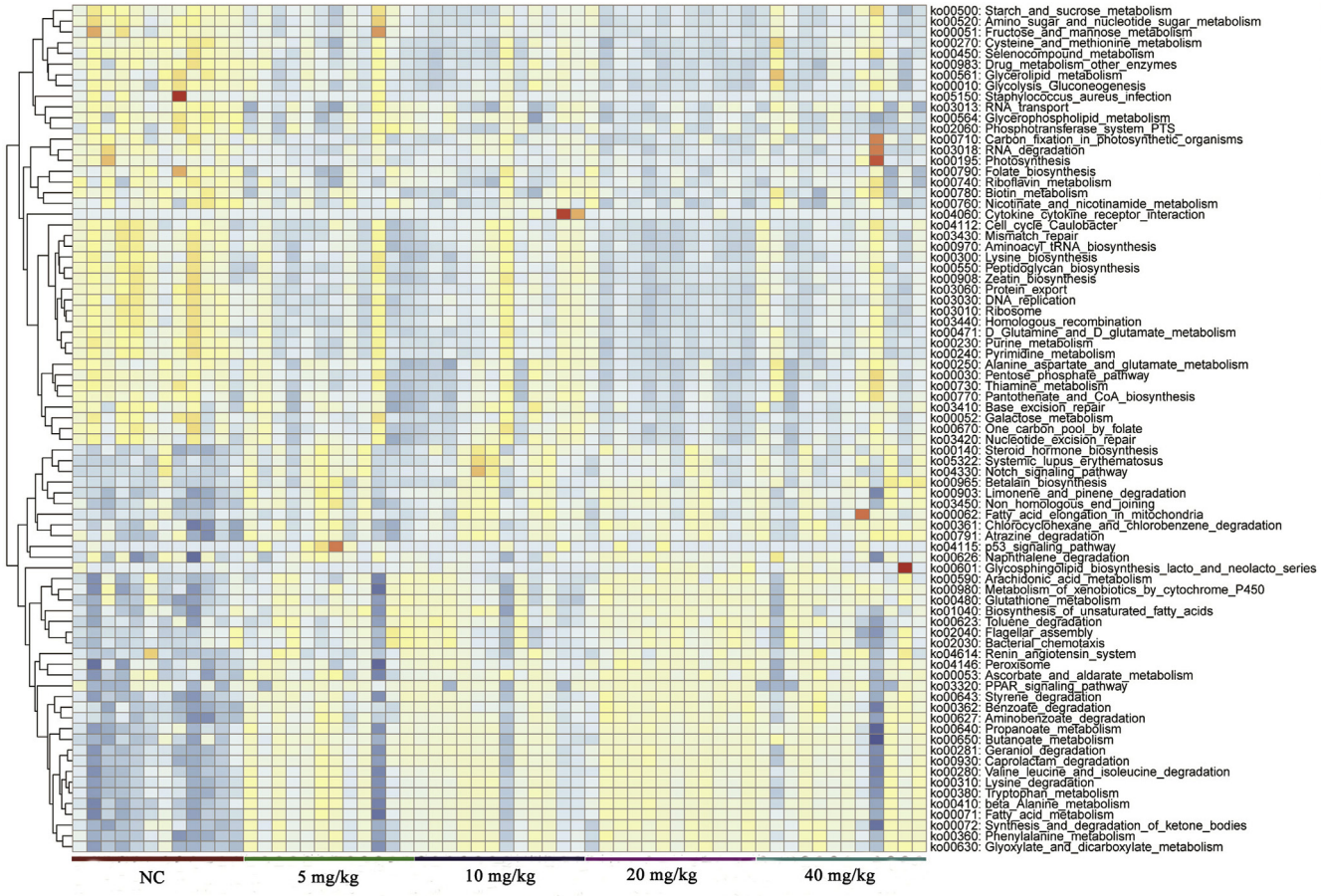

Fig. 11. KEGG pathway heat map comparing the function of differential bacteria among five dietary groups. KEGG pathway annotation information enrichment using Picrust gene function and KEGG comparisons. According to kruskal test, $P < 0.05$ was selected.

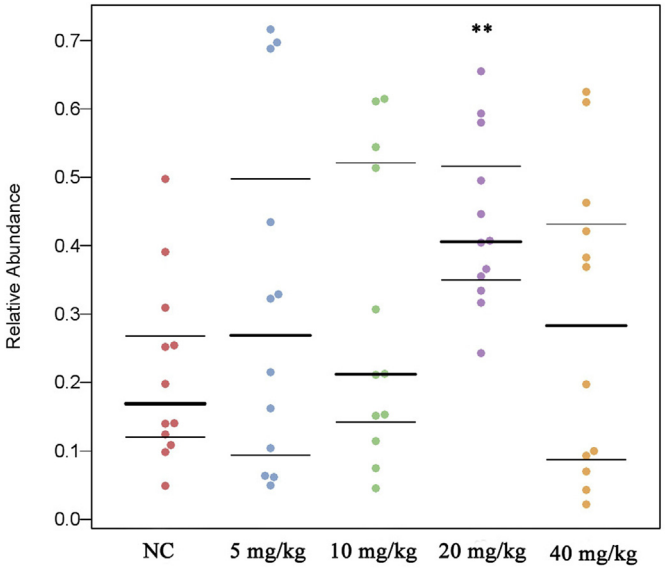

Fig. 12. Oxidative stress tolerance phenotypes among the five dietary groups. Phenotypic analysis of microbial samples using BugBase software based on OTU and mapping data. *, $P \leq 0.05$; **, $P \leq 0.01$.

analyses revealed that gut bacterial communities were altered by the different doses of *G. elegans* alkaloids. We found significant differences between the experimental groups and control group especially between 20 mg/kg and the other groups. This indicated that the diversity and composition of gut microbiota were greatly affected by *G. elegans* alkaloids in *M. amblycephala*.

Previous studies have shown that the core gut microbiota in fish is primarily composed of *Proteobacteria* and *Firmicutes* members [57,58]. We found similar results despite the obvious plasticity in the bacterial diversity and composition. The gut microbiota in all our intestinal samples were dominated by the two phyla *Proteobacteria* and *Firmicutes*, possibly constituting the core gut microbiota of *M. amblycephala*. Furthermore, these two phyla showed significant differences between the five dietary groups suggesting that *G. elegans* alkaloids may affect the core microbiota in the gut of *M. amblycephala*. In particular, the alkaloids increased the abundance of *Proteobacteria* and decreased the abundance of *Firmicutes*. In addition, the phylum *Bacteroidetes* also displayed a high relative abundance. However, the functions played by *Proteobacteria*, *Firmicutes* and *Bacteroidetes* in the fish intestine microbiota have not been extensively explored. In general, the ratio of *Firmicutes* to *Bacteroidetes* had been associated with obesity and weight gain in the human gut microbiota [59]. This suggests that *G. elegans* alkaloids may affect the obesity and weight of *M. amblycephala* and this result corresponds to our previous research [17].

At the genus level we observed large differences in the microbiota among our five dietary groups. *Rhodobacter* and *Staphylococcus* were the most abundant and were identified as microbiological markers of the gut microbiota between the experimental groups and control. In previous studies, *Rhodobacter* extracts (Lycogen) activated macrophages and prolonged the survival of mice with severe colitis [60]. After cyprinid herpesvirus 2 infections, the abundance of *Rhodobacter* decreased significantly suggesting that *Rhodobacter* is important for disease resistance [61]. *Rhodobacter* is also potential probiotic bacteria and

contributed to the enhanced growth of grass carp [62] and improved the fatty acid composition of chicken meat [63]. In addition, *Rhodobacter* exhibits strong antioxidant activities in Caco-2 cells [64]. Overall, these studies have demonstrated that *Rhodobacter* is a candidate probiotic for fish.

In contrast, *Staphylococcus* is not a probiotic bacterium and most species are pathogenic, especially *Staphylococcus aureus*. *S. aureus* infections can cause autoimmune disease and excessive inflammation while increasing the risk of invasion by other pathogens [65]. Increased intestinal level of *S. aureus* has been correlated with intestinal damage [66]. Moreover, the increased abundance of intestinal *Staphylococcus epidermidis* most likely reflects increased exposure of these subjects to opportunistic pathogens [67] and *S. epidermidis* gut colonization can cause serious pathological changes in the gut [68]. We found that *G. elegans* alkaloids increased the abundance of *Rhodobacter* while decreasing *Staphylococcus*, which indicated the alkaloids can resist the invasion of exogenous pathogens and maintain intestinal health. Additionally, the PICRUSt functional predictions and the KEGG pathway enrichment results showed that most of the differential pathways were related to amino acid metabolism, fatty acid metabolism, carbohydrate metabolism and oxidative stress. This suggested that the metabolic capacity of gut microbiota was affected by *G. elegans* alkaloids. Furthermore, the BugBase bacterial phenotype prediction results further showed that adding *G. elegans* alkaloids to diets at the 20 mg/kg level can significantly improve the antioxidant capacity of intestinal microbiota in *M. amblycephala*. This study is only a preliminary step in discovering the effects that *G. elegans* alkaloids have on the intestinal microbiota of *M. amblycephala*.

5. Conclusions

Our study showed for the first time that dietary *G. elegans* alkaloids improved intestinal morphology by increasing villus length, muscle thickness and villus number. Dietary *G. elegans* alkaloids improved intestine antioxidant status *via* increasing antioxidant enzyme activities and expression of related genes. Furthermore, dietary *G. elegans* alkaloids improved intestine immunity by regulating expression of cytokine-related genes. Lastly, this study showed the changes in the intestinal microbiota of *M. amblycephala* after feeding the *G. elegans* alkaloids. The *G. elegans* alkaloid feed additive may be beneficial to enhance fish immune functions and reduce the invasion of pathogenic bacteria. Further studies would be required to investigate the specific action mechanism of *G. elegans* alkaloids in the intestine of *M. amblycephala*.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2019.09.048.

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