Heliyon 10 (2024) e29008

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon

Research article

Effect of dietary supplementation *Ampelopsis grossedentata* extract on growth performance and muscle nutrition of *Megalobrama hoffmanni* by gut bacterial mediation

Qiuling Yang [a,c,1], Yunfan Wang [b,1], Geng Li [b], Xiaoying Huang [b], Lingyan Zheng [b], Mijun Peng [b], Yong Cao [a], Xuesong Wang [b,*]

[a] *Guangdong Provincial Key Laboratory of Nutraceuticals and Functional Foods, College of Food Science, South China Agricultural University, Guangzhou, 510642, China*

[b] *Chinese Academy of Inspection & Quarantine Greater Bay Area, Zhongshan, 528437, China*

[c] *Guangdong Provincial Key Laboratory of Chemical Measurement and Emergency Test Technology, Guangdong Provincial Engineering Research Center for Ambient Mass Spectrometry, Institute of Analysis, Guangdong Academy of Sciences (China National Analytical Center, Guangzhou), Guangzhou, 510070, China*

ARTICLE INFO

Keywords:
Megalobrama hoffmanni
Ampelopsis grossedentata extract
Growth performance
Fatty acids
Gut microbiota

ABSTRACT

Nowadays, *Megalobrama hoffmanni* is a typical cultured fish in south China due to its resource decline in the Pearl River. Meanwhile, since antibiotics had been banned internationally, Chinese medical herbal plant serving as alternative to antibiotics has been adopted in aquaculture. In the present study, to ensure the health growth of *M. hoffmanni*, extract of traditional medical herbal plant *Ampelopsis grossedentata* was dietary supplemented and a series experiments were performed, including growth performance determination, physiological/biochemical detection, nutrition analysis, histology analysis, and 16S rRNA amplicon sequencing. Growth performance enhancement was determined since the weight gain rate (WGR), specific growth rate (SGR), and condition factor (CF) of *M. hoffmanni* increased as feeding inclusion *A. grossedentata* extract. Interestingly, the total content of muscle fatty acids ascended via supplementing *A. grossedentata* extract at middle level, in which group the activities of superoxide dismutase (SOD) and catalase (CAT) significantly increased and thus retarded the lipid peroxidation process (manifesting as malondialdehyde (MDA) content rising). Additionally, immune response and inflammatory reaction was stimulated in low and high level *A. grossedentata* extract added groups, indicating a suitable dosage of *A. grossedentata* extract benefited in safety production. Moreover, gut microbiota community varied hugely as daily supplementation *A. grossedentata* extract and the keystone species were tightly related to lipid transformation, which ultimately led to fatty acids composition variation. Our results confirmed that dietary supplementation *A. grossedentata* extract at the middle level (0.5‰, w/w) is suitable for serving as feed additive in healthful aquaculture of *M. hoffmanni*.

* Corresponding author.
E-mail address: wangxs@caiqgba.org.cn (X. Wang).

[1] These authors (Q. Yang and Y. Wang) contribute equally to this work.

https://doi.org/10.1016/j.heliyon.2024.e29008
Received 7 January 2024; Received in revised form 19 March 2024; Accepted 28 March 2024
Available online 1 April 2024

1. Introduction

Genus Megalobrama possess kinds of fishes, such as *M. hoffmanni*, *M. amblycephala*, *M. pellegrini*, and *M. skolkovii*. Of these, *M. hoffmanni* is the typical economic freshwater fish in China [1–3]. Commonly, the Pearl River and the drainage area of Hainan Island are the important habitats of *M. hoffmanni* [4], which major locate in the subtropical region. As a typical endemic fish, *M. hoffmanni* is popular in local residents and its product is considered as local specialty. However, due to the Pearl River Delta becoming the economic engine of China, the Pearl River has suffered serious environmental disruption during the past 40 years, such as overfishing, water contamination, vessel navigation, and water conservancy construction [5–8]. According to these reasons, the number of *M. hoffmanni* rapidly decreased since 1980 [9]. Currently, in order to preserve fishery resource and meet the food demands of local residents, *M. hoffmanni* has been cultured on a large scale. However, little research regarding *M. hoffmanni* has been reported by far, and no article is about the healthful aquaculture of *M. hoffmanni*.

Formerly, antibiotic was used to assure the normal production in aquaculture. Meanwhile, antibiotic molecules were well associated with the high subjects' densities in aquaculture based on a horizontal concept, e.g., 100–350 mg/L for oxytetraycline hydrochloride [10]. Currently, antibiotics has been prohibited application in animal industries internationally since plenty of negative effects boomed out as time progressing, e.g., genetic toxicity, reproduction toxicity, three cause effects (teratogenicity, mutagenicity, and carcinogenicity), and antibiotic resistance of superbug [11–13]. Therefore, it is timely needed to find new alternative to antibiotics and Chinese medical plants possessing the merits of safety, green, and efficiency are the ideal candidates [14–16]. Nowadays, traditional medical plants of China have been performed in animal breeding based on two aspects: provide safe and eco-friendly active components for replacing either antibiotics or chemicals; and enhance growth performance and body health of animals [17–19]. For example, diet supplementation *Flos lonicerae* [20] and *Hydrastis canadensis* extracts [21] can elevate the laying rates and body health of hens via modifying gut microbiota in the poultry industry. Therefore, diet addition of medical plant extracts have a huge foreground for serving as feed additives in aquaculture.

Ampelopsis grossedentata (Hand.-Mazz.), generally known as "Tengcha" in China, is a traditional Chinese herbal medicine and has been used as tea for hundreds years [22]. Since 2018, the leaf of *A. grossedentata* is promulgated as feed material in China and the extract of *A. grossedentata* leaf has been applied in the poultry [23] as well as livestock science [24]. Simultaneously, previous evidence has reported that the extract of *A. grossedentata* could enhance the anti-oxidative capacity and alleviate intestinal inflammation of model animals, such as mice and drosophila [25], certifying *A. grossedentata* extract may serve as a valuable dietary supplement. Based on the major component dihydromyricetin (DMY), *A. grossedentata* exhibited multiple pharmacological activities, e.g., lipid-lowering, anti-hypertension, and immunity boosting [26,27]. Moreover, dietary supplementation *A. grossedentata* extract can ameliorate the nutrition level of animal products, such as the serum amino acid composition of pigs and the meat quality of pork improve as daily feeding inclusion *A. grossedentata* extract [28]. Although *A. grossedentata* extract functioned as feed additive has been taken into practice, the relevant feeding validation in real production is still limited, especially in aquaculture. Therefore, real application of *A. grossedentata* extract in aquaculture is meaningful and its effects on growth performance and body health should be investigated.

In the present study, we aimed to evaluate the effect of dietary supplementation *A. grossedentata* extract on *M. hoffmanni*. Growth performance, physiological/biochemical indexes, muscle nutrition, histology, and gut microbiota were assessed by daily supplementation *A. grossedentata* extract. We hypothesis that feeding inclusion *A. grossedentata* extract can ameliorate the growth performance and muscle nutrition level of *M. hoffmanni*. Our results will broaden the prospect of *A. grossedentata* extract serving as feed additive in aquaculture and contribute to fishery resource protection.

2. Materials and methods

2.1. Preparation of A. grossedentata extract

The leaves of *A. grossedentata* were purchased from Maoyanmei Co., Ltd (Zhangjiajie, China) and were dried at 50 °C for 3 d. Subsequently, the obtained dry leaves were extracted twice with 50% edible ethanol (liquid:solid, 1:10, 80 °C, 1 h). Next, the extraction liquid was concentrated to 20 Brix by vacuum enrichment method and the concentrated extraction was dried by spray drying. During spray drying, the inlet and outlet air temperature were 150–170 °C and 90–95 °C, respectively. The content of the characteristic compound DMY in *A. grossedentata* extract was analyzed based on previously described method [23].

2.2. Animal experiment and treatment

Juvenile *M. hoffmanni* (mean body weight 1 g, mean body length 5 cm) were provided by Guangdong Fulong aquaculture company (Guangzhou, China). Basal feed pellets (1038 original pond particles) were provided by Shandong Tongwei Feed Co., Ltd. (Zibo, China). To prepare the compound feed pellets, basal feed pellets and *A. grossedentata* extract were filtered through 200 stainless mesh and then homogenized completely. The ratio between feed pellets and *A. grossedentata* extract were in line with the designed experimental concentration. After homogenization, the obtained compound feed pellets were gently mixed with sterile water (diets: water = 4:1, w/v) and then prepared the final compound feed pellets with a feed pellets mill following the condition of 56 °C for 1 h. The final compound feed pellets were used in animal experiment.

During animal experiments, a total of twelve 50 L food-grade high-density polyethylene aquaculture tanks were used, with a size of 48.7 × 34.3 × 25.8 cm. Meanwhile, a total of 240 acclimated juvenile *M. hoffmanni* were randomly divided into four groups: A group, basal feed pellets (control); B group, basal feed pellets supplementation 0.1‰ *A. grossedentata* extract (w/w); C group, basal feed pellets

supplementation 0.5‰ *A. grossedentata* extract (w/w); D group, basal feed pellets supplementation 1‰ *A. grossedentata* extract (w/w). Each aquaculture tank contained 20 individuals and each group contained three tanks. The feeding period was 28 d based on the guidelines of testing tolerance evaluation for feed and feed additives in aquaculture and feeding inclusion *A. grossedentata* extract was performed. During the feeding period, these juvenile *M. hoffmanni* were allowed free access to feed pellets and excessive feed pellets were supplied. The feed conversion ratio in this study was neglected due to the feed pellets would absorb water, thereby the weight of recovered feed pellets was not accurate after feeding. In addition, the final body lengths as well as the final body weights of *M. hoffmanni* were detected individually after feeding (Table S1), and then euthanized in tricaine methanesulfonate with a purity of 98% (Sigma-Aldrich®, Saint Louis, MO, USA). Tissues (including gill, liver, gut, muscle, and spleen) of *M. hoffmanni* were collected for either histology or physiological/biochemical analysis.

2.3. Growth performance assessment

Growth performance of *M. hoffmanni* was exhibited as five parameters, e.g., weight gain rate (*WGR*, %), specific growth rate (*SGR*, %/d), condition factor (*CF*, %), hepatosomatic index (*HSI*, %), and viserosomatic index (*VSI*, %).

$$WGR = \frac{(final\ weight - initial\ weight)}{initial\ weight} \times 100;$$

$$SGR = 100 \times (\ln(final\ weight) - \ln(initial\ weight)) \div (feeding\ days);$$

$$CF = 100 \times weight / (length)^3;$$

$$HSI = 100 \times (liver\ weight / body\ weight);$$

$$VSI = 100 \times (visceral\ weight / body\ weight)$$

Of these, final weight is the body weight after feeding; initial weight is the body weight at the beginning of feeding; feeding days is 28 d; gram (g) is the unit of weight; centimeter (cm) is the unit of length.

2.4. Histology analysis

Four tissues (gill, liver, gut, and muscle) of *M. hoffmanni* were fixed with Bouin's solution. After dehydration with graded ethanol (99% purity) and rinse with xylene (Guangzhou chemical reagent factory, Guangzhou, China), the four selected tissues were embedded in paraffin individually and cooled down at room temperature. Then, 4 μm-thick section was cut from the solid paraffin by a microtome (Leica Biosystems Nussloch GmbH, Nussloch, Germany). Subsequently, hematoxylin and eosin mixture (Sigma-Aldrich®, Saint Louis, MO, USA) was used to stain. The morphology of the four selected tissues was observed by an inverted microscope (ECHO, Chicago, Illinois, USA).

2.5. Physiological and biochemical indexes determination

The activities of two antioxidant enzymes (superoxide dismutase (SOD) and catalase (CAT)) and the content of antioxidant compound (malondialdehyde (MDA)) in gill, liver, gut, and muscle of *M. hoffmanni* were assayed by using the relative chemical colorimetric kits (Beijing Solarbio Science & Technology Co.Ltd., Beijing, China). Additionally, the contents of immunoglobulins (IgM, IgG, and IgA) and interleukins (IL-1β, IL-2, IL-4, IL-6, IL-10, and IL-12) in spleen of *M. hoffmanni* were also monitored by using the relative commercial kits (Shanghai Enzyme-linked Biotechnology Co. Ltd., Shanghai, China). All of these physiological and biochemical indexes were determined following the manufacturer's protocols. The activities of antioxidant enzymes and contents of antioxidant compound, immunoglobulins as well as interleukins were analyzed with three biological replicates and three technique replicates.

2.6. Muscle nutrition detection

Muscle fatty acids and muscle amino acids of *M. hoffmanni* were detected in this study. For muscle fatty acids, gas chromatographic analysis was conducted, which was performed on GC-2010 plus (Shimadzu, Kyoto, Japan) and equipped with a HP-88 column (100 m × 0.25 mm × 0.2 μm, Agilent, California, USA). During detection, a flame ionization detector was necessary. For muscle amino acids, L-8900 amino acid analyzer (Hitachi, Kyoto, Japan) was used. The detailed information of muscle fatty acids and muscle amino acids detection were serious following the standards of GB 5009.168 and GB 5009.124, respectively.

2.7. Safety inspection of muscle

For safety inspection, muscle of *M. hoffmanni* was adopted. All of the detected indexes were selected according to the national food safety standards of China. Two typical food pathogenic bacteria (*Escherichia coli* and *Salmonella*) in muscle were monitored following the standards of GB 4789.3–2016 and GB 4789.4–2016, respectively. Meanwhile, four typical heavy mental (As, Hg, Pb, and Cd) in

muscle were evaluated seriously according to standard of GB 5009.74–2014. In addition, three typical pesticides (hexachlorocyclohexane (HCH), dichlorodiphenyl trichloroethane (DDT), and quintozene (PCNB)) in muscle were also assayed following standards of GB/T 5009.19–2008 and GB/T 5009.136–2003, respectively.

2.8. 16S rRNA amplicon sequencing analysis

The DNA sample of gut contents was extracted by using the relative isolation kit (Qiagen NV, Germany). During amplication, typical forward primer (341F 5′-CCTACGGGNGGCWGCAG-3′) and reverse primer (805R 5′-GACTACHVGGGTATCTAATCC-3′) were added and the specific regions of extracted DNA were amplified. Simultaneously, the thermocycling program was formulated as follow: first, denaturation at 98 °C for 30 s; second, amplication at 98 °C for 10 s, drop to 54 °C for 30 s, then 72 °C for 45 s, with 35 cycles; last, elongation at 72 °C for 10 min. Subsequently, the obtained amplicons were purified and quantified, and target region (V3–V4) was applied to 16S rRNA sequencing. The 16S rRNA amplicon sequencing was performed on an Illumina HiSeq 2500 platform (Illumina, San Diego, USA) and the obtained data had been deposited into a publicly available repository (GSA:CRA015097). Divisive amplicon denoising algorithm (DADA2) method was used to collect clean data and amplicon sequence variants (ASVs) were clustered based on the sequences with 100% similarity, which then matched with SILVA database. To detect the microbial diversity, α-diversity (Chao1, Simpson, and Shannon) were evaluated based on diversity plugin. Meanwhile, β-diversity based on permutational multivariate analysis of variance (PERMANOVA) was also evaluated according to Bray-Curthis distances and principal coordinate analysis (PCoA). To screen the potential keystone species of microbial community, co-occurrence networks were constructed. Generally, $r \geq 0.7$ and $P < 0.05$ were set as the threshold of Spearman's correlations analysis during co-occurrence networks construction. Furthermore, random forest test equipped with ten-fold cross validation was carried out to infer potential keystone species. Among random forest test, *A. grossedentata* extract supplemented groups (B, C, and D groups) were designated as "NOT" in comparison of A group. Lastly, multiple correlations between keystone species, imputed functional profiles as well as fatty acids were calculated. Generally, $r \geq 0.3$ and $P < 0.05$ were set as the threshold during multiple correlations. The obtained data was visualized as the Sankey plot. RStudio software (version 2022.07.0) was used to graph all plots by using the package EasyMicroPlot.

2.9. Statistical analysis

Data was expressed as the mean ± Standard Deviation (SD) in this study. Normality and homoscedasticity of data were evaluated via combination Kolmogorov-Smirnov test and Bartlett's test together. When data was normally distributed, the significant difference among the four tested groups was examined by one-way analysis of variance (ANOVA) coupled with Tukey multiple comparison test. When parametric assumptions were not met, the significant difference among the four tested groups was examined by nonparametric Kruskal-Wallis test coupled with Dunn's multiple comparisons test. In multiple comparisons, groups with $p < 0.05$ were considered statistically significance. WPS office (Kingsoft office, Beijing, China) together with GraphPad Prism version 9.4.0 (GraphPad Software,

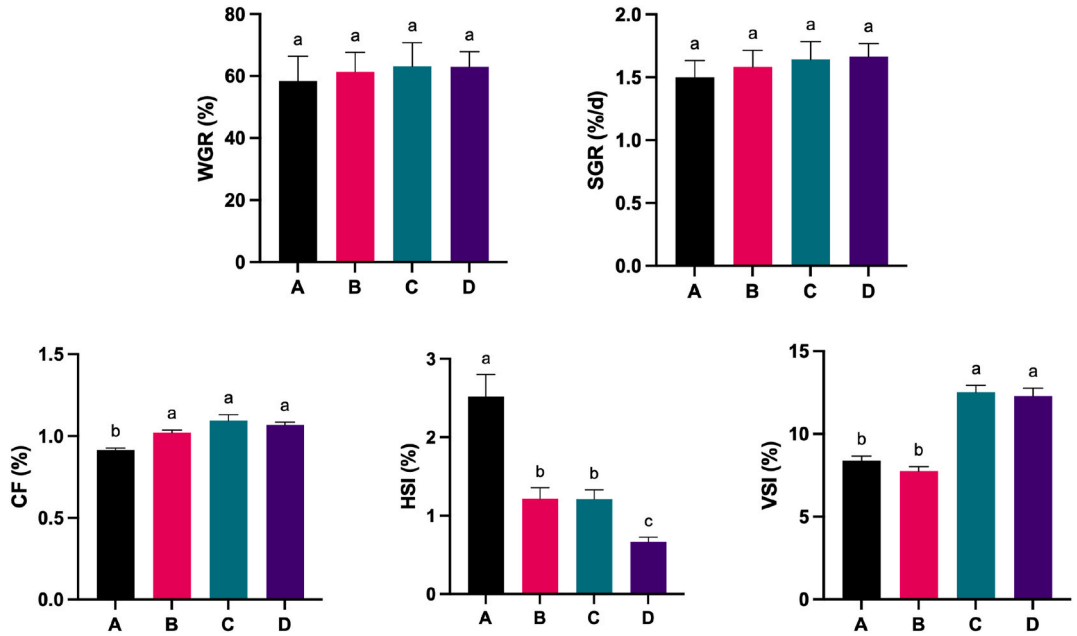

Fig. 1. Variation in morphological indices of *Megalobrama hoffmanni*. WGR is weight gain rate; SGR is specific growth rate; CF is condition factor; HSI is hepatopancreas somatic indices; VSI is visceral somatic indices. Different small letters indicate significant differences at $p < 0.05$ level under different treatments.

San Diego, CA, USA) were used to draw pictures and tables.

3. Results

3.1. Content of DMY

The chromatogram of DMY in *A. grossedentata* extract is shown in Fig. S1. The DMY content in the prepared *A. grossedentata* extract was 38.02 g/100 g. Subsequently, the obtained *A. grossedentata* extract was used for feed pellets preparation.

3.2. Growth performance of M. hoffmanni

Although no significant difference was detected ($p > 0.05$), slightly elevation in WGR and SGR of *M. hoffmanni* were determined by daily supplementation *A. grossedentata* extract in comparison with control (A group) after 28 d feeding (Fig. 1). Simultaneously, CF of *M. hoffmanni* significantly increased in all of the *A. grossedentata* extract supplemented groups (B, C, and D groups) when compared to control ($p < 0.05$), indicating feeding inclusion *A. grossedentata* extract was growth performance promoting. However, CF of *M. hoffmanni* did not reveal significantly difference among the three *A. grossedentata* extract supplemented groups ($p > 0.05$). Interestingly, HSI of *M. hoffmanni* significantly decreased as diet addition of *A. grossedentata* extract in comparison to control ($p < 0.05$) and feeding inclusion 1‰ *A. grossedentata* extract (D group) exhibited the lowest HSI index among the three *A. grossedentata* extract added groups. Additionally, VSI significantly increased in middle (C group) and high (D group) level *A. grossedentata* extract added groups ($p < 0.05$) when compared to control (A group) as well as low level *A. grossedentata* extract added group (B group). These results suggested that dietary supplementation *A. grossedentata* extract had benefits in fatty liver lowering and potentially contributed to fat transferring to the mesentery.

3.3. Muscle fatty acids and amino acids of M. hoffmanni

The contents of muscle fatty acids of *M. hoffmanni* are shown Table 1. Of these, oleic acid (C18:1n9c) was the predominant fatty acids among the four tested groups with a content higher than 1.2 g/100 g FW (fresh weight). Surprisingly, the contents of total fatty acids (TFA) in *A. grossedentata* extract supplemented groups significantly changed and fatty acids were the richest in the middle level added group (C group), followed by control (A group), and were poor in the other groups (B and D groups). Besides, nine fatty acids (including pentadecanoic acid (C15:0), palmitic acid, (PA; C16:0), palmitoleic acid (C16:1n7), heptadecaenoic acid (C17:1n7), elaidic acid (C18:1n9t), eicosapentaenoic acid (EPA; C20:5n3), arachidonic acid (AA; C22:4n6), docosapentaenoic acid (DPA; C22:5n3), and docosahexaenoic acid (DHA; C22:6n3)) were significant higher in middle level *A. grossedentata* extract added group (C group) than that of the other groups ($p < 0.05$), indicating daily feeding *A. grossedentata* extract at a suitable level contributed to fatty acids

Table 1
The fatty acids contents in fish muscles of the four tested groups. TFA is total fatty acids content; FW is fresh weight. Different small letters indicate significant differences at $p < 0.05$ level under different treatments. "-" represent no detection.

Item (g/100 g FW)	A	B	C	D
C4:0	0.737 ± 0.025^{a}	0.738 ± 0.022^{a}	0.749 ± 0.020^{a}	0.721 ± 0.030^{a}
C8:0	0.011 ± 0.001^{a}	0.009 ± 0.001^{a}	0.009 ± 0.001^{a}	0.011 ± 0.002^{a}
C14:0	0.111 ± 0.015^{a}	0.099 ± 0.014^{a}	0.131 ± 0.030^{a}	0.097 ± 0.028^{a}
C15:0	0.018 ± 0.002^{b}	0.016 ± 0.002^{b}	0.024 ± 0.002^{a}	0.019 ± 0.003^{b}
C16:0	0.820 ± 0.40^{b}	0.765 ± 0.082^{b}	0.960 ± 0.043^{a}	0.759 ± 0.050^{b}
C16:1n7	0.351 ± 0.021^{b}	0.303 ± 0.043^{b}	0.395 ± 0.010^{a}	0.302 ± 0.033^{b}
C17:0	0.018 ± 0.002^{a}	0.015 ± 0.001^{a}	0.017 ± 0.001^{a}	0.015 ± 0.001^{a}
C17:1n7	0.025 ± 0.004^{b}	0.026 ± 0.003^{b}	0.033 ± 0.002^{a}	0.025 ± 0.002^{b}
C18:0	0.190 ± 0.016^{a}	0.177 ± 0.013^{b}	0.212 ± 0.018^{a}	0.183 ± 0.029^{a}
C18:1n9t	0.010 ± 0.000^{b}	–	0.012 ± 0.001^{a}	0.010 ± 0.001^{b}
C18:1n9c	1.400 ± 0.252^{a}	1.259 ± 0.142^{a}	1.498 ± 0.054^{a}	1.209 ± 0.073^{a}
C18:2n6c	0.497 ± 0.062^{a}	0.474 ± 0.034^{a}	0.496 ± 0.037^{a}	0.449 ± 0.027^{a}
C20:0	0.012 ± 0.002^{a}	0.011 ± 0.001^{a}	0.013 ± 0.002^{a}	0.011 ± 0.000^{a}
C18:3n6	0.011 ± 0.002^{a}	0.011 ± 0.002^{a}	0.012 ± 0.000^{a}	0.011 ± 0.001^{a}
C18:3n3	0.032 ± 0.003^{a}	0.028 ± 0.003^{a}	0.033 ± 0.003^{a}	0.027 ± 0.002^{a}
C20:1n9	0.040 ± 0.005^{a}	0.038 ± 0.004^{a}	0.045 ± 0.003^{a}	0.035 ± 0.002^{a}
C20:2n6	0.022 ± 0.002^{a}	0.020 ± 0.001^{a}	0.022 ± 0.003^{a}	0.020 ± 0.001^{a}
C20:3n6	0.041 ± 0.003^{a}	0.042 ± 0.004^{a}	0.044 ± 0.002^{a}	0.037 ± 0.003^{a}
C20:4n6	0.098 ± 0.003^{a}	0.098 ± 0.011^{a}	0.106 ± 0.001^{a}	0.094 ± 0.009^{a}
C20:5n3	0.076 ± 0.005^{b}	0.071 ± 0.009^{b}	0.093 ± 0.007^{a}	0.071 ± 0.006^{b}
C24:1n9	0.008 ± 0.001^{a}	0.006 ± 0.005^{a}	0.008 ± 0.001^{a}	0.002 ± 0.004^{a}
C22:4n6	0.009 ± 0.001^{b}	0.009 ± 0.001^{b}	0.012 ± 0.000^{a}	0.009 ± 0.001^{b}
C22:5n6	0.035 ± 0.003^{a}	0.036 ± 0.004^{a}	0.041 ± 0.002^{a}	0.037 ± 0.005^{a}
C22:5n3	0.037 ± 0.003^{b}	0.037 ± 0.007^{b}	0.048 ± 0.003^{a}	0.036 ± 0.004^{b}
C22:6n3	0.261 ± 0.024^{b}	0.255 ± 0.031^{b}	0.325 ± 0.018^{a}	0.250 ± 0.025^{b}
TFA	4.862 ± 0.272^{b}	4.548 ± 0.388^{b}	5.347 ± 0.163^{a}	4.321 ± 0.402^{b}

accumulation. Unfortunately, as shown in Table 2, the muscle amino acids contents exhibited no significant difference among the four tested groups ($p > 0.05$), indicating daily feeding *A. grossedentata* extract could not induce muscle amino acids variation.

3.4. Safety inspection of the muscle of M. hoffmanni

Along with nutrition level detection, safety inspection of the muscle of *M. hoffmanni* was conducted (Table S2). In muscles of *M. hoffmanni*, the two compulsory testing food pathogenic bacteria (*Escherichia coli* and *Salmonella*) and the three common used pesticides (HCH, DDT, and PCNB) were not detected after feeding. Meanwhile, the contents of four selected heavy metals (As, Cd, Hg, and Pb) were lower than 0.1, 0.05, 0.05, and 0.2 mg/kg in muscles of *M. hoffmanni* among the four tested groups, which met the standard limits of GB 2762-2022 and GB 2763-2021 (national food safety standards of China). These results manifested that feeding inclusion *A. grossedentata* extract did not bring any food safety issues (e.g., pathogenic bacteria, pesticide residues, and heavy metals) in healthful aquaculture of *M. hoffmanni*.

3.5. Antioxidant indexes of M. hoffmanni

Due to the huge alterations in fatty acids that occurred by daily supplementation *A. grossedentata* extract, three lipid related antioxidant indexes was analyzed (Fig. 2). Intriguingly, the MDA concentrations in four tested tissues (gill, liver, gut, and muscle) significantly increased in *A. grossedentata* extract added groups when compared to control ($p < 0.05$), indicating *A. grossedentata* extract might potentially trigger lipid peroxidation. Meanwhile, apparent up-regulation in SOD activities was determined in gill and muscle in *A. grossedentata* extract added groups, while visible down-regulation in SOD activities was monitored in liver and gut, especially supplementation *A. grossedentata* extract at the middle level (C group). Similar to SOD, the CAT activities increased in gill and muscle, and decreased in gut as diet addition of *A. grossedentata* extract. However, the CAT activities in the liver was significantly elevated if supplementation *A. grossedentata* extract at low (B group) or middle (C group) level. Therefore, elevation in MDA content might be the result of antioxidant system disruption as feeding inclusion *A. grossedentata* extract.

3.6. Immunoglobulins/interleukins indexes in spleen of M. hoffmanni

Except antioxidant indexes, three typical immunoglobulins (IgG, IgM, and IgA) and six typical interleukins (IL-1β, IL-2, IL-4, IL-6, IL-10, and IL-12) were evaluated (Fig. 3). Except IgA in B group, significant elevation in IgG, IgM, and IgA contents were noted when supplementation *A. grossedentata* extract at a relative low (B group) or high (D group) level if compared with the other two groups, implying low or high level *A. grossedentata* extract would stimulate immune response. Additionally, the contents of six interleukins indexes were almost all higher in low (B group) or high (D group) levels *A. grossedentata* extract added groups than control (A group) as well as the middle level added group (C group). Thus, dietary supplementation *A. grossedentata* extract at a suitable level would not bring out strong *in vivo* inflammatory reaction.

3.7. Tissue histology of M. hoffmanni

The tissue histology of *M. hoffmanni* is shown in Fig. 4. Four tissues (gill, liver, gut, and muscle) were analyzed and no inflammatory

Table 2
The amino acids contents in fish muscles of the four tested groups. DAA is delicious amino acids; SAA is sweet amino acids; BAA is bitter amino acids; EAA is essential amino acids; TAA is total amino acids; FW is fresh weight. Different small letters indicate significant differences at $p < 0.05$ level under different treatments.

Item	Amino acid (g/100 g FW)	A	B	C	D
DAA	Asp	1.59 ± 0.08^a	1.57 ± 0.09^a	1.54 ± 0.07^a	1.71 ± 0.08^a
	Glu	2.58 ± 0.15^a	2.49 ± 0.05^a	2.48 ± 0.08^a	2.58 ± 0.03^a
	Phe	0.70 ± 0.07^a	0.67 ± 0.03^a	0.67 ± 0.02^a	0.73 ± 0.03^a
	Gly	1.06 ± 0.08^a	1.01 ± 0.12^a	0.95 ± 0.10^a	0.95 ± 0.04^a
	Tyr	0.56 ± 0.05^a	0.58 ± 0.03^a	0.61 ± 0.09^a	0.65 ± 0.09^a
	Ala	1.06 ± 0.05^a	1.02 ± 0.04^a	1.01 ± 0.02^a	1.04 ± 0.07^a
SAA	Lys	1.40 ± 0.06^a	1.36 ± 0.04^a	1.35 ± 0.10^a	1.36 ± 0.02^a
	Pro	0.66 ± 0.11^a	0.66 ± 0.04^a	0.59 ± 0.06^a	0.55 ± 0.33^a
	Ser	0.67 ± 0.04^a	0.64 ± 0.02^a	0.64 ± 0.02^a	0.71 ± 0.04^a
	Thr	0.66 ± 0.04^a	0.69 ± 0.04^a	0.66 ± 0.07^a	0.72 ± 0.13^a
BAA	Val	0.75 ± 0.03^a	0.72 ± 0.02^a	0.71 ± 0.04^a	0.75 ± 0.03^a
	Leu	1.26 ± 0.08^a	1.22 ± 0.03^a	1.23 ± 0.03^a	1.28 ± 0.07^a
	Met	0.32 ± 0.27^a	0.29 ± 0.22^a	0.16 ± 0.25^a	0.34 ± 0.25^a
	Arg	1.09 ± 0.11^a	1.10 ± 0.09^a	1.13 ± 0.11^a	1.15 ± 0.12^a
	His	0.44 ± 0.05^a	0.36 ± 0.01^b	0.43 ± 0.02^a	0.45 ± 0.01^a
	Ile	0.69 ± 0.03^a	0.69 ± 0.06^a	0.68 ± 0.02^a	0.69 ± 0.00^a
Total		15.49 ± 1.18^a	15.35 ± 0.23^a	15.05 ± 0.58^a	15.69 ± 0.33^a
EAA/TAA (%)		37.26	36.75	36.32	37.52
DAA/TAA (%)		38.52	37.63	37.96	37.97

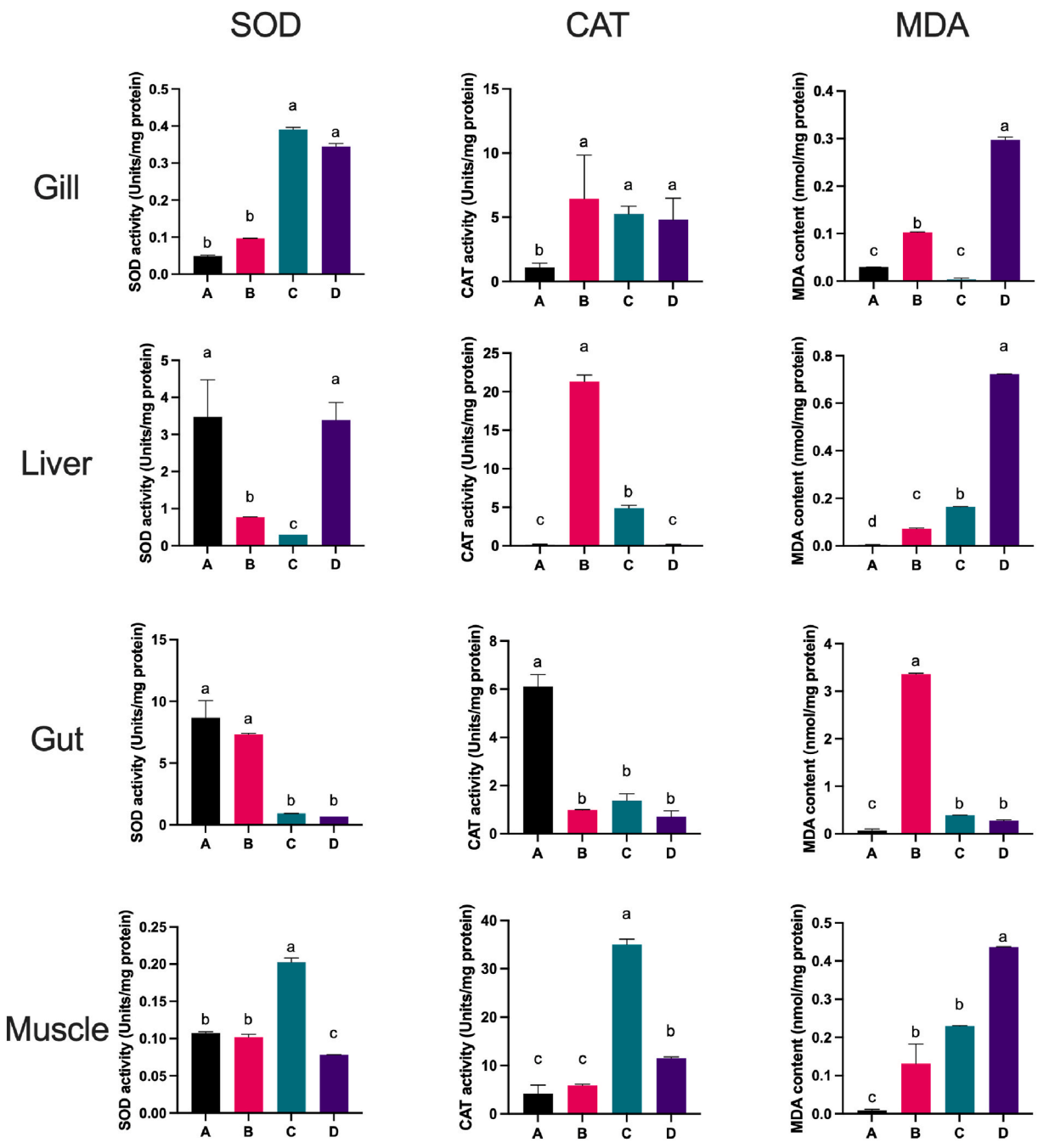

Fig. 2. Antioxidant enzymes (SOD and CAT) and component (MDA) of gill, liver, gut, and muscle in *Megalobrama hoffmanni* of the four tested groups. Different small letters indicate significant differences at $p < 0.05$ level under different treatments.

cell coverage as well as vacuolization of the these tissues were observed among the four tested groups, suggesting that although inflammatory reaction was activated, no *in vivo* oxidative damage was induced by dietary supplementation *A. grossedentata* extract.

3.8. Gut microbiota compositions of *M. hoffmanni*

Significant variations in bacterial community was observed as dietary supplementation *A. grossedentata* extract (Fig. 5). The α-diversity indexes (Chao 1, Simpson, and Shannon) of bacterial community increased as daily supplementation *A. grossedentata* extract, with significant difference in low (B group) and high (D group) level *A. grossedentata* extract added groups in comparison with control (A group) ($p < 0.05$) (Fig. 5A–C). Meanwhile, the β-diversity of gut microbial separated clearly and showed significant

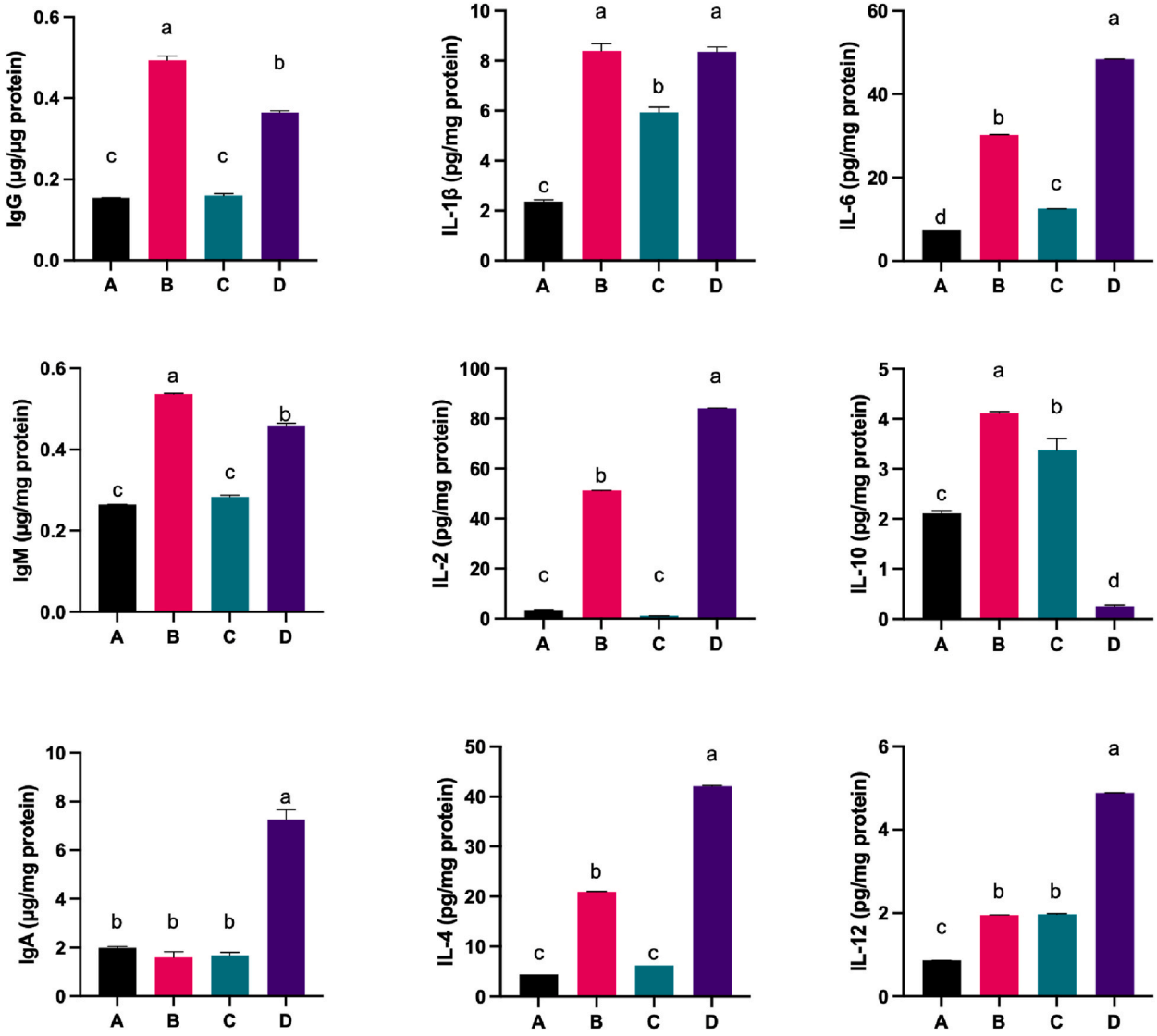

Fig. 3. Immunoglobulins (IgM, IgG, and IgA) and interleukins (IL-1β, IL-2, IL-4, IL-6, IL-10, and IL-12) in *Megalobrama hoffmanni* of the four tested groups. Different small letters indicate significant differences at $p < 0.05$ level under different treatments.

difference among the four tested groups ($p < 0.05$) (Fig. 5D). In addition, the relative abundance of ten top bacterial category changed apparently at class level, e.g., the relative abundance of Actinobacteria, Alphaproteobacteria, and CK-1C4-19 increased as daily feeding *A. grossedentata* extract, whereas the relative abundance of Bacilli decreased (Fig. 5E). These results pointed out that dietary supplementation *A. grossedentata* extract was conducive to gut microbiota alteration.

3.9. Key bacteria involvement with fat accumulation of M. hoffmanni

The top 25 gut microbiota at genus level were selected out (Fig. 5F). Of these, five probiotics (*Propionibacteriaceae*, *Lactobacillales*, *Lactobacillus*, *Enterococcus*, and *Bacteroides*) were observed and no opportunistic pathogen was found. Additionally, according to Random forest test, eleven keystone species were screened out (Fig. 5G), including *Propionibacteriaceae* (V55), *Microbacterium* (V35), *Actinomycetales* (V64), *Rhodobacter* (V264), *Chlamydiia* (V118), *Gemmataceae* (V213), *Rhizobiales* (V256), *Demequina* (V11), *Acidimicrobiales* (V7), CK-1C4-19 (V361), and TM7-1 (V356). Moreover, 25 predicted functional profiles imputed by PICRUSt2 (fatty acid biosynthesis (ko00061), fatty acid degradation (ko00071), steroid biosynthesis (ko00100), primary bile acid biosynthesis(ko00120), secondary bile acid biosynthesis (ko00121), steroid hormone biosynthesis (ko00140), alanine, asparate and glutamate metabolism (ko00250), glycine, serine and threonine metabolism (ko00260), cysteine and methionine metabolism (ko00270), valine, leucine and isoleucine degradation (ko00280), general degradation (ko00281), valine, leucine and isoleucine biosynthesis (ko00290), lysine biosynthesis (ko00300), lysine degradation (ko00310), arginine and proline metabolism (ko00330), histidine metabolism (ko00340),

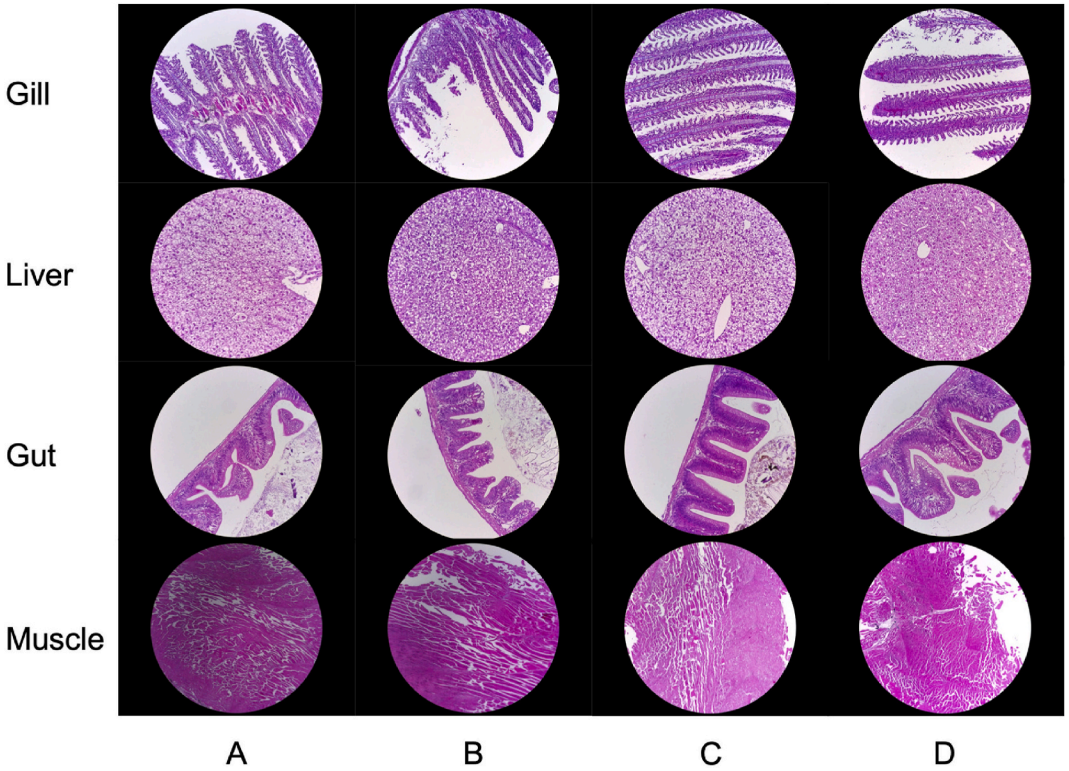

Fig. 4. Histological sections of gill (× 200), liver (× 400), gut (× 400), and muscles (× 100) tissues in *Megalobrama hoffmanni* of the four tested groups (A, B, C, and D).

tyrosine metabolism (ko00350), phenylalanine metabolism (ko00360), tryptophan metabolism (ko00380), phenylalanine, tyrosine and tryptophan biosynthesis(ko00400), glycerolipid metabolism (ko00561), glycerophospholipid metabolism (ko00564), linoleic acid metabolism (ko00591), sphingolipid metabolism(ko00600), and biosynthesis of unsaturated fatty acids (ko01040)) were applied to construct the correlations with keystone species (Fig. 5H). Ten keystone species and six predicted functional profiles exhibited significant positive correlations, which would positively mediate the composition of fatty acids and contributed to fatty acids accumulation (Fig. 5I).

4. Discussion

4.1. Dietary supplementation A. grossedentata extract is growth-promoting

It is well known that medical plants inclusion active compounds contribute to growth performance enhancement in aquaculture [29], such as dietary supplementation with *Panax notoginseng* extract [30], *Origanum majorana* extract [31], and *Crataegus monogyna* extract [32] can significantly elevate the growth performance of *Epinephelus*spp., *Cyprinus carpio*, and *Trachinotus ovatus*, respectively. As a traditional Chinese health food, *A. grossedentata* had been applied in poultry field and exhibited a good potential of laying performance promoting [23]. However, literature regarding the effect of *A. grossedentata* extract application in aquaculture is still scanty. In the present study, slightly elevation in weight growth rates (both WGR and SGR) of *M. hoffmanni* were observed as daily supplementation *A. grossedentata* extract for 28 d, despite no significant difference was detected. Besides, CF increasing supported that feeding inclusion *A. grossedentata* extract was growth performance facilitating. Although this is the first report of *A. grossedentata* enhancing the growth performance of fish, there are little cases reflecting the growth promoting role of *A. grossedentata* in other animals, e.g., broiler [33] and goat [24], which might co-certify that diet addition of *A. grossedentata* extract is growth-promoting in aquaculture.

Apart from growth performance, immune response induced by plant extract is the another key point in aquaculture due to disease is likely outbreaks in intensive aquaculture [34]. Since *A. grossedentata* extract had been certified to stimulate immune response in hens of our previous work [23], the immunoglobulins indexes of *M. hoffmanni* was therefore monitored, together with interleukins indexes. Overall, elevation in both immunoglobulins and interleukins indexes were determined in this study, suggesting dietary supplementation *A. grossedentata* extract would strike inflammatory reaction and improve the immunocompetence of fish. Moreover, no *in vivo* histologic changes of tissues were observed, indicating although immune response and inflammatory reaction had been stimulated in fish, daily feeding *A. grossedentata* extract was safety in aquaculture. Furthermore, no microorganisms, pesticide residues, and heavy

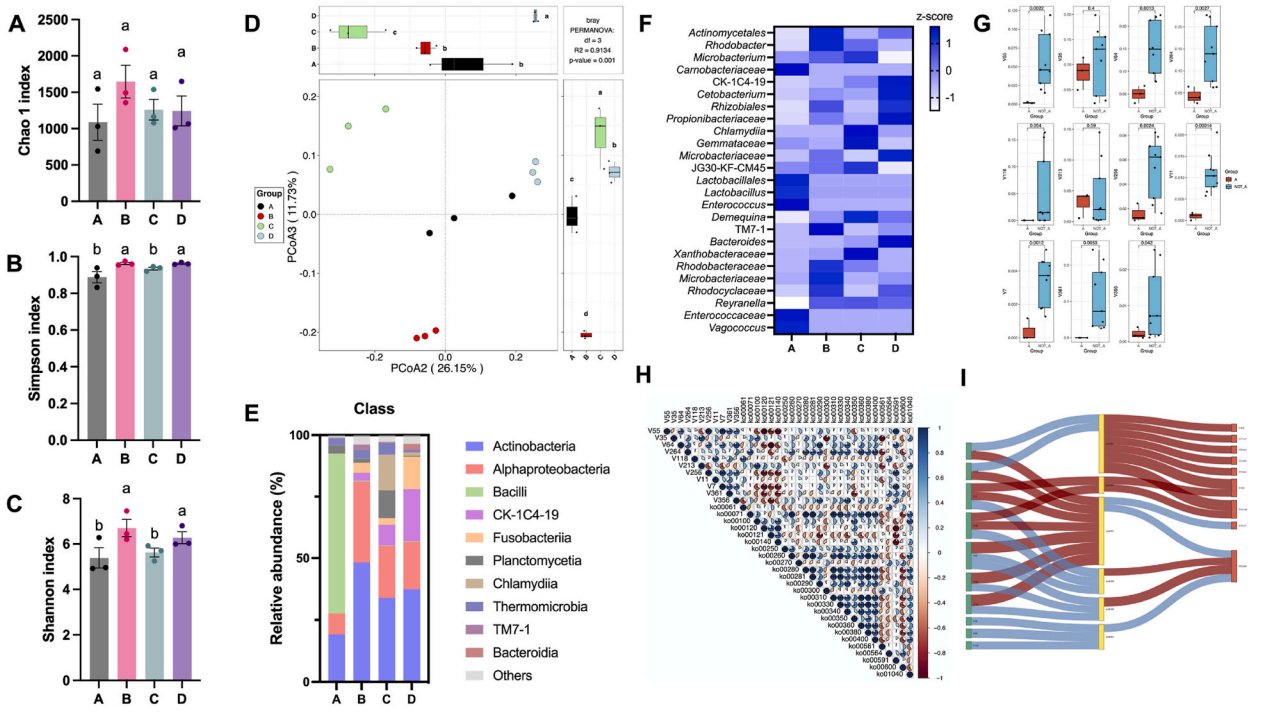

Fig. 5. Variations in gut microbiota of the four tested groups. (A)–(C), alpha diversity. (A), Chao 1; (B) Simpson; (C), Shannon. (D), PCoA coupled with PERMANOVA revealed the beta diversity between groups. (E), top 10 abundant bacterial taxa at the class level. (F), heatmap showing the relative abundance of top 25 genera of bacteria. (G), random forest analysis coupled with ten-fold cross validation revealed the potential keystone taxa after treatment. (H), ternary diagram showing the Spearman's correlations between keystone taxa abundance and functional profiles imputed by PICRUSt2 involved in lipid and amino acid metabolism. (I), Sankey plot showing the relationship between keystone bacterial taxa, imputed functional profiles in gut, and nutrient content in muscle. Only the Spearman's correlation of coefficient $r > 0.3$ and $p < 0.05$ was visualized. The lines in red indicate the positive relationship whereas those in blue mean the negative relationship. Note: Different letters in histogram for alpha diversity indicate the significance between groups under non-parametric Kruskal-Walli's test under Dunn's multiple comparisons correction. Significance of keystone taxa abundance between groups were measured by nonparametric *t*-test under least significant difference (LSD) corrections. Abbreviations: V55, *Propionibacteriaceae*; V35, *Microbacterium*; V64, *Actinomycetales*; V264, *Rhodobacter*; V118, *Chlamydiia*; V213, *Gemmataceae*; V256, *Rhizobiales*; V11, *Demequina*; V7, *Acidimicrobiales*; V361, CK-1C4-19; V356, TM7-1. ko00061, fatty acid biosynthesis; ko00071, fatty acid degradation; ko00100, steroid biosynthesis; ko00120, primary bile acid biosynthesis; ko00121, secondary bile acid biosynthesis; ko00140, steroid hormone biosynthesis; ko00250, alanine, asparate and glutamate metabolism; ko00260, glycine, serine and threonine metabolism; ko00270, cysteine and methionine metabolism; ko00280, ko00281, general degradation; valine, leucine and isoleucine degradation; ko00290, valine, leucine and isoleucine biosynthesis; ko00300, lysine biosynthesis; ko00310, lysine degradation; ko00330, arginine and proline metabolism; ko00340, histidine metabolism; ko00350, tyrosine metabolism; ko00360, phenylalanine metabolism; ko00380, tryptophan metabolism; ko00400, phenylalanine, tyrosine and tryptophan biosynthesis; ko00561, glycerolipid metabolism; ko00564, glycerophospholipid metabolism; ko00591, linoleic acid metabolism; ko00600, sphingolipid metabolism; ko01040, biosynthesis of unsaturated fatty acids. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

metals were detected in muscle of *M. hoffmanni*, indicating that diet addition of *A. grossedentata* extract did not affect growth performance via bringing in adverse effects, which confirmed that *A. grossedentata* extract could serve as feed additive in aquaculture.

4.2. Middle level of A. grossedentata extract contribute to muscle fatty acids accumulation

Liver is an important metabolic organ in vertebrates and HSI reflects energy storage and reproductive activities of fishes [35], which is well associated with lipid transformation [36]. In the present study, down-regulation in HSI of *M. hoffmanni* was recorded, while VSI up-regulated as daily supplementation *A. grossedentata* extract, implying diet addition of *A. grossedentata* extract was fatty liver lowering and participated in fat transferring from liver to the mesentery. Due to muscle is the edible part of fish, the major nutrition level is analyzed. Interestingly, TFA content increased as supplementation *A. grossedentata* extract at middle level if comparison with control, whereas TFA decreased in low and high level *A. grossedentata* extract added groups. The reason of this phenomenon might be ascribed to *A. grossedentata* extract caused antioxidant system disruption, so lipid peroxidation of muscle happened under low and high level *A. grossedentata* extract treatments. Former work had reported that MDA content could reflect the degree of lipid peroxidation as well as the integrity of cell membrane, which were caused oxidative stress [37]. Currently, MDA contents elevation was observed in muscle of *M. hoffmanni* as feeding inclusion *A. grossedentata* extract. Hence, lipid peroxidation happened in muscle inevitably. Meanwhile, SOD and CAT are the scavenger enzymes of free radical and can reduce the lipid peroxidation degree of

animals [38]. Therefore, significantly higher SOD and CAT activities in the muscle of middle level *A. grossedentata* extract added group contribute to retard the lipid peroxidation process, thus the lipid content in the middle level *A. grossedentata* extract added group is higher than the other groups. Furthermore, above-mentioned results had announced that no immune response was stimulated and only slightly inflammatory reaction occurred in middle level *A. grossedentata* extract added group, which ultimately led to the highest CF in the same group. Comprehensive these aspects, we can therefore assure that dietary supplementation *A. grossedentata* extract at middle level (0.5‰, w/w) is conducive to growth-promoting and fatty acids accumulation.

4.3. A. grossedentata extract participation in fatty acids transformation via gut microbiota mediating

Lipid transferring to mesentery was pointed out by us, however, the specific mechanism of lipid transformation in gut was still limited, which subsequently caused fatty acids accumulation in muscle. Former study has reported that both taxonomic and functional compositions of gut microbiota varied by daily supplementation plant extract, and were involvement with amino acids and fatty acids metabolism in aquatic animals, e.g., grass carp (*Ctenopharyngodon idella*) [39]. Therefore, the gut microbiota of *M. hoffmanni* had been analyzed. In our research, the gut microbiota communities significantly altered at class and genus levels, together with α- and β-diversity increasing as feeding inclusion *A. grossedentata* extract. Additionally, the relative abundance of top 10 category microbiota significantly varied at class level. Previous evidence had verified that probiotics were closely involvement with fatty acids metabolism [40], which might bring variations in fatty acids. For instance, *Propionibacteriaceae* enriched in *A. grossedentata* extract added groups and was well associated with the major cellular fatty acid (pentadecanoic acid, C15:0) transformation [41]. Consistently, *Propionibacteriaceae* (v55) was the keystone specie based on random forest test. Meanwhile, other keystone species also revealed relationships with fatty acids transformation, such as *Acidimicrobiales* related to PA (C16:0) [42], *Demequina* related to C15:0 and C16:0 [40], *Rhodobacter* related to C18:1ω7c/C18:1ω6c [43], *Gemmataceae* related to C18:1ω7c, C18:0, and βOH-C16:0 [44], and *Microbacterium* related to C15:0, C17:0, iso-C17:0, and iso-C16:0 [45]. Moreover, functional profiles imputed by PICRUSt2 were selected out and six functional profiles showed significant correlations with keystone species, which were involvement with fatty acid biosynthesis (ko00061), steroid biosynthesis (ko00100), tyrosine metabolism (ko00350), glycerolipid metabolism (ko00561), glycerophospholipid metabolism (ko00564), and linoleic acid metabolism (ko00591). These functional profiles were all tightly related to lipid transformation and eventually led to fatty acid composition significant change as Sankey plot showing. Specifically, dietary supplementation 0.5‰ *A. grossedentata* extract regulated the composition of intestinal microbiota by increasing the relative abundance of keystone bacterial genus *Microbacterium* (V35), and enhanced the imputed functional profile related to glycerolipid metabolism (ko00561) in gut microbiota, thereby increasing the long-chain fatty acid C22:4n6 accumulation in muscle. Therefore, a dose-effect is responsible for gut microbiota variation, which modulated specific metabolic pathway to adapt diet addition of Chinese herbal extract [46]. Comprehensive the above-mentioned aspects, dietary supplementation *A. grossedentata* extract could alter the community composition of gut microbiota and thus medicated lipid transformation, so that the fatty acids composition changed. As these processes occurring, fatty acids in muscle would vary too, which might contribute to fatty acids accumulation in muscle of *M. hoffmanni*.

5. Conclusions

Here, the effect of dietary supplementation *A. grossedentata* extract on *M. hoffmanni* is explored. First, growth performance of *M. hoffmanni* enhanced as daily supplementation *A. grossedentata* extract. Secondly, activities of antioxidant enzymes up-regulation was major responsible for ROS-dependent lipid peroxidation, which benefited in muscle fatty acids accumulation. Thirdly, low or high level *A. grossedentata* extract would stimulate *in vivo* immune response and inflammatory reaction, implying a suitable dosage of *A. grossedentata* extract benefited in safety production. Lastly, *A. grossedentata* extract participates in lipid transformation via regulating the community of gut microbiota, which mediates fatty acids accumulation in muscle of *M. hoffmanni*. Our findings illustrated that diet addition of *A. grossedentata* extract has benefits in healthful aquaculture and provided a scientific basis for *A. grossedentata* extract serving as feed additive.

Declarations

The animal (fishes) experiments were approved by the Animal Welfare and Ethics Committee of China National Analytical Center, Guangzhou (Approval code: No. 2023015).

Data availability statement

Data will be made available on request.

CRediT authorship contribution statement

Qiuling Yang: Writing – original draft, Resources, Investigation, Conceptualization. **Yunfan Wang:** Writing – review & editing, Writing – original draft, Project administration, Funding acquisition. **Geng Li:** Validation, Methodology. **Xiaoying Huang:** Validation, Methodology. **Lingyan Zheng:** Visualization, Formal analysis, Data curation. **Mijun Peng:** Supervision, Resources. **Yong Cao:** Supervision, Project administration. **Xuesong Wang:** Writing – review & editing, Writing – original draft, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

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

Acknowledgements

This study was supported by the Science and Technology Project of Guangdong Province (2021B0909050002) and Innovative Research Team Project of Zhongshan (CXTD2023011). We acknowledge Miss Junzhi Yang (College of Pharmacy, University of Arizona) for her helps in checking English grammar.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e29008.

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