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The differences in intestinal microbiota between common and orange-muscle of *Haliotis gigantea* and dietary influences on abalone's intestinal microbiota

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Abstract

Compared with common abalone, the orange-muscle abalone (*Haliotis gigantea*) has an enhanced ability to accumulate carotenoids. Feeding on asparagus (*Gracilaria lemaneiformis*) was critical for maintaining carotenoid contents in *H. gigantea* muscles. However, whether intestinal microbes play the role in carotenoid accumulation in different colored abalone, and the effects of different diets on intestinal microbes in abalone are unknown. In the study, the differences in intestinal microbiota between abalone with different colors and the effects of different diets on the intestinal microbiota of the abalone were analyzed. The results showed that (1) there was no significant difference in intestinal microbial community composition between the orange-muscle abalone and the common abalone, and (2) the intestinal microbial community composition of abalone fed on asparagus was significantly different from that of abalone fed on kelp (*Laminaria japonica*). At the genus level, the relative abundance of *Psychrilyobacter* in the intestinal tract of abalone fed with asparagus was significantly higher than that of kelp-fed groups. *Stenotrophomonas*

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and *Vibrio* were the dominant bacteria at the genus level in abalone fed with the kelp. This result excluded the role of intestinal microbes in different colored abalone and provided clues for further study of the coloration mechanism of abalone.

KEYWORDS
carotenoid, *Gracilaria lemaneiformis*, *Haliotis gigantea*, intestinal microbiota, *Laminaria japonica*

1 | INTRODUCTION

Carotenoids are the source of color for many important fish and shellfish, as they contribute to the formation of yellow, orange, and red in aquatic animals' tissues. Most expensive seafood such as shrimp, crabs, trout, salmon, and red snapper have orange skin or muscle containing carotenoids (Haard, 1992). Carotenoids are considered the major pigments that not only make animals have attractive colors but also give animals high economic and nutritional value. However, as animals cannot synthesize carotenoids from scratch, they accumulate carotenoids directly from food or symbionts, or alter compounds to accumulate carotenoids in specific tissues through metabolic reactions (Matsuno, 2001).

Microbes in the processes of animal growth, nutrition, and health have formed a close host–microbe interaction with animals referred to as a "symbiosis." As symbionts closely related to animals, microbes in the host intestinal tract play important roles in the processes of host growth and development via helping the host digest and decompose food, providing essential nutrients, and resisting pathogens. The effects of intestinal microbes are manifold; to some extent, intestinal microbes play an important role in animal nutrition metabolism, immunity, maintenance of an internal environment, and stability of behavior (Nicholson et al., 2012; Ussar et al., 2015). The intestinal microflora of mice can increase the fat stored in adipose tissue (Rabot et al., 2010). Intestinal microbes can synthesize nutrients such as carotenoids, vitamins, growth factors, and digestive enzymes, in addition to playing critical roles in nutrient absorption and energy conversion (Ray et al., 2012). Furthermore, animals that feed on the single nutrient are more dependent on the nutritional contribution of intestinal microbes (Harris, 1993). For example, it has been confirmed that bacteria can synthesize different C30 carotenoids and corresponding C30 apocarotenoids that have good stability and anti-inflammatory properties (Steiger et al., 2012; Takaichi et al., 1997).

Although microorganisms can affect the dietary nutrition, diet can also affect the composition and function of the microbial community. The intestinal microbial community structure of animals is affected by many factors, including varieties, feeding habits, and diet. Bifidobacterium is abundant in the intestinal tract of reptiles, while insects are the main food source of reptiles, and these bacteria are ubiquitous in insects (Kopečný et al., 2010). In the marine environment, herbivores feed on macroalgae that live within a community of epiphytic microbes. These microorganisms play important roles in the health and physiology of macroalgae (Fox, 1955), for example, in host adaptation to changing environmental conditions (Tabunoki et al., 2004). As marine herbivorous shellfish, abalone feed on macroalgae that include the brown algae *L. japonica* and *Undaria pinnatifida* and the red alga asparagus. The results of studies of intestinal microbes of *Haliotis discus hannai* cultured in Japan showed that Mollicutes were the dominant group in abalone cultured with seaweed as the diet (Tanaka et al., 2004). A study on the composition of the intestinal flora of *Haliotis diversicolor* found that Mollicutes and Deltaproteobacteria were the dominant intestinal flora of *H. diversicolor* (Huang et al., 2010).

Haliotis gigantea is distributed in the coastal areas of Japan and South Korea. The color of its muscle is usually brownish yellow, but our group found that a small number of mutant individuals in the same breeding colony are

bright orange. Compared with common abalone (H0) with normally colored muscle, orange-muscle abalone (R0) had an enhanced ability to accumulate carotenoids, especially zeaxanthin. Feeding on asparagus was critical for maintaining carotenoid contents, especially zeaxanthin, in *H. gigantea* muscles. However, the absorption, transformation, and metabolism of natural pigments, including carotenoids in diets, are rather complicated processes. The reason why *H. gigantea* with orange muscle had an enhanced ability to accumulate carotenoids in the diet and the related mechanism are still unclear. The phenomenon thus required an exploration of how the diet affected the changes in carotenoid composition and content in abalone. Whether microorganisms play a role in the absorption, transportation, and metabolism of carotenoids in abalone diets needed further exploration.

Therefore, the present study compared the differences in intestinal microbiota between abalone with different colors, and the effects of different diets on the intestinal microbial composition of *H. gigantea*. It tried to explore the role of intestinal microbes in different colored abalone, and provide the clue for further study of the coloration mechanism of abalone.

2 | MATERIALS AND METHODS

2.1 | Animal treatment

Two-year-old orange-muscle and common *H. gigantea* were cultured in ponds under the same conditions at Fuda Aquaculture Co. Ltd., Fujian Province, China (24°31′42.2″ N, 118°36′01.7″ E). Before the experiment, abalone were randomly divided into different diet groups: asparagus (GL) and fresh kelp (FK) after fasting for a week. Each diet group included an orange-muscle group (mean a* 15.17 ± 2.04) and a common group (mean a* 6.04 ± 1.21) with similar average shell length. The experiment was designed using four treatments, with three replicates in each treatments and 60 abalone in each replicate, for a total of 720 abalone used in the experiment. The barrels of the same specification were used for culturing each replicate for 6 months, and the volume of each barrel was 600 L (100 × 100 × 60 cm). Flowing seawater was used for the experiment. During 6 months, the normal light was maintained everyday. The salinity, pH and the water temperature varied with the season. The dead abalone and the extra feeds were removed in time.

2.2 | Sample collection

Before the culture experiment, six orange-muscle and common abalone were taken to sample the intestinal tract (including both intestine and contents) and muscle. After 6 months of the experiment, six orange-muscle abalone and six common abalone were selected from the asparagus and fresh kelp groups. The intestines were quickly frozen in liquid nitrogen and then stored in a −80°C refrigerator prior to the extraction of intestinal DNA. Before and after the experiment, the contents of zeaxanthin, β-carotene, and fucoxanthin in abalone muscles of each group were detected by HPLC. The contents of major carotenoids zeaxanthin, β-carotene, and fucoxanthin in asparagus and fresh kelp were measured as well.

2.3 | DNA extraction

The microbial DNA of abalone intestinal tract (including both intestine and contents) was extracted using a FastDNA™ Spin Kit for Soil (MP Biomedicals, Santa Ana, California, USA) according to the manufacturer's protocol, and the extracted DNA was stored at −20°C for use. The sample was added to a Lysing Matrix E tube. Sodium phosphate buffer (978 μL) and 122 μL of MT buffer were used to lyse samples. The sample was mixed evenly with

FastPrep for 40 s. The lysing matrix E tubes were centrifuged for 5–10 min at 14,000×g. The supernatant was pipetted into a 2.0-mL centrifuge tube; 250 μL PPS was added, and then the tube was shaken manually 10 times. The supernatant was centrifuged at 14,000×g for 5 min and was transferred to a 15-mL centrifuge tube, and 1 mL binding matrix suspension was added to the centrifuge tube. The centrifuge tube was manually rotated for 2 min to combine the DNA with the substrate and was left to stand for 3 min to precipitate the substrate adsorbed to the DNA. About 500 μL of the supernatant was removed carefully to avoid touching the precipitated binding matrix. About 600 μL of the mixture was transferred to a SPINTM Filter and was centrifuged for 1 min at 14,000×g. The catch tube was emptied, and the remaining supernatant was added to the SPINTM Filter and centrifuged. The 500 μL of SEWS-M was added to the SPINTM Filter and centrifuged for 1 min at 14,000×g. The filtrate was discarded, and the SPINTM Filter was replaced into the catch tube and centrifuged for 2 min. The remaining EWS-M in the SPINTM Filter was emptied. The SPINTM Filter was transferred to a new catch tube and air-dried for 5 min at room temperature. The 50–100 μL DES was added into the SPINTM Filter and was gently sucked by gun head, then resuspended in high-efficiency DNA eluting silica matrix. The DNA solution was obtained by centrifugation for 1 min at 14,000×g, then stored at −20°C or 4°C. A NanoDrop 2000 was used to test the concentration and purity of the DNA. A 1 μL sample was used for detection, and the blank was 1 μL of ultrapure water.

2.4 | High-throughput sequencing and bioinformatic analysis

The 515F-806R primer was used to amplify 16S V3-V4 gel electrophoresis PCR products, and the fragments with a size of 400–450 bp were intercepted for DNA recovery and database building. A Qubit 2.0 fluorometer (Invitrogen, Carlsbad, CA) and Agilent Bioanalyzer 2100 (Agilent, Palo Alto, California, USA) were used for quality control. An Illumina HiSeq 2500 platform (Illumina, San Diego, CA, USA) was used for double-end sequencing, and 250 bp was the size of the sequenced fragments.

The low-quality reads in the raw data obtained by sequencing were removed by splicing and filtering to obtain reliable and effective data. Then, the effective data were analyzed for identification of OTUs (operational taxonomic units), and then the abundance, species annotation, alpha diversity, and other analyses were carried out, and a phylogenetic tree was constructed. PCoA, PCA, NMDS, and other dimension reduction methods were used to examine the differences in community structure among different samples or groups. The t test and LEfSe statistical analysis methods were used to examine differences in species composition and community structure of the samples. The Tax4Fun software was used to predict and analyze the functions of microbial communities in the samples.

2.5 | Determination of carotenoid contents

A normal phase high performance liquid chromatograph with a YMC-Pack SIL column (250 × 4.6 mm, 5 μm, 12 nm, YMC CO., Ltd., Kyoto, Japan) was used to determine the carotenoid contents of abalone. In the column procedure, n-hexane (a) and ethyl acetate (b) formed the mobile phase, using a linear gradient method: (1) 40% A and 60% B were used to elute for 10 min; (2) 100% A was used to elute for 5 min, and then (3) the elution was balanced by 40% A and 60% B for 5 min. The total elution time was 20 min. The flow rate and the injection volume were 1.5 mL/min and 20 μL, respectively. The detection wavelength was 446 nm, and the column temperature was 25°C.

A 200 μg/mL zeaxanthin stock solution was prepared by dissolving 1.0 mg analytical standard solution in 5.0 mL ethyl acetate. The standard curve working solution was prepared by diluting the stock standard solution with ethyl acetate. The concentrations of 1.25, 2.5, 5, 10, 25, 50, 100, and 200 μg/mL zeaxanthin solution constituted the working standard curve. A 50 μg/mL β-carotene stock solution was prepared by dissolving 1.0 mg analytical standard in 20 mL ethyl acetate. The concentrations of 0.25, 1.25, 2.5, 5, 10, 15, 25, and 50 μg/mL β-carotene solution constituted the working standard curve. A 50 μg/mL fucoxanthin stock solution was prepared by dissolving 1.0 mg

analytical standard in 20 mL ethyl acetate. The concentrations of 0.05, 0.25, 1.25, 2.5, and 5 μg/mL fucoxanthin solution constituted the working standard curve. The standard curves were established by normal phase high performance liquid chromatography to determine the contents of zeaxanthin, β-carotene, and fucoxanthin.

2.6 | Data analysis

Microsoft Excel was used to preprocess the data, and GraphPad Prism 6.0 was used to draw the graphs. The results were statistically analyzed using SPSS 20.0, and the results in all charts were expressed as mean ± standard deviation. Independent *t* tests and non-parametric tests were used to compare the differences among experimental groups. When $p < 0.05$, the differences among experimental groups were considered significant (marked by * or different lowercase letters in the figures and tables); when $p < 0.01$, the differences between groups were considered extremely significant (marked by ** or different lowercase letters in the figures and tables), and when $p > 0.05$, there was no significant difference (marked by ns or same lowercase letters in the figures and tables).

3 | RESULTS

3.1 | Effects of various diets on carotenoid contents of *H. gigantea* with different colored muscles

The differences in carotenoid contents between the *H. gigantea* with different colored muscles after 6 months were compared, as shown in Table 1. The carotenoid contents in the orange-muscle abalone were significantly higher than those in the common abalone ($p < 0.01$), regardless of whether they were fed with asparagus or fresh kelp. Compared with the initial contents, there were no significant differences in β-carotene contents in abalone after feeding on asparagus for 6 months ($p > 0.05$). However, in the fresh kelp group, the contents of β-carotene in the muscles of orange-muscle and common abalone all decreased after culturing for 6 months. After being cultured for 6 months, the contents of β-carotene in muscles of abalone fed with asparagus, including orange-muscle and common abalone, were significantly higher than those of abalone fed with fresh kelp ($p < 0.01$) (Table 1). In the asparagus group, the contents of zeaxanthin in abalone increased for both color types of abalone, and the contents of zeaxanthin in abalone were significantly higher than the initial levels ($p < 0.01$). However, in the fresh kelp group, the contents of zeaxanthin in both abalone showed a decreasing trend with time, and the contents of zeaxanthin were significantly lower than those of the initial experimental groups ($p < 0.01$). The zeaxanthin contents of abalone fed with asparagus were significantly higher than those of fresh kelp ($p < 0.01$) (Table 1). The fucoxanthin contents in abalone fed with asparagus for 6 months had no significant change ($p > 0.05$). In the fresh kelp group, the fucoxanthin contents

TABLE 1 The contents of carotenoids in abalone under different feedings (mean ± SD).

		β-Carotene (μg/g)		Zeaxanthin (μg/g)		Fucoxanthin (μg/g)	
Diets		**R**	**H**	**R**	**H**	**R**	**H**
0 m		47.6 ± 15.23Aa	12.21 ± 4.90Ab	290.90 ± 150.20Aa	7.5 ± 3.51Ab	9.02 ± 4.44Aa	0.57 ± 0.17Ab
6 m	GL	34.80 ± 10.89Aa	13.5 ± 11.65Ab	485.83 ± 138.66Ba	22.11 ± 14.21Bb	8.35 ± 3.81Aa	1.31 ± 1.25Ab
	FK	10.32 ± 1.82Ba	2.42 ± 0.76Bb	56.54 ± 29.73Ba	1.64 ± 0.53Bb	2.69 ± 0.72Ba	0.44 ± 0.07Ab

Note: Different lowercase letters in the same row in different abalone under the same carotenoid showed significant difference ($p < 0.05$); different uppercase letters in the same column in same abalone under the same feeding showed significant difference ($p < 0.05$).
Abbreviations: FK, fresh kelp; GL, asparagus.

in orange-muscle abalone decreased with culture time, and the fucoxanthin contents in the abalone were significantly lower than those in the initial experimental group ($p < 0.01$). The fucoxanthin contents in common abalone had no obvious change trend with culture time ($p > 0.05$). The fucoxanthin contents in abalone fed with asparagus were significantly higher than those fed fresh kelp ($p < 0.05$) (Table 1).

3.2 | The species distribution of gut microbiota

In this study, the 16S rRNA sequences of 36 intestinal microbial samples of *H. gigantea* were determined. Among the 36 samples, an average of 85,699 reads were measured in each sample. After quality control, an average of 80,547 valid data records were obtained, and the effective rate of quality control was 94.05%. The sequencing quality of samples was high, meeting the requirements of downstream analysis. Based on 97% identity, the sequences were clustered into OTUs, and 2152 OTUs were obtained. The OTU sequences were species annotated based on the Silva132 database, annotating the OTU data to microorganisms of 1824 phyla, 1786 classes, 1668 orders, 1552 families, and 1021 genera.

At the phylum level, the dominant bacteria in the intestinal tract (including both intestine and contents) of *H. gigantea* were Tenericutes, Fusobacteria, Proteobacteria, and Bacteroidetes (Figure 1a). In the initial group, there was a difference in the composition of the intestinal microflora between orange-muscle abalone and common abalone; however, there was little difference in the composition of intestinal microflora between the two kinds of abalone in the asparagus and kelp groups. There were significant differences between the intestinal microbial compositions of abalone fed with different diets, among which the most abundant phylum in the asparagus group was Tenericutes, and its average relative abundances in R6G and H6G were 48.22% and 38.93%, respectively. This was followed in order by Fusobacteria, Proteobacteria, and Bacteroidetes. However, the order of abundance of the intestinal flora of abalone in the kelp group was Proteobacteri, Tenericutes, Bacteroidetes, Fusobacteria, among which Proteobacteria had the highest abundance at 39.46% and 42.11% in R6K and H6K, respectively.

At the class level, the dominant bacteria in the intestinal tract (including both intestine and contents) of *H. gigantea* were Mollicutes, Fusobacteria, Gammaproteobacteria, and Bacteroidia (Figure 1b). Except for the initial group, there was no significant difference in the structure of intestinal bacteria between orange-muscle and common abalone in the asparagus group or fresh kelp group at the class level. Similar to the phylum level, there were clear differences in the structure and composition of abalone intestinal bacteria among different diets. In the asparagus group, whether R6G or H6G, the order of abundance of bacteria in abalone was Mollicutes, Fusobacteria, Gammaproteobacteria, and Bacteroidia, while in the kelp group, the order of abundance was Mollicutes, Gammaproteobacteria, Bacteroidia, and Fusobacteria. The largest differences in abundance were in Fusobacteria and Gammaproteobacteria. The average relative abundance of Fusobacteria in the asparagus group was 28.99% (R6G) and 37.27% (H6G), while those of the *L. japonica* group were 5.01% (R6K) and 4.40%(H6K). The average relative abundances of Gammaproteobacteria in the asparagus group were 3.58% (R6G) and 2.92% (H6G), and in the *L. japonica* group were 31.98% (R6K) and 30.64% (H6K).

At the genus level, the dominant genera in the intestinal tract (including both intestine and contents) of abalone-fed asparagus were *Mycoplasma*, *Psychrilyobacter*, *Stenotrophomonas*, and *Vibrio*. Except for the initial group, there was no significant difference in the structure of intestinal flora between orange-muscle and common abalone in the asparagus and kelp groups at the genus level. The diets had a significant influence on the intestinal flora of *H. gigantea*, and the abundance of intestinal flora of abalone varied among different diets (Figure 1c). The abundance of intestinal flora of abalone in the asparagus group was in the order *Mycoplasma*, *Psychrilyobacter*, *Stenotrophomonas*, and *Vibrio*, while that of the kelp group was in the order *Mycoplasma*, *Stenotrophomonas*, *Vibrio*, and *Psychrilyobacter*. There was a large difference in abundance between the two groups of *Psychrilyobacter*, *Stenotrophomonas*, and *Vibrio*, among which the average relative abundances of *Psychrilyobacter* in the asparagus group were 28.83% (R6G) and 37.15% (H6G), while those in the *L. japonica* group were 4.88% (R6K) and 4.34%

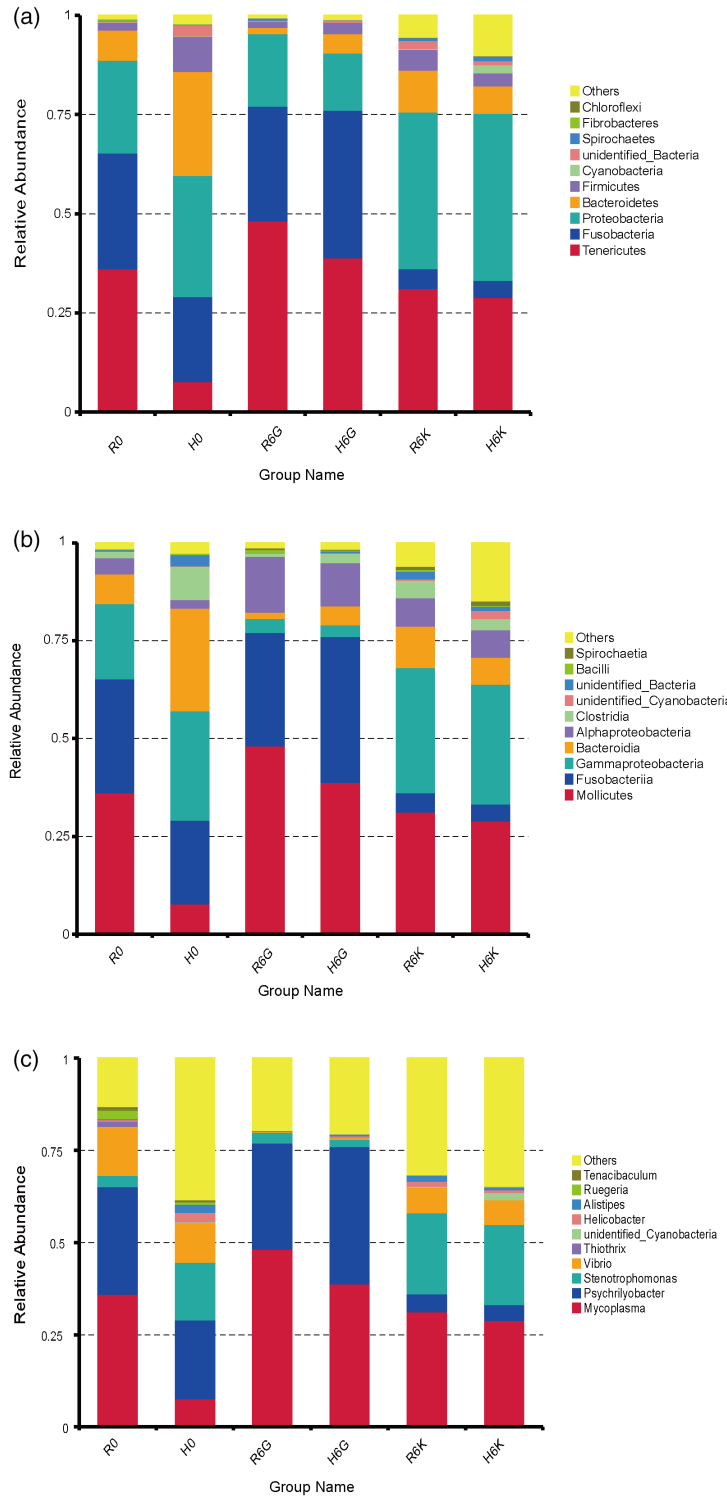


FIGURE 1 Column diagrams illustrating the top 10 microbiota with the highest relative abundances. (a) The column diagram of the top 10 phyla with the most relative abundance. (b) The column diagram of the top 10 class with the most relative abundance. (c) The column diagram of the top 10 genus with the most relative abundance.

(H6K). The abundance of *Stenotrophomonas* in the kelp group was higher than that in the asparagus group, and its average relative abundances in the kelp group were 22.08% (R6K) and 21.75% (H6K), while those in the asparagus group were only 3.03% (R6G) and 2.01% (H6G). The abundance of *Vibrio* in the kelp group was also higher than that in the asparagus group, and its average relative abundances in the kelp group were 6.96% (R6K) and 6.68% (H6K), while those in the asparagus group were only 0.17% (R6G) and 0.48% (H6G).

In summary, there was no significant difference in the species composition of the intestinal microbial community between orange-muscle and common abalone, but different diets had a significant influence on the species composition of the intestinal microbial community of abalone. The composition of the intestinal flora of abalone fed with asparagus was clearly different from that of abalone fed with kelp.

3.3 | Alpha-diversity of gut microbiota

As shown in Figure 2a, the trend of dilution curves of each sample was relatively flat, and the amount of sequencing data in our experiment was sufficient for reliable results. Comparing samples of *H. gigantea* with different colored muscles, there was no significant difference in α-diversity of intestinal microbes between orange-muscle and common abalone, whether in the initial group, the asparagus group, or the kelp group (Figure 2b, Table 2). Taking the Shannon index as an example, the value of the orange-muscle abalone (R0) in the initial group was 4.618, while for the common abalone (H0), this was 5.422; in the asparagus group, the value for the orange-muscle abalone (R6G) was 3.632, while for the common abalone (H6G) this was 4.133. In the kelp group, the index for the orange-muscle abalone (R6K) was 5.598, while for the common abalone (H6K) this was 5.401. The Shannon index had no significant difference between R0-H0, R6G-H6G, or R6K-H6K groups ($p > 0.05$). Comparing the α-diversity of intestinal microbe communities among different diets groups of the same abalone, the indices of intestinal microbes of different abalone in the asparagus group were lower than those in the kelp group, and the Shannon indices of R6G-R6K and H6G-H6K groups were significantly different ($p < 0.05$). The Shannon indexes of R6G and H6G in the asparagus group were 3.632 and 4.133, respectively, values that were significantly lower than those of R6K and H6K in the kelp group of 5.598 and 5.401, respectively.

3.4 | Beta-diversity analysis of gut microbiota

The NMDS analysis based on Bray–Curtis distance showed that different diets had a significant influence on intestinal microorganisms of *H. gigantea*, and the overall NMDS diagram showed a clear aggregation trend of the same diet group. The different diet groups could be well distinguished, and the samples from the asparagus group and the kelp group were clearly separated. Moreover, in the initial group, there was no clear separation trend between the orange-muscle and the common abalone between the asparagus and kelp groups (Figure 3). Adonis analysis showed that in the initial group, asparagus group, and kelp group, the explanatory degrees of the changes in intestinal microbial structure of orange-muscle and common abalone were 32.60%, 23.4%, and 23.02%, respectively. However, the explanation degree of the diets for the change in intestinal microbial structure of orange-muscle abalone was 43.60%, and that of common abalone was 53.13%. The influence of diets on intestinal microbial structure of abalone was significant.

3.5 | Differential microbiota analysis

According to LEfSe analysis, the bacteria with inter-group differences in the abundance of orange-muscle abalone in the asparagus group and the kelp group from phylum to genus are displayed in a bar graph (Figure 4a), and the

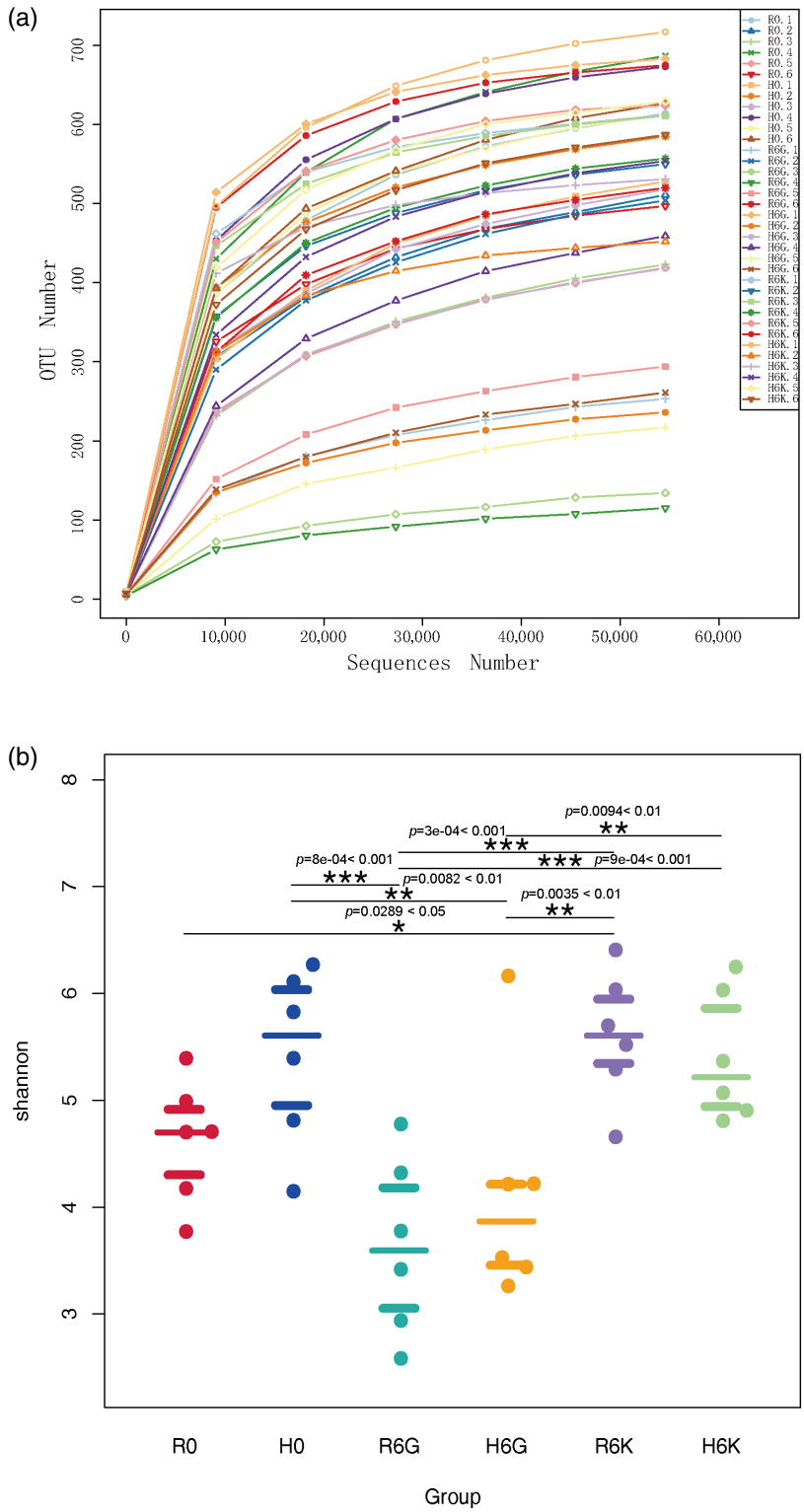


FIGURE 2 The alpha-diversity of gut microbiota. (a) Alpha rarefraction curves of all samples. (b) The shannon index of gut bacteria of different abalone. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

TABLE 2 The α-diversity indexes of gut microbiota in *H. gigantea*.

Group	Observed_species	Shannon	Simpson	Chao 1	ACE
R0	525	4.618	0.878	583.291	586.427
H0	590	5.422	0.906	638.394	641.825
R6G	303	3.632	0.826	333.269	346.767
H6G	379	4.133	0.867	418.889	431.259
R6K	604	5.598	0.929	625.905	626.942
H6K	578	5.401	0.931	608.136	606.464

Note: R0: orange-muscle abalone, $n = 6$; H0: common abalone, $n = 6$; R6G: orange-muscle abalone fed on asparagus, $n = 6$; H6G: common abalone fed on asparagus, $n = 6$; R6K: orange-muscle abalone fed on kelp, $n = 6$; H6K: common abalone fed on kelp, $n = 6$.
Abbreviation: ACE, analysis of community ecology.

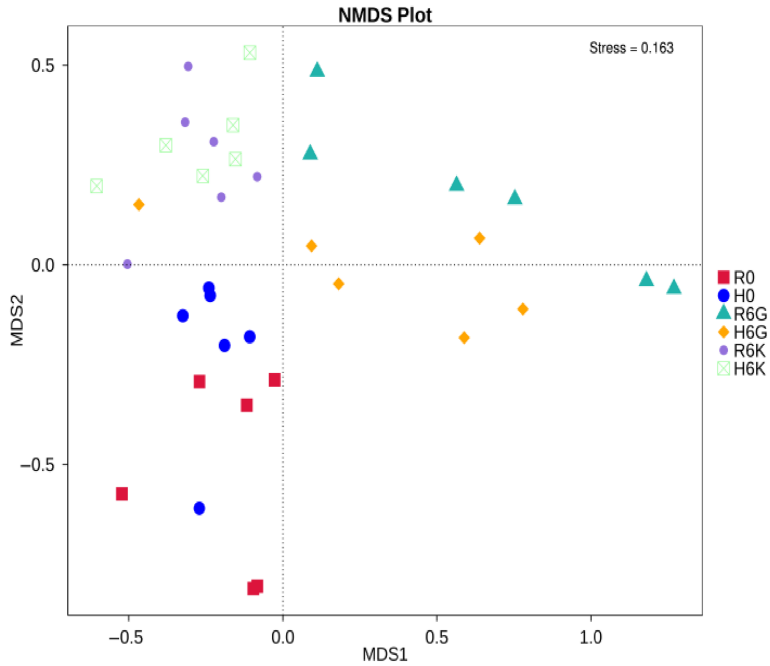


FIGURE 3 The NMDS plot of gut microbiota of different abalone reared in different feeding.

bacteria with inter-group differences in the abundance of common abalone are also displayed in a bar graph (Figure 4b). The green band represents the bacteria of abalone whose abundance was significantly higher in the kelp group than that of abalone in the asparagus group, and the red band represents the bacteria of abalone whose abundance was significantly higher than that of abalone in the asparagus group. A total of 16 kinds of biomarkers were found in the orange-muscle abalone fed with asparagus in the R6G group, and 11 kinds of biomarkers were found in the orange-muscle abalone fed with fresh kelp in the R6K group. A total of 10 biomarkers were found in the H6G group, and seven biomarkers were found in the H6K group. In the kelp group, the biomarkers in both orange-muscle and common abalone were *Stenotrophomonas*, *Vibrio*, and *Vibrio halioticoli*. However, for abalone in the asparagus group, including orange-muscle and common abalone, the biomarker of intestinal microbes included *Psychrilyobacter*.

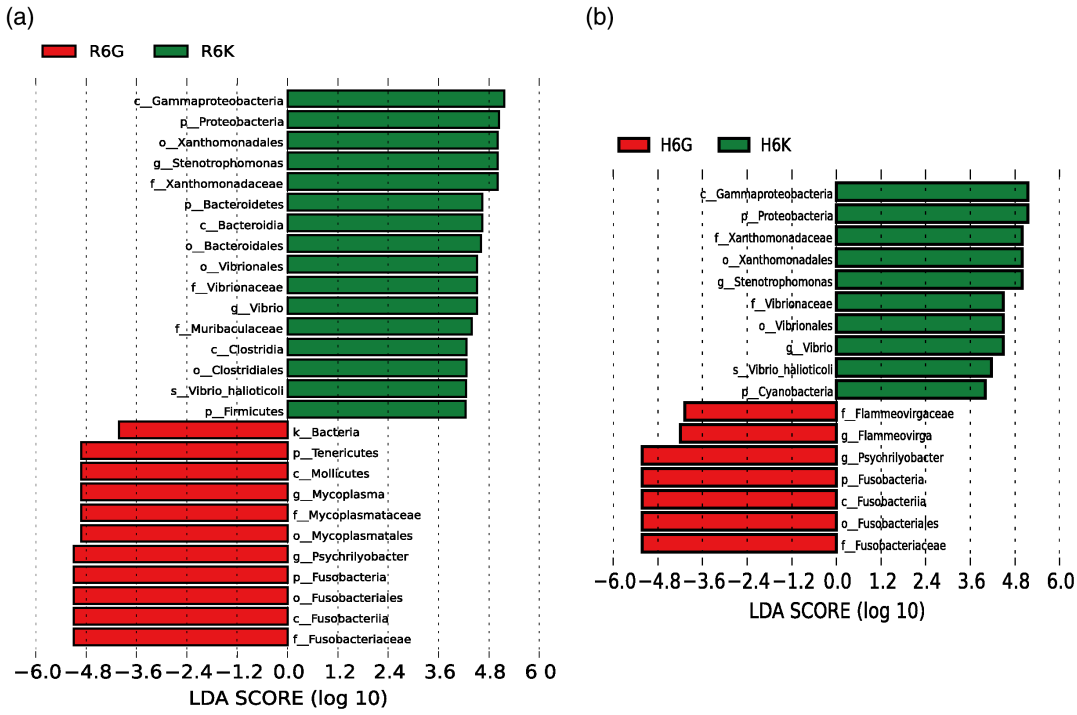


FIGURE 4 The results of LEfSe analysis among abalone with different diets. (a) The LDA bar plot of different bacteria among orange muscle abalone with different diets. (b) The LDA bar plot of different bacteria among common muscle abalone with different diets.

Bacteria with differences in abundance between orange-muscle and common abalone in the initial group, asparagus group, and kelp group are displayed in a bar chart (Figure 5). In the initial group, there were eight biomarkers in orange-muscle abalone, while 16 biomarkers were found in common abalone, as shown in Figure 5a. In the asparagus group, only five biomarkers were found in the orange-muscle abalone (Figure 5b); these were Tenericutes, Mollicutes, Mycoplasmatales, Mycoplasmataceae, and *Mycoplasma*. However, in the kelp group, no biomarkers were found in either orange-muscle or common abalone. This showed that the difference in intestinal microbial flora between orange-muscle or common abalone in each diet group gradually decreased and tended to be similar to the stability of the culture environment and diets.

3.6 | Functional prediction of abalone intestinal microbes

The Tax4Fun program was used to predict the intestinal flora functions of abalone with different colored muscles fed with different diets, and all KOs were annotated to the level 1 and level 2 functional levels. The number of genes under each metabolic pathway was counted, and a KEGG pathway annotation bar chart was drawn (Figure 6). The functional pathways of intestinal flora in abalone included genetic information, metabolism, and environmental information transmission. Genetic information, translation, replication and repair, membrane transport, DNA nucleotide metabolism, energy metabolism, the expression levels of carbohydrate metabolism, and amino acid metabolism were significantly more enriched than other pathways. The PCA dimensionality reduction analysis based on the abundance statistics of functional notes in the database showed that the functional composition of intestinal microbes in orange-muscle and common abalone was similar; however, the functional composition of intestinal microbes in abalone between the asparagus and kelp groups could be clearly separated (Figure 7).

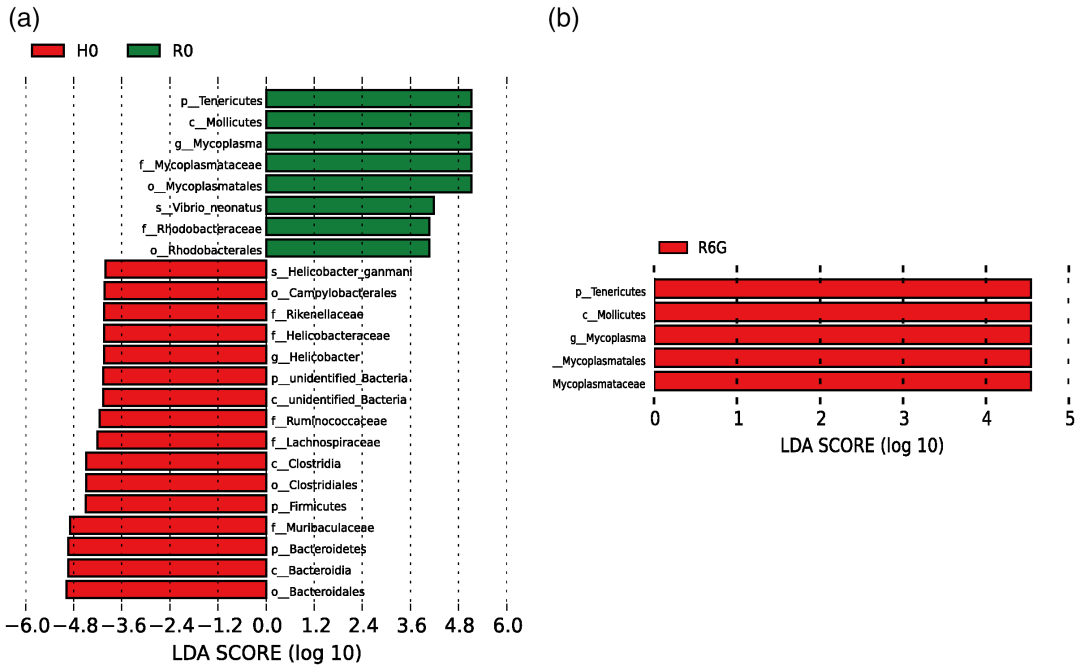


FIGURE 5 The results of LEfSe analysis between orange and common muscle abalone in different groups. (a) The LDA bar plot of different bacteria between orange and common muscle abalone in the beginning. (b) The LDA bar plot of different bacteria between orange and common muscle abalone with *Gracilaria*.

Through the comparative analysis of metabolic pathway abundance, the drug resistance of intestinal microbes in orange-muscle abalone in the asparagus group was significantly lower than that in the kelp group ($p < 0.05$), as shown in Figure 8a. The bar chart of intestinal microbial function difference of abalone in various diets is shown in Figure 8b. Amino acid metabolism, signal transduction, drug resistance, poorly characterized pathway, cell motility, xenobiotics biodegradation and metabolism, and neurodegenerative disease pathways of intestinal microbes of common abalone in the asparagus group were significantly lower than those in the kelp group ($p < 0.05$). The functions of genetic information processing, endocrine system, and viral protein family of intestinal microbes of common abalone in the asparagus group were significantly higher than those of the kelp group ($p < 0.05$).

4 | DISCUSSION

In the study, the differences in intestinal microbiota between abalone with different colors and the effects of different diets on the intestinal microbiota of the abalone were analyzed. The results showed that (1) there was no significant difference in the composition of the intestinal microbial community between orange-muscle abalone and common abalone, and (2) the intestinal microbial community structures of abalone were significantly different under different diets. There was a significant difference between the intestinal flora of abalone fed with asparagus and that of abalone fed with kelp.

At the class level, the most abundant microorganisms in the intestinal tract of *H. gigantea* were Mollicutes, Fusobacteria, Gammaproteobacteria, and Bacteroidia, among which *Mycoplasma* was dominant in *H. gigantea*. The results were similar to the research findings for *Haliotis discus hannai* and *Haliotis diversicolor* by Huang (2018) and Tang (2019). As a kind of facultative anaerobic bacteria, *Mycoplasma* can adapt to special environmental conditions

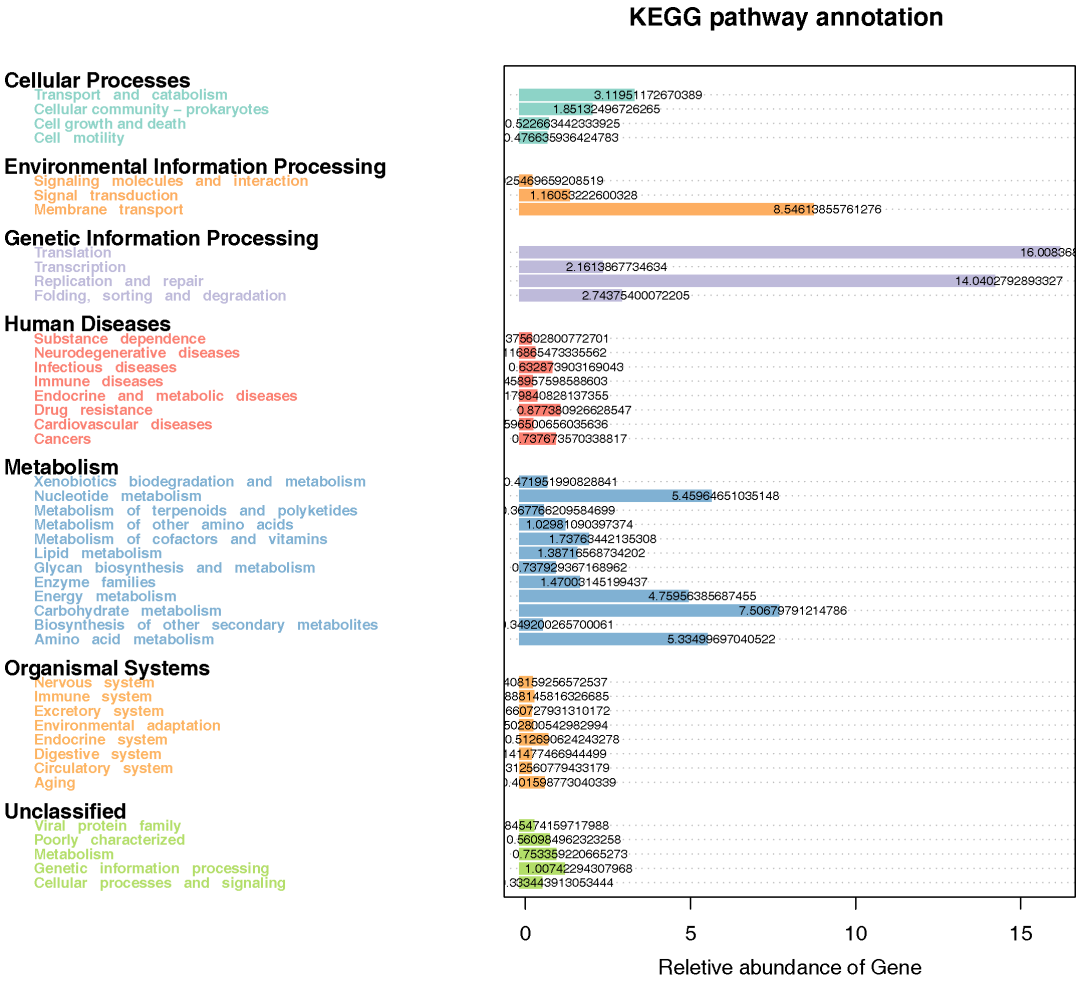


FIGURE 6 The KEGG pathway annotation of gut bacteria in *Haliotis gigantea*.

such as low oxygen content and mucus in the abalone intestinal tract, and they can decompose and utilize nutrients such as glucose and arginine (Miles, 1992), participate in various metabolic pathways, and play an important role in the abalone intestinal microbial community. In addition, the relatively abundant microorganisms *Psychrilyobacter*, *Stenotrophomonas*, and *Vibrio* were present. *Psychrilyobacter* could metabolize and decompose sugars, organic substances, and amino acids as carbon sources to produce H_2 and acetate that participate in many metabolic processes. At present, there has been little research on the related functions of *Stenotrophomonas*, especially concerning biological functions. Microbes dominated by the *Vibrio* group have been demonstrated to have the function of degrading seaweed polysaccharides such as laminarin and alginic acid, and they play an important role in helping the host to digest food in the abalone intestine, as had been reported in *H. discus hannai*, *H. diversicolor*, and *H. gigantea* (Erasmus et al., 1997; Huang et al., 2010; Sawabe et al., 1995; Sawabe et al., 1998; Tanaka et al., 2016). This study confirmed that *Vibrio* was the first of four genera in relative abundance of intestinal microorganisms of the abalone *H. gigantea*, indicating that *Vibrio* played an important role in abalone.

Comparing the structural composition of intestinal microorganisms of *H. gigantea* with different colored muscles, there was no significant difference between orange-muscle and common abalone. No matter whether it was the asparagus or the kelp group, there was no significant difference in intestinal microorganisms between orange-muscle

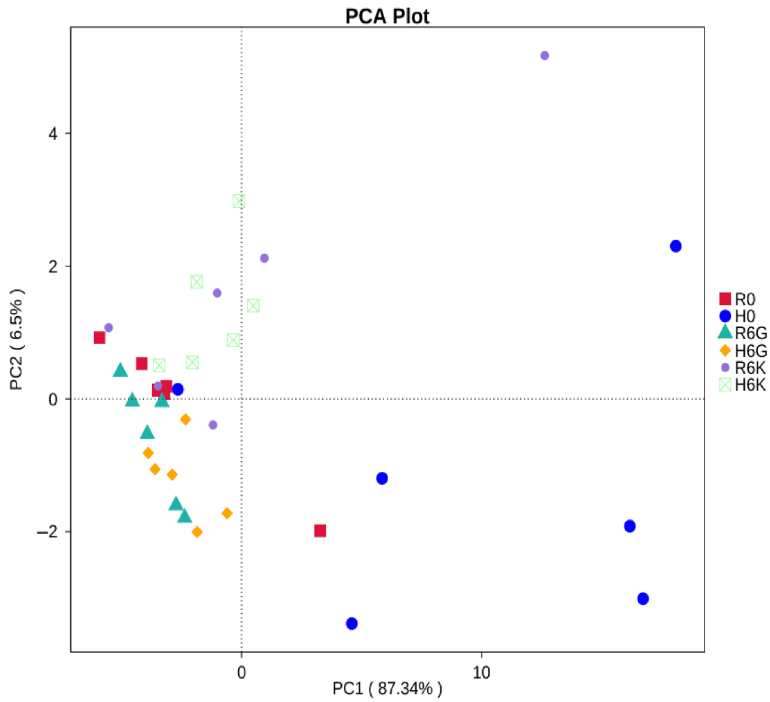


FIGURE 7 The results of the PCA analysis of Tax4Fun function annotation.

and common abalone. Studies have confirmed that the variety of abalone has a significant impact on its intestinal microbes. The research on abalone intestinal microorganisms showed that host genes could affect the composition of its intestinal microbes to a certain extent (Cicala et al., 2017). Previous experiments showed that compared with the common abalone, the orange-muscle abalone had an enhanced ability to accumulate carotenoids in the diet, especially zeaxanthin. However, this study showed that the difference in carotenoid content between orange-muscle and common abalone did not change the composition of intestinal microorganisms. It has been speculated that the difference in carotenoid content between orange-muscle and common abalone may not be directly related to intestinal microorganisms, but the specific relationship needed further exploration.

The intestinal microorganisms of abalone are affected by many factors, including temperature, feed, season, and salinity (Gobet et al., 2018), among which diet is an important factor. By comparing the intestinal microbial communities of *Haliotis rufescens* feeding on different species of algae, it was found that the dominant microbial communities in the abalone intestinal tract were relatively stable and not affected by the diet, but the colony structures were significantly different among different diet groups (Caporaso et al., 2010). As a marine herbivorous shellfish, abalone mainly feed on large algae, including the brown alga *L. japonica* and the red alga asparagus. Different algae species have different components; kelp contains alginic acid, kelp polysaccharide, and cellulose, while the main components of asparagus include agar, carrageenan, and cellulose (Goecke et al., 2013; Popper et al., 2011). It was significant for the carotenoid enrichment ability of *H. gigantea* to study whether the intestinal microbes would be affected by different diets. This study confirmed that different diets influenced the intestinal microbial structure of abalone. There was a significant difference between the intestinal microbial structure of abalone fed with asparagus and that of abalone fed with kelp. At the genus level, whether fed kelp or asparagus, the dominant genera in the intestinal microbial communities of *H. gigantea* were *Mycoplasma*, *Psychrilyobacter*, *Stenotrophomonas*, and *Vibrio*. However, the abundance of intestinal microorganisms of abalone varied among different diet groups (Figure 4). There were significant differences in abundance between the two groups regarding *Psychrilyobacter*, *Stenotrophomonas*, and *Vibrio*.

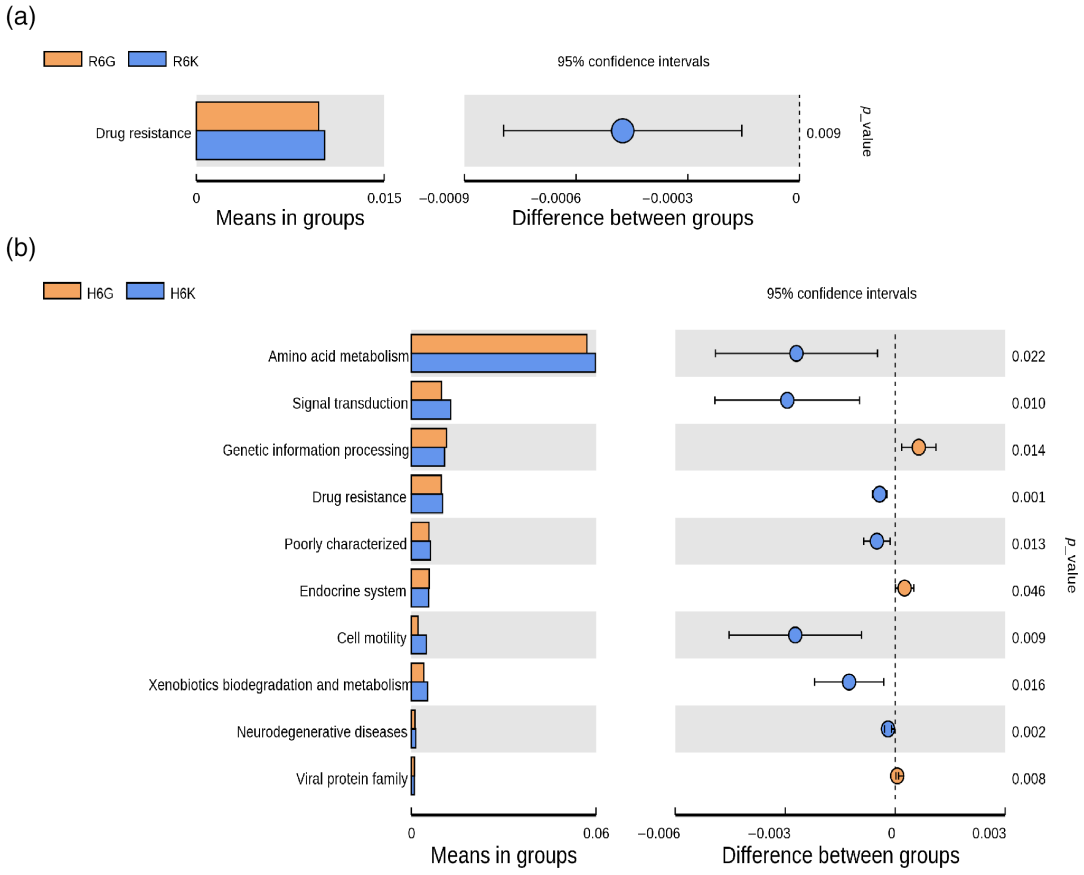


FIGURE 8 Functional prediction of gut bacteria among the same abalone under different feeding conditions. (a) Functional prediction of gut bacteria among orange muscle abalone under different feedings. (b) Functional prediction of gut bacteria among common muscle abalone with different diets.

Microbes such as *Vibrio* and *Vibrio halioticoli* had been demonstrated to have the function of degrading many kinds of seaweed polysaccharides, especially kelp polysaccharides and alginic acid, attributes that played an important role in helping the host abalone to digest food. Tanaka and colleagues cultivated the algae polysaccharide (alginic acid) degrading bacteria *Vibrio* group in the intestine of the abalone *H. gigantea* and found that the bacteria played an important role in the process of abalone digestion (Tanaka et al., 2016). It was found that the relative abundances of *Psychrilyobacter* in the intestines of abalone fed with asparagus were significantly higher than those of the kelp group, indicating that the bacteria might have unique biochemical functions in the intestines of abalone fed with asparagus, different from those of abalone fed with kelp. Studies have shown that Fusobacteria can decompose proteins, metabolize carbohydrates, and produce butyric acid (McCoy et al., 2013), processes closely related to the development of digestive organs and nutritional functions of the host (Keenan et al., 2013). Butyrate can promote intestinal barrier function and the balance of the intestinal microbial community, thereby helping to maintain the host intestinal tract in a healthy state (Leonel & Alvarez-Leite, 2012). In addition, butyric acid can maintain the integrity of animal intestinal morphology and structure, expand the absorption area of the intestinal tract, and promote the regeneration, proliferation, and maturation of intestinal cells, thereby enhancing the digestion and absorption ability of the intestinal tract to nutrient elements (Guilloteau et al., 2010; Hornby & Moore, 2011). Studies have shown that a calf diet supplemented with 3% sodium butyrate was beneficial to the growth of calves and could improve the utilization rate of feed nutrients (Ślusarczyk et al., 2010). The culture experiments with different diets showed that

feeding asparagus played an important role in maintaining the carotenoid content, especially the zeaxanthin content, in *H. gigantea*, whether in orange-muscle or common abalone. Whether this was related to the higher relative abundance of Fusobacteria in the intestinal tract of abalone fed with *G. lemaneiformis*, or whether the increased abundance of Fusobacteria enhanced the absorption ability of nutrients such as carotenoids in the intestinal tract of abalone is unknown. At present, little is known about the functions of these microbial flora, and further functional research of related flora is needed.

5 | CONCLUSION

In summary, our study analyzed that the differences in intestinal microbiota between abalone with different colors and the effects of different diets on the intestinal microbiota of abalone. The results showed that there was no significant difference in intestinal microbial community composition between the orange-muscle abalone and the common abalone. And the intestinal microbial community composition of abalone fed on asparagus was significantly different from that of abalone fed on kelp (*L. japonica*). At the genus level, the relative abundance of *Psychrilyobacter* in the intestinal tract of abalone fed with asparagus was significantly higher than the kelp-fed groups. There may be unique biochemical functions in the intestinal tracts of abalone fed on asparagus, functions that are different from those of abalone fed on kelp. *Stenotrophomonas* and *Vibrio* at the genus level were the dominant bacteria in the abalone fed with the kelp. When kelp was used as a feed, the Vibrio group had a specific type of bacteria in the intestine, thereby possibly playing an important role in abalone. This provides insight for further study of the coloration mechanism of abalone.

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CONFLICT OF INTEREST STATEMENT

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

DATA AVAILABILITY STATEMENT

The authors declare that the data used in Table 1 has published in article "Carotenoid accumulation in common and orange-muscle mutant of abalone, *Haliotis gigantea*, fed with different macroalgae."

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