Effects of scallop visceral mass and mantle as dietary supplements on the physiology, immune response and intestinal microflora of sea cucumber Apostichopus japonicus

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Abstract

To find a way to reuse scallop visceral mass and mantle, these aquatic byproducts were used as dietary supplementation and their effects on sea cucumber *Apostichopus japonicas* culture were investigated, including the growth performance, fatty acid and amino acid compositions, non-specific immune responses and intestinal microflora. The results indicated that the specific growth rate (SGR) of *A. japonicas* was significantly improved within 20 days by dietary supplementation of scallop visceral mass. Scallop visceral mass supplementation also markedly increased the contents of ω-3 fatty acids including EPA and DHA and ω-3/ω-6 ratio of sea cucumber tissue, which is beneficial to the health of sea cucumber and its commercial value. Furthermore, it is found that supplementation of scallop visceral mass and mantle both enhanced the non-specific immunity and optimized the composition of intestinal microflora of *A. japonicas* by increasing microbial diversity and promoting the abundance of beneficial taxa. This study reveals the promising prospect of high-value utilization of these scallop “wastes” in sea cucumber culture industry.

1. Introduction

The annual aquaculture production of scallop is nearly 2 million tons in China. The scallop processing is increasing with the increase scale of scallop production. The scallop visceral mass and mantle are the main byproduct which are considered as low value and usually discarded as waste during processing (Han et al., 2018). However, the visceral mass and mantle of scallop also contain plenty of protein and bioactive substances which are regarded as good resource for anti-virus, anti-tumor and anti-aging. It was reported that scallop muscle contained tropomyosin and actin (Wu et al., 2019), and functional products have been extracted from scallop mantle by using enzymatic hydrolysis technology, such as the protein hydrolysates from scallop mantle exhibited abilities of reducing hydroxyl and DPPH radicals. Therefore, the reuse of scallop visceral mass and mantle has attracted wide attention, which is also needed to lessen the burden on the environment.

The sea cucumber *Apostichopus japonicas* is one of the most commercially important species in Asia countries especially in China due to its nutritional value and therapeutic properties (Xia and Wang, 2015). The annual production was 196,564 tons with a value of more than 50 billion US dollars in 2021 (Ministry of Agriculture and Rural Affairs of the People's Republic of China, 2021). With the development of aquaculture scales, diseases outbreaks that result in high mortality have become a bottleneck restricting the sustainable development of this industry. Antibiotics such as furans quinolones and oxytetracyclines are commonly used for the prevention and control of bacterial infections in sea cucumber culture (Zhang et al., 2021). Farmers realized that disease prevention is more important than cure. Therefore, immunostimulants have been use to enhance the immune responses of *A. japonicas*. Dietary intervention through an environmental friendly approach is an effective way of health management in sea cucumber aquaculture from both the nutritional as well as immunological perspective.
Scallop mantle could be used as protein source and replace the fishmeal in the diet of *A. japonicas*. It was reported that the scallop mantle subject to enzymolysis could modulate the nonspecific immune responses of sea cucumber (Song et al., 2021). Scallop visceral mass was also reported to be rich in unsaturated fatty acids (Xing et al., 2011), which is supposed to be beneficial for the growth and immunity of sea cucumber (Yu et al., 2016). However, the comprehensive study on the effects of scallop mantle and visceral mass as dietary supplements on the growth and intestinal microflora of sea cucumber has not been studied. *Chlamys farreri* is a high-economic shellfish species commonly cultured in East Asian coast and full utilization of *C. farreri* has attracted increasing attention. In this study, the effects of scallop (*C. farreri*) visceral mass and mantle as dietary supplements on the growth, fatty acid and amino acid compositions, immune response and intestinal microflora of *A. japonicas* was studied, which is of great significance for the sustainable utilization of scallop bio-resources as well as guidance on the healthy aquaculture of sea cucumber.

2. Material And Methods

2.1 Diet preparation

Scallop *C. farreri* was purchased from local market in Qingdao City, China. The visceral mass and mantle of the *C. farreri* were separated, homogenized and then dried at 45 °C. Then they were grounded into fine powder for diet preparation. A powder composed of *Sargassum thunbergii* and sea mud (4:6) was used as the basal diet. Three feeding groups were designed: (1) CK with the basic diet; (2) SV with the basal diet adding 3.5% of scallop visceral mass powder; (3) SM with the basal diet adding 3.5% of scallop mantle powder. The protein and crude lipid contents of three diet and the diet ingredients were listed in Table 1.

<table>
<thead>
<tr>
<th></th>
<th>Diet-CK</th>
<th>Diet-SV</th>
<th>Diet-SM</th>
<th>Scallops visceral mass</th>
<th>Scallops mantle</th>
<th><em>S. thunbergii</em></th>
<th>Sea mud</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein (%)</td>
<td>50.7 ± 1.2</td>
<td>49.1 ± 1.6</td>
<td>50.9 ± 0.0</td>
<td>53.2</td>
<td>66.2</td>
<td>19.0</td>
<td>2.7</td>
</tr>
<tr>
<td>lipid (%)</td>
<td>2.6 ± 0.1</td>
<td>2.5 ± 0.2</td>
<td>2.1 ± 0.1</td>
<td>12.5</td>
<td>2.4</td>
<td>2.0</td>
<td>0.3</td>
</tr>
</tbody>
</table>

2.2 Feeding experiment

Sea cucumbers (*A. japonicas*) with average body weight of 3.8 ± 2.3 g were cultured in opaque plastic tanks of 250 L with diameter of 0.26 m and height of 1.2 m. Three replications were designed for each feeding treatment with all replicates being fully randomized. Eighty sea cucumbers were cultured in each tank. During the experiment, the water was continuously aerated and maintained at 16.0 ± 1.0°C, pH 8.0 and salinity of 30–32 psu. The sea cucumbers were fed at 9:00 am and 16:00 pm every day and the diet amount was about 5.0% of the total sea cucumber body weight in each tank. Half volume of the water
was exchanged with seawater after sand-filtering and UV-treatment every day. The experiment lasted for 40 days. On days 0, 20 and 40 during the experiment, all of the sea cucumbers in each tank were weighted to calculate the specific growth rate (SGR). Six sea cucumbers were randomly selected on day 0, 15, 30 and 40. They were dissected and their intestinal tract, respiratory tree and body wall were collected, mixed and frozen at -80 ºC for further analysis for tissue fatty acid and amino acid compositions and intestinal gene expressions and microflora.

2.3 Fatty acid and amino acid analysis

Tissues of sea cucumbers collected on day 40 were homogenized, frozen-dried and then grounded into fine powder. The dry sea cucumber tissue powder, scallop visceral mass powder, mantle powder and S. thunbergii powder were analyzed for fatty acid and amino acid compositions. The samples were weighted and extracted for total lipids using chloroform/methanol (2:1) with 0.01% butylated hydroxytoluene (BHT) as an antioxidant and 19:0 FAME (fatty acid methyl ester) as an internal standard. The extracts were hydrolyzed using 6% KOH methanol, acidified with HCl to pH 2 and then esterified using 14% boron trifluoride-methanol. Fatty acid methyl esters were quantified using a gas chromatography (Agilent 7890A) equipped with a DB-FFAP capillary column (30 m×0.32 mm×0.25µm). The temperature programs were set as 150°C for 1.0 min, rate of 3°C/min to 220°C for 33 min.

For amino acid analysis, about 20 mg samples were weighted and put into ampoule bottle. Then 10 ml 1 mol/L HCl was added, and the bottle was filled with nitrogen gas and then sealed. The sample in the bottle was hydrolyzed at 110 ºC for 24 hours, and then evaporated to dryness. Pre-column derivation of 200 µL sample was conducted by adding 100 µL trimethylamine and 100 µL phenyl isothiocyanate for 1 hour, and then extracted by hexane. The filtered sample was analyzed for amino acids with a high performance liquid chromatograph (Agilent 1100) equipped with Venusil-AA column (4.6×250 mm, 5 µm). The mobile phase A was 0.1 mol/L sodium acetate with 7% acetonitrile and mobile phase B was 80% acetonitrile. Flow velocity was 1 mL/min. The wave length was 254 nm.

2.4 Immune related gene expression

The expressions of immune related genes including TLR3, AjToll, MyD88, TRAF6, p50, p105, rel, MKK36 and p38 in intestine of sea cucumber collected on day 40 were analyzed. AjToll is one of the toll-like receptor genes identified from sea cucumber A. japonicas, which is functionally involved in the immune responses of A. japonicas (Sun et al., 2013). Total RNA from the intestine was extracted using a SPARK easy Improved Tissue/Cell RNA Kit (SparkJade, China). The cDNA was generated by Prime Script™ RT reagent Kit (Takara, Japan). The genes were determined using qPCR performed with SYBR® Green Premix Pro Taq HS qPCR Kit (Accurate Biology, China) in a CFX96 Touch Real-Time PCR Detection System (Bio-Rad, USA). The primers were listed in Supplementary Table S1. qPCR was run in triplicate with the reference gene using the following protocols: 30 s at 95°C followed by 39 cycles of 5 s at 95°C and 30 s at 57°C. Data were quantified using $2^{-\Delta \Delta CT}$ method.

2.5 Intestinal microflora analysis
Intestinal microflora of the sea cucumber collected on day 40 were analyzed. The sample DNA was isolated from the intestine of sea cucumber using CTAB method. 16S rDNA amplicon PCR was performed targeting the V3-V4 region using the primers of 341F-806R. Sequencing libraries were generated using TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA). The library quality was assessed on the Qubit® 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system. At last, the library was sequenced on an Illumina NovaSeq platform and 250 bp paired-end reads were generated.

Paired-end reads were merged using FLASH (V1.2.7, http://ccb.jhu.edu/software/FLASH/). Quality filtering on the raw tags was performed under specific filtering conditions to obtain the high-quality clean tags according to the QIIME (V1.9.1) quality controlled process (Caporaso et al., 2010). Sequence analysis was performed by Uparse software (V7.0.1001, http://drive5.com/uparse/). Sequences with ≥ 97% similarity were assigned to the same OTU. The OTU sequence was made taxonomic annotations using Silva Database (http://www.arb-silva.de/).

2.6 Calculation and statistical analysis

Specific growth rate (SGR, %/d) of sea cucumber was calculated as:

$$SGR(\% / d) = \frac{\ln \left( \frac{W_f}{W_i} \right)}{t} \times 100$$

In which $W_i$ and $W_f$ are the initial and final body weight (g), respectively, $t$ is the duration time of the experiment (d).

One-way ANOVA analysis followed by Turkey and Duncan's multiple comparison test ($P < 0.05$) was used to assess the significant difference of SGRs, fatty acid compositions, amino acid compositions and immune related gene expressions among different dietary groups. ANOVA analysis was performed with software SPSS 22.0.

Shannon and Simpson index of the intestinal microflora were calculated using Qiime (V1.7.0). The significance of Shannon and Simpson index was determined using a Kruskal-Wallis pairwise test ($P < 0.05$) by agricolae package in R platform (V3.5.3). Linear discriminate analysis effect size (LEfSe) was performed to identify the potential biomarkers of microbial. The threshold on the linear discriminant analysis (LDA) score for biomarkers was 3.0. Spearman correlations between the intestinal microbes and gene expressions were conducted by “psych” package in R platform (V3.5.3).

3. Results

3.1 Growth performance of *A. japonicas*

*A. japonicas* in CK, SV and SM dietary groups had grown to an average weight of 4.00, 4.78 and 4.45 g by day 20 and 5.19, 6.41 and 5.83 g by day 40, respectively. The SGR in SV group was the highest, followed
by SM group, and the SGR in CK group was the lowest on day 20 and day 40 (Fig. 1). SGR of SV group on day 20 is significantly higher than that of the CK group ($P<0.05$), with an increase by 356%.

### 3.2 Fatty acid compositions

Fatty acid compositions of sea cucumber tissue and the diet gradients were expressed as the percentage proportion of each fatty acid to total amount of fatty acids (Table 2). Sea cucumbers in the CK and SV groups had significantly higher proportions of polyunsaturated fatty acid (PUFA) than those in SM group, while the latter had higher proportion of saturated fatty acid (SFA) and monounsaturated fatty acid (MUFA) ($P<0.05$). Sea cucumbers in SV group had the highest proportion of total $\omega$-3 fatty acid, followed by group CK, and then group SM (Fig. 2). Specifically, 20:3$\omega$3, 20:5$\omega$3 (EPA) and 22:6$\omega$3 (DHA) all exhibited the highest proportion in SV group, which increased by 18.5, 17.5 and 48.3% than those in the CK group, respectively (Fig. 2). In contrast, 18:3$\omega$6, 20:3$\omega$6, 20:4$\omega$6 and total $\omega$-6 fatty acids displayed comparable proportions in groups CK and SV, but higher than those of SM group ($P<0.05$). The ratio of $\omega$-3/$\omega$-6 fatty acids in SV group reached up to 0.83, and it was significantly higher than 0.6 and 0.52 in the CK and SM groups, respectively.
Table 2
Fatty acid composition in tissue of sea cucumber and feed ingredients (% dry mass)

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>Tissue-CK</th>
<th>Tissue-SV</th>
<th>Tissue-SS</th>
<th>Scallop visceral mass</th>
<th>Scallop mantle</th>
<th>S.thunbergii</th>
</tr>
</thead>
<tbody>
<tr>
<td>C12:0</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.25</td>
<td>0.10</td>
<td>0.20</td>
</tr>
<tr>
<td>C13:0</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>0.00</td>
<td>0.00</td>
<td>0.50</td>
</tr>
<tr>
<td>C14:0</td>
<td>1.75 ± 0.09b</td>
<td>1.53 ± 0.27b</td>
<td>2.29 ± 0.39a</td>
<td>6.34</td>
<td>ND</td>
<td>6.68</td>
</tr>
<tr>
<td>C14:1</td>
<td>0.56 ± 0.17b</td>
<td>0.58 ± 0.04b</td>
<td>0.81 ± 0.28a</td>
<td>0.04</td>
<td>0.20</td>
<td>0.00</td>
</tr>
<tr>
<td>C15:0</td>
<td>0.18 ± 0.01b</td>
<td>0.17 ± 0.05b</td>
<td>0.32 ± 0.03a</td>
<td>0.56</td>
<td>1.39</td>
<td>0.41</td>
</tr>
<tr>
<td>C16:0</td>
<td>11.79 ± 1.19b</td>
<td>9.76 ± 0.89c</td>
<td>14.81 ± 0.7a</td>
<td>22.01</td>
<td>28.26</td>
<td>31.47</td>
</tr>
<tr>
<td>C16:1</td>
<td>8.77 ± 0.27b</td>
<td>9.11 ± 0.95c</td>
<td>11.36 ± 1.54a</td>
<td>19.01</td>
<td>5.46</td>
<td>5.23</td>
</tr>
<tr>
<td>C17:0</td>
<td>1.09 ± 0.02b</td>
<td>0.98 ± 0.09c</td>
<td>1.83 ± 0.19a</td>
<td>0.67</td>
<td>2.27</td>
<td>0.22</td>
</tr>
<tr>
<td>C17:1</td>
<td>0.36 ± 0.04</td>
<td>0.36 ± 0.04</td>
<td>0.44 ± 0.04</td>
<td>0.00</td>
<td>0.00</td>
<td>0.18</td>
</tr>
<tr>
<td>C18:0</td>
<td>7.15 ± 0.27b</td>
<td>7.36 ± 0.08b</td>
<td>10.78 ± 0.2a</td>
<td>4.06</td>
<td>8.78</td>
<td>1.15</td>
</tr>
<tr>
<td>C18:1</td>
<td>10.08 ± 0.42b</td>
<td>8.84 ± 0.66c</td>
<td>12.82 ± 1.43a</td>
<td>6.09</td>
<td>4.50</td>
<td>6.85</td>
</tr>
<tr>
<td>C18:2ω6</td>
<td>3.06 ± 0.08a</td>
<td>2.82 ± 0.21b</td>
<td>2.47 ± 0.15b</td>
<td>3.03</td>
<td>1.77</td>
<td>2.48</td>
</tr>
<tr>
<td>C18:3ω6</td>
<td>0.23 ± 0.00a</td>
<td>0.23 ± 0.02a</td>
<td>0.13 ± 0.00b</td>
<td>0.35</td>
<td>0.11</td>
<td>0.21</td>
</tr>
<tr>
<td>C18:3ω3</td>
<td>0.9 ± 0.05a</td>
<td>0.99 ± 0.08a</td>
<td>0.52 ± 0.08b</td>
<td>0.10</td>
<td>0.20</td>
<td>1.56</td>
</tr>
<tr>
<td>C20:0</td>
<td>1.79 ± 0.05b</td>
<td>1.77 ± 0.07b</td>
<td>2.54 ± 0.2a</td>
<td>0.92</td>
<td>0.69</td>
<td>0.45</td>
</tr>
<tr>
<td>C20:1ω9</td>
<td>5.81 ± 0.41b</td>
<td>5.52 ± 0.34b</td>
<td>7.84 ± 0.38a</td>
<td>0.61</td>
<td>1.21</td>
<td>2.64</td>
</tr>
<tr>
<td>C20:2</td>
<td>1.73 ± 0.12a</td>
<td>1.86 ± 0.1a</td>
<td>1.41 ± 0.13b</td>
<td>0.30</td>
<td>0.41</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND: not detected. Different letter denote significant differences between data by one-way ANOVA analysis (p < 0.05).
<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>Tissue-CK</th>
<th>Tissue-SV</th>
<th>Tissue-SS</th>
<th>Scallop visceral mass</th>
<th>Scallop mantle</th>
<th>S.thunbergii</th>
</tr>
</thead>
<tbody>
<tr>
<td>C20:3ω6</td>
<td>0.72 ± 0.05a</td>
<td>0.7 ± 0.14a</td>
<td>0.49 ± 0.03b</td>
<td>0.21</td>
<td>0.23</td>
<td>0.33</td>
</tr>
<tr>
<td>C21:0</td>
<td>0.79 ± 0.09a</td>
<td>0.83 ± 0.28ab</td>
<td>1.19 ± 0.25b</td>
<td>0.00</td>
<td>0.00</td>
<td>ND</td>
</tr>
<tr>
<td>C20:4ω6</td>
<td>23.12 ± 0.32a</td>
<td>21.73 ± 2.1a</td>
<td>13.6 ± 1.96b</td>
<td>0.00</td>
<td>0.00</td>
<td>6.27</td>
</tr>
<tr>
<td>C20:3ω3</td>
<td>0.27 ± 0.02b</td>
<td>0.32 ± 0.03a</td>
<td>0.16 ± 0.04c</td>
<td>0.31</td>
<td>0.55</td>
<td>ND</td>
</tr>
<tr>
<td>C20:5ω3EPA</td>
<td>9.21 ± 1.26b</td>
<td>10.82 ± 0.04a</td>
<td>5.22 ± 0.64c</td>
<td>20.65</td>
<td>16.31</td>
<td>13.43</td>
</tr>
<tr>
<td>C22:0</td>
<td>1.84 ± 0.06b</td>
<td>1.81 ± 0.1b</td>
<td>3.03 ± 0.39a</td>
<td>0.09</td>
<td>0.54</td>
<td>19.73</td>
</tr>
<tr>
<td>C22:1ω9</td>
<td>1.1 ± 0.16b</td>
<td>0.98 ± 0.04b</td>
<td>2.24 ± 0.58a</td>
<td>0.15</td>
<td>0.23</td>
<td>ND</td>
</tr>
<tr>
<td>C22:2</td>
<td>0.85 ± 0.1</td>
<td>1.18 ± 0</td>
<td>ND</td>
<td>0.12</td>
<td>0.08</td>
<td>ND</td>
</tr>
<tr>
<td>C23:0</td>
<td>0.6 ± 0.08</td>
<td>0.59 ± 0.03</td>
<td>0.65 ± 0.31</td>
<td>0.08</td>
<td>0.14</td>
<td>ND</td>
</tr>
<tr>
<td>C24:0</td>
<td>0.21 ± 0.03b</td>
<td>0.25 ± 0.01b</td>
<td>0.32 ± 0.07a</td>
<td>0.15</td>
<td>0.55</td>
<td>ND</td>
</tr>
<tr>
<td>C22:6ω3DHA</td>
<td>6.01 ± 0.71b</td>
<td>8.91 ± 0.15a</td>
<td>2.74 ± 0.26c</td>
<td>13.92</td>
<td>26.04</td>
<td>ND</td>
</tr>
<tr>
<td>SFA</td>
<td>27.2 ± 0.94b</td>
<td>25.04 ± 1.14c</td>
<td>37.75 ± 0.64a</td>
<td>34.87</td>
<td>42.63</td>
<td>60.11</td>
</tr>
<tr>
<td>MUFA</td>
<td>26.68 ± 1.27b</td>
<td>25.39 ± 1.4b</td>
<td>35.51 ± 2.96a</td>
<td>25.90</td>
<td>11.58</td>
<td>14.91</td>
</tr>
<tr>
<td>PUFA</td>
<td>46.11 ± 2.05a</td>
<td>49.57 ± 2.48a</td>
<td>26.74 ± 3.03b</td>
<td>38.99</td>
<td>45.69</td>
<td>24.28</td>
</tr>
<tr>
<td>ω-3</td>
<td>16.39 ± 1.99b</td>
<td>21.04 ± 0.19a</td>
<td>8.65 ± 0.95c</td>
<td>34.97</td>
<td>43.09</td>
<td>14.98</td>
</tr>
<tr>
<td>ω-6</td>
<td>27.14 ± 0.19a</td>
<td>25.48 ± 2.2a</td>
<td>16.69 ± 1.95b</td>
<td>3.59</td>
<td>2.11</td>
<td>9.30</td>
</tr>
<tr>
<td>ω-3/ω-6</td>
<td>0.6 ± 0.08b</td>
<td>0.83 ± 0.06a</td>
<td>0.52 ± 0.01b</td>
<td>9.73</td>
<td>20.43</td>
<td>1.61</td>
</tr>
</tbody>
</table>

ND: not detected. Different letter denote significant differences between data by one-way ANOVA analysis (p < 0.05).

### 3.3 Amino acid compositions
Amino acid compositions of sea cucumber tissue from different dietary groups and the diet gradients are shown in Supplementary Table S2. The mass percentage of total amino acids summed up to 41.16 ± 2.32%, 41.38 ± 1.99% and 41.19 ± 1.41% in the CK, SV and SM groups, respectively. Essential amino acid accounted for 30 ± 1, 30 ± 1 and 29 ± 1% of the total amino acids in the CK, SV and SM groups, respectively. There are no significant differences for most of the amino acid component, total amino acid, total essential amino acid and total nonessential amino acid contents among three dietary groups, except histidine. Histidine contents in the CK and SV groups were significantly higher than that in SM group \((P<0.05)\).

### 3.4 Immune related gene expressions

The expression levels of immune related genes including TLR3, AjToll, MyD88, TRAF6, p50, p105, rel, M KK36 and p38 in the intestine of sea cucumber were determined (Fig. 3). The expression levels of most of these genes in SV and SM groups were higher than those in the CK group \((P<0.05)\). For SM group, the expression levels of these genes on day 15 except TLR3 increased by 2.3–24.6 times compared with those in the CK group. The expression of p50, p105 and rel genes in SV group on day 30 increased by 47.1, 30.6 and 44.8 times compared with those in the CK group, respectively. The expression levels of TRAF6, M KK36 and p38 genes in SV group on day 15 were 4.1, 0.7 and 0.4 times higher than those in the CK group.

### 3.5 Intestinal microbial community

#### 3.5.1 Microbial abundance and composition

Relative abundances of the top 10 phylum, class and top 15 order, family and genus microbes were presented in Fig. 4 and Supplementary Fig. S1. Phylum Proteobacteria was the dominant abundant microbe in the intestine of sea cucumber, accounting for 29.1–80.3% of the entire community. Then it was followed by Bacteroidota and Firmicutes, accounting for 3.0-36.7% and 2.8–27.1%, respectively. These three phylum accumulated to 71.8–90.6% of the entire community. The most four abundant microbial classes were Alphaproteobacteria, Gammaproteobacteria, Bacteroidia and Clostridia, accounting for 18.3–51.0, 10.8–42.4, 2.7–27.1 and 1.1–30.5% of the total microbe, respectively. At family level, Rhodobacteraceae was the most abundant, accounting for 16.1–45.4%, followed by Vibrionaceae, Propionibacteriaceae, Flavobacteriaceae, etc. The most five abundant genus were *Loktanella*, *Vibrio*, *Colwellia*, *Cutibacterium* and *Lentibacter*.

#### 3.5.2 Alpha diversity of microbial community

Venn diagram (Fig. 5) shows that intestine microbe in SV group exhibited the largest number of unique OUT during the whole experimental process, while microbe in SM group displayed the smallest number of unique OUT. Shannon diversity index of the microbial community in SV group on days 30 and 40 and that in SM group on day 30 were significantly higher than their synchronous Shannon index in the CK group.
Simpson index in the SV group on day 40 and in the SM group on day 30 were also significantly higher than their synchronous Simpson index in the CK group ($P<0.05$).

### 3.5.3 LEfSe analysis

The LEfSe was used to identify differentially abundant taxa (biomarkers) among treatments, whose abundances in a certain treatment are significantly higher than those in other treatments (Fig. 7, Supplementary Fig. S2). On day 15, the biomarkers in the CK group were more than those in SV and SM groups, while on days 30 and 40, the biomarkers were mainly found in SV and SM groups. On day 30, family Bifidobacteriaceae, Streptomycetaceae, Tannerellaceae, Clostridiaceae, Lachnospiraceae, Monoglobaceae, Ruminococcaceae, Methylobacteriaceae and order Rhizobiales were identified as biomarkers in the SV group, while family Bifidobacteriaceae, Lachnospiraceae, Ruminococcaceae, Hyphomonadaceae, Rhizobiaceae, Pseudoalteromonadaceae, Comamonadaceae and Methylobacteriaceae were biomarkers in the SM group. On day 40, biomarkers in the SV group included family Nocardioidaceae, Granulosicoccaceae and Holomonadaceae and order Oceanospirillales and Rhizobiales. Biomarkers in the SM group included family Acrobacteraceae, Comamonadaceae, Pseudomonadaceae, Vibrionaceae, etc.

### Discussion

Scallop visceral mass and mantle are generally considered as inedible portions of aquatic products. However, they are also rich in protein, amino acids, fatty acids and other nutrient substances (Li et al., 2014; Wu et al., 2016; Xing et al., 2011), which is a potential source for the feed supplement of precious marine products. In this case, we turned our attention towards the effects of dietary scallop visceral mass and mantle on growth, amino acid and fatty acid profiles, immune responses and intestinal microbiota of sea cucumber ($A. japonicus$). Growth performance is one of the most important indices to evaluate the effects of sea cucumber aquaculture. Our results show that the dietary supplementation of scallop visceral mass could promote the SGR of sea cucumber, which was increased by 356% on day 20 compared with that in the CK group. This indicates a beneficial effect of dietary supplementation of scallop visceral mass on the growth of juvenile $A. japonicas$. Furthermore, scallop viscera was also found to improve the nutrition composition of sea cucumber. Fatty acids, especially highly unsaturated fatty acids (HUFAs), have been reported to play important roles in physiology and reproductive processes of both plants and animals (Bergé and Barnathan, 2005; Liu et al., 2007). Tissue fatty acid composition is an important index of the metabolism and growth of sea cucumber (Dalsgaard et al., 2003). In this case, the results show that dietary supplementation of scallop visceral significantly increased the contents of $\omega$-3 fatty acids including $20:3\omega3$, EPA, DHA, as well as $\omega$-3/$\omega$-6 ratio. $\omega$-3 fatty acid is essential to the growth and reproduction of sea cucumber. Study in $Parastichopus californicus$ indicated that high level of EPA and DHA in diet resulted in higher fecundity of female broodstock and better survival rate of descendant larvae (Whitefield et al., 2018). Higher $\omega$-3 fatty acid content in $A. japonicas$ tissue indicates the beneficial effect of scallop visceral mass on the health of sea cucumber. On the other hand, $\omega$-3 PUFAs such as EPA and DHA bring more nutritional benefits to human health, and a diet with high ratio of $\omega$-3/$\omega$-6 fatty acid is more desirable in reducing the risk of many chronic diseases, such as...
cardiovascular disease, cancer and inflammatory (Simopoulos, 2008). Therefore, higher ω-3 fatty content and ω-3/ω-6 ratio also suggest higher commercial value of *A. japonicas*.

The increase of tissue ω-3 fatty acids in the SV group likely results from the extremely high content of total lipid as well as ω-3 fatty acids in the scallop visceral mass (Table 1). In addition, the protein contents of scallop visceral mass and mantle approach to that of fish powder (about 70%) (Yu et al., 2015) and far exceed algae (Table 1). Therefore, scallop visceral mass and mantle, as aquatic product waste, could be used as the protein source substituting fish powder in sea cucumber diet, and increase the ω-3 fatty acid content of sea cucumber tissue at the same time.

Previous studies on sea cucumber culture have indicated that dietary supplementation of probiotics and biologically active substances could regulate intestinal microbiota and immunity (Wei et al., 2015; Yang et al., 2019; Yang et al., 2015). Hence, we further investigated the effect of dietary supplementation of scallop visceral mass and mantle on the immune related gene expressions and intestine microbial community of *A. japonicas*. Sea cucumbers are invertebrates that lack adaptive immune responses, and their defense mechanisms mainly rely on the nonspecific immune system of pattern recognition receptors (PRRs) and signal transduction (Xue et al., 2015). Toll-like receptors (TLRs) are well-characterized among the various types of PRRs in *A. japonicus*, which could specifically recognize conserved molecular structures and activate immune system (Kongchum et al., 2011). TLRs can recruit adaptor molecules MyD88 and TRAF6 for signal transduction to activate nuclear factor-kappa B (NF-κB) and mitogen activated protein kinases (MAPKs) pathways (Akira and Takeda, 2004; Lu et al., 2013a; Lu et al., 2013b).

Here, it is found that the expression of some genes related to the Toll-like receptor signal transduction in nonspecific immune system was up-regulated by dietary supplementation of scallop visceral mass and mantle, including AjToll, MyD88 and TRAF6 (two key adaptor molecules), p50, p105 and rel (three NF-κB proteins), MKK36 and p38 (two MAPK proteins). These results suggest a promising effect of dietary scallop visceral mass and mantle on the enhancement of immune defense in sea cucumber. Additionally, it is shown that the benefit for the immune system resulting from dietary scallop visceral mass and mantle have time effect. For SM group, the up-regulation of these immune related genes showed the most remarkable effect on day 15, while SV group exhibited the best immunostimulatory activity on day 30, suggesting different action modes of scallop visceral mass and mantle on immunity regulation of the sea cucumber.

Furthermore, dietary supplementation of scallop viscera and mantle are also found to manipulate the diversity of microbial community in intestine of *A. japonicas*. Dietary scallop viscera markedly increased the specific OUT of intestinal microbe during the whole process (Fig. 5), suggesting higher microbial diversity and more potential functions of microbial community. The microbial diversity index and the number of biomarkers were significantly increased by dietary supplementation of scallop viscera and mantle (Fig. 6, 7), especially on day 30, when the abundance of Bifidobacteriaceae, Streptomycetaceae, Clostridiaceae, Lachnospiraceae, Monoglobaceae and Rhizobiales in group SV and SM markedly increased compared with the CK group.
Family Bifidobacteriaceae, Streptomycetaceae, Clostridiaceae in the gut of *A. japonicas* in this case are dominantly contributed by genus *Bifidobacterium*, *Streptomyces* and *Clostridium*, respectively. *Bifidobacterium* is one of the best known probiotic bacteria, exhibiting antagonistic activities against microbial pathogens, immunomodulatory, antimutagenic and anticarcinogenic activities, and the effects of prevention and cure of pathogen induced diarrheas (Divya et al., 2012; Kim et al., 2012; Servin, 2004; Zorriezhahra et al., 2016). *Streptomyces* could produce many kinds of metabolites which have been used as antibiotics and drug primers with strong inhibition of bacteria and pathogens (Bi and Yu, 2016; Bibb, 2013). *Streptomyces* has been used in aquaculture and demonstrated the potential of bioremediation and improving animal growth and water quality (Babu et al., 2018; Das et al., 2006). *Clostridium* is one of the richest bacterial cluster in the intestine of human and animals. *Clostridium* species have been reported to attenuate inflammation and maintain the intestinal health via their cellular components and metabolites including butyrate, secondary bile acids and indolepropionic acid (Guo et al., 2020). It has been used in aquaculture and exhibited beneficial effects of promoting the growth performance, immune response, and digestive enzyme activities (Wang et al., 2019). *Rhizobiales* has been found in the intestine of zebrafish and shark and is associated with nitrogen fixation (Sapountzis et al., 2015; Stoll et al., 2007). The existence of *Rhizobiales* could alleviate nitrogen limitation through nitrogen fixation, producing bacterial nifH protein and enhance the growth of colony (Sapountzis et al., 2015).

It is shown that supplementation of scallop visceral mass and mantle increased the microbial diversity and the abundance of beneficial microbes, which might facilitate to establish a heather microbial ecosystem in the intestine of *A. japonicus*. Existing studies show that the dietary supplementation of probiotics might improve the innate immunity of sea cucumber (Lu et al., 2021; Sonnenburg et al., 2006; Wang et al., 2021). The enhancement of immune response of *A. japonicus* probably is also associated with the optimization of microbial community with dietary supplementation of scallop visceral mass and mantle (Supplementary Fig. S3).

**Conclusion**

The effect of dietary supplementation of scallop visceral mass and mantle on the physiology, immunity and intestinal microflora of *A. japonicas* has been determined. This study revealed the beneficial effects of dietary supplementation of scallop visceral mass and mantle on the health and growth of *A. japonicas*, including improving the growth rate, increasing the tissue ω-3 fatty acid content, enhancing the immunity and optimizing the composition of intestinal microbiota of sea cucumber. This study is the first time demonstrating the comprehensive effect of dietary supplementation of scallop visceral mass on sea cucumber culture. It is of great significance to reveal the promising application of scallop visceral mass and mantle in feed development in sea cucumber culturing industry, and the potential of high-value utilization of these scallop “wastes” as well.

**Declarations**

**Ethics approval**
All applicable international, national, and/or institutional guidelines for the care and use of animals were followed by the authors.

**Human and Animal Ethics**

Not applicable.

**Consent to participate**

All the authors agree to participate in this experiment.

**Consent for publication**

All the authors have approved the publication of this manuscript.

**Availability of supporting data**

The supporting data of this work are available upon request.

**Competing interests**

The authors declare no competing interests.

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**Author's contributions**

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Yu Yu, Mengshu Wang, Xin Wang and Xiangyun Ge. The first draft of the manuscript was written by Yu Yu and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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References


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Figures
Figure 1

Specific growth rate (SGR, %/d) of *A. japonicas* in different dietary groups on days 20 and 40. Different letters above bars denote significant differences among different groups (*P* < 0.05).

Figure 2
Contents of $\omega$-3 and $\omega$-6 fatty acid components (%) in *A. japonicas* tissue from different groups. Different letters above bars denote significant differences among different groups ($P < 0.05$)

**Figure 3**

Expression levels of immune related genes in intestine of *A. japonicas* from CK, SV and SM dietary groups on day 15, 30 and 40, respectively. Different lowercase letters denote significant difference ($P < 0.05$). SV: dietary supplementation with 3.5 % of scallop visceral mass powder; SM: dietary supplementation with 3.5 % of scallop mantle powder; CK: control group
Figure 4

Composition and relative abundance (%) of microbial community in each group at (A) Phylum, (B) Family and (C) Genus level, respectively.
Figure 5

Venn diagrams of out in the CK, SV and SM dietary groups on days 15, 30 and 40, respectively.
Figure 6

Alpha diversity of microbial community in group CK, SV and SM on days 0, 15, 30 and 40. (A) Shannon index, (B) Simpson index. Wilcox test was used. * $P < 0.05$, ** $P < 0.01$
Figure 7

Cladogram of the microbial communities in different dietary supplement groups on day 30. The color mode means differentially abundant taxa identified as biomarkers in different treatments. The six rings of the cladogram from inner to outside stand for phylum, class, order, family, genus and species.
Supplementary Files

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