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# Effects of *Bacillus halophilus* on growth, intestinal flora and metabolism of *Larimichthys crocea*

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ABSTRACT

The incorporation of probiotics into the diet of large yellow croaker has been demonstrated by several studies to confer partial disease resistance. *Bacillus halophilic* isolated from the intestinal flora was used to study its effects on performance growth indicators, intestinal tissue structure, intestinal flora and the metabolism of *Larimichthys crocea*. A total of 180 fishes with an initial body weight of (164.00 ± 54.00) g were fed diets with three different concentrations of *Bacillus halophilic*: 0 cfu/mL (FC0, control group), $10^8$ cfu/mL (FC8, treatment group), and $10^{12}$ cfu/mL (FC12, treatment group). The results showed that there were no significant differences in specific growth rate among all groups ($P > 0.05$). Compared to the FC0 group, the final body weight and Weight gain rate were significantly higher in FC8 and FC12 groups ($P < 0.05$). The Survival of the FC12 group significantly improved ($P < 0.05$). Compared to the FC0 group, crude protein content in muscle of the FC8 group significantly increased ($P < 0.05$), crude fat content significantly increased in the FC12 group ($P < 0.05$), crude protein content in whole fish experimental groups significantly increased ($P < 0.05$), and ash content significantly increased in the FC8 group ($P < 0.05$). In terms of antioxidant ability, the content of LZM in blood increased significantly in the FC8 group ($P < 0.05$), GSH content in liver of the FC12 group increased significantly ($P < 0.05$), while the content of MDA and AKP in blood and liver had no significant difference ($P > 0.05$). At the level of intestinal structure, there were no significant differences in villus height, crypt depth and goblet cell number between control group and treatment groups ($P > 0.05$). At the phylum level, Firmicutes was the dominant phylum, and the genus level, *Lactobacillus* and *Bacteroides* were the dominant bacteria in FC8 and FC12. A total of 1070 metabolites were identified, among which lipid metabolites accounted for 46.7%. Metabolites were involved in six main ways, mainly related to the metabolism of amino acids and lipids. The correlation analysis between microbes and metabolites showed that the intestinal flora of *Larimichthys crocea* could promote the synthesis of metabolites, among which *Bacteroides* and *Megamonas* could promote the synthesis of beneficial metabolites such as amino acids and vitamins. Through this study, we found that *Bacillus halophilic* can significantly improve growth, the antioxidant immunity ability and promote the expression of growth related metabolites, with the FC12 group being the better successful.

## 1. Introduction

*Larimichthys crocea* belongs to the family of Totoaba; the genus Croaker, with croaker, cucumber fish and other commonly known species, has the advantages of delicate and delicious meat, golden scales and lips, and a beautiful shape and thus is favored by consumers [1]. It is a unique mariculture fish in China. In 2015, the national production of large yellow croaker was 1.48 × $10^5$ t, mainly distributed in coastal areas of Fujian, Zhejiang and Guangdong, making it the largest mariculture fish [2]. The rapid development of the large yellow croaker industry has become an important link in our country's fishery economic growth [3], which not only leads to a serious abuse of resources but also to an immense amount of pollution and destruction the aquaculture environment. At the same time, it also causes frequent diseases to appear in the process of breeding large yellow croaker [4]. The main diseases of *Larimichthys crocea* are enteritis and ulcers, caused by *Vibrio harveyi* [5];

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gill rot, caused by *Campylobacter* [6]; *Nocardia* disease caused by seriolae; and white sarcoidosis of internal organs, caused by *Pseudomonas* gallinaceae [7,8]. Therefore, it is a feasible approach for the aquaculture industry to develop and utilize environment-friendly feed that can promote the growth of aquaculture organisms and prevent diseases [9,10].

Probiotics are a general term for active microorganisms that are beneficial to the host organism [11,12]. There are many kinds of probiotics, and 34 kinds of probiotics can be used as feeding microbial additives for cultured animals, recent studies have proven that when given supplementation with probiotics, farmed fish showed improved disease prevention and growth promotion [13,14]. As safe additive in aquaculture, probiotics have been shown to improve animal immunity and disease resistance, adjust the structure of intestinal flora, as well as provide nutrients and promote the growth of animals [15]. *Bacillus* is one of the most important alternatives to antibiotics in fish. It is unique in that it has good stability, can settle in the gut and produce a range of digestive enzymes. Compared to other candidates, *Bacillus* is considered a reliable probiotic and stands out among many probiotics for its positive effects in promoting growth, immune response and disease resistance. For example, when Bacillus isolated from soil was cultured and treated with Japanese *Anguilla japonica*, it was found that mortality was greatly reduced [16]. Dietary supplementation with *Bacillus* sp. improved protease and lipase activities, the contents of total protein and fat of proximate carcass and regulated the intestinal flora [17]. Similar results showed by *Channel Ictalurus punctatus* [18], *Carassius auratus* var. *Pengze* [19], *Ctenopharyngodon Idella* [20], and *Oreochromis niloticus.L* [21]. These examples confirm that *Bacillus* has a positive influence on the growth of the host. *Bacillus* can affect the intestinal flora and metabolites of aquatic animals. For example, in the study of *JadePerch* and *Tilapia*, it was found that *Bacillus* reduced the content of harmful bacteria in the intestinal tract and increased the number of beneficial bacteria [22,23]. In addition, *Bacillus* can not only improve the nutritional composition of turbot carcass by promoting the probiotics in turbot intestine, such as *Acinetobacter*, *Ralstonia*, *Lactobacillus*, and *Bifidobacterium*, and reducing the relative abundance of the bacteria, such as *Vibrio* and *Bacteroides* in turbot intestine [24], but also can play a desirable role in removing waste products from aquaculture environments, maintaining optimum water quality, and reducing stress, which can lead to an improved immuno-physiological balance, better growth and enhanced survival in target aquatic animals [25].

In this study, *Bacillus halophilus* isolated from the intestine of large yellow croaker was added to the feed to study its effects on growth, intestinal flora and metabolism in order to provide a scientific basis for green and healthy culture and make contributions to the development and application of healthy and environmentally friendly feed that promotes the quality improvement of large yellow croaker.

## 2. Materials and methods

### 2.1. Strain culture

#### 2.1.1. Strain isolation

Internal intestine was collected from each large yellow croaker and streaked onto LB agar and seawater agar (LB, 2216E; Haibo, Qingdao, China). Plates were incubated at 28 °C in aerobic conditions and colony formation was observed every 12h for three days. Based on colony morphology, the intestinal isolates were then cultured on 2216E to obtain pure colonies. The bacterial strain was stored at −80 °C in 25% glycerol until further analysis [26].

#### 2.1.2. Purification and screening of strains

For phenotypical characterization, isolates were grown on 2216E at 28 °C for 48 h. Biochemical and phenotypic characterizations of the isolates were analyzed as *Bacillus* are large (4–10 μm) Gram-positive, strictly aerobic or facultative anaerobic bacteria with capsules; the bacteria's morphology was studied by Gram staining following the manufacturer's instructions; and cell motility was examined by cultivation microscopy on LB [27]. Ten well-grown pure slides were selected from the microscopic examination results, and the corresponding culture medium was taken out on the ultra-clean workbench. Single colonies were inoculated in the corresponding autoclaved liquid culture medium and then placed in a thermostatic oscillator at 28 °C for overnight culture [28]. One mL of the overnight cultured bacterial solution was centrifuged at 9, 000×*g*/min for 10 min to discard the supernatant, and the bottom sediment was stored in an ice pack and sent to Shanghai Yuanshen Biotechnology for 16SrRNA sequencing. Among the purified strains, only *Bacillus halophilus* belongs to *Bacillus*, so *Bacillus halophilic* was chosen for the experiment.

### 2.2. Feed preparation

*Bacillus halophilus* was stored in glycerol and taken out for extended culture, and the single colony was inoculated in a beef paste peptone liquid culture medium and then incubated in a constant-temperature shaking room at 28 °C. Every hour, 0.1 mL of bacterial solution was absorbed to determine the concentration of bacterial solution in the bateria chamber [29]. According to the preliminary experiment, the experimental feed was prepared by evenly spraying the bacterial solution (in the process of spraying, constantly stirring to make the bacterial solution penetrate into the feed) on the basic feed (the basic feed consisted of Tianbang No. 3 large yellow croaker compound feed, which contained 17.03% crude protein, 2.43% crude fat, 1.59% crude ash, and 4.92% moisture). Except for the control group, there were two treatment groups, and the basic feed group was named the control group (FC0). The expression was $10^8$ cfu/mL (FC8) in the low concentration group and $10^{12}$ cfu/mL (FC12) in the high concentration group (after pre-experimental treatment, it was found that the bacteria concentrations of $10^8$ cfu/mL and $10^{12}$ cfu/mL had certain effects on the growth of large yellow croaker). The corresponding concentration of the bacteria solution was sprayed onto the feed and then air dried in a cool place until the activity of *Bacillus halophilus* was detected.

### 2.3. Aquaculture Management

The experiment was carried out at Zhoushan Peninsula Aquaculture Base and Changzhi Island Aquaculture Base. Before the formal experiment, 180 healthy and uniform (164 ± 54 g) fish were randomly divided into nine groups. Twenty fishes in each group were put in a cage of 1 × 1 × 1 m for two weeks, during which they were fed a basic diet to adapt to the diet and feeding time. After the end of temporary rearing, nine cages were numbered in three groups and three parallel sets were set in each group [29]. The treated feed was fed twice a day (at 5:00 and 17:00), and the feeding amount was about 3% of body weight. The feeding experiment lasted for 56 days. Water temperature was measured with a hydrometer (Mittel) every two days, and water quality was tested every three days by detecting the activities of ammonia nitrogen, nitrite and dissolved oxygen (all tested according to the instructions of the Yujiawang kit).

The water temperature was 24.1 ± 1 °C, nitrite was 0.005 g/mL, ammonia was 0.2 g/mL, and dissolved oxygen was >6.5 mg/L.

## 3. Data acquisition and measurement

### 3.1. Sample collection and processing

After the breeding experiment, according to the experimental method of Huang Jun [28], a 24-h starvation experiment was conducted on the experimental fish. Before sampling, the large yellow croaker was anesthetized with water and eugenol at a ratio of 1000:1 (volume ratio, in liters). Each group was counted and weighed, and basic data such as body length and body width were measured. Under sterile conditions,

three fish were dissected on ice, the abdominal cavity was cut open from the anus, the entire intestine was removed, and the connective tissue was carefully treated. The samples were placed in EP tubes, immediately stored in liquid nitrogen, and then immediately stored in a −80 °C refrigerator for intestinal microbial diversity and metabolome sequencing. The intestinal tissue cut in an aseptic operation was fixed in 4% paraformaldehyde (PFA), and the appropriate volume ratio of tissue to 4% paraformaldehyde was 1:7 (unit: L). The fixed tissues were sent to Hangzhou Houai Biotechnology for the follow-up production of tissue sections. The intestinal samples were fixed in a 4% multistage formaldehyde solution for 24 h, then dehydrated through a series of alcohol concentrations and cleaned with xylene. The sample is then embedded in paraffin. Cross sections (4–6 μm thick) were stained with hematoxylin using a microtome (HistoCoreBIOCUT, Leica Microsystems, Shanghai). An imaging microscope (NIKON ECLIPSE C1, Japan) was used to examine the morphology.

### 3.2. Growth index analysis

Survival (%) $= 100 \times N_t / N_0$;

Weight gain rate, $WGR\ (\%) = 100 \times (W_t - W_0)/W_0$;

Specific growth rate, $SGR\ (\%) = 100 \times (\ln W_t - \ln W_0)/t$;

$W_0$ and $W_t$ represent the initial and final weights of the experimental fish, respectively. $N_0$ and $N_t$, respectively, represent the number of fish in the cage at the beginning and the number of fish at the end of the aquaculture experiment. The t represents the number of days in the experiment.

### 3.3. Routine body composition determination

The crude protein, crude fat, moisture and ash content in whole fish and muscle of large yellow croaker were determined by routine after sample collection.

### 3.4. Analysis of serum and liver biochemical indexes

The activities of complement C4, alkaline phosphatase (AKP), glutathione peroxidase (GSH-PX), malondialdehyde (MDA) and lysozyme (LZM) in serum and liver were tested according to the instructions of the kit.

### 3.5. Statistical analysis

One-way analysis of variance (ANOVA) was carried out with SPSS 19.0 software, and the significance level was set at $P < 0.05$.

### 3.6. Determination of intestinal flora

The nine intestinal samples of fish—each treatment group had three samples processed in parallel—were sent to Biomarker Technologies. After the total DNA of the samples was extracted using specific primers, *Bacillus halophilus* was isolated as described in the previous study (forward: 5′-AGAGTTTGATCCTGGCTCAG-3′ and reverse: 5′-GGTTACCTTGTTACGACTT-3′). PCR (Labcycler 48, SensoQuest) amplification, purification, quantification and homogenization of the products were carried out to form a sequencing library. Illumina Novaseq 6000 was used to sequence the qualified library. The raw image data files obtained by high-throughput sequencing were transformed into raw sequencing sequences by base recognition analysis, and then the Raw Reads obtained by sequencing were filtered by Trimmomatic v0.33 software. Cut Adapt 1.9.1 software was used to identify and remove primer sequences, and Clean Reads without primer sequences were obtained. Usearch v10 software was used to splice the Clean Reads of each sample through overlap, and then the length of the spliced data was filtered according to the length range for different regions. UCHIME v4.2 software was used to identify and remove chimeric sequences and to obtain the final Effective Reads. Mothur version 1.30 was used for Alpha diversity analysis, and binary Jaccard, Bray Curtis, and (un)weighted unifrac (limited to bacteria) algorithms were used to present the species diversity matrix for Beta diversity analysis. After standardized processing according to OTU data, analysis results at various classification levels can be obtained.

### 3.7. Metabolome determination

The main process flow was as follows: the intestines of each treatment group were ground into homogenate samples, we transferred 100 μL of sample to a 1.5 mL EP tube, added 300 μL of methanol, then added 20 μL of internal standard, and vortex mixed for 30 s; we used an ultrasonic treatment for 10 min (ice water bath); let the sample stand for 1 h at −20 °C; the samples were centrifuged at 13, 000 rpm for 15 min at 4 °C; 200 μL of supernatant was carefully put into a 2 mL injection bottle; 20 μL of each sample was mixed into the QC sample, and then 200 μL was put on the machine. The LC-MS system for metabolomics analysis was composed of a water-age Acquity I-Class PLUS ultra-high performance liquid chromatography tandem water-age Xevo G2-XS QTof high-resolution mass spectrometer, which used an Acquity UPLC HSS T3 column purchased from a water-age chromatographic column (1.8 μm 2.1*100). According to different research needs, we introduce personalized metabolome analysis content, for example, Principal component Analysis (PCA), Partial Least Squares Discriminant analysis (PLS-DA), Difference multiple histogram analysis, correlation analysis, correspondence analysis, etc.

Notes: Positive ion mode: mobile phase A: 0.1% formic acid solution; Mobile phase B: 0.1% acetonitrile formate. Anion mode: Mobile phase A: 0.1% formic acid solution; Mobile phase B: 0.1% acetonitrile formate injection, volume 1 μL.

### 3.8. Screening criteria for significantly different substances

Metabonomics data has the characteristics of "high dimension and huge quantity". After qualitative and quantitative analysis of the detected metabolites, the multiple changes of metabolites in each group were compared, and different substances were selected: metabolites with multiple change≥2 and multiple change≤0.5 were identified as significant differences.

## 4. Results

### 4.1. Effects of Bacillus halophilus on growth performance of Larimichthys crocea

In Table 1, the results showed that there were no significant

**Table 1**
Effects of *Bacillus halophilus* on growth performance of *Larimichthys crocea*.

| project | control group | FC8 group | FC12 group | *P*-value |
|---|---|---|---|---|
| Initial body weight/g | 164.00 ± 53.95 | 164.00 ± 53.95 | 164.00 ± 53.95 | 1.000 |
| Final body weight/g | 222.24 ± 3.72$^{b}$ | 233.16 ± 6.18$^{a}$ | 237.55 ± 5.81$^{a}$ | 0.031 |
| WGR/% | 35.51 ± 2.27$^{b}$ | 42.17 ± 3.76$^{a}$ | 44.85 ± 3.54$^{a}$ | 0.031 |
| Survival/% | 93.33 ± 3.33$^{ab}$ | 90.00 ± 5.00$^{b}$ | 100 ± 0.00$^{a}$ | 0.032 |
| SGR/% | 0.85 ± 0.04 | 1.01 ± 0.10 | 1.00 ± 0.04 | 0.058 |

Note: Data are mean ± SD, n = 3. Different letters (a, b) above indicate significant differences between groups ($P < 0.05$, ANOVA and Duncan's multiple comparison test), the same as below.

differences in Specific growth rates among the treatment groups ($P > 0.05$). The final body weight and Weight gain rate significantly higher than those in the control group ($P < 0.05$). The Survival of the FC12 group was significantly improved ($P < 0.05$).

### 4.2. Effects of Bacillus halophilus on body composition of Larimichthys crocea

According to Table 2, in muscle components, crude protein content in the FC8 group was significantly increased ($P < 0.05$), crude lipid content in the FC12 group was significantly increased ($P < 0.05$), while moisture and ash content had no significant differences ($P > 0.05$). In whole fish components, crude protein content in the experimental group was significantly increased compared with the control group ($P < 0.05$), ash content in the FC8 group was significantly increased ($P < 0.05$), water content in the experimental group was significantly decreased ($P < 0.05$), and crude lipid content had no significant difference ($P > 0.05$).

### 4.3. Effects of Bacillus halophilus on serum antioxidant and immune indexes of Larimichthys crocea

According to Table 3, after the addition of *Bacillus halophilus*, the lysozyme content in the blood of large yellow croaker in FC8 group significantly increased ($P < 0.05$), and the lysozyme content in FC12 group was also on the rise, while the MDA content and AKP content were not significantly different ($P > 0.05$).

### 4.4. Effects of Bacillus halophilus on liver antioxidant and immune indexes of Larimichthys crocea

According to Table 4, the GSH-PX content of the FC12 group in the liver of large yellow croaker after treatment with *Bacillus halophilus* was significantly increased ($P < 0.05$), LZM showed an increasing trend after treatment with *Bacillus halophilus* although there was no significant difference ($P > 0.05$), and AKP content and MDA content were not significantly different ($P > 0.05$).

### 4.5. Analysis of intestinal structure

According to Table 5, there were no significant differences in villus height, crypt depth and goblet cell number between control group and treatment groups ($P > 0.05$).

### 4.6. Analysis of intestinal flora

The effective sequence length of all samples was mainly distributed around 420 bp. There were 415 OTUs in the control group, 405 OTUs in the FC8 group and 340 OTUs in the FC12 group. The species diversity and abundance of a single sample were reflected by alpha diversity. The general analysis consisted of Simpson, Shannon, Coverage, and Chao 1 indices. In Table 6, there is no significant difference in Shannon and Simpson between the control group and the treatment groups ($P > 0.05$),

**Table 2**
Effects of *Bacillus halophilus* on body composition of *Larimichthys crocea*.

| project | control group | FC8 group | FC12 group | *P*-value |
|---|---|---|---|---|
| Muscle composition (%) | | | | |
| Crude protein | $11.67 \pm 0.41^{b}$ | $12.82 \pm 0.72^{a}$ | $11.46 \pm 0.45^{b}$ | 0.045 |
| Crude lipid | $3.22 \pm 0.26^{b}$ | $3.45 \pm 0.10^{b}$ | $4.35 \pm 0.28^{a}$ | 0.002 |
| Moisture | $78.84 \pm 0.65$ | $76.68 \pm 1.69$ | $79.46 \pm 1.54$ | 0.103 |
| Ash | $1.88 \pm 0.03$ | $1.92 \pm 0.01$ | $1.89 \pm 0.007$ | 0.089 |
| Whole fish composition (%) | | | | |
| Crude protein | $12.33 \pm 0.01^{c}$ | $16.42 \pm 0.09^{a}$ | $15.67 \pm 0.25^{b}$ | 0.000 |
| Crude lipid | $17.13 \pm 0.74$ | $19.13 \pm 1.74$ | $18.21 \pm 0.85$ | 0.204 |
| Moisture | $66.79 \pm 0.13^{a}$ | $61.63 \pm 0.34^{b}$ | $62.16 \pm 1.29^{b}$ | 0.000 |
| Ash | $1.817 \pm 0.004^{b}$ | $1.864 \pm 0.016^{a}$ | $1.811 \pm 0.013^{b}$ | 0.003 |

**Table 3**
Effects of *Bacillus halophilus* on serum antioxidant and non-specific immune indexes of *Larimichthys crocea*.

| project | control group | FC8 group | FC12 group | *P*-value |
|---|---|---|---|---|
| MDA (nmol/mL) | $13.04 \pm 2.12$ | $15.34 \pm 2.80$ | $14.97 \pm 1.461$ | 0.439 |
| AKP | $17.71 \pm 3.54$ | $14.14 \pm 6.26$ | $15.98 \pm 7.06$ | 0.763 |
| LZM | $3.10 \pm 0.63^{b}$ | $4.57 \pm 0.36^{a}$ | $3.87 \pm 0.46^{ab}$ | 0.031 |

**Table 4**
Effects of *Bacillus halophilus* on liver antioxidant and non-specific immune indexes of *Larimichthys crocea*.

| project | control group | FC8 group | FC12 group | *P*-value |
|---|---|---|---|---|
| GSH-PX | $9.24 \pm 0.78^{b}$ | $9.34 \pm 0.44^{b}$ | $12.25 \pm 2.06^{a}$ | 0.049 |
| MDA (nmol/mg) | $1.43 \pm 0.04$ | $1.48 \pm 0.02$ | $1.57 \pm 0.08$ | 0.075 |
| AKP | $2.34 \pm 0.50$ | $4.53 \pm 1.14$ | $3.36 \pm 1.38$ | 0.117 |
| LZM | $46.39 \pm 2.64$ | $69.81 \pm 19.62$ | $57.88 \pm 13.49$ | 0.198 |

**Table 5**
Effects of *Bacillus halophilus* on intestinal microstructures of *Larimichthys crocea*.

| project | FC0 group | FC8 group | FC12 group | *P*-value |
|---|---|---|---|---|
| Villus height (μm) | $1158.63 \pm 45.07$ | $978.67 \pm 262.08$ | $1017.64 \pm 119.21$ | 0.439 |
| Crypt depth (μm) | $262.99 \pm 28.85$ | $226.73 \pm 9.27$ | $259.46 \pm 30.10$ | 0.220 |
| gobletcell quantity | $27.87 \pm 4.48$ | $19.13 \pm 2.26$ | $28.90 \pm 8.50$ | 0.149 |

**Table 6**
Alpha diversity analysis.

| Groups | Chao1 | Simpson | Shannon | Coverage |
|---|---|---|---|---|
| FC0 group | $1348.18 \pm 78.63^{a}$ | $0.9945 \pm 0.0009$ | $8.74 \pm 0.19$ | $0.9978 \pm 0.0002^{b}$ |
| FC8 group | $1193.27 \pm 90.56^{ab}$ | $0.9939 \pm 0.0002$ | $8.51 \pm 0.02$ | $0.9979 \pm 0.0001^{b}$ |
| FC12 group | $1128.53 \pm 45.55^{b}$ | $0.9939 \pm 0.0002$ | $8.73 \pm 0.03$ | $0.9985 \pm 0.0003^{a}$ |
| *P*-value | 0.027 | 0.361 | 0.077 | 0.015 |

but the Chao1 index of the FC0 group significantly improved, while the Coverage index of the FC12 group significantly improved ($P < 0.05$), which indicates that the richness of the FC0 group is higher and the probability of species detection in the FC12 group is higher. The higher the probability of detecting a species in the sample, the higher the coverage value. The coverage rate of each group was close to 1, which indicates that almost all sequences in the sample have been detected.

As shown in Fig. 1, the distribution of intestinal microbial species in different groups of large yellow croakers in all treatment groups was analyzed. At the phylum level, Firmicutes was the dominant microbial community, among which Firmicutes accounted for 31.31% on average. The average proportion of Firmicutes was 27.63% in the control group, 27.63% in the FC8 group, and 33.81% in the FC12 group. At genus level, *Lactobacillus* was the dominant genus with the highest average proportion of 3.87%, followed by *Bacteroides* (3.23%), *Colibacillus* (2.91%), and *Faecalibacterium* (2.54%) (Fig. 2).

### 4.7. Analysis of metabolite species

In order to know more about the changes of metabolites under different treatments, 1070 metabolites were identified from 9 samples by LC-MS platform non-targeted metabolomics technology. As shown in Fig. 3, the samples contained lipids (46.7%), organic acids and their derivatives (20.28%), organic monocyclic compounds (10.38%),

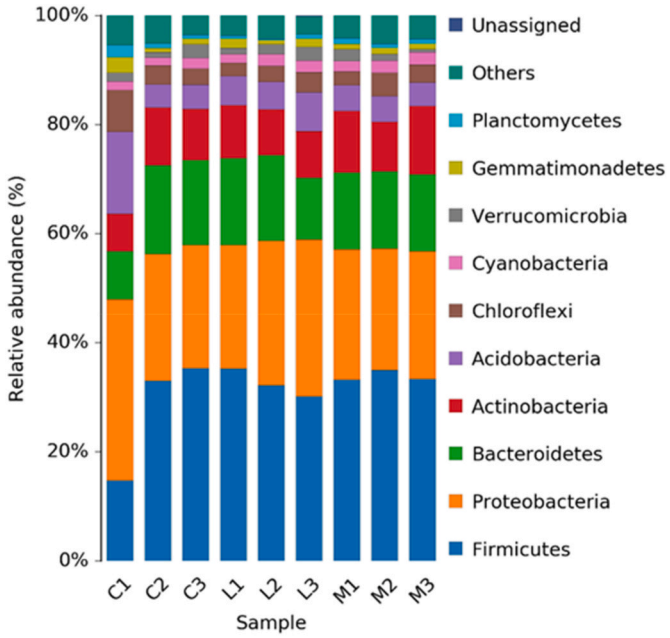



**Fig. 1.** Species distribution (phylum level).

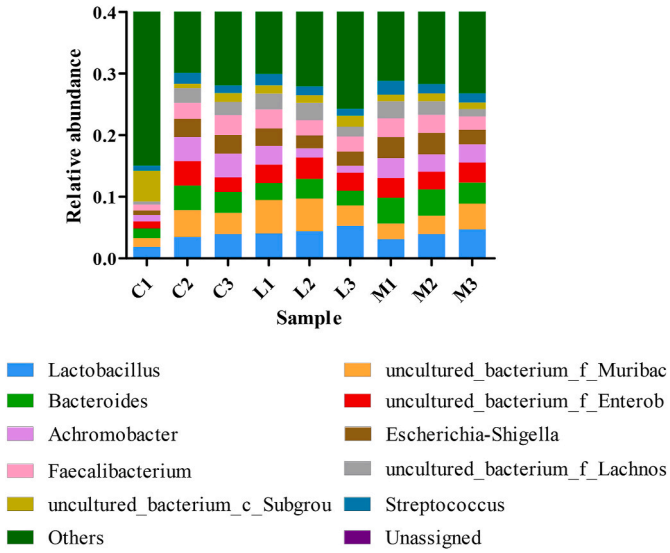



**Fig. 2.** Species distribution (genus level).

nucleotides and their analogues (5.66%), and ketones (5.66%) with the rest being organic compounds. There were 190 pathways involved, the metabolite analysis of all samples is shown in Fig. 4, among which 69.95% were related to metabolism, 17.03% to the biological system, 6.84% to human disease, 5.51% to environmental information processing, 0.5% to cell processes, and 0.17% to genetic information processing.

The principal component analysis (PCA) reflects the characteristics of multi-dimensional metabolomics data through several principal components, so we can observe the differences between different groups through principal component diagrams. As shown in Fig. 5, among the different results of this study, PCA treatment groups were obviously separated, which indicated that the metabolites of the sample changed after treatment. You can see the first and second principal components are separate. Among them, the first principal component (PC 1) can explain 50.5% of the original data set, and the second principal component (PC 2) can explain 22.6% of the original data set. The first

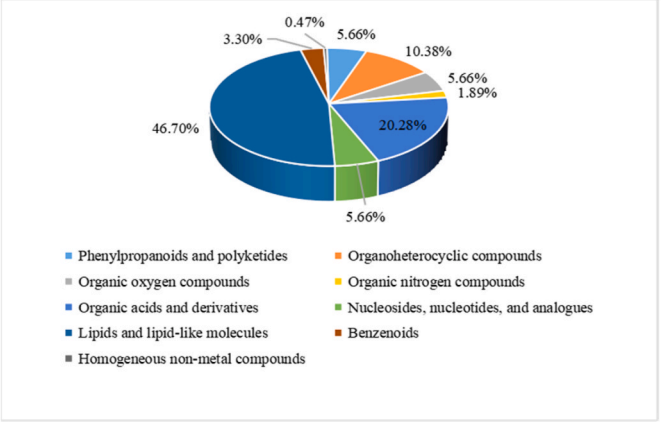



**Fig. 3.** Distribution of metabolite species.

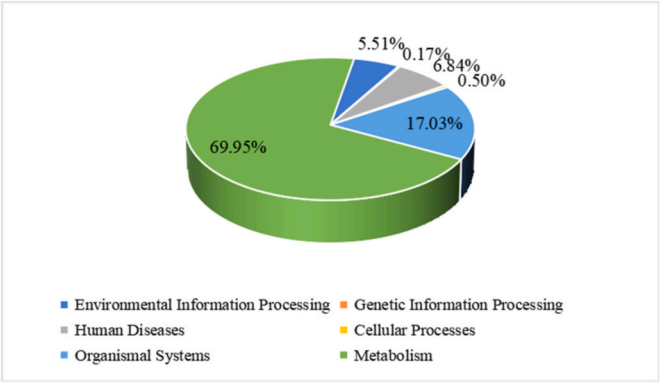



**Fig. 4.** Metabolic pathway distribution.

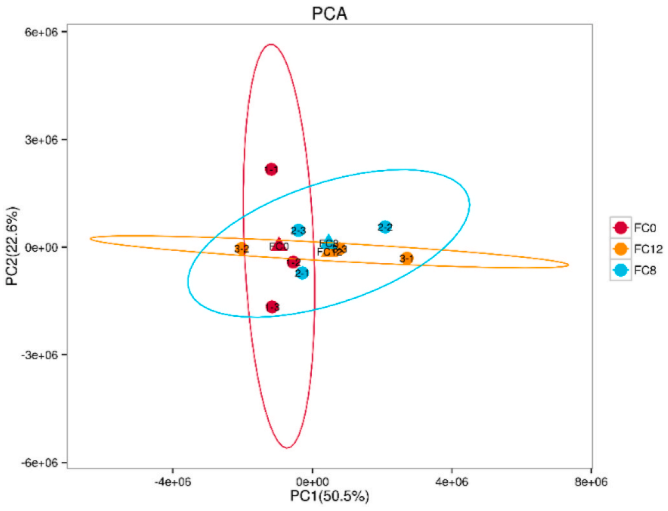



**Fig. 5.** PCA analysis plot.

and second principal components accounted for 73.1% of the total variance between samples. Generally speaking, cluster analysis and principal component analysis can show the differences in metabolites produced in different groups.

### 4.8. Analysis of differential metabolites

Partial Least Squares Discriminant Analysis (PLS-DA) is a

multivariate statistical analysis method with supervised pattern recognition that can effectively eliminate irrelevant influences and screen differential metabolites. An unsupervised PCA analysis was used to observe the overall contour distribution of the control group and the treatment group. Then, a PLS-DA analysis was carried out to evaluate the fitting degree ($R^2$) and prediction ability ($Q^2$) of the model, and permutation and combination tests were carried out (200 cycles). The closer the values of $R^2$ and $Q^2$ are to 1.0, the better the difference, fitting and predictability of the model. As shown in Fig. 6, the values of $R^2X$ (0.792), $R^2Y$ (0.999), and $Q^2Y$ (0.799) were all close to 1.0, which indicates that the model was reasonable.

After qualitative and quantitative analysis of the detected metabolites, the Fold Change of the quantitative information of the metabolites in each group was compared. Fig. 7 shows the logical results of the first 10 up-regulated and down-regulated metabolites in the experimental group compared with the control group after multiple treatments.

According to Fig. 7-A, the top 10 differentially up-regulated and down-regulated metabolites between the FC0 group and FC8 groups were mainly amino acids and lipids, among which amino acids included Argininosuccinic acid, Asp Phe, phenylalanyl-Glutamine, Histidinyl-Lysine, 5-Hydroxylysine, phenylalanyl-Histidine, and saccharopine. According to Fig. 7-B, the top 10 differentially up-regulated and down-regulated metabolites between the FC0 and FC12 groups were mainly amino acids and lipids, among which lipids included PA (18:0/18:2 (9z,12z)), Melibiitol, and C16 sphingosine. According to Fig. 7-C, the top 10 differentially up-regulated and down-regulated metabolites between the FC8 and FC12 groups were mainly lipids and purines, among which lipids included Lithocholic acid, L-palmitoylcarnitine, C16 sphingosine, and Trimethylammonioacetate.

### 4.9. Combined analysis of microorganisms and metabolism

#### 4.9.1. Correlation analysis between gut microbiota and metabolites

The Operational Taxonomic Unit (OTU) can be obtained based on the microbial diversity analysis. Combined with metabonomics, we can determine the relationship between metabolites and the OTU and further analyze the population structure, physiological metabolism, and genetic variation of microorganisms.

A heat map, or a thermogram, is one of the most widely used data mining methods in recent years. Its principle is based on aggregating a large number of test data and using a progressive blue-red band to visually display the results so as to see the density and frequency of the data and the regional differences of key research objectives. Correlations between intestinal microflora and metabolites can be analyzed by using a thermogram, as shown in Fig. 8.

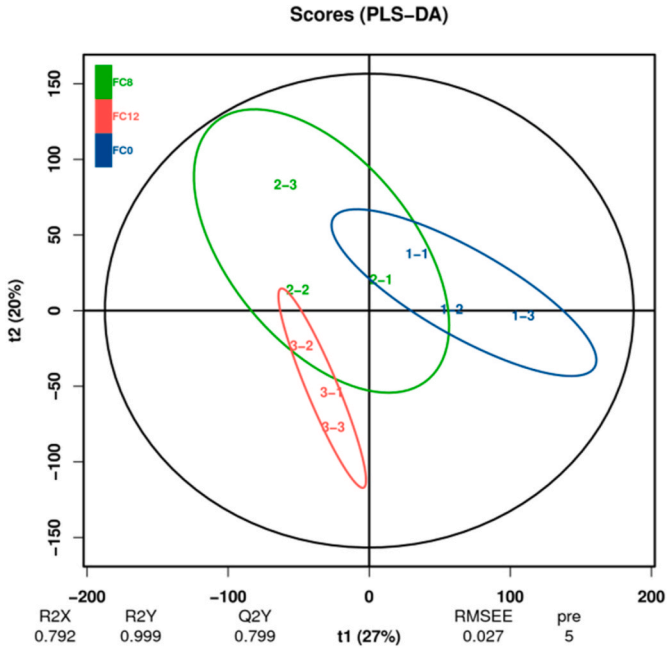



**Fig. 6.** Analysis of metabolites PLS-DA by group.

According to Fig. 8-A, 8-B and 8-C, at $P < 0.05$,*Luteimonas* has a positive correlation with Tetrahydroperidine and PG(16:0/20:3 (5z,8z,11z)); it was negatively correlated with 4-Aminobutyraldehyde, Tryptophyl-Glutamine, and Histidinyl-Lysine. *Lachnospiraceae-NK4A136-group* was negatively correlated with L-Glutamic-gamma-semialdehyde, PI (14:1(9z)/16:0), 5-Aminolevulinic acid, and fructoselysine-6-phosphate. *Bacteroides* was positively correlated with PI (14:1(9z)/16:0), Tryptophyl-Glutamine, PC (24:1(15z)/P-18:1 (11z)), PC (18:0/20:5(5z, 8z, 11z, 14z, 17z)), PC (18:1(11z)/20:5(5z, 8z, 11z, 14z, 17z)), PC (16:1(9z)/22:4(7z, 10z, 13z, 16z)), PC (20:3(5z, 8z, 11z)/P-18:1(11z)), and Palmitoleoyl-EA; it was negatively correlated with 4-alpha-Methyl-5-alpha-clolest-7-en-3-beta-ol. *Bilophila* was positively correlated with 4-Aminobutyraldehyde, Tryptophyl-Glutamine, PC (24:1(15z)/P-18:1(11z)), PC (18:0/20:5(5z, 8z, 11z, 14z, 17z)), PC (18:1(11z)/20:5(5z, 8z, 11z, 14z, 17z)), PC (16:1(9z)/22:4(7z, 10z, 13z, 16z)), and PC (20:3(5z, 8z, 11z)/P-18:1(11z)). There was a positive correlation between *Megamonas* and PI(14:1(9z)/16:0),5-Aminolevulinic acid, fructoselysine-6-phosphate and beta-ketophosphonate. *Solobacterium* was positively correlated with beta-ketophosphonate.

At $P < 0.01$, there was a negative correlation between *Luteimonas* and PI(14:1(9z)/16:0), Aminolevulinic acid, beta-ketophosphonate, PC (24:1(15z)/P-18:1(11z)), PC(18:0/20:5(5z, 8z, 11z, 14z, 17z)), PC(18:1 (11z)/20:5(5z, 8z, 11z, 14z, 17z)), PC(16:1(9z)/22:4(7z, 10z, 13z, 16z)), and PC(20:3(5z, 8z, 11z)/P-18:1(11z)). *Lachnospiraceae-NK4A136-group* was positively correlated with Tetrahydroperidine and 4-alpha-Methyl-5-alpha-clolest-7-en-3-beta-ol; it was negatively correlated with 4-Aminobutyraldehyde.*Bacteroides* was positively correlated with L-Glutamic-gamma-semialdehyde; it was negatively correlated with Tetrahydroperidine. *Bilophila* was positively correlated with 5-Aminolevulinic acid, beta-ketophosphonate and Palmitoleoyl-EA. *Megamonas* was positively correlated with 4-Aminobutyraldehyde. *Solobacterium* was positively correlated with fructoselysine-6-phosphate and PC (18:1(11z)/20:5(5z, 8z, 11z, 14z, 17z)).There was a positive correlation between *Dialister* and fructoselysine-6-phosphate.

#### 4.9.2. Correspondence analysis

The Correspondence Analysis, also known as the correlation analysis, is a multivariate statistical analysis technique. By analyzing the interaction summary table composed of qualitative variables, the relationship between variables can be determined. It can reveal the differences between different categories of the same variable, and the corresponding relationship between different categories of different variables. Restrictive correspondence analysis of metabolites and microflora can show the distribution characteristics of microflora and mine related metabolites, as shown in Fig. 9.

Note: pos – 772 is L-Glutamic-gamma-semialdehyde; pos-743 is 4-Aminobutyraldehyde;

pos-5986 is Tetrahydroperidine; pos-13371 is 4-alpha-Methyl-5-alpha-clolest-7-en-3-beta-ol;

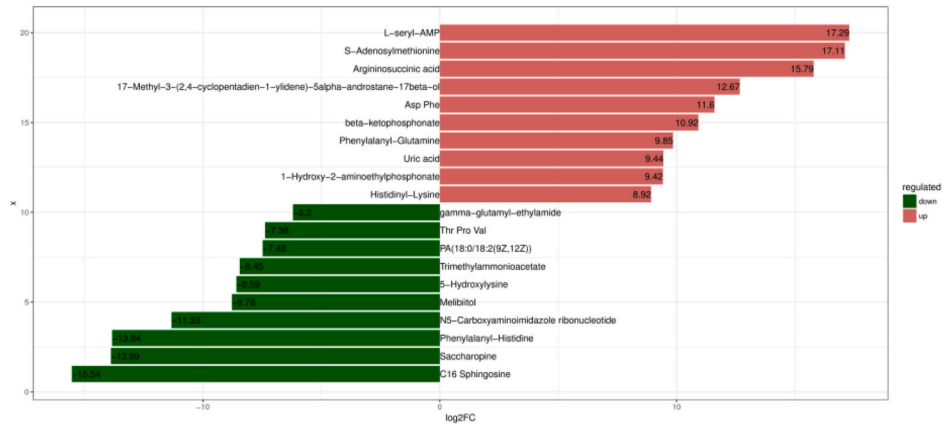
pos-10950 is fructoselysine-6-phosphate; pos-3080 is 5-Aminolevulinic acid;

pos-13938 was beta-ketophosphonate; pos-2385 is Tryptophyl-Glutamine;

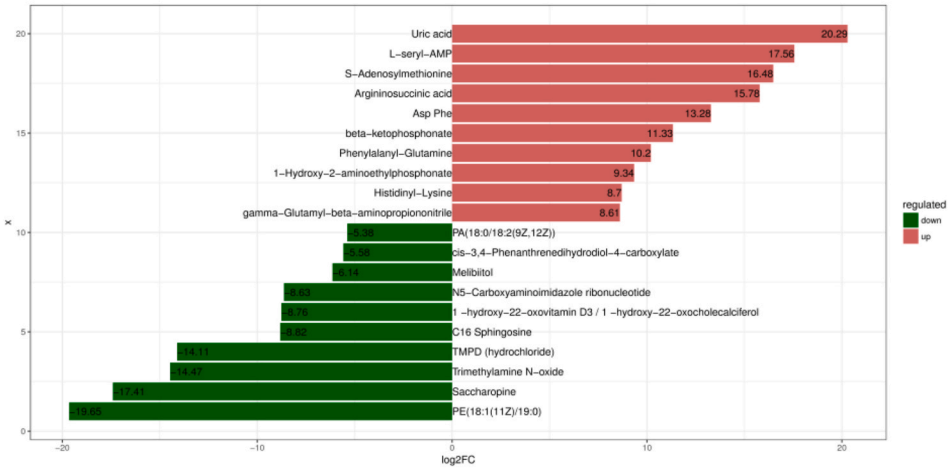
pos-3342 is Histidinyl-Lysine; pos-12971 is Palmitoleoyl-EA;

pos-2675, pos-13710, pos-14376 and pos-14048 are all poly-methylmethacrylate(PC)

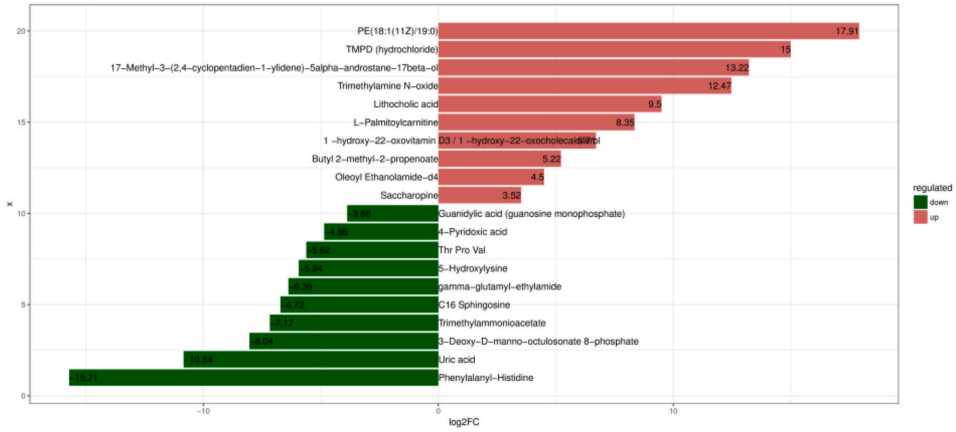
It can be seen in Fig. 9-A that L-Glutamic-gamma-semialdehyde is positively correlated with ASV14, ASV17, and ASV27, and negatively correlated with ASV78 and ASV83; 4-alpha-Methyl-5-alpha-clolest-7-




A




B




C

**Fig. 7.** Difference multiple histogram. A: FC0 vs FC8 B: FC0 vs FC12 C: FC8 vs FC12.

en-3-beta-ol is positively correlated with ASV78 and ASV83, and negatively correlated with ASV14, ASV17 and ASV27; 4-Aminobutyraldehyde is negatively correlated with ASV14 and ASV83; Tetracyanpteridine is positively correlated with ASV14 and ASV83. Fig. 9-B shows that ASV25, ASV37, and ASV38 are positively correlated with fructoselysine-6-phosphate, 5-Aminolevulinic acid, beta-ketophosphonate, Tryptophyl-Glutamine, and Histidinyl-Lysine; ASV7 was negatively correlated with fructoselysine-6-phosphate, 5-Aminolevulinic acid, beta-ketophosphate, Tryptophyl-Glutamine, and Histidinyl-Lysine; ASV15 is positively correlated with Histidinyl-Lysine, and negatively correlated with fructoselysine-6-phosphate, 5-Aminolevulinic acid, beta-ketophosphonate, and Tryptophyl-Glutamine. Fig. 9-C shows that ASV14, ASV6, ASV4 and ASV36 are positively correlated with Palmitoleoyl-EA and polymethylmethacrylate (PC); ASV7 is

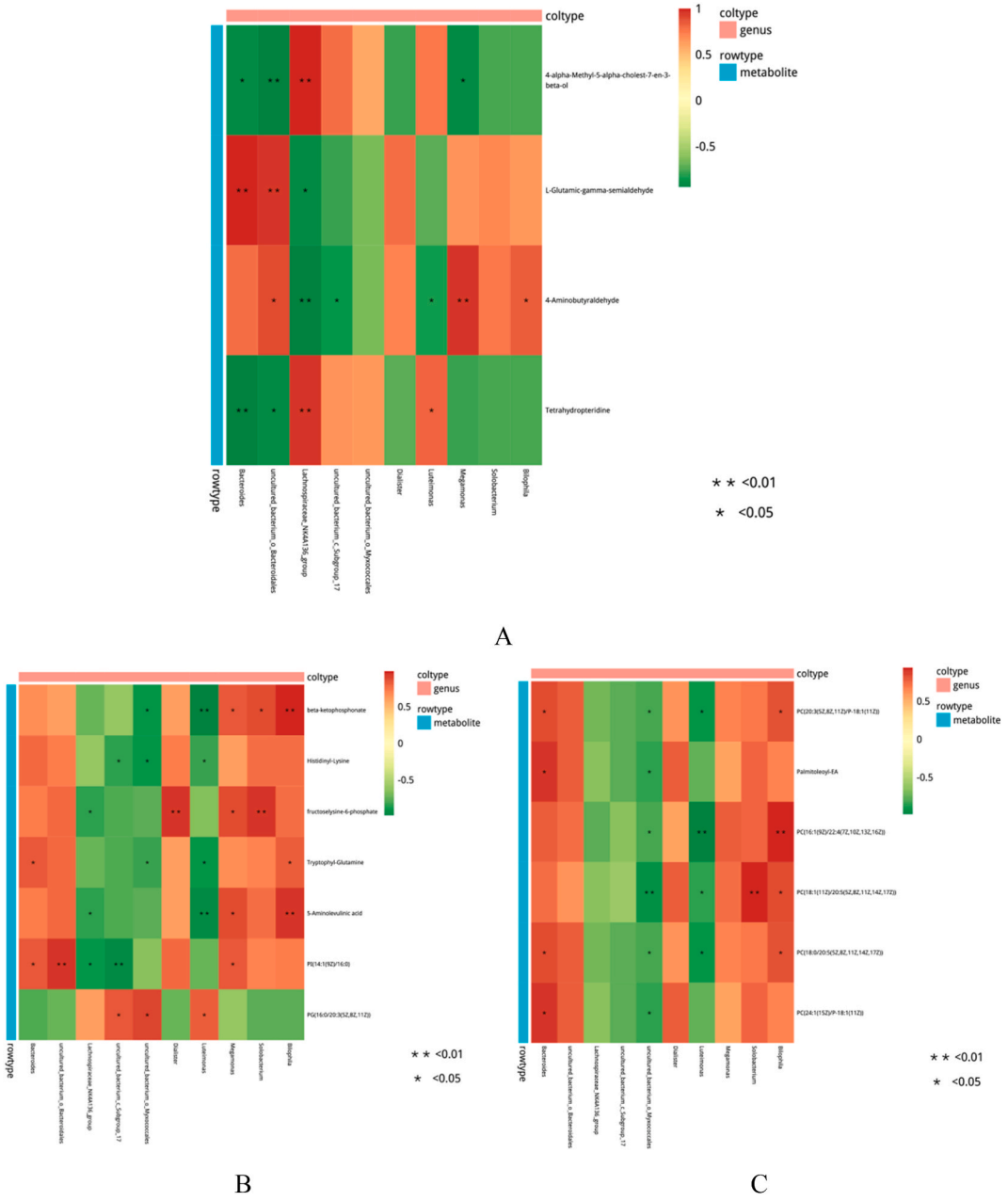



**Fig. 8.** Heat map of gut microbiota and metabolite correlation A: FC0 vs FC8 B: FC0 vs FC12 C: FC8 vs FC12.

negatively correlated with Palmitoleoyl-EA and poly-methylmethacrylate (PC).

## 5. Discussion

### 5.1. Effects of Bacillus halophilus on growth performance of Larimichthys crocea

Probiotics are widely used in aquaculture, and most of them are directly added to the diet for feeding [30]. Most studies have shown that probiotics can promote the growth of aquatic animals. For example, compound probiotics can improve the Weight gain rate and Specific growth rate of juvenile *Scophthalmus maximus* [31]. It also promotes the growth of *Cyprinus carpio* L. [32]. There are many reports on the effects of *Bacillus* on the growth and performance of aquatic animals. For example, studies on juvenile *Penaeus monodon*, *Ctenopharyngodon idella*, and *Takifugu obscurus* were conducted [33]. The *Bacillus halophilus* used in this experiment was isolated from the intestinal flora of juvenile *Larimichthys crocea* in our lab. When *Bacillus halophilus* was added to the diet for 56 days, its growth-promoting effect was mainly reflected in the Weight gain rate. The results showed that compared with the control group, the weight gain rates of both treatment groups were significantly improved. Compared with previous studies, the results of this study are consistent. In this study, it was found that the Survival of the FC12 group was significantly improved, it shows that *Bacillus halophilus* can enhance the disease resistance and risk resistance of large yellow croaker, and can effectively promote its growth.

### 5.2. Effects of Bacillus halophilus on body composition of Larimichthys crocea

The body composition of fish is affected by its growth environment, dietary conditions, health status, etc. The body composition of fish reflects the overall situation of the body, and the change of each

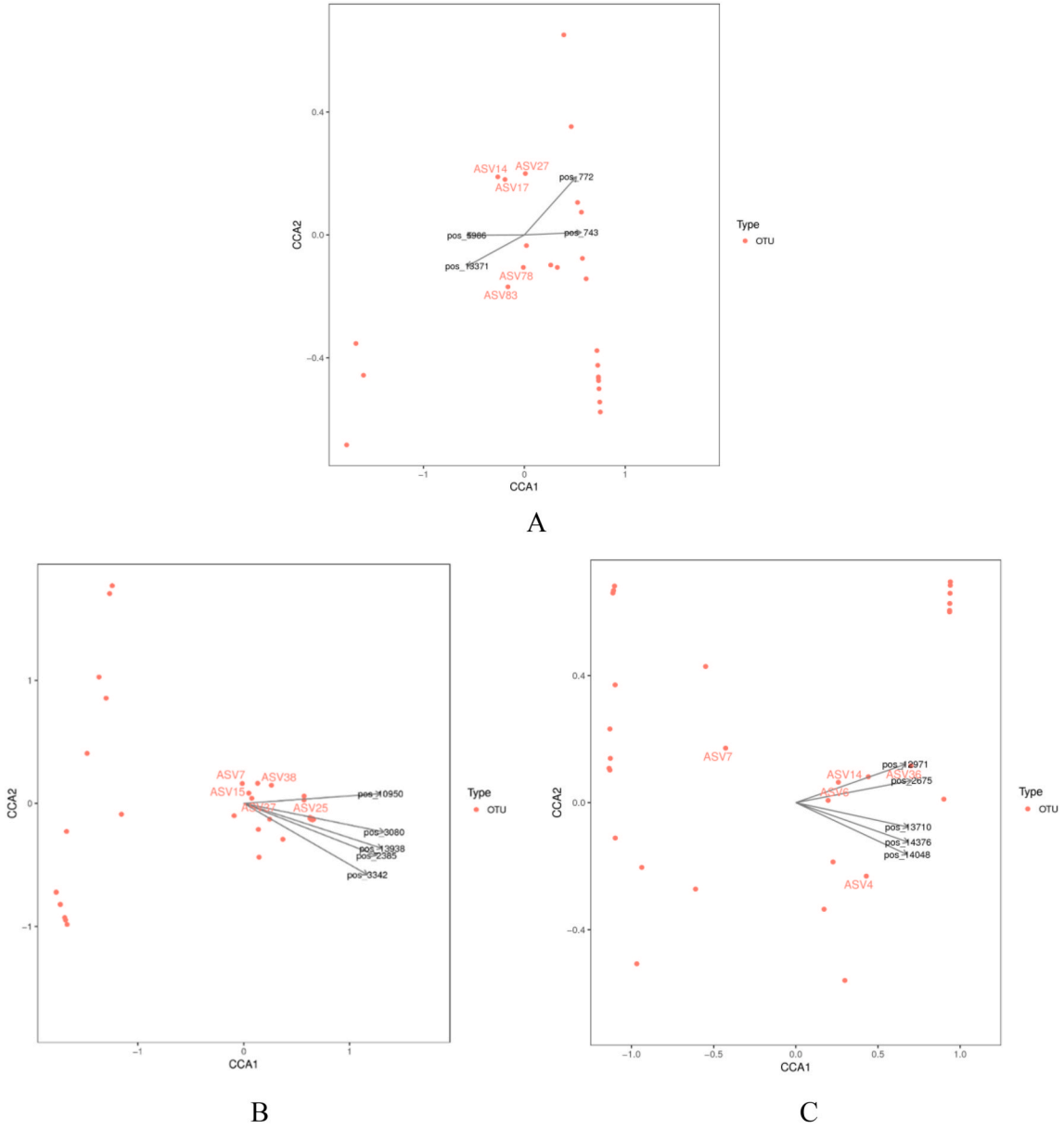



**Fig. 9.** Restrictive Correspondence Analysis Results A: FC0 vs FC8 B: FC0 vs FC12 C: FC8 vs FC12.

component affects the quality and nutritional value of meat [34]. Protein and fat content determine the nutrient composition of fish muscle. As the main source of energy of fish, fat produces energy several times higher than protein and sugar after decomposition, so the content of fat can reflect the growth and development status of fish [35]. The crude fat content in muscle of FC12 group significantly increased ($P < 0.05$), the crude protein content in FC8 group significantly increased ($P < 0.05$), and the crude protein content in whole fish of experimental groups significantly increased ($P < 0.05$), indicating that the addition of *Bacillus halophilus* promoted the growth and development of large yellow croaker. The ash content of FC8 group in the whole fish significantly increased while the water content significantly decreased ($P < 0.05$), indicating that the absorption ability of mineral elements was gradually enhanced after the addition of Bacillus halophilus, thus promoting bone development and making the body more robust.

### 5.3. *Effects of Bacillus halophilus on antioxidant and immunity in Larimichthys crocea*

In fish, glutathione peroxidase (GSH-PX) is a common antioxidant enzyme that can remove excess damaging reactive oxygen species (ROS) and protect cells from damage caused by ROS through lipid peroxidation [36–38]. Malondialdehyde (MDA) is used as a biomarker of lipid peroxidation in tissues, which can reflect the degree of oxidative damage in vivo, and high levels of MDA lead to high cytotoxicity [39,40]. GSH-PX plays a crucial role in scavenging free radicals and derivatives and reducing the formation of lipid peroxides [41]. In the liver of large yellow croaker, the GSH-PX activity of the FC12 group significantly increased ($P < 0.05$), while the MDA activity of all groups was not significantly different ($P > 0.05$), indicating that the addition of *Bacillus halophilus* did not cause damage to the liver of large yellow croaker and that the antioxidant activity of the liver was improved.

Alkaline phosphatase (AKP) is a common immune factor in the fish immune system that can kill and eliminate microorganisms or foreign substances. The increase in enzyme activity indicates an enhancement of

fish immune ability. For example, in the study of *Apostichopus japonicus*, AKP activity was improved under the regulation of *Bacillus subtilis* D1-2 [42]. In this experiment, there was no significant difference in blood and liver alkaline phosphatase (AKP) among all groups ($P > 0.05$), but the activity of AKP in the liver of FC8 group was higher than that in the control and the FC12 groups. The results showed that *Bacillus halophilus* could improve liver non-specific immunity in large yellow croakers, and the lack of AKP in the liver FC12 group was probably related to the high supplemental level of *Bacillus halophilus*. LZM is a mucolytic enzyme that also acts as an opsonin to activate the complement system [43]. Peptidoglycans acting on bacterial walls promote hydrolysis of the link between n-acetyl-D-glucosamine and n-acetyluric acid and play a role in mediating resistance to microbial invasion [44]. Examples include *Cyprirnus Carpio Songpa Linnaeus Mirror* [45], olive flounder (*Paralichthys olivaceus*) [46], and tilapia (*Oreochromis niloticus*) The study of niloticus [47] showed that feed addition of *Bacillus subtilis* could increase the activity of LZM in aquatic animals. In this experiment, the activity of lysozyme (LZM) in the blood of the FC8 group significantly increased ($P < 0.05$), while there was no significant difference between the liver control and the experimental groups ($P > 0.05$), which was similar to the results of feeding juvenile cobia with *Bacillus subtilis*. The increase in lysozyme activity by *Bacillus* may be due to its cell wall components or the active substances produced by *Bacillus*, which stimulate the host intestinal immune cells to enhance the expression of related immune genes, thus enabling the host to improve the innate immune capacity of the body [48]. The results of this study indicate that *Bacillus halophilus* can improve the non-specific immunoenzyme activity of large yellow croakers, but the optimal supplemental level needs to be further studied.

### 5.4. Effect of Bacillus halophilus on intestinal structure and flora of Larimichthys crocea

Fish intestinal structure can be divided into four layers, namely the mucosal layer, the submucosal layer, the muscle layer and the serous layer. The mucosal epithelium consists of single columnar epithelial cells with goblet cells scattered among them. Intestinal epithelial cells secrete digestive enzymes and mucus to protect the intestine, lubricate food, and promote the passage of food through the intestine [49], and they also produce a trifolate protein that repairs mucosal epithelial cells and accelerates intestinal mucosal repair [50]. Goblet cells secrete digestive enzymes and mucus that protect epithelial cells and lubricate food for easy passage through the intestine [51]. Goblet cells can also produce a trilobate protein that can repair mucosal epithelial cells and accelerate the repair of intestinal mucosa [52]. In this study, there was no significant difference in the number of goblet cells between the control group and the treatment group ($P > 0.05$), but the number of goblet cells in the treatment group was less than that in the control group, which was more obvious in the FC8 group. The number of goblet cells decreased, indicating that the integrity of the intestinal mucosa in the treatment group was better than that in the control group. The higher the villi, the larger the area of the absorption site and the stronger the digestive ability. The reduction of villi height means that the intestinal absorption area is reduced and the supply of nutrients is reduced [53]. Crypt depth reflects the ability of intestinal cells to differentiate villi [54]. In this study, villi height was reduced in the treatment group compared to the control group. This is contrary to the findings of *European lobster* studies [55]. It can be inferred that the addition of bacteria will affect the stability of intestinal flora, thereby affecting intestinal absorption capacity and resulting in a reduction in the height of intestinal villi. At present, the reason why *Bacillus halophilus* affects the intestinal structure of large yellow croakers is not clear, and its specific mechanism needs to be further studied and evaluated.

The intestine is an important part of a fish's body [56]. Intestine can provide a barrier to the body, promote the absorption of nutrients and enhance immunity [57]. They form an interdependent and mutually restrictive microecosystem with the host and its environment and play a very important role in the nutrition, development and immunity of the host [58]. In this study, it was found that the intestinal flora of *Larimichthys crocea* in different treatment groups had the same dominant flora species at phylum and genus levels, among which Firmicutes was the dominant phylum and *Lactobacillus* was the dominant genus. The results of this phylum level study are similar to those of *Monopterus albus* which was treated with probiotic compound fermented feed [59], and the intestinal flora of *Monopterus albus* was fed with a probiotic compound that was dominated by Firmicutes. However, the results of probiotics on *Rachycentron canadum* showed that the dominant bacteria group after probiotic addition was the genus *Luminescence* [60], which belongs to the phylum Gracilicutes. This is different from the results of our study, which may be related to the experimental objectives and feeding environment.

*Lactobacillus*, a Gram-positive bacterium belonging to the phylum Firmicutes, belongs to the family *Bacillus* and has many beneficial effects on its host [61]. The application of *Lactobacillus* bacteria in aquatic products can enhance the immunity of aquatic organisms, maintain the microecological balance of animal intestines, improve the water environment, promote the growth of organisms, and prevent and inhibit harmful substances [62,63]. In this experiment, *Lactobacillus* was the main species of fish fed by *Bacillus halophilus*. It can be inferred that the feeding of *Bacillus halophilus* caused the growth of other strains, and the main growing bacteria was *Lactobacillus* of the same genus. It is speculated that *Bacillus halophilus* has a certain resistance to harmful substances and maintains intestinal stability to prevent bacterial damage. *Bacteroides* is the main synthetic bacteria for vitamin K. *In vivo*, vitamin K is mainly involved in blood coagulation. The carboxylase system dependent on vitamin K not only plays an important role in blood coagulation but is also related to the formation of the calcium binding protein osteocalcin [64,65]. *Bacteroides* can prevent or treat osteoporosis by increasing bone density [66]. The addition of vitamin K to fish feed has no effect on the quality of haddock bone matrix. When vitamin K is deficient, haddock first shows a decrease in bone mineralization levels, then a decrease in total bone mass, and an increase in the deformity rate [67]. In this study, *Bacteroides* belongs to the predominant genus of intestinal microbial diversity. It can be speculated that the addition of *Bacillus halophilus* leads to an increase in *Bacteroides*, which promotes the synthesis of vitamin K in large yellow croakers and keeps the bones of *Pseudosciaena crocea* growing normally.

### 5.5. Effects of Bacillus halophilus on metabolism of Larimichthys crocea

Forty-six percent of the metabolites detected in the intestinal microbiota of *Larimichthys crocea* were lipids, indicating that the lipid metabolism in the intestinal microbiota was the most robust, and the lipid metabolites were mainly Glycerophospholipid (Glycerophospholipid), fatty acyl (FA), and prenol lipid (PRs). Lipids are mainly divided into eight categories: fatty acids (FAs), triglycerides (GL), glycerophospholipids (GPs), sphingolipids (SP), glycolipids (SL), polyketides (PK), sterol lipids (SP), and prenol lipids (PRs) [68]. From the analysis of differential metabolites, it can be seen that the detected metabolites are not only lipids but also amino acids.

Lipids are mainly composed of Lithocholic acid, a type of sterol lipid (SP), which is a secondary bile acid that participates in the digestion and absorption of fat and fat-soluble vitamins, regulates intestinal flora, and maintains the barrier function of intestinal mucosa [69]. Studies have shown that Lithocholic acid can stimulate liver cells to establish an insulin resistance model and improve the hypoglycemic activity of insulin [70]. Through this experiment and from the analysis of differential metabolites, it can be seen that Lithocholic acid (also known as lipid compounds) is up-regulated, which indicates that *Bacillus halophilus* has a significant promoting effect on lipids and that lithocholic acid in lipids can improve intestinal flora and antioxidant capacity to some extent. To sum up, *Bacillus halophilus* can enhance the ability to regulate intestinal flora and promote the content of crude fat in muscle and whole fish to

some extent.

Among them, the main amino acid metabolites detected are Arginine, Aspartic acid and Phenylalanine. Arginine, as an essential amino acid, can be involved in regulating physiological functions such as protein synthesis, immunity, intestinal barrier function, wound repair, anti-inflammatory, anti-oxidation and so on [71,72]. For example, dietary arginine supplementation can promote the proliferation of intestinal epithelial cells and protein synthesis of intestinal epithelial cells, thus improving the morphology of intestinal mucosa, reducing the injury of intestinal mucosa, and playing an important role in regulating the barrier function of intestinal mucosa [73,74]. As a functional amino acid, Aspartic acid (Asp) plays an important biological role in glucose metabolism, the urea cycle, mitochondrial function, etc. [75,76]. It can improve the immune and antioxidant functions of animals and promote their growth and development [77,78]. Phenylalanine (Phe) is an aromatic amino acid that is necessary for fish growth [79]. *In vivo*, phenylalanine is mainly converted into tyrosine, which is the precursor of the thyroid hormones norepinephrine and epinephrine [80]. The results showed that proper phenylalanine can promote the growth of *Ctenopharyngodon idella* in the middle growth stage, improve its meat quality, enhance the antioxidant capacity of muscle tissue and improve the intestinal mucosal immune response of *Ctenopharyngodon idella* [81]. Through the analysis of differential metabolites, it was found that the contents of amino acids such as Arginine, Aspartic acid and Phenylalanine in intestinal metabolites of *Larimichthys crocea* were up-regulated, which indicated that *Bacillus halophilus* could promote the production of amino acid metabolites in the intestinal tract, thus increasing the protein content of the whole large yellow croaker.

### 5.6. Combined analysis of gut microbiota diversity and metabolism

L-Glutamic-gamma-semialdehyde is the metabolic intermediate stage of arginine and proline, proline is an effective osmotic regulation substance, can maintain the cell membrane structure and the stability of the protein subcellular structure, and has the role of scavenging reactive oxygen [82] when the animal body is experiencing stress, injury, pregnancy, postpartum, etc. The demand for proline increases dramatically [83]. Proline plays an important role in the growth and health of fish. Studies have shown that dietary proline can promote the deposition of collagen in the body of *Nibea coibor* [84]. Many fish have a high demand for proline. In order to attain the highest growth, enough proline must be obtained from the diet, so it is often classified as a conditionally essential amino acid or functional amino acid for fish [85]. L-Glutamic-gamma-semialdehyde was positively associated with *Bacteroides*, as *Bacteroides* increase. L-Glutamic-gamma-semialdehyde increases, followed by the metabolic of arginine and proline. *Bacteroides* abundance increases with the increase of *Larimichthys crocea* in the intestinal flora advantage bacterium group *Bacteroides (3.23%)*. It is speculated that *Bacillus halophilus* can enhance the metabolic pathway of arginine and proline, thus promoting the growth of *Larimichthys crocea* and improving the feed utilization rate.

5-Aminolevulinic acid is a necessary substance for the biosynthesis of chlorophyll, heme and vitamin B12 [86]. On the other hand, the intestinal microbiota produce hundreds of bioactive compounds [87], including B vitamins, which play important physiological roles in the host by supporting the adaptability of symbiotic species and inhibiting the growth of competing species [88]. Studies have shown that *Megamonas* [89], *Bifidobacterium* [90,91], *Lactococcus* [92], and *Bacteroides* [93] fragilis participate in the synthesis and metabolism of vitamin B12 [94]. In this experiment, *Megamonas* is positively correlated with 5-Aminolevulinic acid. Therefore, it can be speculated that *Bacillus halophilus* has a promoting effect on 5-Aminolevulinic acid and has enhanced significance for the growth physiological indices of large yellow croakers.

## 6. Conclusion

Through this study, we found that *Bacillus halophilus* could significantly improve the WGR and Survival of large yellow crocks, increase the crude fat and crude protein content, enhance the antioxidant and immune capacity of the blood and liver, maintain the stability of intestinal flora, and promote the expression of growth-related metabolites. It was found that the effect of the high concentration treatment group (FC12 group) was relatively good. It has certain guiding significance for large yellow croaker culture, but it still needs to be optimized and improved to guide the limitations of production practice.

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## Author contributions

Ling Huang, Xiaomei Shui, Ping Wang conceived and designed research. Ling Huang, Xiaomei Shui, Hanying Wang, Haoyu Qiu, Chenzhi Tao, Ruoyu Chai, Heng Yin, Ping Wang conducted experiments, analyzed data, and wrote the manuscript. The authors critically reviewed and approved the manuscript.

## Ethical standards

All international, national, and institutional guidelines for the care and use of animals were followed. This study was approved by Animal Experimental Ethical Inspection, Institutional Animals Care and Use Committee of Zhejiang Ocean University(2023035).

## Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Wangping reports financial support was provided by Zhejiang Ocean University.

## Data availability

The data that has been used is confidential.

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