# Effects of weaning American glass eels (*Anguilla rostrata*) with the formula diet on intestinal microbiota and inflammatory cytokines genes expression

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ABSTRACT

This study aimed to investigate the effects of weaning American glass eels (*Anguilla rostrata*) with the formula diet on intestinal microbiota and the expression of inflammatory cytokines genes. During the feeding trial, the control group (termed IF group) was fed with initial feed for 34 days, and the experimental group (termed FF group) was fed with initial feed for 30 days, and then weaned with the formula diet for 4 days. After feeding trial, intestines were subjected to microbiota analysis using 16S rDNA high-throughput sequencing, and expression of three inflammatory cytokines genes in gut were examined by qPCR. The results indicated that the species richness and diversity of intestinal microbiota exhibited significantly higher in FF group than that in IF group $(P < 0.05)$. At the phylum level, the core intestinal microflora was the same for two groups. The most abundant phylum was Firmicutes in IF group, while it was Proteobacteria in FF group. Five genera were significantly higher in the IF group compared with the FF group, and *Bacillus* was the most major enriched biomarker at genus level. Nine genera were significantly higher in the FF group compared with the IF group, and *Acidovorax* was the most major enriched biomarker. Weaning from initial feeding diet to formula feeding diet enhanced the expression levels of *TNF-α* and *IL-8*, and there was no significant change in *IL-1β* expression between the two groups. These findings would be very useful to improve the diet formulation for weaning stage of American glass eels.

## 1. Introduction

The eel is a catadromous fish species that spends most of their life in freshwater, and they complete their spawning by migration from freshwater environments into marine [1]. Eel farming has long been a worldwide aquaculture industry base on rearing specimens from glass eel to commercial size [2]. So far, eel farming strictly relies on stocks of wild glass eels [3]. However, eel stocks and species were dramatically endangered in recent decades due to many reasons, such as reproduction, overfishing for aquaculture and barriers to

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migration [4,5]. In addition, Japanese eel (*Anguilla japonica*) had exhibited drastic declines in recruitment and had been listed as Endangered on the IUCN red list in 2014 [6], and the European eel (*Anguilla anguilla*) had been considered to be out of safe biological limits and inserted in Appendices II of the Convention on International Trade in Endangered Species of wild Flora and Fauna (CITES) [7]. These limits resulted in a sharp rise in eel fry price, an increase of the product price and a strong reduction of eel production. Therefore, the development of techniques in artificial rearing of eel larvae for sustainability of this aquaculture industry is becoming necessary.

It is well known that weaning is the most important time in fish farming [8]. Due to the poor ability for protein absorption of fish larvae, live foods, which can provide soluble proteins and fatty acids to larval fish, are optimally selected as initial diets [9,10]. Traditionally, in Europe, fish roes are chosen as the main initial foods for the first stage of *A. anguilla* glass eels feeding [8]. In China and Japan, the main initial diets for most eel larvae species are *Tubifex* sp. and minced fish flesh [11]. However, basing on the previous studies, the use of live foods has been proven to be generally expensive and unreliable, such as *Tubifex* sp. often resulted in pathogen infection [12,13]. Furthermore, previous studies have highlighted that larval growth rates cannot be maintained by using live foods due to their poor nutritive composition [14]. Notably, to get acceptable growth and survival rates in aquaculture conditions, glass eels must be gradually weaned from natural diets to formulated feed when they have been fed with initial diets like *Tubifex* sp. for about 40 days at the body specification between 800 and 1000 p/kg [15]. Gisbert and Mozanzadeh (2019) found that glass eels fed with cod roe for 90 days showed a lower growth than those weaned onto compound diets, and this strategy had negative effects on their meta-morphosis and digestive function [2]. In addition, it has been found that though some commercial micro-diets have been employed for larval development with their economical and palatable characteristic, micro-diets can't completely take place of live foods because they might not match nutritional requirements of larvae fish [13,16]. Therefore, there is a need to find suitable weaning diets for glass eels to adapt aquaculture conditions successfully.

There are a number of important issues which can determine the success of weaning protocols. It is generally known that the intestinal tract contains trillions of commensal bacteria and forms a complex ecosystem [17]. As an important organ of digestion and absorption in the host fish, it is directly related to the growth, reproduction, nutrition, diet, immune response and other important metabolic processes of fish [18–21]. Generally, the diversity and abundance of the bacterial community can contribute to determining the direct effects of changing in diet and nutritional status. Recent advances in modern biotechnology technique like 16S rDNA high throughput sequencing, along with the development of bioinformatics tools, have provided faster results and high-resolution insights into the characteristic of the microbial communities [18,22].

American eel (*Anguilla rostrata*) was originated from the Western North Atlantic basin from Greenland to Venezuela, including the Caribbean and Gulf of Mexico drainages [23]. American eel was introduced into Fujian province, China in 2015 due to its great development potentials and its advantages of lower fry price and enough resources compared with Japanese eel or European eel [24]. At present, the research is very limited about weaning processing at glass eel stage of this newly introduced Anguilla species and how the weaning strategies affect the intestinal microbiota and immunity. The objective of this study was to evaluate the effects of weaning American glass eels with the formula diet on gut microbiota and the expression of inflammatory cytokines genes.

## 2. Materials and methods

The animal study protocol was approved by the guidelines of the Ethics Committee of Animal Research Institute Committee, Jimei University, China (Approval No. 2019–32, Approval date: 5 March 2019).

### 2.1. Diets, experimental design and sample collection

Two commodity feeding diets were supplied by Fujian Tianma Science and Technology Group Co., Ltd., Fuzhou, China but different in composition. The nutrition ingredients of the initial feed: moisture ≤78%, crude protein ≥15%, crude lipid ≥2.0%, based on high-quality fresh fish, while the formula feed contained about 10% moisture, 47% crude protein, 4.0% crude lipid, 3.0% crude fiber, based on fish meal, fish oil and puffed soybean. In addition, vitamins and minerals were added to both diets. The proximate composition of experimental diets is listed in Table 1.

The purpose of the experimental design was to assess the effects of weaning American glass eels (*Anguilla rostrata*) with the formula diet. The glass eels feeding trial was performed in an eel farm of Sanming, Fujian, China under the stable aquatic environment

**Table 1**
Proximate composition of experimental diets.

| Proximate composition (%) | IF | FF |
| --- | --- | --- |
| Moisture | ≤78.0 | ≤10.0 |
| Crude protein | ≥15.0 | ≥47.0 |
| Crude lipid | ≥2.0 | ≥4.0 |
| Crude fiber | – | ≤3.0 |
| Crude ash | ≤3.0 | ≤17.0 |
| Ca | ≤0.5 | 2.0–5.0 |
| Total phosphorus | ≤0.5 | ≥1.0 |
| Lysine | – | ≥2.5 |
| Methionine | ≥0.3 | – |

conditions. Glass eels were divided into two groups. Glass eels were fed with initial feed for 30 days, then eels were weaned from initial feeding diet to formula feeding diet for 4 days, and in this weaning period, initial feed was progressively replaced by the formula diet according to Xu et al. (2013) and Fan (2002) [25,26], which was as experimental group (termed FF group). Experimental group was compared to the control group (termed IF group) that was only fed with initial feed for 34 days. In this study, authors used the term glass eel for the whole experiment, and actually specimens at the stage feeding for 34 days were considered as elvers.

At the end of the feeding trial, healthy American glass eels weighing about 2 g were obtained and cultured in aerated tanks at 25–27 °C. All eels were fasted for 2 days to empty their intestines as much as possible before sampling. Six fish from each group were anesthetized with 100 mg/L MS-222 for 5–10 min, then the whole intestinal tract was removed using a sterile scalpel and forceps and stored at −80 °C immediately until further intestinal microbiology analysis. Another three fish from each group were dissected and the whole intestinal tract was removed and immediately immersed in Trizol reagent (Invitrogen, USA) and then preserved at −80 °C for cytokine gene expression.

### 2.2. Extraction of DNA and pyrosequencing of 16S rDNA

Genomic DNA of intestinal microbiota was extracted from each intestinal sample using PowerSoil DNA Isolation Kit (MoBio Laboratories, Carlsbad, CA). DNA from two intestinal samples were randomly chosen and mixed together. The IF group samples were labeled with A1-A3, and the FF group samples were labeled with B1–B3. The quality and purity of DNA were checked by agarose gel electrophoresis.

The V3–V4 hypervariable region was amplified by PCR using 16S rDNA universal primer 338F and 806R (Table 2) on a Master-cycler Gradient system (Eppendorf, Germany). The 25 μL reaction volume contained: 12.5 μL KAPA 2G Robust Hot Start Ready Mix, 1 μL 5 μM each primer, 1 μL 30 ng DNA sample and 9.5 μL ddH$_2$O. The thermal cycling procedure was 95 °C for 5 min; followed by 28 cycles of 45 s at 95 °C, 50 s at 55 °C and 45 s at 72 °C; 72 °C for 10 min. The products were sequenced on the MiSeq Genome Sequencer (Illumina, San Diego, CA, USA) by Beijing Allwegene Tech., Ltd. (Beijing, China).

### 2.3. Bioinformatic analysis of intestinal microbiota

To ensure the quality of the data, the original data were screened by QIIME software to remove primer mismatches, barcode sequences and ambiguous base sequences. Moreover, truncated reads with less than 50 bp and a quality score ≤20 were discarded. Using the sample-specific barcode sequences and Illumina Analysis Pipeline Version 2.6 separated and trimmed the qualified data. In order to plot rarefaction curves and measure the alpha diversity indexes, the sequences with 97% similarity level were clustered into operational taxonomic units (OTUs) and cut off from all sequences. The diversity of bacterial community was analyzed by calculating the diversity indices (i. e. Chao1, Shannon) of samples using QIIME version v.1.8. To examine the similarity between different samples, the evolution distances between microbial communities from each sample were calculated using the Bray Curtis algorithms. Then the hierarchical clustering tree and PCoA were analyzed by R (v3.6.0). The abundance of intestinal microbial components was analyzed using a linear discriminant analysis effect size (LEfSe).

### 2.4. Cloning of TNF-α, IL-1β and IL-8

Total RNA was extracted from 100 mg liver of a healthy American eel using Trizol reagent (Invitrogen, USA). 4 μg of total RNA was used for cDNA synthesis using GoScrip™ Reverse Transcription System kit (Promega, USA). Primers (Table 2) were designed according to the conserved regions obtained from several teleost fish using tblastn tool. Thermal cycling parameters were 94 °C for 3 min; followed by 35 cycles of 30 s at 94 °C, 30 s at 57 °C (*TNF-α*) or 55 °C (*IL-1β*) or 52 °C (*IL-8*) and 2 min at 72 °C; 72 °C for 10 min. The PCR

**Table 2**
Sequences and applications of primers used in this study.

| Primer name | Sequence (5′–3′) | Application |
| --- | --- | --- |
| 338F | GTACTCCTACGGGAGGCA | 16S rDNA |
| 806R | GTGGACTACHVGGGTWTCTAAT | |
| TNF-αF | GCTGCATCTGGAGGAGGACA | cDNA cloning |
| TNF-αR | ATCAAGTAATCAAGATAAGAACCTGT | |
| IL-1βF | ATGGAATCCAACGCTGTTT | |
| IL-1βR | CCGTCAAACCGCTCTGA | |
| IL-8F | ATGAAGATCACAATCACAGC | |
| IL-8R | AGAAAATCCTGGCCAAGTAA | |
| TNF-αrealF | TGCGGCACCTCTGCG | Real-time PCR |
| TNF-αrealR | GAGAGATGGGGGATGGACATT | |
| IL-1βrealF | GGCGGCAACATGGATCT | |
| IL-1βrealR | TGCTGAAGACCATCGACG | |
| IL-8realF | CTGGCCGCTCTCAGCAC | |
| IL-8realR | TGGTCAGCAGATCTGTCTGG | |
| ß-actin-F | CCGAGGGCCCTGTTCCA | |
| ß-actin-R | GGAGGCACCACCATGTACC | |

fragments were sequenced by Bo Rui (Xiamen, China).

### 2.5. Quantitative real-time PCR analysis

To investigate the expression level of *TNF-α*, *IL-1β* and *IL-8* under weaning conditions, the quantitative real-time PCR (qPCR) was performed on LigthCycler 480 II PCR instrument (Roche, Germany). Total RNA was extracted from each intestinal sample of the IF group and FF group as described above, and then treated with RNase-free DNase I (New England Biolabs Inc, USA). The specific primers used for the qPCR were designed based on the full-length DNA sequences of the three genes and shown in Table 2 *β-actin* of *Anguilla rostrata* was used as the endogenous control. Amplification reactions were carried out at a final 20 μL volume containing 4 μL diluted cDNA sample, 10 μL 2 × LightCycler 480 SYBR Green I Master (Roche, Germany), 0.5 μL of each primer and 5 μL nuclease-free water.

The reaction parameters were one cycle of 5 min at 95 °C, 40 cycles of 95 °C/20 s, 58 °C (*TNF-α* and *IL-8*), 54 °C (*IL-1β*)/20 s and 72 °C/25 s. Fluorescence outputs were measured and recorded at 82 °C (*TNF-α*) and 84 °C (*IL-8* and *IL-1β*) after each extension step. All qPCR experiments were performed in triplicate in a 96-well plate.

### 2.6. Statistical analysis

All statistical analysis was performed using SPSS 22.0 software (SPSS, Chicago, IL, USA). Differences in the gut microbiota of two groups were analyzed using one-way ANOVA. All data was given as means ± SD. The Student's *t*-test was used to evaluate the expression of three inflammatory factors. The value of $p < 0.05$ was set for statistical significance and indicated with (*).

## 3. Results

### 3.1. 16S rDNA sequencing data processing

Finally, 273,094 sequences in total were produced from the 6 samples (A1-A3, B1–B3) belonging to two groups by filtering and trimming the sequencing reads, with 28,334 to 47,529 effective sequences each sample. The sequences with the 97% similarity level were clustered into the OTUs. As shown in Fig. 1, each sample OUTs ranged from 100 to 400. It can be seen from the rarefaction curve that the number of OTUs increased with the increase of sequencing depth. Finally, the rarefaction curves reached the saturation plateau. It implies that the sequencing data is reasonable and sufficient which can reflect the information of most microorganisms from the sample (Fig. 1).

### 3.2. Alpha diversity analysis of microbial communities

In this study, the alpha diversity indices including OTUs, Chao1, Shannon and Good's coverage, which can measure the bacterial richness and diversity, were calculated. As shown in Table 3, in the IF group, the OTUs, Chao1 and Shannon indices were 173, 225.677 and 3.48, respectively, while in the FF group, the three indices were 355, 412.096 and 4.859, respectively. It can be seen from the result that OTUs, Chao1 and Shannon indices were increased when weaning from initial diet to formula diet. In alpha diversity analysis, Good's coverage index can reflect the integrity of the sequencing. In our study, although no significant difference was found in the good's coverage value between the two groups ($P > 0.05$), with 0.998 in the IF group and 0.999 in the FF group, 99% Good's
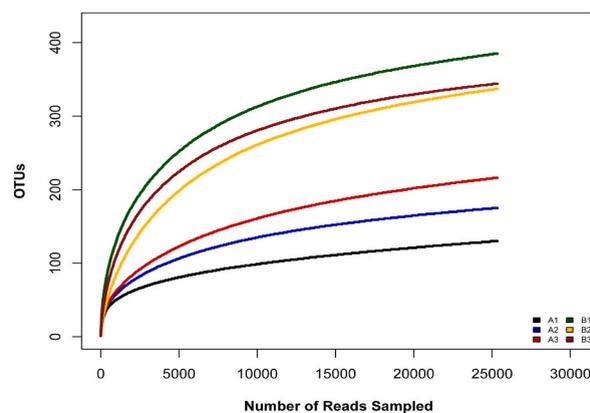


**Fig. 1.** Rarefaction curve of six intestinal samples. The x-coordinate represents the number of reads sampled and the y-coordinate represents the number of OTUs. Each curve labeled with a different color represents a sample. The number of OTUs increases with the sequencing depth and finally becomes stable, indicating the amount of sequencing data is reasonable. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

**Table 3**
OTUs, good's coverage and alpha diversity indices of intestinal microbiota composition of two groups.

| Item | IF group | FF group |
|---|---|---|
| OTUs | $173 \pm 43^a$ | $355 \pm 26^b$ |
| Chao1 | $225.677 \pm 36.128^a$ | $412.096 \pm 30.747^b$ |
| Shannon | $3.481 \pm 0.055^a$ | $4.859 \pm 0.763^b$ |
| Good's coverage | $0.998 \pm 0.0004^a$ | $0.999 \pm 0.0012^a$ |

Note: Means in the same row with different superscripts are significantly different ($P < 0.05$).
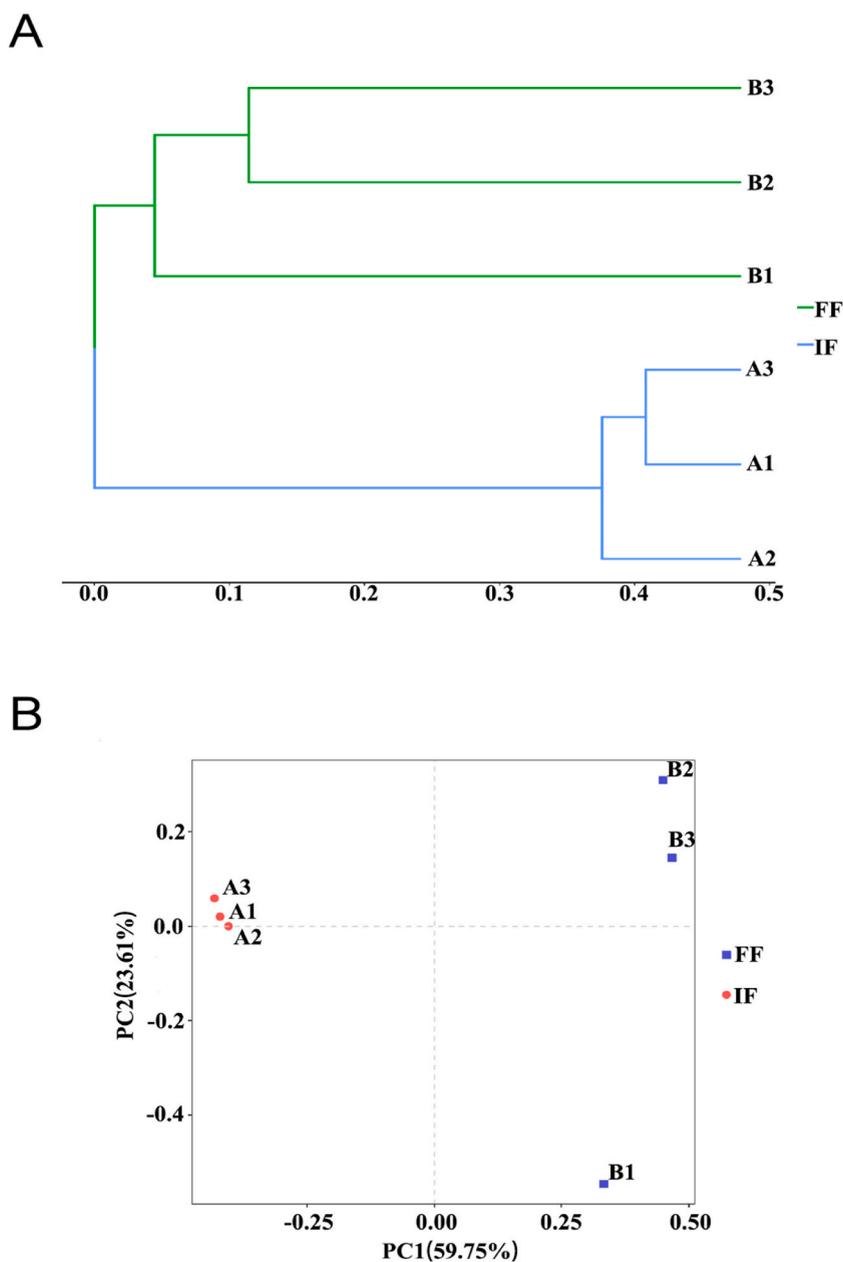


**Fig. 2.** (A) Hierarchical clustering tree analysis based on UPGMA (Unweighted Pair-group Method with Arithmetic Mean) algorithm between intestinal microbiota samples from IF and FF groups. (B) Principal Coordinates analysis (PCoA) of intestinal microbial community samples based on unweighted Unifrac distances. Red circle represents the IF group; blue square represents the FF group. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

coverage points that the sequences detected can represent most bacterial species presented in samples.

### 3.3. Beta diversity analysis of microbial communities

The beta diversity analysis was used for investigating the similarity or dissimilarity between microbial community samples from the IF and FF group. The hierarchical clustering tree of all 6 samples is shown in Figs. 2A and 3 samples of IF group clustered together into one group and 3 samples of FF group also gathered into another group base on the similarity of OTUs. Moreover, as shown in Fig. 2B, the similarity matrix of the IF and FF groups were analyzed by OTU-based principal coordinate analysis (PCoA). The result revealed that the two groups significantly separated, which was consistent with the result of hierarchical clustering tree. The PCA score plot showed that A1-A3 libraties clustered to the left of the graph along the PC2 axis, which accounted for 23.61% of the total variations. The B1–B3 libraties grouped together along the PC1 axis, which accounted for 59.75% of the total variations. In each libraries, points clustered closer indicated the higher similarity between two samples. Moreover, no intersection was observed between the samples meant significant difference between the IF and FF group.

### 3.4. Analysis of the differences in intestinal microbiota at the phylum level

The comparison of the main bacterial phyla in the intestines between the two groups was shown in Fig. 3. At the phylum level, the core microflora in two groups was similar, including Firmicutes, Proteobacteria, Bacteroidetes, Actinobacteria, Saccharibacteria, Acidobacteria and Planctomycetes. The dominant phylum of the IF group was found to be Firmicutes with the 83.98% relative abundance, followed by Proteobacteria (10.08%), and the other dominant phyla with relative abundance <5% were Bacteroidetes (4.12%), Actinobacteria (1.09%) Saccharibacteria (0.01%), Acidobacteria (0.01%) and Planctomycetes (0.02%) (Fig. 3A). Feeding with formula diet resulted in a significant increase ($P < 0.05$) of the abundance of Proteobacteria (52.70%), Bacteroidetes (6.05%) and
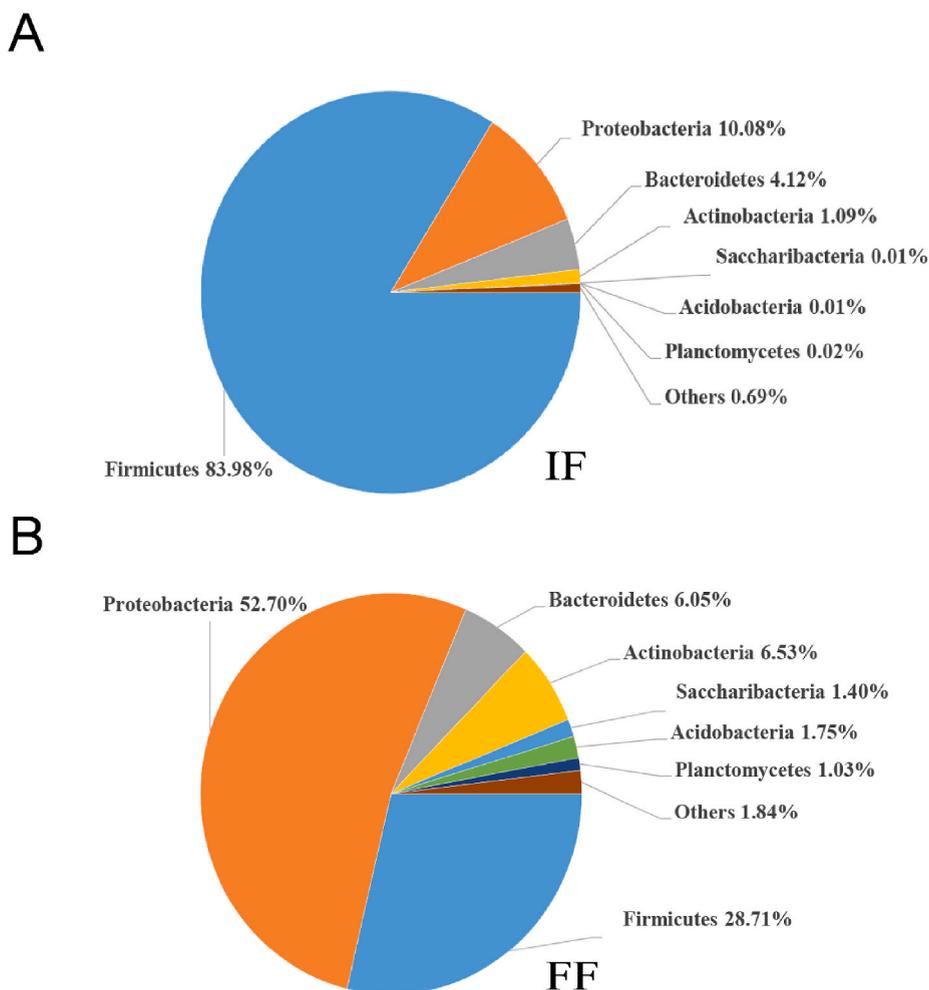


**Fig. 3.** Pie charts show the most abundant taxa in the IF (A) and FF (B) group.

# A

## Cladogram



Legend:
- IF (green)
- FF (red)

Phyla labeled: P__Cyanobacteria, Chloroflexi, Chlorobi, P__Actinobacteria, P__Acidobacteria, P__Saccharibacteria, P__Firmicutes, P__Proteobacteria

a: f__ABS_19
b: o__Subgroup_10
c: c__Holophagae
d: f__Intrasporangiaceae
e: f__Micrococcaceae
f: f__Propionibacteriaceae
g: o__Propionibacteriales
h: c__Actinobacteria
i: f__Coriobacteriaceae
j: o__Coriobacteriales
k: c__Coriobacteriia
l: f__Cytophagaceae
m: o__Cytophagales
n: c__Cytophagia
o: f__NS9_marine_group
p: o__Sphingobacteriales
q: c__WCHB1_32
r: f__SJA_28
s: f__Chlorobiales
t: c__Chlorobia
u: f__Anaerolineaceae
v: o__Anaerolineales
w: c__Anaerolineae
x: f__Chloroflexaceae
y: o__Chloroflexales
z: c__Chloroflexia
a0: c__Chloroplast
a1: f__Bacillaceae
a2: f__Thermoactinomycetaceae
a3: o__Bacillales
a4: c__Bacilli
a5: c__Ignavibacteria
a6: f__Nitrospiraceae
a7: o__Nitrospirales
a8: c__Nitrospira
a9: f__Planctomycetaceae
b0: o__Planctomycetales
b1: c__Planctomycetacia
b2: f__Unknown_Family
b3: o__Alphaproteobacteria_Incertae_
b4: f__Bradyrhizobiaceae
b5: f__Methylobacteriaceae
b6: f__Phyllobacteriaceae
b7: f__Xanthobacteraceae
b8: o__Rhizobiales
b9: f__Rhodobacteraceae
c0: o__Rhodobacterales
c1: c__Alphaproteobacteria
c2: f__Oxalobacteraceae
c3: f__Nitrosomonadaceae
c4: o__Nitrosomonadales
c5: f__Rhodocyclaceae
c6: o__Rhodocyclales
c7: o__SC_I_84
c8: c__Betaproteobacteria

# B



LDA SCORE(log 10)

Legend: IF (red), FF (green)

IF:
- Bacillus
- Anaerobacillus
- Flavobacterium
- Acinetobacter
- Plesiomonas

FF:
- Acidovorax
- Bosea
- Sphingobium
- Xanthobacter
- Ruminococcaceae_UCG_013
- Pseudolabrys
- Aquicella
- Collinsella
- Sphingomonas

*(caption on next page)*

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**Fig. 4.** Comparison of microbial community abundance using linear discriminant analysis effect size (LEfSe) analysis. (A) Cladogram showing taxa with significant difference between two groups. The circles from the inside to the outside represent the classification from the phylum to the species level. The dot colors match with two groups (green, IF group; red, FF group), and the color yellow represents species with no significant differences. The diameter of the dots is proportional to the relative abundance. (B) Differences among IF and FF groups after LDA score using a threshold score >3.0. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Actinobacteria (6.53%) when compared with the initial diet group. However, feeding with formula diet decreased the abundance of Firmicutes (28.71%) in the intestines of eels from the FF group ($P < 0.05$) (Fig. 3B).

*3.5. Analysis of the differences in intestinal microbiota at the genus level*

LEfSe analysis of 16S rDNA sequencing showed differences in the abundance of taxa between the IF and FF group (Fig. 4A). The identified bacteria with significant differences of abundance in two groups at the genus level were shown in Fig. 4B. Compared with the FF group, five genera including *Bacillus*, *Anaerobacillus*, *Flavobacterium*, *Acinetobacter* and *Plesiomonas* were significantly increased in the IF group, and *Bacillus* was the most major enriched biomarker at genus level. Nine genera including *Acidovorax*, *Bosea*, *Sphingobium*, *Xanthobacter*, *Ruminococcaceae_UCG_013*, *Pseudolabrys*, *Aquicella*, *Collinsella* and *Sphingomonas* were significantly higher in the FF group, and *Acidovorax* was the most major enriched biomarker at genus level, which was significantly difference with the IF group.

*3.6. Expression of TNF-α, IL-1β and IL-8 mRNA in intestine tissue*

The full-length cDNA sequences of American eel *TNF-α* (GenBank accession No. MT861110), *IL-1β* (GenBank accession No. MT861112) and *IL-8* (GenBank accession No. MT861113) were composed of a 687 bp, 750 bp and 285 bp open reading frame (ORF), respectively. The expression of *TNF-α*, *IL-1β* and *IL-8* in intestine tissue from IF group and FF group individuals were determined by qPCR analysis. The specific primers used for the qPCR were designed based on the full-length DNA sequences of the three genes. The results showed that the constitutive mRNA expression of *TNF-α*, *IL-1β* and *IL-8* was observed in 6 samples tested. As shown in Fig. 5, the transcription of *TNF-α*, *IL-1β* and *IL-8* was up-regulated in the FF group, with 2.25-fold ($P < 0.05$), 1.28-fold and 4.24-fold ($P < 0.05$) higher than the expression level of the IF group, respectively, and among which, *IL-8* displayed the highest expression level, then by *TNF-α*.

## 4. Discussion

In order to industrialize the farming processes of American eel, several significant factors including feeding behavior, disease control, farming density, especially weaning strategies, which had been carried out to find the optimum conditions to maximize its farming production in China. Generally, fish intestinal microbiota, which is accompanied by diet, is regarded to be an important parameter to evaluate the success of weaning process [27]. However, seldom studies have been conducted to understand the relationship between the intestinal microbiota and weaning regimes in American glass eels. In this study, we focused on analysis of changes of gut microbiota according to diet using the high-throughput sequencing technology and the expression of inflammatory genes in the intestines by qPCR, which would be very important to improve the weaning regime for American glass eels.

The present study disclosed the IF and FF group had obviously differences in the alpha diversity in terms of OTUs, Chao1 and Shannon indices (Table 3). Studies on other fish species such as Atlantic salmon (*Salmo salar* L.), rainbow trout (*Oncorhynchus mykiss*) and puffer fish (*Takifugu obscurus*) demonstrated that intestinal microbiota in fish were affected significantly by diets [28–30]. Furthermore, it has been mentioned in our study that the diversity of intestinal microbiota was much higher in formula diet group (FF group: containing 10% moisture and 47% crude protein base on fish meal and plant ingredients of puffed soybean) than that in the
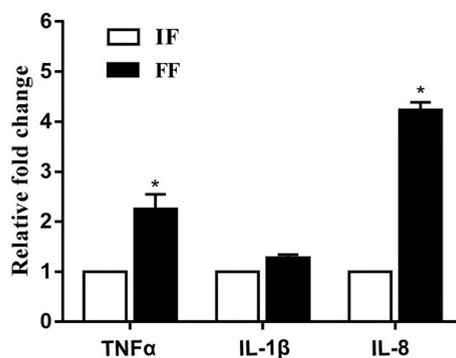


**Fig. 5.** Relative expression of *TNF-α*, *IL-1β* and *IL-8* in intestine tissue of American glass eels from IF and FF groups. *β-actin* of *Anguilla rostrata* was used as an endogenous reference gene to normalize the relative expression level of the three genes. All data was presented as means ± S.D. (N = 3). * means p < 0.05.

initial diet group (IF group: containing about 78% moisture and 15% crude protein base on fresh fish). This result was similar with the study of Feng et al. (2010) who reported that compared with natural diet containing ice-fresh fish and shrimp as the protein source, the complete diet (crude protein >45%, $H_2O$ < 10% based on fish meal, fish oil and wheat) increased the richness of intestinal bacterial species in yellow grouper [31]. A previous study in rainbow trout also reported that the significant higher diversity of gut microbial was observed in the fish fed with two different commercial diets, including a marine diet containing fish meal and a plant-based diet containing pea meal after first feeding [32]. It has been shown that high bacterial diversity was beneficial for the gut health because communities with rich species could resist pathogen invasion and intestinal infection by overcoming pathogens for nutrients and colonization [33]. Therefore, the higher diversity of intestinal microbiota in formula diet group may indicate that American glass eels can be weaned from initial feeding diet to formula feeding diet and accept the formula diet progressively over a certain weaning period of four days.

We examined the gut microbiota at phyla level. As seen in Fig. 3, the four phyla Firmicutes, Proteobacteria, Bacteroidetes and Actinobacteria were the main microbiota compositions of IF and FF group regardless of whether the glass eels weaned with the formula diet, which was consistent with three earlier studies in rainbow trout [32,34,35]. Moreover, Huang et al. (2018) found that the core bacterial phyla in the three life stages of European eel were Proteobacteria, Fusobacteria and Bacteroidetes [36]. The phyla Proteobacteria, Firmicutes, Bacteroidetes, Actinobacteria and Fusobacteria have been previously reported to be generally resident in the gut of many fish species [37]. In our study, the abundance of the Firmicutes decreased in FF group, whereas the Proteobacteria was significantly higher than that of IF group (Fig. 3). In a previous study, by evaluating the influence of two different diets on the intestinal microbiota of rainbow trout, a pattern was shown that the plant-based diet containing fish meal, rape-seed oil and pea meal contributed to the presence of Firmicutes, whereas the marine based diet containing fish meal and fish oil contributed to the presence of Proteobacteria [32]. This result was generally in agreement with a previous report on rainbow trout that a diet containing proteins from plants such as pea and soy caused a higher abundance of Firmicutes relative to the phylum Proteobacteria compared to a fish meal based diet, which is likely to be that Firmicutes could promote the catabolic metabolism and degradation of cellulose in the host intestinal tract [34,38]. Our result that the abundance of Firmicutes decreased when glass eels have been fed with the formula diet base on fish meal and plant ingredients of puffed soybean, to some extent, seems to be different from the above views. However, other report indicated the intermediate abundance of Firmicutes was found in salmon fed with intermediate levels of both fish meal and plant components compared to more extreme diets that mainly contained either fish meal or plant ingredients [39]. Another study showed that the abundance of Firmicutes seemed to be lower in salmon fed high fish meal diets, whereas the Proteobacteria dominated in intestinal contents [40]. In our study, the FF group showed significantly increased Proteobacteria compared to the IF group (Fig. 3B), which might be due to adding the fish meal in the formula diet. Thus, it can be concluded that the type and origin of the feed ingredients (marine and plant) may influence the intestinal microbial diversity by different patterns. Taken together, the difference in the abundance of Firmicutes and Proteobacteria in fish fed with different diets may be speculated that the gut bacterial community of fish species is plastic and can be manipulated by diets. Further study is required to validate the close relationship between diets and gut microbiota.

Another result showed that the species diversity of intestinal microbiota at genus level in two groups was markedly different (Fig. 4B). Compared with the FF group, five genera were significantly increased in the IF group, whereas nine genera were significantly higher in the FF group, indicating that more bacteria genera were enriched in the FF group when eels fed with the formula diet. Previous studies have reported that *Acidovorax*, an acid degrading member of the phylum Proteobacteria was identified in freshwater invertebrates and many earthworm species [41,42], and also was noted in vertebrate gut microbial communities, such as trout and marine sea bream, which was related to the metabolism of nitrogenous compounds [43,44]. A recent study has reported that *Acidovorax* was significantly enriched in the fermented soybean meal group compared to the nontreated group [45]. In the current study, similar results showed that the relative abundance of *Acidovorax* was significantly increased in the FF group fed with the formula diet base on fish meal and puffed soybean, which may be beneficial for performing some complex functions, such as digestive function within eel gut. In addition, the present study showed that the relative abundance of *Bacillus* was significantly higher in IF group than that in FF group (Fig. 4B). This result was in some extent consistent with the previous study, which reported that the relative abundance of *Bacillus* was increased in Beluga sturgeon when they fed with lower fish meal [46]. The previous studies discovered that *Bacillus*, as a typical probiotic, could enhance host immunity, nutrients extracting, digestive function and disease resistance against pathogenic [47,48]. Our results showed that *Bacillus* mainly colonized the gut of eels fed with the initial diet without fish meal, but not of eels fed with the formula diet base on fish meal. Therefore, further studies will be needed to determine whether the change in species of *Bacillus* is associated with fish meal supplementation and whether this change has an impact on the immune system response.

In general, *TNF-a, IL-1β, IL-4, IL-6, IL-8* and *IL-10* family cytokines are commonly used as reference genes in studies of immune regulation. In these cytokines, *IL-1β, IL-8* and *TNF-α* are pro-inflammatory cytokines, and the up-regulated high expression of *IL-1β, IL-8* or *TNF-a* is often associated with Inflammatory Bowel Disease (IBD) [49–52]. In the current study, the expression of the *TNF-α* and *IL-8* was significantly up-regulated in the FF group compared to the IF group, and there was no significant change in *IL-1β* expression level between the two groups (Fig. 5), which suggested that these genes might be involved in the regulation of inflammatory responses. The previous studies have shown that changes in microbial composition due to dietary changes might result in imbalance of the microbial community and increase the sensibility of fish to inflammation reaction induced by different pathogens [53]. Some researchers also revealed that the metabolites or components of gut microbial community could induce the immune cells to secrete cytokines to regulate the immune response [54,55]. These findings may explain that the expression levels of inflammatory genes were up-regulated in the FF group may be due to its high diversity of intestinal microbiota. In addition, another possible explanation may be the change of ingredients in the formula diet. Previous studies revealed that soybean meal (SM) could induce intestinal inflammation responses by regulating the expression level of pro-inflammatory and anti-inflammatory cytokines, and had adverse effects on gut

health, though it was an alternative protein source for fish meal (FM) replacement [56,57]. In our study, the up-regulation of *TNF-α* and *IL-8* in eels fed with formula diet containing fish meal and puffed soybean are in line with the observations in zebrafish [58], Japanese seabass [59] and turbot [60]. Moreover, recent studies showed that the expression levels of pro-inflammatory cytokines increased with the SM level in diet, indicating dose-dependent increases in severity of the inflammation [57,60]. According to these results, further studies are required for understanding the molecular mechanisms of intestine inflammation associated with dietary changes.

## 5. Conclusions

The results in this study indicated that intestinal microbial diversity, composition, and inflammatory cytokine genes expression of American glass eels were obviously different when weaning from initial feeding diet (IF group) to formula feeding diet (FF group). The diversity of intestinal microflora was much higher in formula diet group than that in the initial diet group. Furthermore, the identified bacteria at the phylum and genus level had significant differences between IF and FF groups. The qPCR analysis revealed that the transcription of *TNF-α* and *IL-8* was significantly up-regulated in the FF group compared to the IF group, and the expression of *IL-1β* was not significantly different between two groups. These findings would be useful to improve the weaning strategies for American glass eels. However, further studies of optimum diet formulation should be conducted for glass eel stage of development.

## Author contribution statement

Ying Liang: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Haizi Liu: Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Shaowei Zhai: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

Lixing Huang: Conceived and designed the experiments; Analyzed and interpreted the data.

Wenshu Huang, Bei Huang, Jisong Xu, Jing Xiong, Bei Wang: Analyzed and interpreted the data.

## Data availability statement

Data included in article/supp. material/referenced in article.

## Declaration of competing interest

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

## References

[[1]  K. Tsukamoto, S. Chow, T. Otake, H. Kurogi, N. Mochioka, M.J. Miller, J. Aoyama, S. Kimura, S. Watanabe, T. Yoshinaga, Oceanic spawning ecology of freshwater eels in the western North Pacific, Nat. Commun. 2 (2011) 1–9, https://doi.org/10.1038/ncomms1174.

[2]  E. Gisbert, M.T. Mozanzadeh, Weaning European glass eels (*Anguilla anguilla*) with plant protein-based diets and its effects on intestinal maturation, Comp. Biochem. Physiol. A 228 (2019) 43–50, https://doi.org/10.13140/RG.2.2.22311. 85923.

[3]  K. Kaifu, Challenges in assessments of Japanese eel stock, Mar. Pol. 102 (2019) 1–4, https://doi.org/10.1016/j.marpol.2019.02.005.

[4]  W. Dekker, Status of the European eel stock and fisheries, in: W. Dekker (Ed.), Eel Biology, Springer, 2003, pp. 237–254.

[5]  J.G. Støttrup, C. Jacobsen, J. Tomkiewicz, H. Jarlbæk, Modification of essential fatty acid composition in broodstock of cultured European eel *Anguilla anguilla* L, Aquacult, Nutrition 19 (2013) 172–185, https://doi.org/10.1111/j.1365-2095.2012. 00967.x.

[6]  IUCN, Anguilla Anguilla, The IUCN red list of threatened species (2014), Version 3.

[7]  D.M.P. Jacoby, J.M. Casselman, V. Crook, M.B. DeLucia, H. Ahn, K. Kaifu, T. Kurwie, P. Sasal, A.M.C. Silfvergrip, K.G. Smith, K. Uchida, A.M. Walker, M. J. Gollock, Synergistic patterns of threat and the challenges facing global anguillid eel conservation, Glob. Ecol. Conserv. 4 (2015) 321–333, https://doi.org/10.1016/j.gecco.2015.07.009.

[8]  S.S. De Silva, R.M. Gunasekera, B.A. Ingram, J.L. Dobson, Weaning of Australian shortfin glass eels (*Anguilla australis*): a comparison on the effectiveness of four types of fish roe, Aquaculture 195 (2001) 133–148, https://doi.org/10.1016/S0044-8486(00)00548-2.

[9]  I. Rønnestad, M. Yu'fera, B. Ueberschär, L. Ribeiro, Y. Sæle, C. Boglione, Feeding behaviour and digestive physiology in larval fish: current knowledge, and gaps and bottlenecks in research, Rev. Aquacult. 5 (2013) S59–S98, https://doi.org/10.1111/raq.12010.

[10]  T.A. Samuelsen, A. Oterhals, Water-soluble protein level in fishmeal affects extrusion behaviour, phase transitions and physical quality of feed, Aquacult. Nutr. 22 (2016) 120–133, https://doi.org/10.1111/anu.12235.

[11]  W.D. Fang, K.J. Guo, C.A. Xu, Research on the diets determination for Initial feeding of Anguilla mormorata glass eel, Animal Husbandry and Feed Science 5 (2013) 82–84.

[12] T.N.D. Khoa, V. Waqalevu, A. Honda, K. Shiozaki, T. Kotani, Comparative study on early digestive enzyme activity and expression in red sea bream (*Pagrus major*) fed on live feed and micro-diet, Aquaculture 519 (2020), 734721, https://doi.org/10.1016/j.aquaculture.2019.734721.

[13] M. Srichanun, C. Tantikitti, P. Utarabhand, T.M. Kortner, Gene expression and activity of digestive enzymes during the larval development of Asian seabass (*Lates calcarifer*), Comp. Biochem. Physiol. B Biochem. Mol. Biol. 165 (2013) 1–9, https://doi.org/10.1016/j.cbpb.2013.02.005.

[14] G. Rosenlund, J. Stoss, C. Talbot, Co-feeding marine fish larvae with inert and live diets, Aquaculture 155 (1997) 183–191, https://doi.org/10.1016/S0044-8486(97) 00116-6.

[15] X.Y. Li, The cultivation techniques of American glass eel, *Anguilla rostrata*, Agricultural Technology Service 34 (2017) 97–98.

[16] M. Yúfera, F.J. Moyano, G. Martínez-Rodríguez, The digestive function in developing fish larvae and fry. from molecular gene expression to enzymatic activity, Emerging Issues in Fish Larvae Research 3 (2018) 51–86, https://doi.org/10.1007/978-3-319-73244-2_3.

[17] I. Sekirov, S.L. Russell, L.C.M. Antunes, B.B. Finlay, Gut microbiota in health and disease, Physiol. Rev. 90 (2010) 859–904, https://doi.org/10.1159/000481627.

[18] M. Ghanbari, W. Kneifel, K.J. Domig, A new view of the fish gut microbiome: advances from next-generation sequencing, Aquaculture 448 (2015) 464–475, https://doi.org/10.1016/j.aquaculture.2015.06.033.

[19] Y.X. Jin, S.S. Wu, Z.Y. Zeng, Z.W. Fu, Effects of environmental pollutants on gut microbiota, Environ. Pollut. 222 (2017) 1–9, https://doi.org/10.1016/j.envpol.2016.11.045.

[20] S.K. Nayak, Role of gastrointestinal microbiota in fish, Aquacult. Res. 41 (2010) 1553–1573, https://doi.org/10.1111/j.1365-2109.2010.02546.x.

[21] V. Tremaroli, F. Backhed, Functional interactions between the gut microbiota and host metabolism, Nature 489 (2012) 242–249, https://doi.org/10.1038/nature 11552.

[22] D.J. Davis, E.C. Bryda, C.H. Gillespie, A.C. Ericsson, 16S rRNA amplicon sequencing dataset for conventionalized and conventionally raised zebrafish larvae, Data Brief 8 (2016) 938–943, https://doi.org/10.1016/j.dib.2016.06.057.

[23] J. Benchetrit, J.D. McCleave, Current and historical distribution of the American eel *Anguilla rostrata* in the countries and territories of the wider Caribbean, ICES J. Mar. Sci. 73 (2016) 122–134, https://doi.org/10.1016/icesjms/fsv043.

[24] X.D. Yang, X.L. Jiang, P.M. Le, M.J. Gong, Studies on oxygen consumption rate (ROC), asphyxial point (AP) and optimum water temperature range (RT) of *Anguilla rostrata* and LC50 and SC of non-ionic ammonia (NIA) and nitrite in water, Oceanol. Limnol. Sinica 50 (2019) 455–464.

[25] C.A. Xu, J.C. Shi, X. Tang, W.D. Fang, K.J. Guo, Determination of weaning regimes for *Anguilla mormorata* glass eels, Agricultural Science & Technology 14 (2013) 527–529.

[26] H.P. Fan, Key techniques for breeding glass eels, Scientific fish farming 2 (2002) 11–12.

[27] C.D. Filippo, D. Cavalieri, M.D. Paola, M. Ramazzotti, J.B. Poullet, S. Massart, S. Collini, G. Pieraccini, P. Lionetti, Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa, Proc. Natl. Acad. Sci. USA 107 (2010) 14691–14696, https://doi.org/10.1073/pnas.1005963107.

[28] A.M. Bakke-McKellep, M.H. Penn, P.M. Salas, S. Refstie, S. Sperstad, T. Landsverk, E. Ringø, Å. Krogdahl, Effects of dietary soyabean meal, inulin and oxytetracycline on intestinal microbiota and epithelial cell stress, apoptosis and proliferation in the teleost Atlantic salmon (*Salmo salar* L.), Br. J. Nutr. 97 (2007) 699–713, https://doi.org/10.1017/S0007114507381397.

[29] D. Huyben, A. Vidaković, S.W. Hallgren, M. Langeland, High-throughput sequencing of gut microbiota in rainbow trout (*Oncorhynchus mykiss*) fed larval and pre-pupae stages of black soldier fly (*Hermetia illucens*), Aquaculture 500 (2019) 485–491, https://doi.org/10.1016/j.aquaculture.2018.10.034.

[30] G.M. Yang, B.L. Bao, E. Peatman, H.R. Li, L.B. Huang, D.M. Ren, Analysis of the composition of the bacterial community in puffer fish *Takifugu obscurus*, Aquaculture 262 (2007) 183–191, https://doi.org/10.1016/j.aquaculture.2006.11. 031.

[31] J.B. Feng, C.Q. Hu, P. Luo, L.P. Zhang, C. Chen, Microbiota of yellow grouper (*Epinephelus awoora* Temminck & Schlegel, 1842) fed two different diets, Aquacult. Res. 41 (2010) 1778–1790, https://doi.org/10.1111/j.1365-2109.2010.02481.x.

[32] H.C. Ingerslev, L.V.G. Jørgensen, M.L. Strube, N. Larsen, I. Dalsgaard, M. Boye, L. Madsen, The development of the gut microbiota in rainbow trout (*Oncorhynchus mykiss*) is affected by first feeding and diet type, Aquaculture 424–425 (2014) 24–34, https://doi.org/10.1016/j.aquaculture.2013.12.032.

[33] R. Cerezuela, M. Fumanal, S.T. Tapia-Paniagua, J. Meseguer, M.Á. Moriñigo, M.Á. Esteban, Changes in intestinal morphology and microbiota caused by dietary administration of inulin and *Bacillus subtilis* in gilthead sea bream (*Sparus aurata* L.) specimens, Fish Shellfish Immunol. 34 (2013) 1063–1070, https://doi.org/10.1016/j.fsi.2013.01.015.

[34] A.R. Desai, M.G. Links, S.A. Collins, G.S. Mansfield, M.D. Drew, A.G. Van Kessel, J.E. Hill, Effects of plant-based diets on the distal gut microbiome of rainbow trout (*Oncorhynchus mykiss*), Aquaculture 350–353 (2012) 134–142, https://doi.org/10.1016/j.aquaculture.2012.04.005.

[35] P. Navarrete, F. Magne, C. Araneda, P. Fuentes, L. Barros, R. Opazo, R. Espejo, J. Romero, PCR-TTGE analysis of 16S rRNA from rainbow trout (*Oncorhynchus mykiss*) gut microbiota reveals host-specific communities of active bacteria, PLoS One 7 (2012), e31335, https://doi.org/10.1371/journal.pone. 0031335.

[36] W. Huang, Z.Q. Cheng, S.N. Lei, L.Y. Liu, X. Lv, L.H. Chen, M.H. Wu, C. Wang, B.Y. Tian, Y.K. Song, Community composition, diversity, and metabolism of intestinal microbiota in cultivated European eel (*Anguilla anguilla*), Appl. Microbiol. Biotechnol. 102 (2018) 4143–4157, https://doi.org/10.1007/s00253-018-8885-9.

[37] M.S. Llewellyn, S. Boutin, S.H. Hoseinifar, N. Derome, Teleost microbiomes: the state of the art in their characterization, manipulation and importance in aquaculture and fisheries, Front. Microbiol. 5 (2014) 1–18, https://doi.org/10.3389/fmicb.2014.00207.

[38] J.M. Brulc, D.A. Antonopoulos, M.E.B. Miller, M.K. Wilson, A.C. Yannarell, E.A. Dinsdale, R.E. Edwards, E.D. Frank, J.B. Emerson, P. Wacklin, P.M. Coutinho, B. Henrissat, K.E. Nelson, B.A. White, Gene-centric metagenomics of the fiber-adherent bovine rumen microbiome reveals forage specific glycoside hydrolases, P. Natl. Acad. Sci. USA. 106 (2009) 1948–1953, https://doi.org/10.1073/pnas. 0806191105.

[39] K. Gajardo, A. Rodiles, T.M. Kortner, Å. Krogdahl, A.M. Bakke, D.L. Merrifield, H. Sørum, A high-resolution map of the gut microbiota in Atlantic salmon (*Salmo salar*): a basis for comparative gut microbial research, Sci. Rep. 6 (2016) 30893, https://doi.org/10.1038/srep30893.

[40] M.S. Llewellyn, P. Mcginnity, M. Dionne, J. Letourneau, F. Thonier, G.R. Carvalho, S. Creer, N. Derome, The biogeography of the Atlantic salmon (Salmo salar) gut microbiome, ISME J. 10 (2015) 1280–1284, https://doi.org/10.1038/ISMEJ.2015.189.

[41] M. P. Stief, L.P. Poulsen, H. Nielsen, A. Brix, Schramm, Nitrous oxide emission by aquatic macrofauna, P. Natl. Acad. Sci. USA 106 (2009) 4296–4300, https://doi.org/10.1073/pnas.0808228106.

[42] P.K. Wüst, M.A. Horn, G. Henderson, P.H. Janssen, B.H.A. Rehm, H.L. Drake, Gut-Associated denitrification and in vivo emission of nitrous oxide by the earthworm families megascolecidae and lumbricidae in New Zealand, Appl. Environ. Microbiol. 75 (2009) 3430–3436, https://doi.org/10.1128/AEM.00304-09.

[43] K.A. Kormas, A. Meziti, E. Mente, A. Frentzos, Dietary differences are reflected on the gut prokaryotic community structure of wild and commercially reared sea bream (*Sparus aurata*), MicrobiologyOpen 3 (2014) 718–728, https://doi.org/10.1002/mbo3.202.

[44] S. Wong, T. Waldrop, S. Summerfelt, J. Davidson, F. Barrows, P.B. Kenney, T. Welch, G.D. Wiens, K. Snekvik, J.F. Rawls, C. Good, Aquacultured Rainbow Trout (*Oncorhynchus mykiss*) possess a large core intestinal microbiota that is resistant to variation in diet and rearing density, Appl. Environ. Microbiol. 79 (2013) 4974–4984, https://doi.org/10.1128/AEM.00924-13.

[45] L. Li, J.J. Song, C.Y. Peng, Z.C. Yang, L. Wang, J.C. Lin, L.B. Li, Z.C. Huang, B. Gong, Co-occurrence network of microbes linking growth and immunity parameters with the gut microbiota in Nile tilapia (*Oreochromis niloticus*) after feeding with fermented soybean meal, Aquacult. Rep. 26 (2022), 101280, https://doi.org/10.1016/j.aqrep.2022.101280.

[46] G.L. Xu, G.L. Xing, T.L. Li, M. Xue, Z.H. Ma, N. Jiang, L. Luo, Comparative study on the effects of different feeding habits and diets on intestinal microbiota in *Acipenser baeri* Brandt and *Huso huso*, BMC Microbiol. 19 (2019) 297, https://doi.org/10.1186/s12866-019-1673-6.

[47] M.L. González-Félix, D.M. Gatlin, P. Urquidez-Bejarano, R.R. Carolina, R. Duarte-Rodríguez, F. Sánchez, A. Casas-Reyes, F.Y. Yamamoto, A. Ochoa-Leyva, M. Perez-Velazquez, Effects of commercial dietary prebiotic and probiotic supplements on growth, innate immune responses, and intestinal microbiota and histology of *Totoaba macdonaldi*, Aquaculture 491 (2018) 239–251, https://doi.org/10.1016/j.aquaculture.2018.03.031.

[48] K. Hao, Z.Q. Wu, D.L. Li, X.B. Yu, G.X. Wang, F. Ling, Effects of dietary administration of *Shewanella xiamenensis* A-1, *Aeromonas veronii* A-7, and *Bacillus subtilis*, single or combined, on the grass carp (*Ctenopharyngodon idella*) intestinal microbiota, Probiotics Antimicro 9 (2017) 386–396, https://doi.org/10.1007/s12602-017-9269-7.

[49] A. Kołodziejska-Sawerska, A. Rychlik, A. Depta, M. Wdowiak, M. Nowicki, M. Kander, Cytokines in canine inflammatory bowel disease, Pol. J. Vet. Sci. 16 (2013) 165–171, https://doi.org/10.2478/pjvs-2013-0025.

[50] W.S. Liu, P.F. Ren, S.X. He, L. Xu, Y.L. Yang, Z.M. Gu, Z.G. Zhou, Comparison of adhesive gut bacteria composition, immunity, and disease resistance in juvenile hybrid tilapia fed two different *Lactobacillus* strains, Fish Shellfish Immunol. 35 (2013) 54–62, https://doi.org/10.1016/j.fsi.2013.04.010.

[51] P. Manzanillo, C. Eidenschenk, W. Ouyang, Deciphering the crosstalk among IL-1 and IL-10 family cytokines in intestinal immunity, Trends Immunol. 36 (2015) 471–478, https://doi.org/10.1016/j.it.2015.06.003.

[52] L.G. Su, The progress of the relationship between IL-8, IL-10 and inflammation of intestinal tract, Med. Recapitulate 14 (2008) 3378–3380.

[53] S. Brugman, W. Ikeda-Ohtsubo, S. Braber, G. Folkerts, C.M.J. Pieterse, P.A.H.M. Bakker, A Comparative review on microbiota manipulation: lessons from fish, plants, livestock, and human research, Front. Nutr. 5 (2018) 80, https://doi.org/10.3389/fnut.2018.00080.

[54] T. Csak, M. Ganz, J. Pespisa, K. Kodys, A. Dolganiuc, G. Szabo, Fatty acid and endotoxin activate inflammasomes in mouse hepatocytes that release danger signals to stimulate immune cells, Hepatology 54 (2011) 133–144, https://doi.org/10.1002/hep.24341.

[55] M. Ganz, T. Csak, B. Nath, G. Szabo, Lipopolysaccharide induces and activates the Nalp3 inflammasome in the liver, World J. Gastroenterol. 17 (2011) 4772–4778, https://doi.org/10.3748/wjg.v17.i43.4772.

[56] D.M. Gatlin, F.T. Barrows, P. Brown, K. Dabrowski, T.G. Gaylord, R.W. Hardy, E. Herman, G. Hu, Å. Krogdahl, R. Nelson, K. Overturf, M. Rust, W. Sealey, D. Skonberg, E.J. Souza, D. Stone, R. Wilson, E. Wurtele, Expanding the utilization of sustainable plant products in aquafeeds: a review, Aquacult. Res. 38 (2007) 551–579, https://doi.org/10.1111/j.1365-2109.2007.01704.x.

[57] Y.R. Wang, L. Wang, C.X. Zhang, K. Song, Effects of substituting fishmeal with soybean meal on growth performance and intestinal morphology in orange-spotted grouper (*Epinephelus coioides*), Aquacult. Rep. 5 (2017) 52–57, https://doi.org/10.1016/j.aqrep.2016.12.005.

[58] M.I. Hedrera, J.A. Galdames, M.F. Jimenez-Reyes, A.E. Reyes, R. Avendaño-Herrera, J. Romero, C.G. Feijóo, Soybean meal induces intestinal inflammation in zebrafish larvae, PLoS One 8 (2013), e69983, https://doi.org/10.1371/journal. pone.0069983.

[59] C.X. Zhang, S. Rahimnejada, Y.R. Wang, K.L. Lua, K. Song, L. Wang, K.S. Mai, Substituting fish meal with soybean meal in diets for Japanese seabass (*Lateolabrax japonicus*): effects on growth, digestive enzymes activity, gut histology, and expression of gut inflammatory and transporter genes, Aquaculture 483 (2018) 173–182, https://doi.org/10.1016/j.aquaculture.2017.10.029.

[60] M. Gu, N. Bai, Y.Q. Zhang, Å. Krogdahl, Soybean meal induces enteritis in turbot *Scophthalmus maximus* at high supplementation levels, Aquaculture 464 (2016) 286–295, https://doi.org/10.1016/j.aquaculture.2016.06.035.