Dietary bile acid supplementation reveals beneficial effects on intestinal healthy status of tongue sole (*Cynoglossus semiliaevis*)

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ABSTRACT

The aim of this study was to investigate the effects of dietary bile acids (BAs) on intestinal healthy status of tongue sole in terms of immunity, antioxidant status, digestive ability, mucosal barrier-related genes expression and microbiota. Three experimental diets were prepared with BA levels at 0 mg/kg (CT), 300 mg/kg (BA1) and 900 mg/kg (BA2) in a commercial basal diet. Each diet was fed to three replicates with 120 fish (10.87 ± 0.32 g) in each tank. After an 8-week feeding trial, growth parameters were significantly enhanced in both BAs supplementary groups ($P < 0.05$), and compared with CT group, survival rate in BA2 group was significantly improved ($P < 0.05$). Intestinal lysozyme activity and contents of immunoglobulin M and complement 3 were significantly increased in both BAs supplementary groups ($P < 0.05$), suggesting an enhancement effect on the non-specific immune response. BAs inclusion also significantly improved intestinal antioxidant capabilities by increasing antioxidase activities and decreasing malondialdehyde levels. In addition, compared with CT group, intestinal digestive ability was substantially enhanced as indicated by the significantly increased lipase activity in BA2 group ($P < 0.05$) and significantly increased amylase activity in BA1 and BA2 groups ($P < 0.05$). Coincidentally, BAs inclusion significantly upregulated the relative expression of intestinal mucosal barrier-related genes ($P < 0.05$). Further, dietary BAs distinctly remodeled intestinal microbiota by decreased the abundance of some potential pathogenic bacteria. In conclusion, dietary BAs supplementation is an effective way to improve the intestinal healthy status of tongue sole.

1. Introduction

Bile acids (BAs) as amphipathic sterol compounds are synthesized from cholesterol in liver and play an important role in the intestinal digestion and absorption of dietary lipids and fat-soluble vitamins in mammals as well as in other vertebrates [1]. Other roles of BAs include regulating the homeostasis of lipid/glucose and the cholesterol levels, activating receptors in the intestine and accessory digestive organs, and modulating the immune response in liver and intestine [2–5]. Further, BAs possess antimicrobial activities which can influence the intestinal microbiome [6–9], increasing evidences reveal that there is a close correlation between BAs and intestinal microbiota in mammals [10–13]. In livestock and poultry, the usage of bile acids/salts as additives can be traced back to the 1970s [14], while for aquaculture species, to our knowledge, it should be from the 1980s [15]. In China, BAs were

approved as a new feed additive by Ministry of Agriculture in 2014, since then BAs have been extensively used in animal feed. Though dietary BAs has received considerable attention in the past few years in aquaculture, studies are still limited. Recent studies revealed the beneficial effects of dietary BAs on growth, liver function and antioxidant capacity, immunity and intestinal microbiota [16–24]. Besides, dietary BAs supplementation could improve the digestion and absorption of lipids and intestinal health in largemouth bass (*Micropterus salmoides*) [25]. However, till now, reports of BAs functions focused on intestinal health are still scarce in fish.

As we all known, intestine is the major organ of digestion and absorption. Still, the fish intestine has been confirmed that it could regulate immunity and reduce the risk of infection [26]. Some underlying antinutritional factors in feed, hostile environmental stressors, and as well as unbalanced diet (e.g., high-lipid and high-carbohydrate) will

affect the intestinal health, then further decreasing the animal's growth rate, disease resistance and general health [26–28]. Thus, in view of ensuring the sustainability of aquaculture, nutritional methods of improving intestinal health are needed. The local intestinal immunity is increasingly recognized as an important factor in maintaining the general health of aquaculture species [27].

As an indigenous marine flatfish species with high economic values, tongue sole (*Cynoglossus semilaevis*) is widely cultured in China's coastal areas [29,30] The objectives of present study were to investigate the beneficial effects of dietary BAs on intestinal health. A series of intestinal indices (including immune parameters, antioxidant and digestive ability, mucosal barrier-related genes expression and microbiota) were assayed. These results will provide novel insights into the beneficial effects of BAs on intestine health in tongue sole.

2. Materials and methods

2.1. Experimental diets

In this study, three experimental diets with different levels of BAs were added in the basal diet. The proximate compositions of basal diet on dry matter include ~53% crude protein, ~8% crude fat, ~3% crude fiber and ~16% crude ash. Briefly, 0 mg/kg (control group, CT), 300 mg/kg (BA1) and 900 mg/kg (BA2) BAs (Longchang Group, China) was supplemented to the basal diet respectively. The range of additive contents of BAs was determined based on the manufacture's advice. The main ingredients of BAs used in this study are as described in Ref. [31]. The detailed main ingredients and proximate compositions of basal diet are as described in previous studies [32].

2.2. Feeding trial and experimental conditions

Tongue sole juveniles were cultured in our aquaculture and breeding center for flatfish in Tangshan China. A total of 1800 fish were randomly assigned to 9 flow-through tanks (0.8 m^3, each with 200 individuals), each dietary group with three triplicate tanks. After a two-week acclimation, 30 individuals were randomly selected from each tank for measuring the initial body weight and all the fish in each tank were recounted. Thereafter, fish of each tank were hand-fed to apparent satiation with an amount of 1–2% of wet body weight twice daily at 8:00 and 20:00, 6 days per week for 8 weeks. The feeding amount was updated weekly. Died and moribund fish were observed, recorded and removed daily. During the experimental period, the main water parameters were as follows: temperature 23–24 °C, salinity 26–30‰, pH 7.6–8.3, dissolved oxygen 6–8 mg/L and water exchange rate 600% per day.

2.3. Growth performance and sample collection

At the end of 8-week feeding trial, prior to final measurement and sampling, individuals in each tank were deprived for 24 h. All fish from each replicate were counted and 30 randomly selected fish from each replicate were individually weighted to obtain weight gain rate (WGR), specific growth rate (SGR) [33] and survival rate (SR). The calculations were as follow.

$$WGR = \frac{IBW - FBW}{IBW} \times 100\%,$$

$$SGR = \left(e^{\frac{\log_e(FBW) - \log_e(IBW)}{days}} - 1 \right) \times 100;$$

$$SR = \frac{N1}{N0} \times 100\%;$$

where IBW represents initial body weight, FBW represents final body weight, N1 represents the final fish number in each tank, N0 represents the initial fish number in each tank.

Thereafter, for analyzing intestinal immune and antioxidant parameters, digestive enzyme activities and intestinal microbiota, three individuals from each tank were randomly selected for mid-gut sampling under sterile conditions. The mid-guts were removed by using aseptic tools. Then the intestinal mucosa layers were carefully scraped and pooled to 2 ml sterile tubes. Meanwhile, the middle intestines of another three individuals from each tank were collected and pooled in RNase-free tubes for gene expression analysis. The intestine samples for all the analyses were immediately frozen in liquid nitrogen and then stored at −80 °C until use. Fish were anesthetized with MS-222 (30 mg/L) in all the manipulations mentioned above.

2.4. Estimation of immune parameters, antioxidant ability and digestive enzyme activity

In this study, four immune parameters, five antioxidant indices and three digestive enzyme activity indices of middle gut were assayed respectively (Table 1). All the parameters in intestine were assayed by commercial kit (Shanghai Jining Biotechnology Co. Ltd., China) according to the operating instructions. Briefly, intestinal samples were homogenized in cold phosphate buffered saline (0.01 M, pH = 7.4) and centrifuged (5000×g) at 4 °C for 10 min. Then the supernatant was used for biochemical assays using spectrophotometry. All assays were performed in triplicate.

2.5. Quantitative real-time PCR (qRT-PCR) analysis

The mRNA expression of four intestinal mucosal barrier-related genes, claudin 4 like (Claudin-4-like), claudin 7 like (Claudin-7-like), zonula occludens 1 like (ZO-1-like) and myosin light chain kinase (MLCK), were detected by qRT-PCR. Total RNA was extracted from the tongue sole middle intestines using Trizol Reagent (Takara, Japan) according to the manufacturer's instructions. Agarose gel electrophoresis (1.2%) and spectrophotometric analysis (Nanodrop 2000, Thermo Fisher Scientific, USA) were performed to assess RNA quality and quantity. The cDNA was synthesized using a Servicebio® RT reagent kit with gDNA Eraser (Servicebio, China). Primers used in this study were designed by Premier 5.0 and were shown in Supplementary Table 1. The qRT-PCR amplification procedures were described as [34]. The expression level of target gene was normalized by the $2^{-\Delta\Delta CT}$ method [35], and the control group (CT) was used as the reference group.

2.6. Analysis of the intestinal microbiota

2.6.1. DNA extraction and 16S rDNA sequencing

Total microbial DNA was extracted from intestinal samples using the E. Z.N.A. stool DNA Kit (Omega Bio-Tek, Norcross, GA, U.S.) according to the manufacturer's protocols. The 16S rDNA V3–V4 regions of the rRNA gene were amplified by polymerase chain reaction (PCR, programs: 95 °C for 2min, followed by 27 cycles at 98 °C for 10s, 62 °C for 30s, and 68 °C for 30s and a final extension at 68 °C for 10min) using primers 341F (CCTACGGGNGGCWGCAG) and 806R (GGAC-TACHVGGGTATCTAAT). Amplicons were extracted from 2% agarose gels and purified using the AxyPrep DNA Gel Extraction Kit (Axygen

Table 1
The assayed intestinal parameters in this study.

Item	Index (activity or content)
Immune parameter	lysozyme (LZM), alkaline phosphatase (AKP), immunoglobulin M (IgM), complement 3 (C3)
Antioxidant index	superoxide dismutase (SOD), catalase (CAT), total antioxidant capacity (TAOC), glutathione peroxidase (GPX), malondialdehyde (MDA)
Digestive enzyme	protease, lipase, amylase

Biosciences, USA) according to the manufacturer's instructions and quantified using QuantiFluor-ST (Promega, U.S.). Purified amplicons were pooled in equimolar and paired-end sequenced on Illumina HiSeq2500 platform according to the standard protocols (Gene Denovo Biotechnology Co., Ltd., Guangzhou, China). The raw reads were deposited into the NCBI Sequence Read Archive (SRA) database with accession number PRJNA714600.

2.6.2. Bioinformatics analysis

Raw reads were filtered by using FASTP (version 0.18.0) [36] with following rules: (1) removing reads containing more than 10% of unknown nucleotides (N) and (2) those containing less than 80% of bases with quality (Q-value) > 20. Paired end clean reads were merged as raw tags by FLSAH (version 1.2.11) [37] with a minimum overlap of 10 bp and mismatch ratio of 0.02. Subsequently, high-quality clean tags were filtered from the Noisy sequences of raw tags by QIIME (version 1.9.1) [38] pipeline under specific filtering conditions [39]. UCHIME Algorithm was performed to detect and remove chimeric tags [40]. Then the effective tags were clustered into operational taxonomic units (OTUs) using UPARSE (version 7.0.1001) based on 97% sequence similarity [40].

The tag sequence with highest abundance was selected as representative sequence within each cluster. Principal component analysis (PCA) was performed by using Vegan package (version 2.5.3) in R [41]. The unique and common OTUs between groups were identified in R with VennDiagram package (version 1.6.16) [42]. The microbiota composition in each group was visualized by stack maps by using R ggplot2 package (version 2.2.1). Alpha diversity (Chao1, abundance-based coverage estimator (ACE), Shannon and Simpson) and beta diversity were analyzed by using QIIME (version 1.9.1) [38]. Principal coordinates analysis, PCoA of bray-curtis distances were generated by Vegan package (version 2.5.3) [41] and plotted by ggplot2 package (version 3.4.1) in R. Statistical difference of alpha and beta diversity between different dietary groups were tested by Tukey's and Wilcox's test.

For assessing microbial community structure changes and detecting the indicator species, logarithmic linear discriminant (LDA) [43] and LEfSe (linear discriminant analysis effect size) [44] analysis was performed to identify the most differentially abundant taxa between the control and dietary BA groups. The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis of the OTUs was inferred by using PICRUSt (version 2.1.4) [45]. Tukey's test was used to test the statistical difference between different dietary groups.

2.7. Statistical analysis

Quantitative data were presented as mean ± standard error (SE). Prior to statistical analyses, fractional data were arcsine square root transformed and followed by one-way analysis of variance (ANOVA) to determine significant differences in various parameter indices (significance level was set as $P < 0.05$). Pearson correlation coefficients between microbial species and intestinal parameters (i.e., immune, antioxidant and digestive enzyme) were calculated by using psych package (version 1.8.4) in R [46]. Variation partitioning analysis based on redundancy analysis procedure was performed by using the varpart procedure in package Vegan (version 2.5.3) [41] in R to quantify the relative contributions of intestinal immune, antioxidant and digestive enzyme parameters.

3. Results

3.1. Growth and survival

The growth parameters and survival rate are shown in Table 2. Dietary supplementation with BAs had a significant effect on fish growth, parameters including FBW, WGR and SGR were enhanced substantially,

Table 2
Growth performance and survival of tongue sole fed diets supplemented with bile acids for 8 weeks[a].

Group[b]	CT	BA1	BA2
IBW, g	10.77 ± 0.31	10.94 ± 0.34	10.91 ± 0.32
FBW, g	46.37 ± 1.73[c]	55.74 ± 2.25[b]	66.14 ± 1.78[a]
WGR, %	355.28 ± 37.45[c]	483.04 ± 50.92[b]	557.52 ± 58.77[a]
SGR	2.61 ± 0.27[c]	2.92 ± 0.31[b]	3.28 ± 0.35[a]
SR, %	90.34 ± 0.25[b]	93.01 ± 0.83[ab]	96.55 ± 1.80[a]

IBW = initial body weight; FBW = final body weight; WGR = weight gain rate; SGR = specific growth rate; SR = survival rate.
[a, b, c] Values with different letters within the same row are significantly different ($P < 0.05$).
[a] Data are expressed as mean ± standard error (n = 3 for SR, n = 90 for others).
[b] CT: control group with basal diet; BA1, BA2: bile acids were supplemented at the level of 300 and 900 mg/kg of basal diet, respectively.

especially in high addition group ($P < 0.05$). In BA1 group, the SR was only marginally increased ($P > 0.05$), while in BA2 group, it was significantly improved compared to CT group ($P < 0.05$).

3.2. Intestinal immune response

The intestinal immune parameters are presented in Table 3. Compared with CT group, the activity of LZM and contents of IgM and C3 in intestine were significantly improved ($P < 0.05$) in both dietary BAs supplementation groups. However, the activity of AKP was significantly decreased. It is worth mentioning that when the volume of BA addition increased to 900 mg/kg (BA2 group), IgM content was further significantly improved ($P < 0.05$), while there was little difference for the other three immune parameters between BA1 and BA2.

3.3. Intestinal antioxidant status

The antioxidative parameters in intestine are shown in Table 4. SOD and CAT activities were significantly increased when 300 mg/kg of BAs was added in the basal diet (BA1) and was even significantly higher in 600 mg/kg supplementary group (BA2) ($P < 0.05$). However, the improvement of TAOC level was not significant in BA1 group, but significantly enhanced in BA2 group ($P < 0.05$). For GPX activity, it was elevated dramatically at a low level of BAs supplementation (BA1), but with a very limited increasement at a high level of BAs supplementation (BA2). The content of MDA in intestine was significantly decreased with the increasing of dietary BAs supplementation ($P < 0.05$).

3.4. Intestinal digestive enzyme activity

The intestinal digestive enzymes activities are presented in Table 5. The intestinal lipase activity in BA2 group was significantly higher than

Table 3
Intestinal immune parameters of tongue sole fed the experimental diets for 8 weeks[a].

Group[b]	CT	BA1	BA2
LZM, U/g prot	34.86 ± 1.21[b]	46.48 ± 1.27[a]	46.15 ± 1.67[a]
AKP, U/g prot	42.83 ± 1.20[a]	38.83 ± 0.94[b]	35.76 ± 1.77[b]
IgM, µg/g prot	665.31 ± 25.17[c]	755.43 ± 14.33[b]	885.38 ± 34.13[a]
C3, mg/g prot	85.91 ± 4.31[b]	101.69 ± 2.44[a]	106.02 ± 5.71[a]

LZM = lysozyme; AKP = alkaline phosphatase; IgM = immunoglobulin; C3 = complement 3.
[a, b, c] Values with different superscript letters in the same row show significant differences ($P < 0.05$).
[a] Data are expressed as mean ± standard error (n = 3).
[b] CT: basal diet; BA1, BA2: bile acids were supplemented at the level of 300 and 900 mg/kg of basal diet, respectively.

Table 4
Effects of dietary bile acids on intestinal antioxidant capacity of tongue sole fed the experimental diets for 8 weeks[a].

Group[b]	CT	BA1	BA2
SOD, U/mg prot	0.23 ± 0.01^c	0.32 ± 0.01^b	0.47 ± 0.04^a
CAT, U/mg prot	54.02 ± 2.26^c	64.85 ± 1.81^b	87.50 ± 1.67^a
TAOC, U/g prot	122.65 ± 8.72^b	138.68 ± 2.34^b	245.69 ± 10.24^a
GPX, mU/g prot	164.16 ± 9.56^b	206.77 ± 3.13^a	217.08 ± 18.44^a
MDA, pmol/µg prot	44.94 ± 1.62^a	32.70 ± 1.39^b	24.91 ± 0.52^c

SOD = superoxide dismutase; CAT = catalase; TAOC = total antioxidant capacity; GPX = glutathione peroxidase; MDA = malondialdehyde.
[a, b, c] Values with different superscript letters in the same row show significant differences ($P < 0.05$).
   [a] Data are expressed as mean ± standard error ($n = 3$).
   [b] CT: basal diet; BA1, BA2: bile acids were supplemented at the level of 300 and 900 mg/kg of basal diet, respectively.

Table 5
Effect of dietary bile acids on the activity levels of protease, lipase and amylase in the intestine[a].

Group[b]	CT	BA1	BA2
Protease, U/mg prot	3.31 ± 0.22	3.11 ± 0.09	4.00 ± 0.38
Lipase, mU/g prot	94.56 ± 4.03^b	102.39 ± 2.58^b	155.52 ± 14.03^a
Amylase, U/g prot	7.08 ± 0.60^c	9.67 ± 0.19^b	13.85 ± 0.59^a

[a, b, c] Values with different superscript letters in the same row show significant differences ($P < 0.05$).
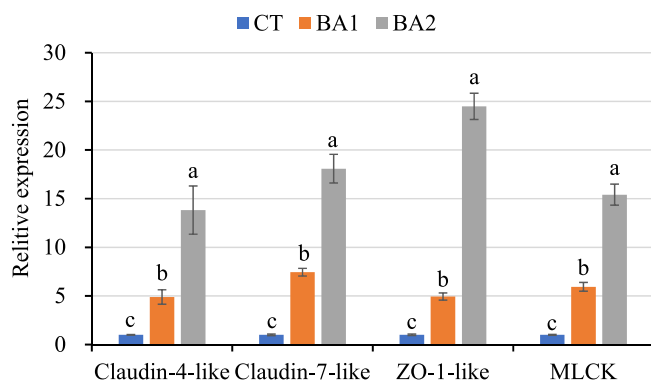   [a] Data are expressed as mean ± standard error ($n = 3$).
   [b] CT: basal diet; BA1, BA2: bile acids were supplemented at the level of 300 and 900 mg/kg of basal diet.

BA1 and CT groups ($P < 0.05$), while the difference between BA1 and CT group was negligible. The amylase activity in intestine was gradually significantly enhanced in low and high levels of additive amount ($P < 0.05$). However, no significant difference was observed in protease activity between CT and two BAs supplementary groups ($P > 0.05$).

3.5. Intestinal mucosal barrier-related gene expression

Fig. 1 shows the effects of BAs on mRNA expression of Claudin-4-like, Claudin-7-like, ZO-1-like and MLCK genes. Compared with the CT group, dietary BAs addition significantly improved ($P < 0.05$) the expression levels of all the four intestinal mucosal barrier-related genes, especially in high additional level group (BA2).

Fig. 1. Effects of dietary bile acids on intestinal mucosal barrier-related gene expressions of tongue sole after an 8-week feeding. CT: basal diet; BA1, BA2: bile acids were supplemented at the level of 300 and 900 mg/kg of basal diet. Claudin-4-like = *C. semilaevis* claudin 4 like (LOC103395016); Claudin-7-like = *C. semilaevis* claudin 7 like (LOC103393253); ZO-1-like = *C. semilaevis* zonula occludens 1 like (LOC103378762); MLCK = *C. semilaevis* myosin light chain kinase (LOC103381494). Values are expressed as mean ± standard error ($n = 3$). Data columns with different letters denote significant difference ($P < 0.05$).

3.6. Intestinal microbiota

High-throughput sequencing generated a total number of 969,150 raw reads, and after quality control and reads assembly, a total of 916,108 effective tags were obtained from nine samples, resulting in a total of 1374 OTUs clustered under 97% sequence similarity (Supplementary Table 2 and Fig. 2). Goods coverage estimators for all groups were close to 1, indicating a sufficient sequencing coverage (Table 6). As shown in Figs. 2 and 268 OTUs were shared by the three dietary groups, the unique OTUs number in group CT, BA1 and BA2 was 428, 183 and 198, respectively. The PCA results showed that samples between dietary treatments were clearly separated (Fig. 3), indicating different supplementary levels of BAs had tremendous effects on the structure of intestinal microbiota in tongue sole.

At genus level, *Vibrio*, *Cetobacterium*, *Brevibacillus*, *Brevinema*, *Chryseobacterium*, *Acinetobacter*, *Photobacterium*, *Propionigenium*, *Shewanella* and *Pseudomonas* composed the top 10 dominant genera of intestinal microbiota in tongue sole from the three groups. Dietary BAs significantly decreased the relative abundance of *Vibrio* and *Brevinema* ($P < 0.05$), however, the relative abundance of *Cetobacterium*, *Brevibacillus*, *Chryseobacterium* and *Photobacterium* was significantly increased ($P > 0.05$) (Fig. 4).

Alpha diversity indices including Shannon, Simpson, Chao1 and ACE were used to compare the intestinal bacterial diversity and richness of tongue sole fed with different dietary BAs levels. Overall, the intestinal bacterial diversity (Shannon and Simpson) was improved substantially by dietary addition of BAs, however, the bacterial richness (Chao1 and ACE) was slightly decreased but not significant ($P > 0.05$) (Table 6). Beta diversity of intestinal microbiota of tongue sole fed different experimental diets was presented in Fig. 5, there was a clear separation among the three groups.

In LEfSe analysis, from domain-to-genus level, total 18 differentially abundant taxa were identified between CT group and BAs groups (BA1 and/or BA2) with LDA scores over 3 (Fig. 6). These taxa were considered as potential biomarkers. No differentially abundant taxa were identified between BA1 and BA2 group.

According to functional enrichment analysis and Tukey's test results, there were six functions of level 2 (involving the metabolism of organic compounds, infectious diseases and membrane transport) showed significant improvement between CT group and BAs addition groups (BA1
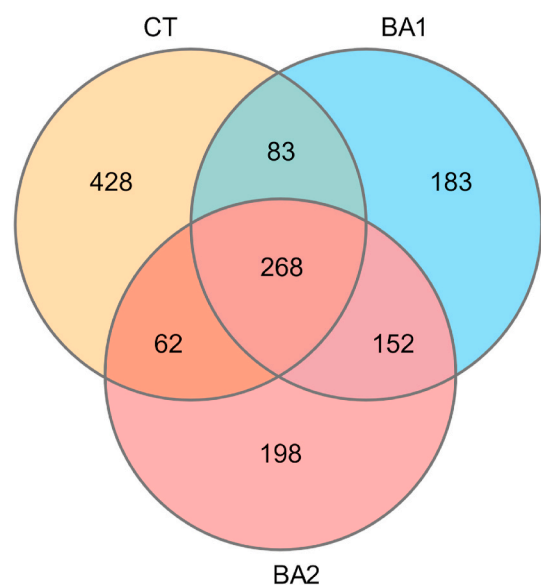
Fig. 2. Venn diagram of the unique and shared operational taxonomic units (OTUs) in different dietary groups. CT: basal diet; BA1, BA2: bile acids were supplemented at the level of 300 and 900 mg/kg of basal diet.

Table 6

Alpha diversity index of intestinal microbiota in tongue sole fed diets with different bile acids levels[a].

Group[b]	Shannon	Simpson	Chao1	ACE[c]	Goods coverage
CT	3.13 ± 0.09[b]	0.69 ± 0.05[b]	1780.82 ± 130.02[a]	1849.80 ± 114.83[a]	0.9950 ± 0.0002[a]
BA1	4.19 ± 0.27[a]	0.87 ± 0.04[ab]	1574.63 ± 59.54[a]	1667.83 ± 66.07[a]	0.9953 ± 0.0001[a]
BA2	4.35 ± 0.03[a]	0.90 ± 0.00[a]	1594.92 ± 176.50[a]	1703.63 ± 165.91[a]	0.9947 ± 0.0004[a]

[a, b, c] Values with different superscript letters in the same row show significant differences ($P < 0.05$).

[a] Data are expressed as mean ± standard error ($n = 3$).

[b] CT: basal diet; BA1, BA2: bile acids were supplemented at the level of 300 and 900 mg/kg of basal diet.

[c] ACE: abundance-based coverage estimator.
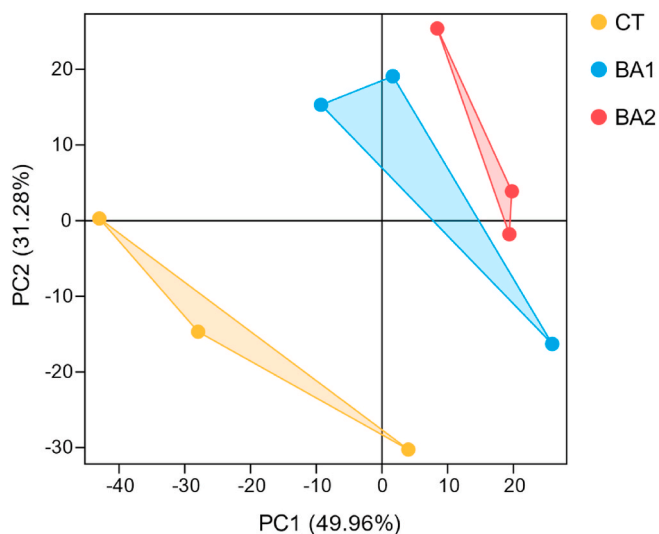
Fig. 3. Comparison of intestinal microbiota of tongue sole fed different experimental diets. CT: basal diet; BA1, BA2: bile acids were supplemented at the level of 300 and 900 mg/kg of basal diet.
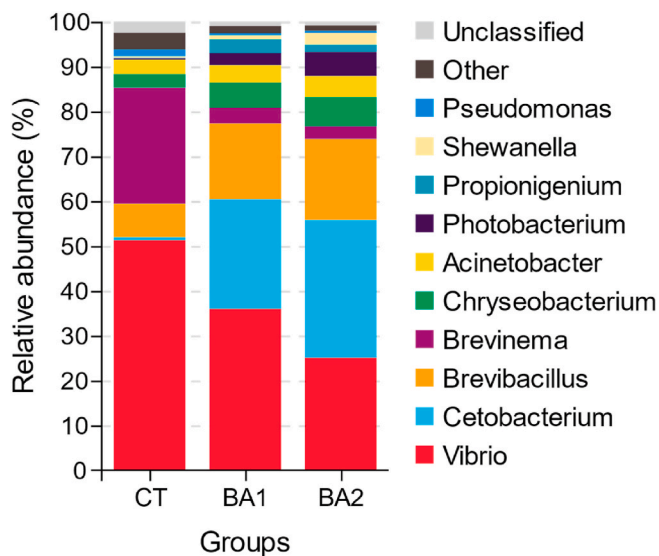
Fig. 4. Relative abundance of intestinal bacteria (at genus level) of tongue sole fed different experimental diets. CT: basal diet; BA1, BA2: bile acids were supplemented at the level of 300 and 900 mg/kg of basal diet.
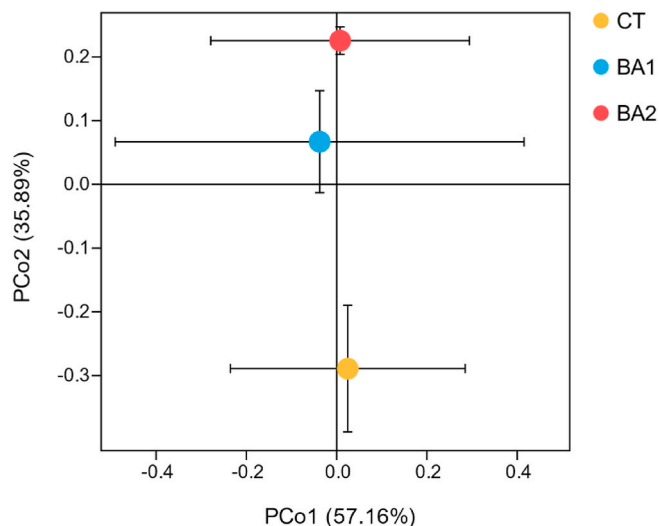
Fig. 5. Principal coordinate analysis (PCoA) of bacterial community structures (based on bray-curtis distance) of tongue sole fed basal diets (CT), low additional bile acids level (300 mg/kg BA1) and high additional bile acids level (900 mg/kg, BA2).

and/or BA2), though the corresponding microbiota abundances were not high (Supplementary Fig. 1).

Correlations between intestinal microbiota abundance at genus level and intestinal physiological and biochemical indicators are presented in Fig. 7. Genera of *Shewanella*, *Photobacterium* and *Cetobacterium* were clustered together, and their abundance were positively correlated with most of the studied intestinal parameters (except MDA). All the four intestinal immune parameters were significantly negatively correlated with the abundance of *Brevinema* genus ($P < 0.05$). High intestinal activities of LZM and AKP extremely decreased the abundance of *Pseudoalteromonas* genus ($P < 0.01$). Among all the intestinal physiological and biochemical parameters, LZM, C3 and AKP occupy the top three relative contributions, the explanatory values were 24.65%, 20.97% and 18.73% respectively (Fig. 8).

4. Discussion

The main contribution of this paper was to uncover the beneficial effects of BAs supplemented in commercial aquafeed on intestinal health in tongue sole. As a first insight, unlike previous studies, we did not set up negative control groups by adding high level lipid or carbohydrate in the basal diet. It is reported that excessive levels (e.g., >0.1%) of dietary BAs supplementation could exhibit a cytotoxic effect with lower growth rate and intestinal injury [17,25]. However, in this study, no side-effect was observed with high level BAs addition (0.09%). Anyway, our results demonstrated valuable firsthand information which is helpful for future disease control and health management in tongue sole aquaculture.

For the effects of dietary BAs in tongue sole, firstly, the growth rate was dramatically enhanced. Similar results were obtained in largemouth bass [21,24], large yellow croaker (*Larimichthys crocea*) [19], grass carp [26], rainbow trout [47], tilapia [25] and turbot [48]. The growth-promotion effect of exogenous BAs can be partially explained by the simultaneously improved digestive enzyme activities. These results are consistent with a previous study in tilapia, that the lipase activity in liver and intestine was significantly increased when fish fed diets with different BAs addition levels [25].

Furthermore, the enhanced antioxidant capacity is another important indicator which can reflect a superior growth performance [49,50], as well as the healthy status and metabolic homeostasis [31,51,52]. In this study, fish fed diets with BAs showed significantly higher SOD, CAT, TOAC and GPX activities and significantly lower MDA content in
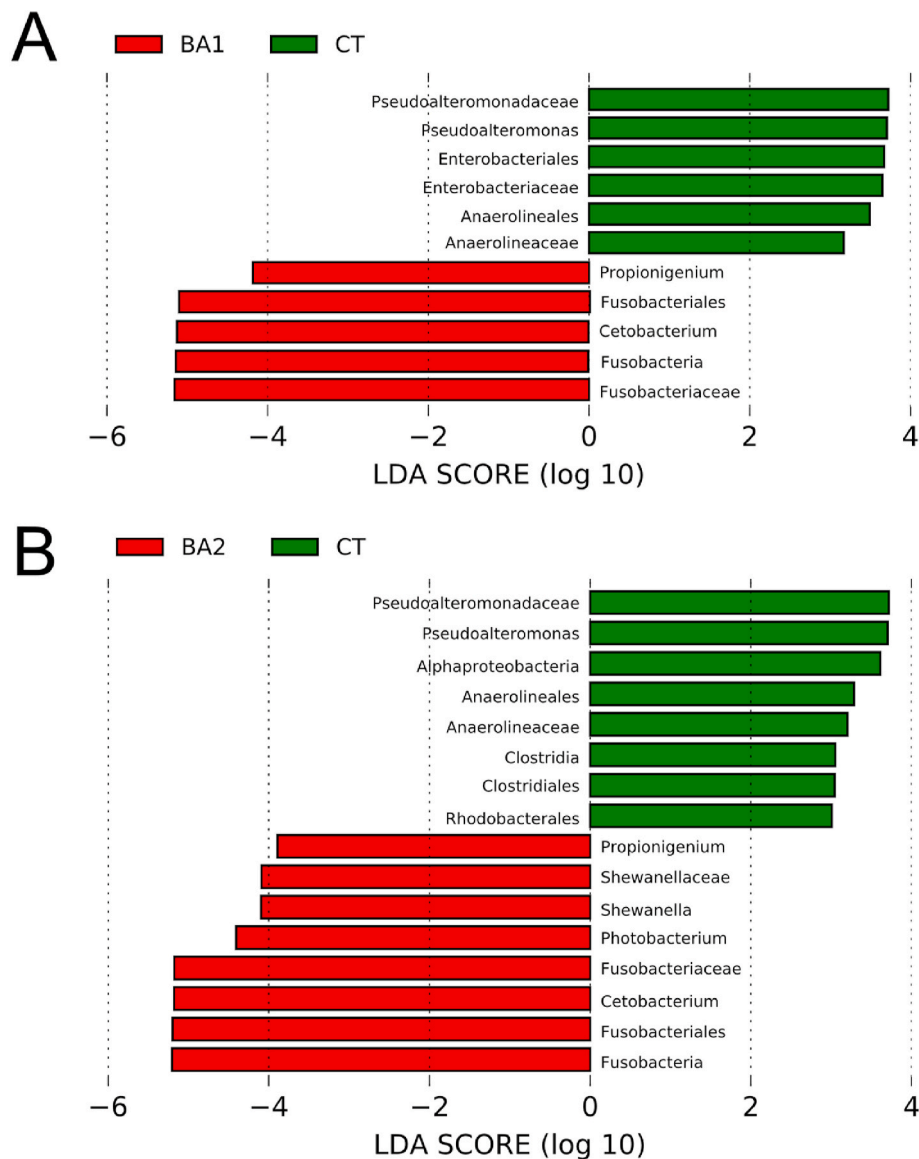
Fig. 6. Linear discriminant analysis (LDA) coupled with effect size (LEfSe) analysis of the intestinal microbiota between control (CT) and bile acids group (BA1 and BA2) of tongue sole. The threshold of the logarithmic LDA score was 3.0 from domain-to-genus level. CT: basal diet; BA1, BA2: bile acids were supplemented at the level of 300 and 900 mg/kg of basal diet.

intestine. Similar results have been reported in fishes, such as large yellow croaker (*L. crocea*) [19], largemouth bass (*M. salmoides*) [21] and black seabream (*Acanthopagrus schlegelii*) [23], and as well as mammals [53,54]. And coincidentally, these studies also revealed an enhanced immune ability in terms of inflammatory responses and dealing with diseases. Thus, the increased ability of intestinal antioxidation may be an explanation for the higher survival rates in dietary BAs supplementation groups.

In this paper, the activity of LZM and contents of IgM and C3 in intestine were significantly promoted when fish were fed diets with BAs, while the AKP activity was significantly decreased. These findings are somewhat consistent with a previous study in largemouth bass, where dietary BAs supplementation elevated the LZM activity and decreased the AKP activity in plasma [21]. However, in another study in black seabream (*A. schlegelii*), when BAs were added to high-fat diet, increasing trends of both AKP and LZM activities in serum and liver were observed [23]. The ambivalent results in AKP activity may ascribe to different experimental designs and different fish species. Anyway, in current study, the higher activity of LZM and higher contents of IgM and

C3, and lower AKP activity in intestine indicated dietary BAs supplementation could improve the healthy status of tongue sole. Correspondingly, BAs inclusion also significantly enhanced the expression levels of mucosal barrier-related genes, indicating that dietary BAs could reinforce the intestine epithelial tight junction then further benefited the intestinal mucosal barrier function in tongue sole. In general, the intact intestinal mucosa as an essential barrier plays a prominent role in inflammatory response and against pathogens from the external environment.

Another important aspect is that, in this study, dietary BAs supplementation improved intestinal barrier function by modulating the intestinal microbiota profile of tongue sole. It has been well acknowledged that the interaction between BAs and gut microbiota contributes to the healthy status of host in humans and other animals. As a novel approach to therapeutics, there is an ever-increasing of interests in the potential for manipulation of the gut microbiota-host BA axis [12,13,55,56]. Compared to mammals, the intestinal immunity of aquaculture species is less developed, the general health and the ability to resist infection and environmental stressors are highly related to intestinal biodiversity
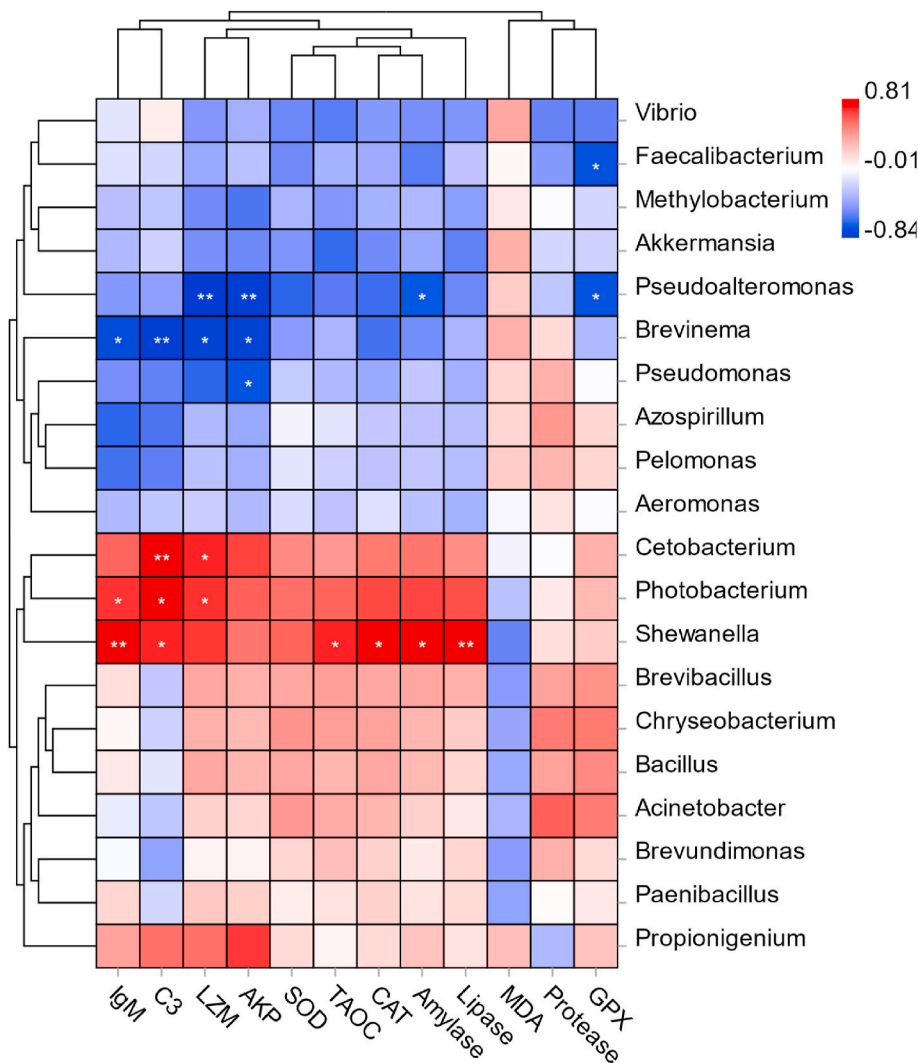
Fig. 7. Correlations between the top 20 predominant genus and immune parameters, antioxidant parameters and digestive enzyme activities in the intestine. Heatmap constructed according to Pearson correlation coefficients. Red represents a positive correlation, and blue represents a negative correlation. "*" represents $P < 0.05$; "**" represents $P < 0.01$. LZM = lysozyme; AKP = alkaline phosphatase; IgM = immunoglobulin; C3 = complement 3; SOD = superoxide dismutase; CAT = catalase; TAOC = total antioxidant capacity; GPX = glutathione peroxidase; MDA = malondialdehyde. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)
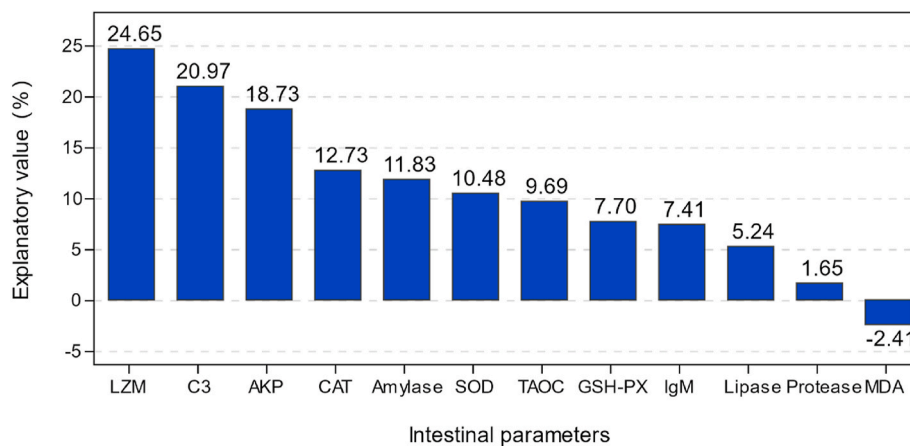
Fig. 8. The relative contributions of different intestinal parameters to the microbiota abundance at genus level in tongue sole fed with different dietary bile acids addition levels.

[57,58].

Recent evidences showed that BAs can influence the intestinal microbiota in grass carp with somewhat contrasting results [26,27]. In this paper, overall, four genera (i.e., *Vibrio*, *Cetobacterium*, *Brevinema* and *Brevibacillus*) showed dominant relative abundance (~80% in total) in three experimental dietary groups. In terms of potential pathogenic bacteria, BAs inclusion significantly decreased the relative abundance of genus *Vibrio*. In veterinary, *Vibrio* spp. are important pathogenic bacteria which have a wide spectrum in aquatic fish hosts [59], and *Vibrio* spp. have emerged as the major disease in tongue sole in China [29,60,61].

Though high levels of relative abundance of *Brevinema* were observed in many fishes [62–64], the knowledge of this genus was very limited. Combined with the significant negative correlations between *Brevinema* and intestinal immune parameters, the significant reduction of relative abundance of *Brevinema* may imply its underlying destructiveness in tongue sole. While, when BAs were included in diets, the significantly increased relative abundance of *Cetobacterium* and *Brevibacillus* should ascribe to the probiotic function of BAs. It is reported that some bile sensitive microbiota in intestine can be directly inhibited by BAs, while the growth of other BA-metabolizing microbiota can be promoted [12, 65]. Anyway, BAs have some antimicrobial and probiotic properties that may influence the gut microbiota and thus intestinal healthy status of fish, however, research in this area is still scarce. Further studies are needed to elucidate the mechanisms.

5. Conclusion

Dietary bile acids supplementation is capable of promoting growth, improving intestinal immunity, antioxidant and digestive ability, enhancing the protective effect of mucosal barrier and remodeling the microbiota in tongue sole. The results will be not only helpful for aquafeed formulating, but also rewarding for healthful aquaculture.

CRediT authorship contribution statement

Yangzhen Li: Methodology, Conceptualization, Formal analysis, Writing – original draft. **Shengpeng Wang:** Resources, Testing and assaying. **Yuanri Hu:** Testing and assaying. **Jiayu Cheng:** Resources, Investigation, Validation. **Xiangming Cheng:** Resources, Investigation. **Peng Cheng:** Resources, Investigation. **Zhongkai Cui:** Resources, Investigation.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fsi.2021.06.020.

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