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Dietary daidzein improved intestinal health of juvenile turbot in terms of intestinal mucosal barrier function and intestinal microbiota

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ABSTRACT

A 12-week feeding trial was conducted to investigate the effect of dietary daidzein on the intestinal mucosal barrier function and the intestinal microbiota profile of juvenile turbot (*Scophthalmus maximus* L.). Three isonitrogenous and isolipidic experimental diets were formulated to contain 0 (FM), 40 (D.40) and 400 (D.400) mg kg^{-1} daidzein, respectively. Fish fed D.400 had significantly lower growth performance than fish fed D.40. Dietary daidzein significantly increased the feed efficiency, while significantly decreased the feed intake. Daidzein supplementation increased the activity of total anti-oxidative capacity and the gene expression of anti-inflammatory cytokine *transforming growth factor-β1*, *Mucin-2* and tight junction proteins (*Tricellulin*, *Zonula occludens-1 transcript variant 1*, *Zonula occludens-1 transcript variant 2* and *Claudin-like* and *Occludin*), and down-regulated the gene expression of pro-inflammatory cytokines *interleukin-1β* and *tumor necrosis factor-α* in the intestine of turbot. Dietary daidzein increased intestinal microbial diversities, the abundance of several short chain fatty acids producers, and decreased the abundance of some potential pathogenic bacteria. However, D.400 had dual effects on lactic acid bacteria and increased the abundance of potential harmful bacterium *Prevotella copri*. Collectively, dietary daidzein at the levels of 40 and 400 mg kg^{-1} could enhance the intestinal mucosal barrier function and alter the intestinal microbiota of turbot. However, high dose of daidzein must be treated with caution for its unclear effects on intestinal microbiota of turbot in the present study.

1. Introduction

Daidzein is one of the dominant and most biologically active isoflavones in soy products [1]. Daidzein has many biological activities, such as anti-inflammatory [2–5], anti-cancer [6,7], and anti-oxidative [4,8–10] activities. Therefore, it has attracted a lot of attention regarding the application in both humans [11] and animals, such as pigs [12], bull calves [10], and poultry [13]. However, few studies on daidzein were conducted in fish and limited information is available at present. Besides, daidzein can also exert antinutritive effects on fish, and the dose is a very important reason [9]. Moreover, soybean meal, the most important alternative protein sources replacing fish meal in commercial aqua-feeds, is rich in daidzein which should be taken in consideration. Therefore, it is necessary to investigate the effect of dietary daidzein on fish to expand the effective use of daidzein in aqua-feed.

The intestine homeostasis is crucial for securing optimal host health and growth. Extracellular stimuli, for example inflammatory cytokines and reactive oxygen species (ROS), can disrupt the intestinal mucosal barrier [14], whose function is closely related to intestinal homeostasis and is tightly associated with the interaction of several barrier components, for example the mucous layer and the tight junctions between adjacent epithelial cells [15]. Suzuki and Hara [14] reviewed that intestinal barrier defects are implicated in some intestinal and metabolic diseases, for example inflammatory bowel disease (IBD), food allergies, obesity and alcoholic liver disease. Soy isoflavones can strengthen intestinal tight junction functions and enhance barrier integrity in human intestinal Caco-2 cells, rats and weaned piglets [14,16–18]. However, studies on the effects of dietary daidzein on intestinal mucosal barrier function in fish have not been carried out yet.

In addition, the homeostasis of intestinal microbiota is indispensable for intestinal health, and the intestinal dysfunction can be

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prevented by the beneficial intestinal bacteria through diverse ways [8,19]. Therefore, intestinal microbiota is also critical for intestinal homeostasis, and its imbalance has been considered as another pivotal factor in the initiation of chronic intestinal disease [20–22]. Several positive health effects attributed to isoflavones could be partly ascribed to their stimulatory or inhibitory effect on some intestinal microbial compositions [23]. It has been reported that daidzein could decrease the abundance of pathogenic bacteria and increase the number of beneficial bacteria in bull calves and rodents [10,24]. However, as far as we know, the effects of dietary daidzein on intestinal microbiota of fish have not been reported.

Turbot (*Scophthalmus maximus* L.) is a highly valued marine carnivorous fish and has been extensively cultured all over the world. The present study was aimed at estimating the influence of dietary daidzein on intestinal health of turbot in terms of intestinal mucosal barrier function and intestinal microbiota. These results are expected to help better understand more precise mechanisms involved in biological functions of dietary daidzein in feed for fish.

2. Materials and methods

2.1. Ethics statement

Animal care and treatment procedures in the current study were approved by the Institutional Animal Care and Use Committee of Ocean University of China.

2.2. Experimental diets

Three isonitrogenous and isolipidic experimental diets were formulated to contain 0 (FM), 40 (D.40) and 400 (D.400) mg kg^{-1} daidzein, respectively (Table 1). The feeds were made, packed and stored following Xu et al. [25]. Briefly, all dietary ingredients were ground into fine powder through 320 μm mesh, and then they were thoroughly mixed with fish oil. After adding water, the stiff dough was produced, and it was pelleted with an experimental single-screw feed mill. Finally, the feeds were dried for about 12 h in a ventilated oven at 45 °C and then stored at −20 °C.

Table 1
Formulation and proximate composition of the experimental diets (% dry matter).

Ingredients	FM	D.40	D.400
Menhaden fish meal[a]	67.00	67.00	67.00
α-starch[a]	16.00	16.00	16.00
Menhaden fish oil[a]	3.50	3.50	3.50
Soybean lecithin	0.50	0.50	0.50
Choline chloride	0.30	0.30	0.30
Vitamin premix[b]	1.00	1.00	1.00
Mineral premix[c]	0.50	0.50	0.50
$Ca(H_2PO_3)_2$	0.50	0.50	0.50
Y_2O_3	0.10	0.10	0.10
Daidzein	0.00	0.004	0.04
Microcrystalline cellulose	10.60	10.596	10.56
Analyzed nutrients compositions			
Crude protein	48.69	48.77	49.17
Crude lipid	9.37	9.41	9.52
Ash	8.20	8.29	8.34

[a] Menhaden fish meal, starch, menhaden fish oil and soybean lecithin were obtained from Great Seven Bio-tech (Shandong, China). Menhaden fish meal, crude protein 74% DM, crude lipid 9.7% DM.

[b] Vitamin premix (mg kg^{-1} diet): thiamin, 25; riboflavin (80%), 45; pyridoxine HCl, 20; vitamin B_{12}, 10; vitamin K_3, 10; inositol, 800; pantothenic acid, 60; niacin acid, 200; folic acid, 20; biotin (2%), 60; retinyl acetate, 32; cholecalciferol, 5; α-tocopherol, 240; ethoxyquin 3; ascorbic acid 2000; Microcrystalline Cellulose, 6470.

[c] Mineral premix (mg kg^{-1} diet): $MgSO_4{\cdot}7H_2O$, 1200; $CuSO_4{\cdot}5H_2O$, 10; $FeSO_4{\cdot}H_2O$, 80; $ZnSO_4{\cdot}H_2O$, 50; $MnSO_4{\cdot}H_2O$, 45; $CoCl_2{\cdot}6H_2O$ (1%), 50; $Ca(IO_3)_2$ (1%), 60; Na_2SeO_3 (1%), 20; Zeolite, 3485.

2.3. Feeding trial

Juvenile turbot (initial body weight 9.99 ± 0.01 g) were bought from a commercial farm in Laizhou, China. After being fed with the commercial diet (Great Seven Bio-Tech Co. Ltd, Qingdao, China) for two weeks to acclimate to the experimental conditions, a total of 270 fish were fasted for 24 h and weighed, then they were randomly distributed to 9 cylindrical fiberglass tanks (200 L) with continuous aeration and circulating water in an indoor rearing system. Each experimental feed was randomly assigned to triplicate tanks. Fish were slowly hand-fed to apparent satiation twice daily (7:30 and 19:30). During the feeding period (12 weeks), the rearing conditions were as follows: water temperature, 15–19 °C; pH, 7.5–8.0; salinity, 30–33‰; ammonia nitrogen, < 0.4 mg L^{-1}; nitrite, < 0.1 mg L^{-1}; dissolved oxygen, > 7.0 mg L^{-1}. The residual feed and feces in each tank were siphoned out daily.

2.4. Sample collection

At the end of the feeding trial, 6 h after the last feeding, fish were anesthetized with eugenol (1:10,000) (purity 99%, Shanghai Reagent Corp, Shanghai, China), and then counted and weighed. The whole sampling process strictly followed the aseptic operation around alcohol flame. To analyze intestinal microbiota, after surface sterilization by using tampon with 70% alcohol, the abdominal cavity of 1 randomly selected fish per tank (3 fish per group) was opened; subsequently, the whole intestine was removed and opened, and the intestinal content was cleaned up carefully, by using sterile scissors and bistoury. The whole intestinal mucosa layer from foregut to hindgut were carefully scraped using sterile rubber spatula, then transferred to 2 ml sterile tubes (Axygen, America). The whole intestinal mucosa layer from other 6 randomly selected fish per tank were collected following the procedure described above, then transferred to RNase-free 1.5 ml tubes (Axygen, America) for the analysis of intestinal gene expression (3) and intestinal oxidative stress status (3). All the above-mentioned samples were instantly put into liquid nitrogen and then stored at −80 °C before further processing.

2.5. Intestinal microbiota DNA extraction and sequencing

The QiAamp DNA Stool Mini Kit (Qiagen, Germany) was used for extracting genomic DNA from intestinal samples on super clean bench around alcohol flame according to the manufacturer's instructions. Subsequently, the quantity and purity of the extracted genomic DNA were evaluated using a Nano-Drop® ND-1000 spectrophotometer (Nano-Drop Technologies, Wilmington, DE, USA). And the integrity of the extracted genomic DNA was assessed via electrophoresis on a 1.2% (w/v) agarose gel. The sole and bright strip around 20,000 bp on the gel of each DNA sample and the similar and stable 260/280 nm absorbance ratios (1.8–2.0) of all DNA samples showed satisfactory quality of all DNA samples. The concentration of DNA was diluted to 1 ng/μl using sterile water.

The 515f/806r primer set was used for amplifying the V4 region of the 16S rRNA gene of intestinal bacteria for the analysis of the diversity and composition of intestinal bacterial community. The sample volume of reactions was 30 μL containing 15 μL Phusion® High-Fidelity PCR Master Mix (New England Biolabs), 0.2 μM forward and reverse primers, 10 ng template DNA, and nuclease-free water. The procedure of PCR was as follow: 98 °C for 1 min, then 30 cycles of "denaturation at 98 °C for 10 s, annealing at 50 °C for 30 s, and extension at 72 °C for 60 s", as well as a final extension at 72 °C for 5 min was applied. PCR products were mixed with same volume of 1X loading buffer (contained SYB green) and then detected by electrophoresis on a 2% (w/v) agarose gel, and samples with bright main strip between 400 and 450bp were

selected for further experiments. PCR products was mixed in equi-density ratios. Next, the Qiagen Gel Extraction Kit (Qiagen, Germany) was used for purification of mixture PCR products. Sequencing libraries were prepared with TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA) according to manufacturer's recommendations and index codes were added. After assessment of quality of libraries on the Qubit@ 2.0 Fluorometer (Thermo Scientific) and Agilent Bioanalyzer 2100 system, the libraries were sequenced on an Illumina HiSeq2500 platform and 250 bp paired-end reads were generated following the manufacturer's recommendations, provided by Beijing Novogene Genomics Technology Co. Ltd (China). Complete data were submitted to the NCBI Sequence Read Archive (SRA) database under accession number PRJNA523564.

2.6. RNA extraction and quantitative real-time PCR (qRT-PCR)

The total RNA was extracted from intestinal samples with Trizol Reagent (Invitrogen, USA) and electrophoresed on a 1.2% (w/v) de-naturing agarose gel to assess the integrity followed by determination of concentration with Nano-Drop® ND-1000 (Nano-Drop Technologies, Wilmington, DE, USA). The 260/280 nm absorbance ratios of all RNA samples were similar and stable (approximately 2.0), indicating that the purity of each RNA sample was satisfactory. Next, according to the manufacturer's instructions, RNA was reversely transcribed to cDNA by PrimeScript® RT reagent Kit with gDNA Eraser (Perfect Real Time, Takara, Japan). And then the cDNA products were diluted to 100 ng/μl using sterilized double-distilled water.

The primers used for quantitative real-time PCR (qRT-PCR) were presented in Table 2. The qRT-PCR was conducted with a quantitative thermal cycler (Mastercycler eprealplex, Eppendorf, Germany) in a total volume of 25 μl including 12.5 μl 2 × SYBR Green Real-time PCR Master Mix (SYBR® Premix Ex Taq™ (Tli RNaseH Plus)) (TaKaRa, Japan), 0.5 μl (10 μM) primer, 1 μl diluted cDNA template and 10.5 μl dH_2O. The procedure of qRT-PCR began with 2 min at 95 °C, followed by 35 cycles of "15 s at 95 °C, 15 s at 58 °C, and 20 s at 72 °C". In order to confirm that only one product was amplified, the melting curve analysis (1.85 °C increment/minute from 58 °C to 95 °C) was implemented after each amplification phase. *β-ACTIN* was selected as reference gene due to its stable expression profile among all groups. The gene expression level was calculated using $2^{-\Delta\Delta CT}$ method [26], and the relative expression level of gene in FM group was used as a calibrator.

Table 3
Effects of dietary daidzein on survival and growth performance of juvenile turbot.

	FM	D.40	D.400
Survival rate (SR, %)	100 ± 0	100 ± 0	100 ± 0
Initial body weight (W_i, g)	9.99 ± 0.01	9.99 ± 0.02	9.99 ± 0.01
Final body weight (W_f, g)	75.99 ± 1.74^{ab}	80.61 ± 0.46^{b}	74.38 ± 0.89^{a}
Weight gain rate (WGR, %)	6.61 ± 0.17^{ab}	7.07 ± 0.05^{b}	6.44 ± 0.09^{a}
Specific growth rate (SGR, %/d)	2.25 ± 0.03^{ab}	2.32 ± 0.01^{b}	2.23 ± 0.01^{a}
Feed intake (FI, %/d)	1.44 ± 0.02^{b}	1.29 ± 0.01^{a}	1.30 ± 0.02^{a}
Feed efficiency ratio (FER)	1.18 ± 0.03^{a}	1.34 ± 0.01^{b}	1.31 ± 0.01^{b}

Values were expressed as means ± S.E. (n = 3), and values within the same row with different letters are significantly different ($P < 0.05$).

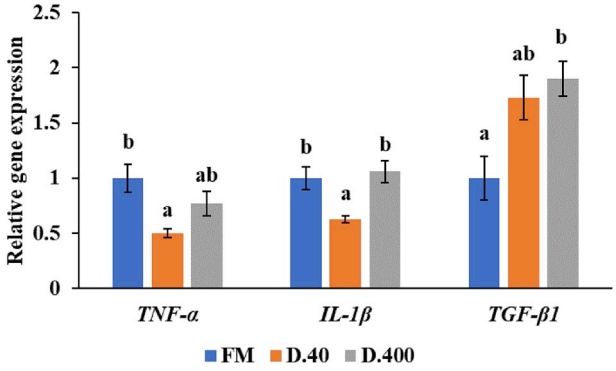

Fig. 1. Effects of dietary daidzein on intestinal inflammatory-related genes expressions of juvenile turbot. Values were expressed as means ± S.E. (n = 3), and values not sharing same letters were significantly different ($P < 0.05$). Abbreviation: *TNF-α*: *tumor necrosis factor-α*; *IL-1β*: *interleukin-1β*; *TGF-β1*: *transforming growth factor-β1*.

2.7. Intestinal oxidative stress status

The TBARS (thiobarbituric acid reactive substances) content [27],

Table 2
Primers used in quantitative real-time PCR (qRT-PCR).

Genes	Primers	Primer sequence (5′-3′)	GenBank accession number
Claudin-3	*Claudin-3*-F[a]	GCCAGATGCAGTGTAAGGTC	KU238180
	Claudin-3-R[a]	CCGTCCAGGAGACAGGGAT	
Claudin-like	*Claudin-like*-F[a]	ATGTGGAGGGTGTCTGCC	KU238181
	Claudin-like-R[a]	CTGGAGGTCGCCACTGAG	
Occludin	*Occludin*-F[a]	ACTGGCATTCTTCATCGC	KU238182
	Occludin-R[a]	GGTACAGATTCTGGCACATC	
Tricellulin	*Tricellulin*-F[a]	GCCTACATCCACAAAGACAACG	KU238183
	Tricellulin-R[a]	TCATTCCCAGCACTAATACAATCAC	
ZO-1 transcript variant 1	*ZO-1tv-1*-F[a]	AGAGAACCTGTCACTGATAGATGC	KU238184
	ZO-1tv-1-R[a]	CTGTCGGAATTGTTGCCTGATG	
ZO-1 transcript variant 2	*ZO-1tv-2*-F[a]	AATGGCACAGTGACAGAGAACC	KU238185
	ZO-1tv-2-R[a]	AGGCGTTGGCTGAAGGAATG	
Mucin-2	*Mucin-2*-R[a]	GTTGGTGCAGCCGCATAG	KU238186
	Mucin-2-F[a]	CACTGGACGCTGGGAATG	
IL-1β	*IL-1β*-F[a]	ATGGTGCGATTTCTGTTC	AJ295836.2
	IL-1β-R[a]	CACTTTGGGTCGTCTTTG	
TGF-β1	*TGF-β1*-F[a]	CTGCAGGACTGGCTCAAAGG	KU238187
	TGF-β1-R[a]	CATGGTCAGGATGTATGGTGGT	
TNF-α	*TNF-α*-F[a]	GGACAGGGCTGGTACAACAC	AJ276709.1
	TNF-α-R[a]	TTCAATTAGTGCCACGACAAAGAG	
β-actin	*β-actin*-F[a]	GCTGTCTTCCCTTCTATCGTCG	AY008305.1
	β-actin-R[a]	TCCATGTCATCCCAGTTGGTC	

Abbreviation: *ZO-1*: *Zonula occludens-1*; *IL-1β*: *interleukin-1β*; *TGF-β1*: *transforming growth factor-β1*; *TNF-α*: *tumor necrosis factor-α*.
[a] The F: forward primer; The R: reverse primer.

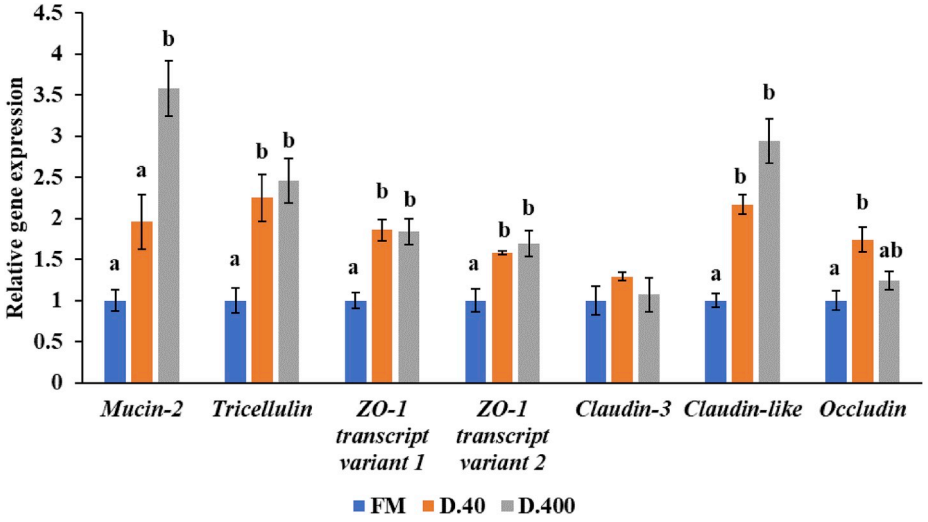

Fig. 2. Effects of dietary daidzein on intestinal mucosal barrier-related genes expressions of juvenile turbot. Values were expressed as means ± S.E. (n = 3), and values not sharing same letters were significantly different ($P < 0.05$). Abbreviation: *ZO-1*: *Zonula occludens-1*.

Table 4
Effects of dietary daidzein on intestinal oxidative stress status of juvenile turbot.

	FM	D.40	D.400
TBARS content (μM malondialdehyde equivalents)	41.33 ± 0.61[b]	35.58 ± 1.74[a]	42.67 ± 0.83[b]
PC content (nmol mg^{-1} protein)	4.92 ± 0.23	4.59 ± 0.28	4.65 ± 0.12
T-AOC (U mg^{-1} protein)	8.12 ± 0.36[a]	10.48 ± 0.28[b]	9.88 ± 0.23[b]

Values were expressed as means ± S.E. (n = 3), and values within the same row with different letters are significantly different ($P < 0.05$). Abbreviation: TBARS: thiobarbituric acid reactive substances; PC: protein carbonyl; T-AOC: total anti-oxidative capacity.

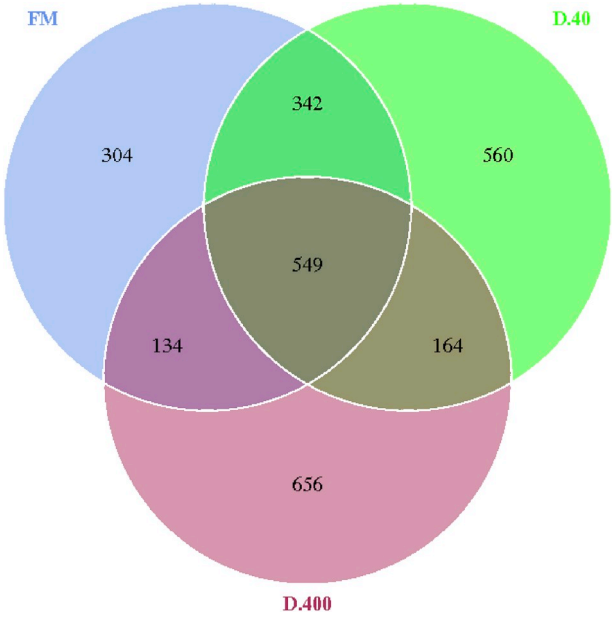

Fig. 3. Venn diagram.

PC (protein carbonyl) content [28] and T-AOC (total anti-oxidative capacity) [29] in the intestine were assayed according to the previously described procedures with slight modifications.

2.8. Microbiota sequencing data analysis

Paired-end reads were assigned to each sample based on the unique barcode of each sample. FLASH software (v. 1.2.7) was used to merge paired-end reads into raw tags [30]. High-quality clean tags were obtained through quality filtration on raw tags under specific filtering conditions [31] according to the QIIME (v. 1.7.0) quality-controlled process [32]. UCHIME algorithm was used to detect and remove chimera sequences in order to obtain the effective tags [33]. The tags were then clustered to OTU (operational taxonomic unit) using Uparse software (v. 7.0.1001) based on 97% sequence similarity [34]. Representative sequence for each OTU was screened for further taxonomic annotation using the GreenGene Database [35] based on RDP classifier (v. 2.2) algorithm [36], and then sequences that could potentially belong to mitochondria, chloroplasts or streptophyta were filtered out. For the analysis of phylogenetic relationship of different OTUs and the difference of the dominant species in different samples (groups), multiple sequence alignment was performed using the MUSCLE software (v. 3.8.31) [37]. Alpha diversity (Chao1 index, observed species number, Shannon index, Simpson index and ACE (abundance-based coverage estimator)) and beta diversity (Non-Metric Multi-Dimensional Scaling (NMDS)) analysis were calculated with QIIME (v. 1.7.0) and displayed with R software (v. 2.15.3). Tukey's test was used to test statistical difference of α diversity and β diversity indices between groups. To determine the changes in bacterial community populations brought by dietary daidzein, differentially abundant taxa between FM group and daidzein-supplemented groups were identified by Metastats analysis [38].

2.9. Calculations and data statistical analysis

Survival rate, SR (%) = 100 × (final amount / initial amount)

Weight gain rate, WGR (%) = 100 × [(final body weight- initial body weight) / initial body weight]

Specific growth rate, SGR (%/d) = ($\ln_{\text{final body weight}}$ - $\ln_{\text{initial body weight}}$) / days × 100

Table 5
Alpha diversity indices of intestinal microbiota of juvenile turbot.

	observed species number	shannon	simpson	chao1	ACE
FM	787.00 ± 33.45	6.81 ± 0.25^{b}	0.97 ± 0.005^{ab}	1116.45 ± 102.77	1213.50 ± 95.50
D.40	1033.33 ± 68.55	7.56 ± 0.38^{ab}	0.97 ± 0.009^{b}	1353.92 ± 96.38	1418.63 ± 104.32
D.400	1016.67 ± 85.11	8.41 ± 0.21^{a}	0.99 ± 0.001^{a}	1244.10 ± 157.52	1253.44 ± 148.67

Values were expressed as means ± S.E. (n = 3), and values within the same row with different letters are significantly different ($P < 0.05$). Abbreviation: ACE: abundance-based coverage estimator.

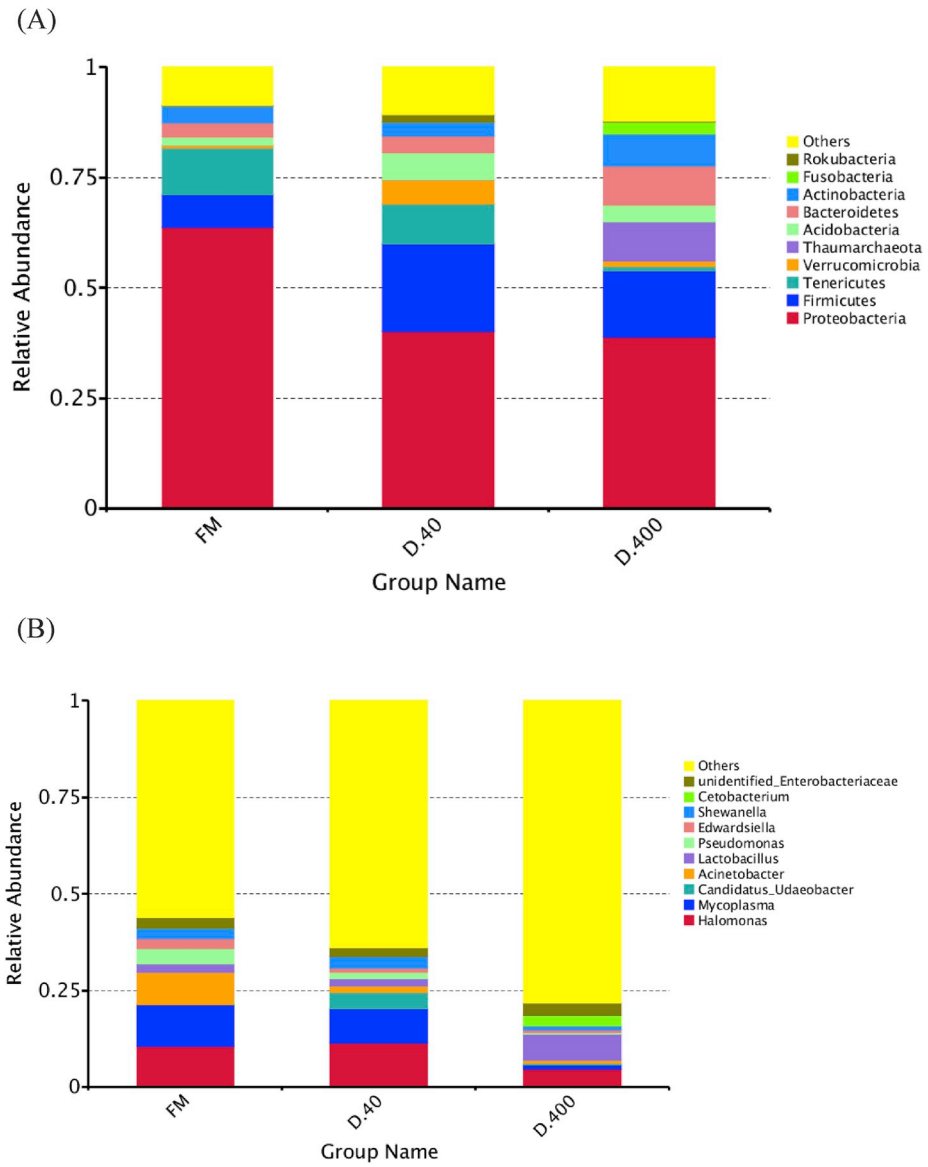

Fig. 4. Top 10 most abundant (based on relative abundance) bacterial phyla (A) and genera (B).

Feed intake, FI (%/d) = 100 × total amount of feed consumption (g) / [(initial body weight + final body weight) / 2] / days

Feed efficiency ratio, FER = weight gain (g) / total amount of feed consumption (g)

All data except the microbiota sequencing data were tested for normality and variance homogeneity using the Shapiro−Wilk *W* goodness of fit test and the Bartlett test, respectively. Then, these data were subjected to one-way analysis of variance (ANOVA) using SPSS 16.0 for windows. When overall difference was significant ($P < 0.05$), Tukey's test was used to compare the means among individual groups. Each result was presented as mean ± S.E. (standard error).

3. Results

3.1. Growth performance

The fish fed with D.400 diet showed significantly lower specific growth rate, weight gain rate and final body weight than that of fish fed with D.40 diet ($P < 0.05$), however, there are no significant differences were observed between FM and D.40 or FM and D.400

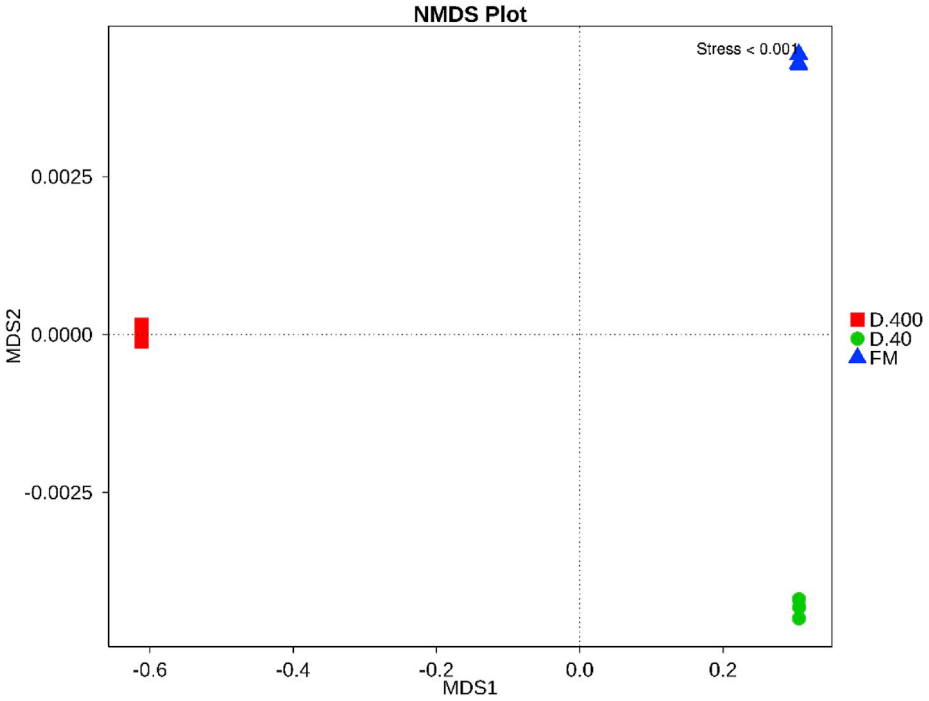

Fig. 5. Non-Metric Multi-Dimensional Scaling (NMDS) analysis of intestinal microbiota of juvenile turbot.

($P > 0.05$). The feed efficiency ratio of fish was significantly increased by dietary daidzein (D.40 and D.400) ($P < 0.05$) whereas the significantly higher feed intake was observed in FM ($P < 0.05$) (Table 3).

3.2. Expression of intestinal inflammatory-related genes and intestinal mucosal barrier-related genes

Compared with FM group, the gene expressions of *tumor necrosis factor-α* (*TNF-α*) and *interleukin-1β* (*IL-1β*) in D.40 group were down-regulated significantly, and the gene expression of *transforming growth factor-β1* (*TGF-β1*) in D.400 group was up-regulated significantly ($P < 0.05$, Fig. 1). The gene expression of *Mucin-2* was significantly higher in D.400 group than that in FM and D.40 group ($P < 0.05$, Fig. 2). As for the tight junction proteins, compared with FM group, dietary daidzein significantly up-regulated the gene expressions of *Tricellulin*, *Zonula occludens-1* (*ZO-1*) *transcript variant 1*, *ZO-1 transcript variant 2* and *Claudin-like* ($P < 0.05$), and D.40 group significantly increased the gene expression of *Occludin* ($P < 0.05$, Fig. 2).

3.3. Intestinal oxidative stress status

The content of TBARS in the intestinal mucosa layer was decreased significantly ($P < 0.05$) by D.40 diet, however, the activity of T-AOC in the intestinal mucosa layer was increased significantly by dietary daidzein ($P < 0.05$, Table 4).

3.4. Intestinal microbiota

For all samples, the rarefied curves for observed species number tended to approach the saturation plateau, indicating complete sequencing efforts for all samples (Supplementary Fig. S1). A Venn diagram showed that 549 OTUs were shared by group FM, D.40 and D.400, and the number of unique OTUs in group FM, D.40 and D.400 was 304, 560 and 656, respectively (Fig. 3). The Shannon index was significantly increased in D.400 group ($P < 0.05$, Table 5). Compared with D.40 group, D.400 had significantly higher Simpson index ($P < 0.05$, Table 5).

Each circle in Venn diagram represent one group, the value from the overlapping part of different circles represent the shared OTUs between these groups, and the value from the non-overlapping part of one circle represent the unique OTUs of that group.

At phylum level, the top ten predominant bacterial phyla in the intestine of turbot contained Proteobacteria, Firmicutes, Tenericutes, Verrucomicrobia, Thaumarchaeota, Acidobacteria, Bacteroidetes, Actinobacteria, Fusobacteria and Rokubacteria (Fig. 4A). At genus level, *Halomonas*, *Mycoplasma*, *Candidatus_Udaeobacter*, *Acinetobacter*, *Lactobacillus*, *Pseudomonas*, *Edwardsiella*, *Shewanella*, *Cetobacterium* and unidentified_Enterobacteriaceae made up the top ten dominant genera of intestinal bacterial community of turbot (Fig. 4B). The Non-Metric Multi-Dimensional Scaling (NMDS) analysis (Fig. 5) was used to access the overall structural changes of intestinal microbiota in response to dietary daidzein. The result of NMDS analysis showed that samples clustered together according to the diets with clear separation between all groups.

Furthermore, MetaStat analysis on genus and species level between different groups showed obvious differences in bacterial communities. At genus level, compared with FM group, D.40 group significantly ($P < 0.05$) up-regulated the relative abundance of *Roseburia*, *Akkermansia* and *Phascolarctobacterium*, and significantly ($P < 0.05$) down-regulated the relative abundance of *Acinetobacter*, *Pseudomonas* and *Helicobacter* (Fig. 6A). At species level, compared with FM group, D.40 group significantly ($P < 0.05$) increased the relative abundance of *Akkermansia muciniphila*, and significantly ($P < 0.05$) decreased the relative abundance of *Acinetobacter lwoffii* and *Pseudomonas stutzeri* (Fig. 6B). Compared with FM group, the significantly ($P < 0.05$) higher abundance of genus *Lactobacillus*, *Bifidobacterium*, *Bacteroides*, *Dialister*, and *Phascolarctobacterium* were observed in D.400 group (Fig. 7A). Compared with FM group, the significantly ($P < 0.05$) lower abundance of genus *Acinetobacter*, *Mycoplasma*, *Shewanella*, *Pseudomonas*, *Helicobacter*, *Arcobacter*, *Halomonas*, *Enterococcus*, *Pediococcus*, *Streptococcus*, *Lactococcus* and *Psychrobacter* were also observed in D.400 group (Fig. 7A). Meanwhile, compared with FM group, D.400 group significantly ($P < 0.05$) increased the relative abundance of species *Megasphaera elsdenii* and *Prevotella copri*, and significantly ($P < 0.05$) decreased the relative abundance of species *Shewanella algae*, *Acinetobacter lwoffii*, and *Pseudomonas stutzeri* (Fig. 7B).

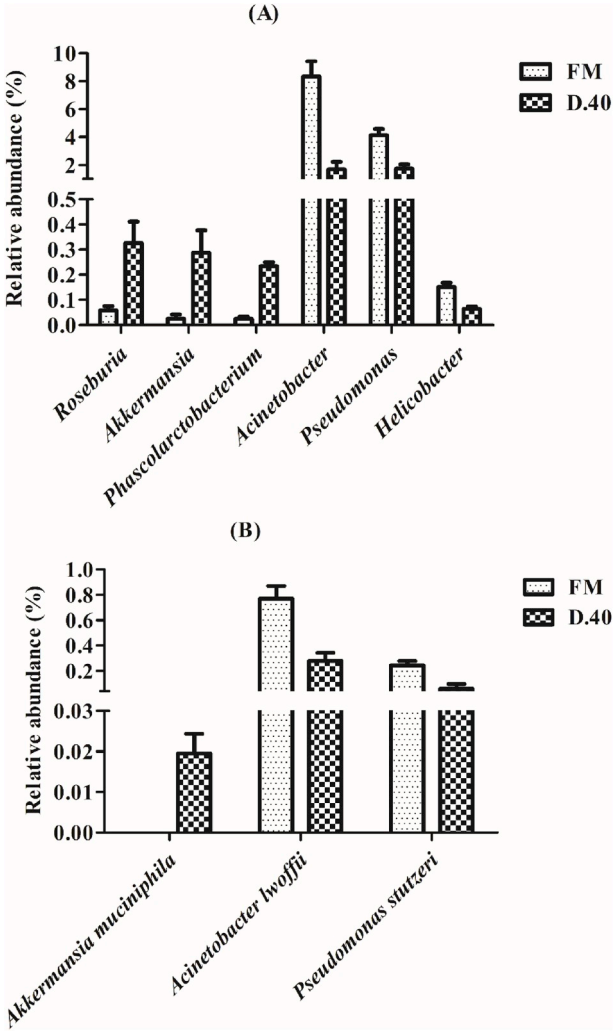

Fig. 6. MetaStat analysis of some bacterial communities with significant difference ($P < 0.05$) between FM and D.40 groups. (A): Genera; (B): Species.

4. Discussion

In the present study, dietary daidzein elevated the feed efficiency and reduced the feed intake of fish. However, fish fed daidzein at 400 mg kg^{-1} showed significantly lower specific growth rate, weight gain rate and final body weight than that of fish fed daidzein at 40 mg kg^{-1}. This result indicated that the dose of daidzein in aqua-feed must be carefully considered. Consistent with previous studies [2–5,8], dietary daidzein showed anti-inflammatory effect on the intestine of fish in the present study via down-regulating the gene expression of pro-inflammatory cytokines *TNF-α* and *IL-1β*, and up-regulating the gene expression of anti-inflammatory cytokine *TGF-β1*. *TNF-α* and *IL-1β* strengthen the inflammatory response via boosting the recruitment and activation of other inflammatory elements, resulting in increased production and release of inflammatory mediators [39]. *TGF-β1* plays an important role in the control of immune homeostasis and prevention of mucosal inflammation [40]. Daidzein could inhibit the nuclear translocation of nuclear factor-κB (NF-κB) which is involved in the activation of several inflammatory genes to exert its anti-inflammatory effects on LPS-treated murine J774 macrophages [3], but the exactly mechanisms need further investigations. The anti-oxidative effect of daidzein has also been well reported by previous studies [4,8,10]. The improved antioxidative status in the intestine of fish, in the present study, was showed by decreased content of TBARS in D.40 and increased activity of T-AOC in both D.40 and D.400. The content of TBARS indirectly reflected the content of malondialdehyde, a product of lipid peroxidation. The increased content of malondialdehyde can cause cell toxicity, facilitating cell and tissue breakdown [41]. The activity of T-AOC is a comprehensive index of antioxidative capacity and stands for the integral level of enzymatic and non-enzymatic antioxidants. Our results were consistent with our earlier study [9] that dietary daidzein could improve the activities of antioxidant enzymes superoxide dismutase (SOD) and catalase in gibel carp. Furthermore, some intestinal bacterial metabolites of daidzein possess strong anti-oxidative property [42,43], which may also contribute to the anti-oxidative effect of daidzein.

The mucus layer, which covers the intestinal epithelium and the tight junctions, enhances the mechanical epithelial barrier, consequently playing a vital role in maintaining the barrier integrity and function [44,45]. In the present study, the daidzein-supplemented groups had higher gene expression of *Mucin-2*, which is a component of the mucus layer and is indispensable in protecting against pathogen infections and inflammation [46]. The tight junction, which regulates paracellular permeability, is composed of the transmembrane proteins (occludin, claudins, junctional adhesion molecule (JAM) and tricellulin), as well as the intracellular proteins (for example, zonula occludens (ZO) and cinglin) [16]. Additionally, defects in the tight junction barrier can disrupt the gut epithelial barrier function, which is related to the pathogenesis of many intestinal diseases [15]. In the current study, dietary daidzein increased intestinal mRNA level of tight junction proteins, thereby promoting stronger epithelial barrier integrity. Noda et al. [16] reported that daidzein enhanced epithelial integrity in human intestinal Caco-2 cells by increasing the cytoskeletal expression of tight junction proteins and their assembly. Besides, pro-inflammatory cytokines such as *IFN-γ* and *TNF-α*, as well as ROS can compromise intestinal tight junction function [14,47,48]. These mediators affect not only the expression but also the cytoskeletal association of tight junction proteins via activating/inhibiting intracellular signaling [14]. Therefore, apart from the direct effect of daidzein on tight junction proteins, its inhibitory effect on the expression of pro-inflammatory cytokines and the production of ROS can also account for the improvement of the intestinal mucosal barrier function.

The current study determined the overall intestinal bacterial community of juvenile turbot in response to dietary daidzein. Consistent with previous studies on turbot intestinal microbiota [46,49–51], Proteobacteria and Firmicutes were the dominant phyla in intestinal mucosa in this study. Besides, dietary daidzein decreased the relative abundance of Proteobacteria. Shin et al. [52] pointed out that Proteobacteria is sensitive to diet, and the prevalence of Proteobacteria in the intestine not only can symbolize an imbalanced and unstable microbial community structure or a state of disease in the host, but also can further facilitate inflammation or invasion by exogenic pathogens. At genus level, *Halomonas* accounted for high proportion in all groups, which was in line with prior studies which reported that *Halomonas* was prevailing in carnivorous fish [53,54]. Compared with FM group, daidzein-supplemented groups had higher Shannon index, indicating the increased intestinal microbial diversities in these groups. Ordinarily, the more diversity of intestinal microbiome is beneficial for community stability and function, revealing better adaptive capacities when facing detrimental situations, such as stress or pathogenic invasion [55,56]. The result of NMDS analysis revealed that intestinal bacterial communities of fish from different groups formed different clusters, indicating that dietary daidzein had a strong effect on the overall structure of intestinal microbiota in turbot and the effect was associated with the dose of daidzein.

Compared with FM group, the relative abundance of some short chain fatty acids (SCFAs) producers (reviewed by Koh et al. [57] and Fernández et al. [58]) were significantly increased by dietary daidzein, i.e., *Roseburia*, *Phascolarctobacterium* and *Akkermansia muciniphila* in D.40 group, meanwhile *Bacteroides*, *Dialister*, *Phascolarctobacterium* and *Megasphaera elsdenii* in D.400 group. Besides the anti-inflammatory

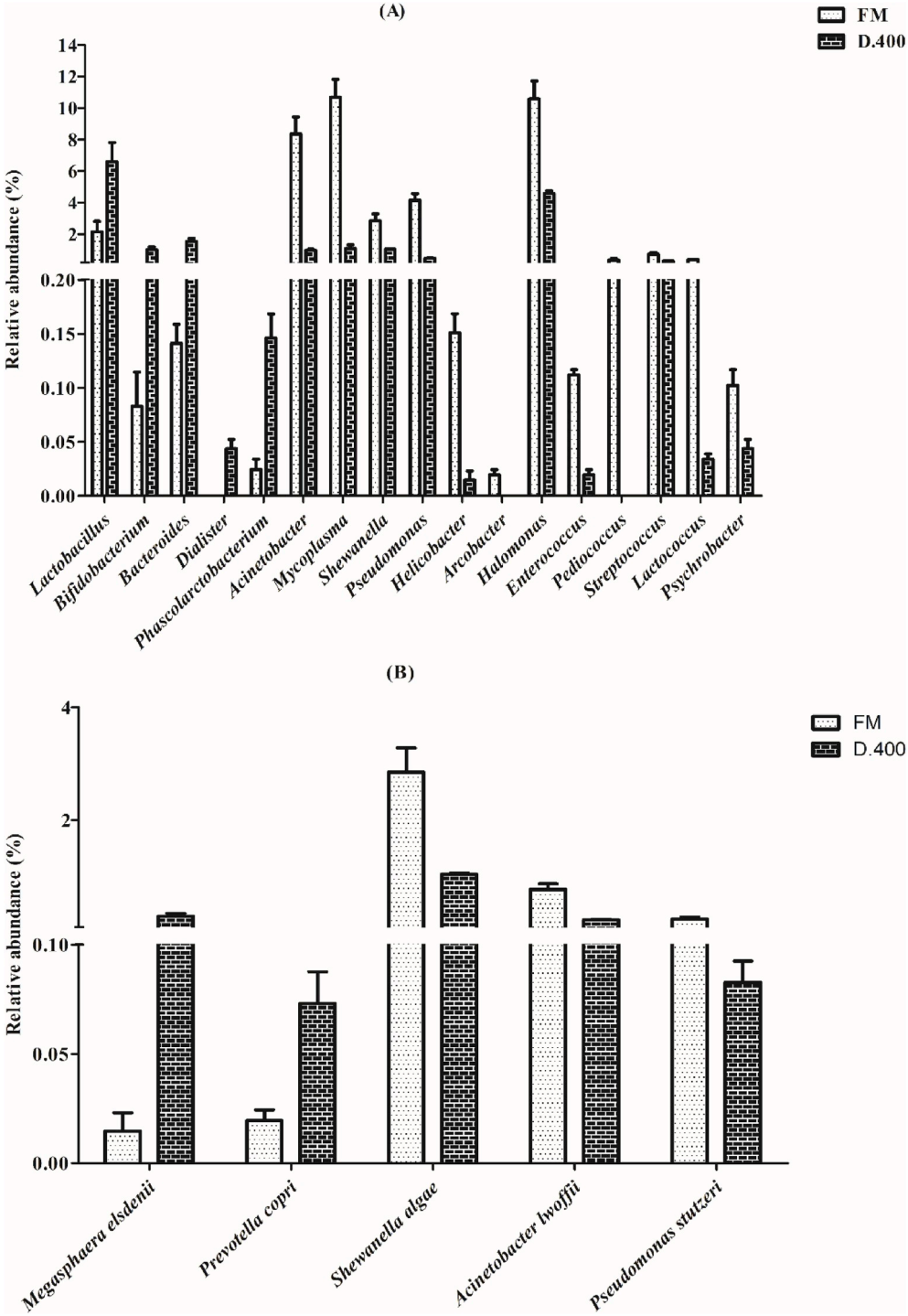

Fig. 7. MetaStat analysis of some bacterial communities with significant difference ($P < 0.05$) between FM and D.400 groups. (A): Genera; (B): Species.

effects [58], SCFAs can improve intestinal barrier integrity and tight junction assembly, as well as increase *Mucin-2* expression and mucin secretion [59]. Moreover, lactic acid bacteria (LAB) are well recognized beneficial organisms with diverse probiotic potentials in fish [60–64]. In the current study, the relative abundance of LAB belonging to genera *Lactobacillus* and *Bifidobacterium* showed a markedly increase in D.400 group. In terms of potential pathogenic bacteria, D.40 and D.400 all significantly down-regulated the relative abundance of genera *Acinetobacter*, *Pseudomonas* and *Helicobacter*. Besides, D.400 significantly down-regulated the relative abundance of genera *Mycoplasma* and *Arcobacter*, as well as species *Shewanella algae*, *Acinetobacter lwoffii* and *Pseudomonas stutzeri*. *Acinetobacter* was associated with bacteraemia, pulmonary infections, meningitis, diarrhea and notorious nosocomial infections [65]. Huddam et al. [66] reported that *Acinetobacter lwoffii* was the pathogen causing meningitis and peritonitis. Some species of *Pseudomonas* may also exert harmful effects on fish health: *Pseudomonas plecoglossicida* and *Pseudomonas anguilliseptica* infections in *Plecoglossus altivelis* and *Gadus morhua* L. have been reported, respectively [67,68]. *Pseudomonas stutzeri* could cause bacteremia, generalized sepsis, and local infections such as meningitis, endocarditis, conjunctivitis [69]. *Helicobacter pylori* infection led to the gastric mucosa inflammation and damage to the gastric epithelium [70]. Other species of *Helicobacter*

were associated with hepatobiliary disease, Crohn's disease, sepsis, and gastric disease [71]. Some species of *Mycoplasma* are well-recognized as etiological agents of disease in human and animals [72]. The members of genus *Arcobacter* have been regarded as emergent entero-pathogens and potential zoonotic agents [73]. *Shewanella algae* could cause gut inflammation in tongue sole [74]. However, D.400 also significantly decreased the abundance of several beneficial bacteria (*Halomonas*, *Psychrobacter,* and LAB belonging to genera *Streptococcus*, *Lactococcus*, *Enterococcus* and *Pediococcus*), and significantly increased the abundance of potential pathogenic bacterium *Prevotella copri. Halomonas* was protease-producing bacteria prevailed in the intestine of carnivorous fish [51,53]. In Atlantic cod, *Psychrobacter* isolates also displayed enzymatic activities, for example, protease, chitinase and phytase [75]. *Prevotella copri* possess a pro-inflammatory potential [76]. Accordingly, the exact effects of daidzein at the high level (400 mg kg^{-1}) on turbot intestinal microbiota need further investigations.

5. Conclusion

In conclusion, dietary daidzein at 40 mg kg^{-1} could improve the intestinal health through mitigating intestinal inflammation, enhancing intestinal anti-oxidative capacity, improving the mucous layer and the tight junction barrier, and positively modulating intestinal bacterial community of turbot. However, fish fed 400 mg kg^{-1} daidzein had lower growth performance than fish fed 40 mg kg^{-1} daidzein. This potential adverse effect of high dose of daidzein (400 mg kg^{-1}) might be partly attributed to its dual effects on intestinal microbiota. Therefore, high dose of daidzein (400 mg kg^{-1}) must be treated with caution.

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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.2019.08.059.

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