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Dietary stachyose altered the intestinal microbiota profile and improved the intestinal mucosal barrier function of juvenile turbot, *Scophthalmus maximus* L.

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ABSTRACT

Recent studies have revealed the beneficial effects of stachyose on intestinal histology and digestive function of fish. However, a comprehensive understanding of stachyose's impact on intestinal health of fish remains unclear, limiting its use in aqua-feed. In the present study, a 12-week feeding trial was conducted to investigate the effects of dietary stachyose on intestinal microbiota and mucosal barrier function of turbot (*S. maximus* L). Three isonitrogenous and isolipidic experimental diets were formulated to contain 0%, 1.25% and 5% stachyose, respectively. Sequencing of bacterial 16s rRNA V_4 region indicated that dietary stachyose altered the intestinal adherent microbiota profile, which was supported by the diet-cluster of PCA, PCoA, beta diversity heatmap and phylogenetic tree. LEfSe and MetaStat analysis indicated that both 1.25% and 5% dietary stachyose significantly elevated the abundance of intestinal cellulose-degrading bacteria. However, the higher level of stachyose (5%) increased the abundance of intestinal beneficial bacteria as well as that of potential pathogenic bacteria. Moreover, 1.25% dietary stachyose significantly up-regulated the genes expression of occludin, claudin-3, and ZO-1, and down-regulated the gene expression of claudin-like in the intestine ($P < 0.05$). Dietary stachyose at 5% significantly increased mucin-2 secretion and the gene expression of ZO-1, while significantly decreased the gene expression of claudin-like in the intestine ($P < 0.05$). Collectively, our study showed that dietary stachyose supplementation could favorably modulate the profile of intestinal microbiota and enhance the intestinal mucosal barrier function in juvenile turbot. Stachyose showed promising potential of being used as prebiotic in diet for enhancing the intestinal health of turbot.

1. Introduction

As an oligosaccharide, stachyose has been used as prebiotics in human and mammal food industry, and it has been reported to be able to promote the growth of specific gut bacteria species and improve the intestinal health (Mussatto and Mancilha, 2007; Li et al., 2013; Li, T., et al., 2017; Pacifici et al., 2017). Studies concerning the effects of stachyose on fish performances are limited. In some cases, contradictory results even existed (Cai, 2006; Cai et al., 2012; Hu et al., 2015). Some recent studies in fish have suggested the potential prebiotic properties of stachyose. Moderate levels of stachyose in diets improved the intestinal development and digestive capability (Mi et al., 2011; Sørensen et al., 2011; Hu et al., 2015). Nevertheless, these studies mainly focused on the gut histological structures and digestive function, whereas more expanded effects of stachyose on intestinal health-related properties is less investigated in fish.

Both the homeostasis of intestinal bacteria and the integrity of intestinal mucosal barrier function are important components of gut health (Maloy and Powrie, 2011). In the healthy state, the intestinal bacteria can exert a series of beneficial effects on the intestinal health of the host, such as providing nutrients and energy to the host via the fermentation of non-digestible dietary components, influencing the development of intestinal mucosal and protecting the gut from invasive pathogens (Sekirov et al., 2010; Flint et al., 2012; de Medina et al., 2014; Liu et al., 2016). Meanwhile, the intact intestinal mucosa provides an essential barrier against harmful substances and pathogens

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from the external environment. Its function is built mainly upon the epithelial layer which constitutes a physical barrier (de Medina et al., 2014; König et al., 2016), and mucus secreted from epithelial goblet cells provides protection for the epithelium layer(Maloy and Powrie, 2011; Sahlmann et al., 2013). Tight junctions (TJs) proteins between intestinal epithelial cells which include members of the Claudin family, Occludin family and Zonula occludens 1 (ZO-1) are also indispensable in the maintenance of barrier integrity and function (Turner, 2009; Jutfelt, 2011). Among the various factors that regulate intestinal bacterial community and barrier function, dietary ingredients were found to play an vital role in influencing gut microbial composition and modifying expression and localization of TJ proteins (Clemente et al., 2003; Drago et al., 2006; Ulluwishewa et al., 2011; Luo et al., 2014; Ringø et al., 2016; Schmidt et al., 2016; Huyben et al., 2017; Li, Y., et al., 2017; Zhou et al., 2017).However, no information has been reported about the effects of dietary stachyose on these two aspects of intestinal health in fish.

To further investigate the expanded effects of dietary stachyose on fish intestinal health, the present study evaluated the effects of a low (1.25%) or high (5%) dose of stachyose on the intestinal microbiota profile and mucosal barrier integrity in turbot, which are both an important fish species for aquaculture industry and a good model marine fish for academic research. The results will gain novel insight into the nutritive property of stachyose.

2. Materials and methods

2.1. Ethics statement

Procedures for animal care and handling in the present study were approved by the Institutional Animal Care and Use Committee of Ocean University of China.

2.2. Experimental diets design

The present experiment is an extension of our precious study (Hu et al., 2015), which was designed to evaluate the effects of dietary stachyose, an oligosaccharide often regarded as an anti-nutritional factor (ANF), on the growth performance and intestinal health of turbot. Among all oligosaccharides, stachyose is one of the most predominant ANFs in plant ingredients, and the content of stachyose in soybean meal is around 5%. In our previous experimental design, five isonitrogenous and isolipidic diets were formulated to contain 0, 0.625%, 1.25%, 2.5% and 5% stachyose respectively, which is equal to the content of stachyose in fish diets when 12.5%, 25%, 50% and 100% soybean meal was used in the diet of turbot. Based on the results our precious study, three diets were chosen in the present experiment to contain 0% (FM) (As the Control group), 1.25% (S-1.25) and 5% (S-5) stachyose, respectively (Table 1).

The feed was made, packed and stored following the standard procedures of our lab. Briefly, dietary ingredients were ground into fine powder through 320 μm mesh. All ingredients were thoroughly mixed with fish oil, then water was added to produce stiff dough. The dough was then pelleted with an experimental single-screw feed mill. After being pelleted, the feeds were dried in a ventilated oven at 45 °C for about 12 h and then stored in a freezer at − 20 °C (Xu et al., 2010).

2.3. Feeding trial

Juvenile turbot (*Scophthalmus maximus* L.) were obtained from a commercial farm in Laizhou, China. Prior to the start of the experiment, fish were acclimated to a commercial diet (Great Seven Bio-Tech Co. Ltd., Qingdao, China) for two weeks. Then the fish were fasted for 24 h and weighed. A total of 270 fish (initial weight 4.63 ± 0.01 g) were randomly distributed to 9 cylindrical fiberglass tanks (200L) in an indoor rearing system with flowing sea water. Each experimental diet was randomly assigned to three tanks. The feeding trial lasted for 12 weeks. During the feeding period, fish were slowly hand-fed to apparent satiation twice daily (7:30 and 19:30). The rearing conditions followed those in our previous studies (Hu et al., 2015).

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

Ingredients (%)	FM	S-1.25	S-5
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[a]	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	0.05	0.05	0.05
Stachyose[d]	0	1.37	5.47
Microcrystalline cellulose	10.65	9.28	5.18
Analyzed nutrients compositions (dry matter basis)			
Crude protein	48.45	48.33	47.99
Crude lipid	9.40	9.02	9.14
Ash	8.41	8.23	8.64

Abbreviations: FM, fish meal diet; S-1.25: 1.25% stachyose diet; S-5: 5% stachyose diet.

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

[b] Vitamin premix (mg/kg 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 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.

[d] 91.45% stachyose, Xi'an Rongsheng Bio Technology Co., Ltd.

2.4. Sample collection

At the end of the feeding trial, six hours after the last feeding, fish were anesthetized with eugenol (1:10,000) (purity 99%, Shanghai Reagent Corp, Shanghai, China), and then counted and weighed. For the analysis of intestinal microbiota, the surface of one randomly selected fish per tank (three fish per treatment) was sterilized by tampon with 70% alcohol, then the abdominal cavity of fish was opened; the whole intestine was removed, opened, and cleared of intestinal content carefully, by using sterile scissors and bistoury, around alcohol flame. The whole intestinal mucosa layer of these three fish per treatment were carefully scraped from foregut region to hindgut region using sterile rubber spatula, then transferred to 2 mL sterile tubes (Axygen, America). To analyze intestinal gene expression the whole intestinal mucosa layer from other six randomly selected fish per tank were collected following the procedure described above, then transferred to 1.5 mL RNase-free tubes (Axygen, America). All the above mentioned samples were immediately stored at liquid nitrogen before further processing.

2.5. Intestinal microbiota DNA extraction and sequencing

Genomic DNA sample was extracted from the whole intestinal mucosa layer of one fish per tank using a QiAamp DNA stool Mini Kit (Qiagen, Germany) on super clean bench around alcohol flame with some modifications as we previous described. Briefly, the intestinal mucosa layer samples were transferred from − 80 °C to ice for a short while, then carefully transferred to a 5 mL sterile tube containing sufficient InhibitEX buffer (proportional to the tissue weight). The tube was subjected to vortex at maximum speed for 1 min, and 1 mL of the homogenate was used for the downstream DNA extraction according to

the manufacturer's instructions (Li, Y., et al., 2017). The quantity and purity of the DNA were assessed using a Nano-Drop®ND-1000 spectrophotometer (Nano-Drop Technologies, Wilmington, DE, USA). The integrity of the extracted genomic DNA was determined by electrophoresis on a 1.2% (w/v) agarose gel. Each sample had only one single and bright strip around 20,000 bp on the gel and the 260/280 nm absorbance ratios of all samples were 1.8–2.0, and ratios were similar and stable, indicating a satisfactory purity of the DNA samples. According to the concentration, DNA was diluted to 1 ng/μL using sterile water.

To determine the diversity and composition of bacterial communities, PCR amplifications were conducted with the 515f/806r primer set that amplifies the V4 region of the 16S rRNA gene of gut microbiota. The reactions were carried out in 30 μL sample volume using 15 μL of Phusion® High-Fidelity PCR Master Mix (New England Biolabs), 0.2 μM of forward and reverse primers, 10 ng template DNA, and nuclease-free water. The PCR was ran as follows: 98 °C for 1 min, followed by 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, after which a final extension at 72 °C for 5 min was applied. The quantity and purity of the PCR product were assessed by Beijing Novogene Genomics Technology Co. Ltd. (China). Sequencing was conducted on an Illumina MiSeq platform according to the manufacturer's recommendations, provided by Beijing Novogene Genomics Technology Co. Ltd. (China). Complete data were submitted to the NCBI Short Read Archive under accession no.SRR3497398, SRR3497400-SRR3497403, SRR3497405-SRR3497408.

2.6. RNA extraction and qPCR

The total RNA was extracted (three intestinal samples per tank and three tanks per treatment) using Trizol Reagent (Invitrogen, USA) and electrophoresed on a 1.2% denaturing agarose gel to evaluate the integrity followed by concentration determination with Nano-Drop®ND-1000 (Nano-Drop Technologies, Wilmington, DE, USA). The 260/280 nm absorbance ratios of all samples were close to 2.0, and ratios were similar and stable, indicating a satisfactory purity of the RNA samples. Then, RNA was reversed transcribed to cDNA by PrimeScript® RT reagent Kit with gDNA Eraser (Perfect Real Time, Takara, Japan) following the manufacturer's instructions.

Quantitative real-time PCR primers (Table 2) were used in the qPCR. The qPCR assays were carried out in a quantitative thermal cycler (Mastercycler eprealplex, Eppendorf, Germany) in a final volume of 25 μL containing 12.5 μL of 2 × SYBR Green Real-time PCR Master Mix [SYBR® Premix Ex Taq™ (Tli RNaseH Plus)] (TaKaRa, Japan), 0.5 μL (10 μM) of each forward and reverse primer, 1 μL of 100 ng/μL complementary DNA template and 10.5 μL of dH_2O, according to (Li et al., 2017). PCR conditions 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. A melting curve analysis (1.85 °C increment/min from 58 °C to 95 °C) was performed after each amplification phase to confirm amplification of one product only. The gene expression stability of reference gene *β-ACTIN* within FM, S-1.25 and S-5 groups was assessed and *β-ACTIN* was used as internal controls. Each sample was run in triplicate. The expression levels of these genes were calculated by $2^{-\Delta\Delta CT}$ method, and the value stood for n-fold difference relative to the calibrator (Livak and Schmittgen, 2001).

2.7. Data statistical analysis

Data of gene expression was tested for normality and variance homogeneity using the Shapiro − Wilk *W* goodness of fit test and the Bartlett test, respectively. These data was subjected to one-way analysis of variance (ANOVA) using SPSS 16.0 for windows. When overall differences are significant ($P < 0.05$), Tukey's test is used to compare the means among individual treatments. Results are presented as means with S.E. (standard error).

2.8. Microbiota sequence data analysis

Sequencing reads was assigned to each sample according to the unique barcode of each sample. FLASH software (v. 1.2.7) was used to merge Pairs of reads from the original DNA fragments into raw tags (Magoč and Salzberg, 2011). Sequences were then analyzed with the QIIME software package (v1.7.0); quality filtering of these raw tags were then performed under specific filtering conditions to obtain the high-quality clean tags (Bokulich et al., 2013) according to the QIIME (v1.7.0) quality controlled process (Caporaso et al., 2010). UCHIME algorithm was used to detect and remove chimera sequences, thus obtaining the effective tags (Edgar et al., 2011).The tags were then clustered to OTU (operational taxonomic unit) using Uparse software (v7.0.1001) based on 97% sequence similarity (Edgar, 2013). Representative sequence for each OTU was screened for further annotation using the GreenGene Database (DeSantis et al., 2006). Alpha diversity (Chao1 index, observed species number, Shannon index, Good's coverage, Simpson and ACE) and beta diversity (PCA and PCoA) analysis were calculated with QIIME (v1.7.0) and displayed with R software (v2.15.3). Tukey's test and wilcox's test were used to test statistical difference of α diversity and β diversity between treatments. To assess the changes in microbial community structure brought by dietary stachyose, differentially abundant taxa between the control group and the treatment groups were identified by the LEfSe analysis (linear discriminant analysis effect size) (Segata et al., 2011) and Metastats analysis (White et al., 2009).

3. Results

3.1. Intestinal microbiota

After assembled, quality screened and trimmed, a total of 184,678 high quality valid reads was obtained, ranging from 12,454 to 30,165, resulting in identification of 2511 OTUs with 97% identity from 9 samples in FM, S-1.25 and S-5 groups. The OTUs were assigned to 355 genera, 267 families, 195 orders, 113 class, and 41 phyla. 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 366 OTUs were shared by groups FM, S-1.25 and S-5, and the number of unique OTUs in group FM, S-1.25 and S-5 was 149, 423 and 735, respectively (Fig. 1). The alpha diversity indexes results indicated that the inclusion of dietary (1.25% and 5%) stachyose led to higher richness of OTU number in turbot intestinal

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

Target genes	Forward primer (5′-3′)	Reverse primer (5′-3′)	AE	SAV	Accession number
Claudin-3	GCCAGATGCAGTGTAAGGTC	CCGTCCAGGAGACAGGGAT	0.91	0.01	KU238180
Claudin-like	ATGTGGAGGGTGTCTGCC	CTGGAGGTCGCCACTGAG	1	− 0.02	KU238181
Occludin	ACTGGCATTCTTCATCGC	GGTACAGATTCTGGCACATC	1.01	0.02	KU238182
ZO1_transcript variant 1	AGAGAACCTGTCACTGATAGATGC	CTGTCGGAATTGTTGCCTGATG	0.95	0.03	KU238184
Muc-2	GTTGGTGCAGCCGCATAG	CACTGGACGCTGGGAATG	0.95	− 0.01	KU238186
β-ACTIN	GCTGTCTTCCCTTCTATCGTCG	TCCATGTCATCCCAGTTGGTC	0.97		AY008305.1

Abbreviations: AE: the primer amplification efficiencies; SAV: the absolute ΔC_T values {Genes C_T – β-actin C_T} of the slopes.

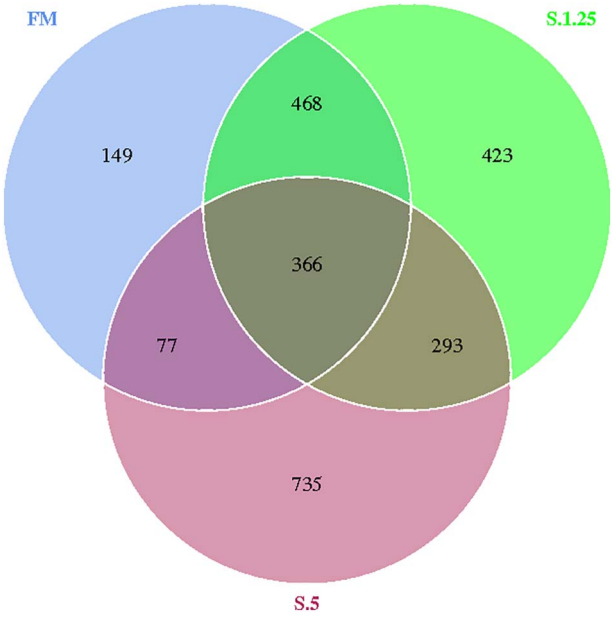

Fig. 1. Venn diagram of unique and shared OTUs.
Every circle in Venn diagram represents one group; the value from the overlapping part of different circles represents the shared OTUs between groups, and the value from the non-overlapping part of one circle represents the unique OTUs of that group.

microbiota ($P < 0.05$). Alpha-diversity including Chao1 index and ACE index were markedly increased in the gut microbiota of fish in S-1.25 group ($P < 0.05$) (Table 3).

At phylum level, Proteobacteria, Firmicutes and Bacteroidetes were detected as predominant bacterial phyla in the intestine of turbot from all groups (Fig.2A). At genus level, *Halomonas*, *Lactobacillus*, *Nitrosopumilus*, *Escherichia*, *Acinetobacter*, *Shewanella*, *Cetobacterium*, *Bacteroides*, *Edwardsiella* and *Loktanella* composed the top ten dominant genera of turbot intestinal microbiota communities (Fig. 2B).

The PCA, PCoA, beta diversity heatmap and phylogenetic tree based on Weighted UniFrac distances and Unweighted UniFrac distances were used to compare the similarity in the microbial community composition of 9 specimens (Supplementary Fig. S2). The PCA results showed that samples clustered together according to the diets with a clear separation between them, indicating dietary stachyose had a strong effect on the overall structure of intestinal microbiota in turbot (Fig. 3).

To compare the differences in gut microbial community composition between fish fed diets FM, S-1.25 and S-5, linear discriminant analysis (LDA) effect size (LEfSe) was performed. The results revealed that there were significant differences in taxonomic distribution of intestinal microbiota communities between FM and stachyose supplemented groups (Supplementary Fig.S3.5A-5D). Dietary stachyose at 1.25% significantly increased the relative abundance of class Clostridiales, genera *Clostridia* and *Halomonas*, but the abundance of orders Rhodobacterales and Vibronales, families Rhodobacteraceae and Vibronaceae, genus *Loktanella* decreased significantly ($P < 0.05$). Dietary stachyose at 5% significantly increased the relative abundance of phyla Actinobacteria, Firmicute and Proteobacteria, classes Clostridia and Gammaproteobacteria, orders Oceanospirillales, Enterobacteriales, Pseudomonadales, Alteromonadales and Actinomycetales, families Halomonadaceae, Enterobacteriaceae, Ruminococcaceae, Moraxellaceae, Psedomonadaceae and Shewanllaceae, genera *Acinetobacter*, *Halomonas*, *Shewanella*, *Edwardsiella*, *Clostridia* and *S. algae* while decreased the relative abundance of phyla Crenarchaeota, Fusobacteria, classes Thaumarchaeota, Fusobacteriia, orders Fusobacteriales, Rhodobacterales, Vibrionales, families Fusobacteriaceae, Rhodobacteraceae, Vibrionaceae, genera *Cetobacterium*, *Loktanella* and *C. somerae* ($P < 0.05$).

Additional MetaStat analysis on genus and species level between different groups showed significant differences in bacterial communities. The inclusion of 1.25% stachyose in diet significantly ($P < 0.05$) increased the relative abundance of *Clostridium*, *Blautia* and *Lachnospira* (Fig.4A, Supplementary Table 1). The addition of 5% dietary stachyose significantly ($P < 0.05$) increased the relative abundance of core genus from the Proteobacteria (*Halomonas*, *Acinetobacter* and *Shewanella*), meanwhile, the significantly higher abundance of gennus *Clostridium*, *Streptococcus*, *Ruminococcus*, *Pediococcus*, *Enterococcus*, *Lactococcus*, *Bacillus* and *Carnobacterium* were also observed (Fig.4B,C). In particular, significant increases in abundance of potential pathogenic *Mycobacterium, Shewanella algae, Prevotella copri, Haemophilus parainfluenzae, Lactococcus garvieae, Acinetobacter lwoffii, Escherichia coli and Clostridium perfringens* were also observed in group S-5 (Fig.4D, Supplementary Table 2).

3.2. Expression of intestinal Muc-2 gene and tight junction related genes

The inclusion of 5% dietary stachyose significantly up-regulated the gene expression of Muc-2 in the intestine compared with group FM (Table 4). The gene expressions of tight junction proteins in the intestine were affected by dietary stachyose. Diet S-1.25 significantly up-regulated the gene expression of Occludin, Claudin-3, ZO1_transcript variant 1, whereas down-regulated Claudin-like's gene expression in turbot intestine (Table 4). The gene expression of ZO1_tv1 in the intestine was significantly up-regulated while the gene expression of Claudin-like was significantly down-regulated by 5% dietary stachyose when compared with group FM (Table 4).

4. Discussion

The present study represents the first analysis of alterations in the intestinal bacterial profiles of turbot by dietary stachyose. In contrast with previous studies on mammals that only quantified the effect of dietary stachyose on certain intestinal bacterial species (such as *Bifidobacteria* and *Lactobacill*) (Zhou et al., 2014; Li, T., et al., 2017; Pacifici et al., 2017), the present study assessed the overall intestinal bacterial community of juvenile turbot in response to dietary stachyose. Compared with FM treatment, higher OTU numbers were observed in the gut of turbot fed diets with stachyose supplementation. Meanwhile, the bacterial communities formed different clusters. FM and S-1.25 clusters were relatively coherent, while S-5 cluster was clearly separated from the FM and S-1.25 clusters. Furthermore, the abundance-dominating phyla Proteobacteria and Firmicutes also showed a significant increase in group S-5, indicating 5% dietary stachyose may have a more profound effect on the intestinal community of turbot than

Table 3
Alpha diversity index of intestinal microbiota of experimental turbot.

Sample name	Richness estimates			Diversity estimates	
	OTUs	Chao1	ACE	Shannon	Simpson
FM	779 ± 85 ^{a}	1029 ± 58 ^{a}	1054 ± 89 ^{a}	6.13 ± 1.01	0.88 ± 0.10
S-1.25	1514 ± 43 ^{b}	1503 ± 110 ^{b}	1600 ± 71 ^{b}	7.01 ± 0.74	0.91 ± 0.04
S-5	1284 ± 61 ^{b}	1308 ± 42 ^{ab}	1337 ± 15 ^{ab}	7.79 ± 0.21	0.98 ± 0.01

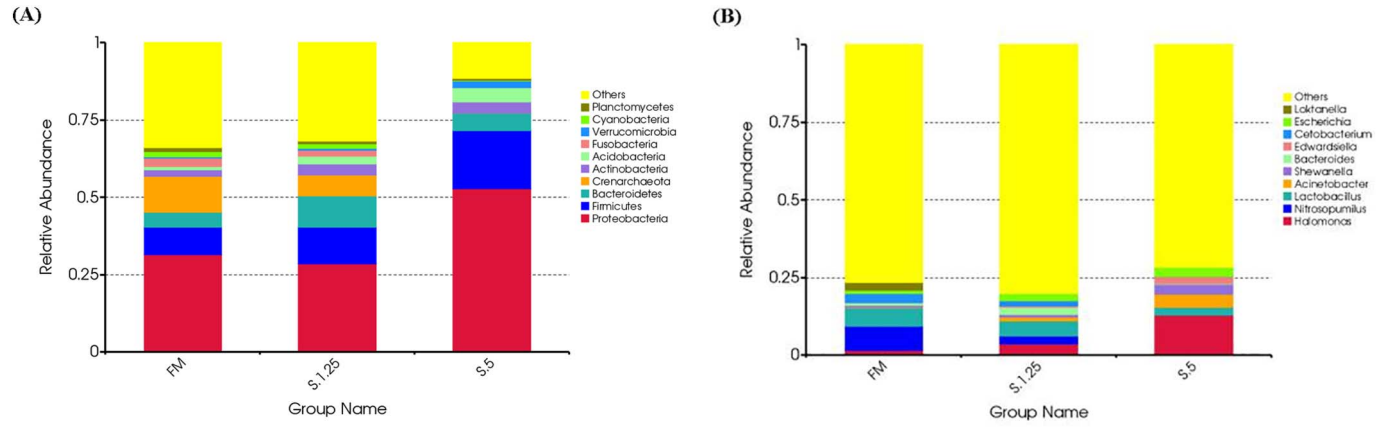

Fig. 2. Taxonomy classification of reads at phylum (A) and genus (B) taxonomic levels.
Only top 10 most abundant (Based on relative abundance) bacterial phyla and genera were shown in the figures. Other phyla and genera were all assigned as 'Others'.

1.25% dietary stachyose.

Overall, the observation that the predominant phyla in the intestinal mucosa belonged to Proteobacteria and Firmicutes was in accordance with previous studies on turbot intestinal microbiome (Xing et al., 2013; Bai et al., 2017; Li, Y., et al., 2017). At genus level, *Halomonas*, *Nitrosopumilu* and *Lactobacillus* were the most abundant genera in all groups, which was also consistent with previous studies which showed that *Halomonas* was dominant in carnivorous fish (Liu et al., 2016; Zhang et al., 2017) and that Lactic acid bacteria (mainly *Lactobacillus*) is the components of gut microflora in both freshwater and marine fish although their abundance are host-dependent (Izvekova et al., 2007; Givens et al., 2015; Gajardo et al., 2016). Despite an overall similarity, the abundance of Proteobacteria and the profile of main genus in different studies with turbot varied. While previous studies reported relative abundance of Proteobacteria of 78.8%–90.6% in turbot intestine (Bai et al., 2017; Li, Y., et al., 2017), the present study showed a relatively low abundance of this phyla, that was 37.54%. Moreover, in the work by Xing et al. (2013), *Vibrio* and *Photobacterium* were found to be the most dominating genera in turbot intestine. A strong distinction in composition and abundance of gut microbiota do occur within a species in various studies (Givens et al., 2015; Sullam et al., 2015; Forberg et al., 2016; Liu et al., 2016), perhaps attributed to the difference in fish source, size, diet, rearing environment and sequencing method.

It is reported that intestinal bacteria can serve the host in two ways: they can represent a nutrient source and/or contribute with enzymes that may improve host digestion (Ray et al., 2012; Clements et al., 2014). Krogdahl et al. (2005) demonstrated that stachyose is non-digestible in fish gut due to the absence of α-galactosidases. Therefore, stachyose digestion depends on the exogenous cellulose. In addition, Saha and Ray (1998) found that the gut cellulase activity was largely contributed by the gut microbiota. As it has been suggested, *Clostridium* and *Bacillus* were important cellulose-degrading bacteria (Silva et al., 2007; Liu et al., 2016); *Blautia*, *Lachnospira* and *Ruminococcus* were recognized as SCFA-producing genera and the most probable candidates for fermentation of carbohydrates (Biddle et al., 2013; De Filippis et al., 2015; Panasevich et al., 2015). In the present study, the abundance of these generas showed a significant increase in stachyose supplemented groups, with *Blautia, Lachnospira* and *Clostridium* increased in group S-1.25, and *Clostridium*, *Ruminococcus* and *Bacillus* increased in group S-5. *Halomonas*, *Acinetobacter* and *Pseudomonas*,

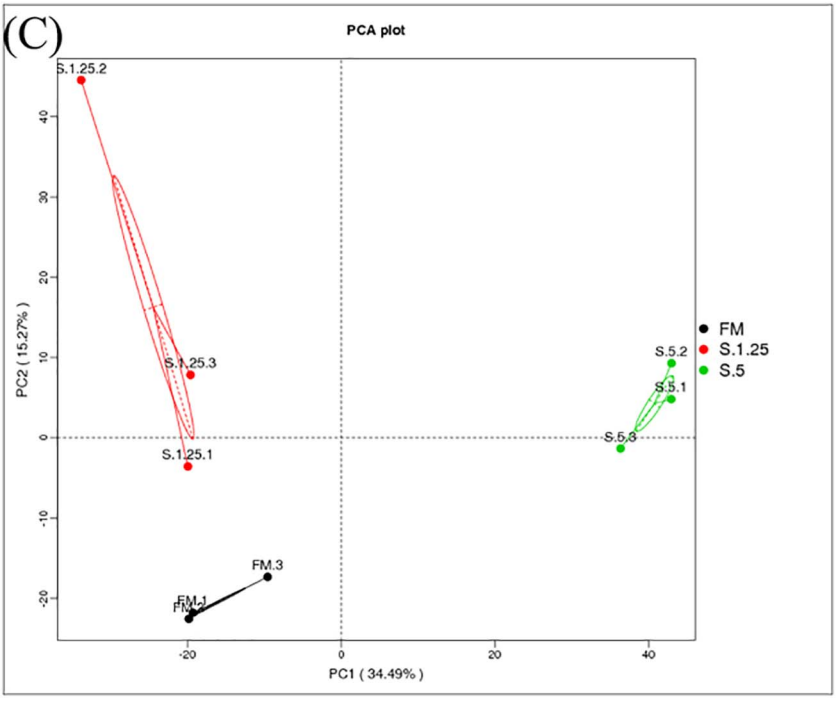

Fig. 3. Beta diversity of intestinal microbiota of juvenile turbot.
Principal Component Analysis (PCA) against PC1 versus PC2 axes based on OTUs.

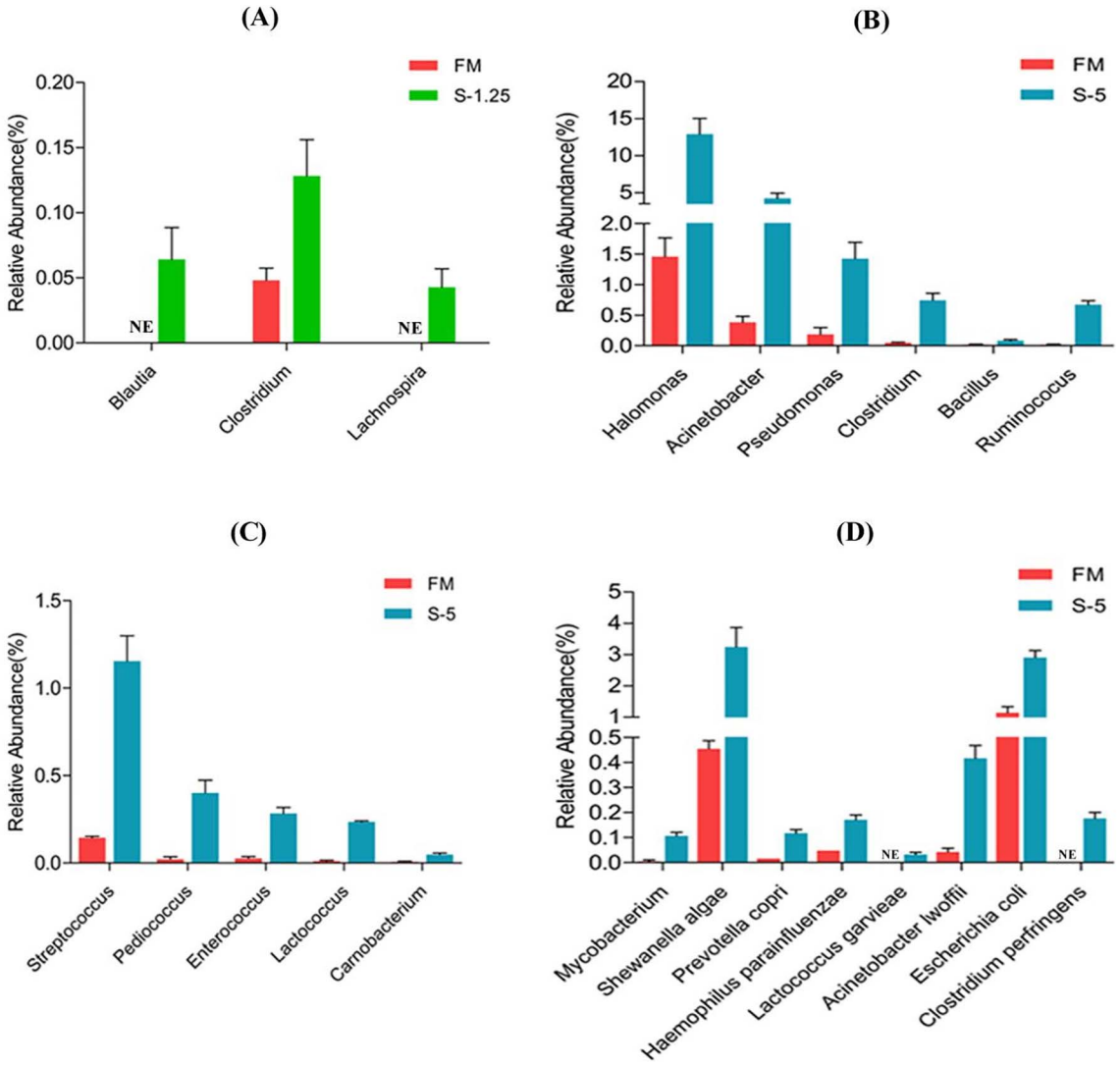

Fig. 4. MetaStat analysis of intestinal microbiota communities of juvenile turbot.
(A): The significantly up-regulated abundance genus in group S-1.25 compared with group FM. (B) and (C): The genus with significantly up-regulated abundance in group S-5. (D): The significant increase in abundance of potential pathogen in group S-5.

Table 4
Effects of 1.25% and 5% dietary stachyose on gene expressions of intestinal Muc-2 and tight junction-related proteins.

Diet	FM	S-1.25	S-5
Muc-2	1.00 ± 0.15 ^{a}	0.94 ± 0.07 ^{a}	1.73 ± 0.08 ^{b}
Occludin	1.00 ± 0.09 ^{a}	1.59 ± 0.14 ^{b}	1.19 ± 0.14 ^{ab}
Tricellulin	1.00 ± 0.18	0.94 ± 0.12	0.99 ± 0.19
Claudin-3	1.00 ± 0.14 ^{a}	1.90 ± 0.12 ^{b}	0.80 ± 0.14 ^{a}
Claudin-like	1.00 ± 0.06^{c}	0.76 ± 0.01 ^{b}	0.48 ± 0.07 ^{a}
ZO-1 transcript variant 1	1.00 ± 0.07 ^{a}	1.29 ± 0.05 ^{b}	1.38 ± 0.06 ^{b}

which were suggested to be enzyme-producing microbiota in fish intestine, displayed a significant increase in abundance in group S-5. Meanwhile, *Halomonas* was also identified as one of the most predominant cultivated protease-producing bacteria (Tsuchiya et al., 2008; Ray et al., 2012). Our previous studies found that 1.25% and 5% dietary stachyose had positive effects on growth performance and significantly increased the intestinal caseinolytic activity in turbot (Hu et al., 2015). Sørensen et al. (2011) indicated in their study that the increased activity of maltase was mediated either directly or indirectly via an increase in SCFA production by intestinal bacteria's degradation of dietary stachyose. Although the functions of enzymes and SCFA produced by gut microbiota are not exactly known, it is possible that the enzyme-producing bacteria and SCFA they produced mentioned above might work in coordination with intestinal digestive enzyme system, thus involving in stachyose and protein digestion in turbot.

Oligosaccharides could serve as live microbial food supplements and beneficially affect the host by selectively stimulating the growth of salutary bacteria in the large intestine of humans (Qiang et al., 2009). Lactic acid bacteria (LAB) are among the generally recognized beneficial species with various health-promoting functions in fish (Ringø and Gatesoupe, 1998; Rastall and Maitin, 2002; Suzer et al., 2008; Dalié et al., 2010; Pan et al., 2017). In the present study, LAB belonging to the genera *Enterococcus*, *Lactococcus*, *Streptococcus*, *Pediococcus* and *Carnobacterium* displayed a significant increase in group S-5. This result suggested that stachyose could profitably regulate the intestinal microbiota in turbot, which is consistent with previous studies in mammals which showed that dietary stachyose could effectively increase the proportion of beneficial intestinal bacteria Bifidobacterium and Lactobacillus, thereby enhancing the intestinal health of host (Li, T., et al., 2013, 2017; Pacifici et al., 2017). Interestingly, in contrast with the inhibitory effects of stachyose on pathogenic bacteria in these studies, the present study found that 5% dietary stachyose increased the abundance of several recognized potential pathogenic bacteria

(*Mycobacterium*, *Shewanella algae*, *Prevotella copri*, *Haemophilus parainfluenzae*, *Lactococcus garvieae*, *Acinetobacter lwoffii*, *Escherichia coli* and *Clostridium perfringens*) (Chen et al., 2002; Hau and Gralnick, 2007; Jacobs et al., 2009; Denizot et al., 2012; Huddam et al., 2013; Scher et al., 2013; Barreau and Hugot, 2014; Merrifield and Ringo, 2014; Saitoh et al., 2015; Meyburgh et al., 2017). An imbalance between commensal bacteria with pathogenic potential and beneficial potential may interrupt intestinal homeostasis and have a role in intestinal diseases (Maranduba et al., 2015). Our previous studies showed that neither growth performance nor intestinal histology was affected by 5% dietary stachyose in turbot (Hu et al., 2015), indicating the adaptive capacity of the endogenous microbial communities may somehow keep a balanced microorganism environment in the intestinal tract during our feeding period. Further studies are needed to assess whether longer feeding trial with high levels of stachyose has adverse effects on the intestinal health of turbot.

The mucus layer covering the intestinal epithelium and TJs (such as Claudins, Occludin and Zo-1) between adjacent epithelial cells strengthens the mechanical epithelial barrier, thus playing a crucial role for the maintenance of barrier integrity and function (McGuckin et al., 2009; Ulluwishewa et al., 2011). Muc-2, as a composition of mucus layer, is important in the protection against bacterial infections and inflammation. In the present study, an increased gene expression of mucin-2 was observed in group S-5. This result was in accordance with previous studies on other prebiotics such as galacto-oligosaccharides and fructooligosaccharides which also showed to be able to increase the mucin content in the intestinal mucosa (Tsukahara et al., 2003; Leforestier et al., 2009). It was reported that the commensal intestinal microbiota played pivotal roles in the maintenance of mucus layer. Furthermore, studies have demonstrated that genes encoding mucins were directly regulated by probiotics and their products (Mattar et al., 2002; Caballero-Franco et al., 2007; Hamer et al., 2008; van Vliet et al., 2010; Engevik et al., 2016). As stated above, 5% dietary stachyose up-regulated the abundance of some lactic acid bacteria. These observations indicated that the gene expression of Muc-2 might be up-regulated by the increased abundance of probiotics, but further studies are needed to make a conclusive statement.

Several studies have supported the barrier-forming roles of ZO-1, occludin and claudin-3 in fish (Duffy et al., 2011; Chasiotis et al., 2012; Wen et al., 2014). Furthermore, the up-regulation of ZO-1 and occludin expression had been shown to attenuate the loss of intestinal barrier function in mice (Sun et al., 2008). In the present study, increases in gene expression of barrier-forming tight junction proteins in the intestine of turbot were observed in groups S-1.25 and S-5, suggesting that dietary stachyose (1.25% and 5%) benefited the intestinal mucosal barrier function. However, the decreased gene expression of claudin-like was also found in groups S-1.25 and S-5. Claudin-like have been found to be essential in septate junctions for the epithelial barrier function in *Drosophila* and *Caenorhabditis elegans,* but its functions remains unclear in fish (Behr et al., 2003; Anderson and Van Itallie, 2008; Mukendi et al., 2016). Notably, besides the barrier tighten claudin proteins, claudins such as claudin-2, − 10, and − 15 were recognized as pore-forming proteins in previous studies with salmonids (Tipsmark et al., 2010; Sundell and Sundh, 2012; Kosińska and Andlauer, 2013). In addition, our previous studies have shown the improvement of turbot intestinal structure by dietary stachyose (Hu et al., 2015). Therefore, we hypothesized that claudin-like might play a pore-forming function in turbot intestine, and its decreased gene expression found in the present study further supported the tightening effects of stachyose on turbot intestinal TJ barrier.

5. Conclusion

In conclusion, the present results suggested that 1.25% and 5% dietary stachyose could exert promoting effects on intestinal cellulose-degrading bacteria and gene expressions of barrier-forming tight junction proteins, and thus improve the digestion of stachyose and enhance the mucosal barrier function in the intestine of juvenile turbot. Notably, 5% dietary stachyose greatly modified the composition of intestinal microbiota community with an increase in abundance of both beneficial and potential pathogenic bacteria. Therefore, stachyose showed promising potential as a dietary supplement to enhance the intestinal health of turbot, but the promoting effect of 5% dietary stachyose on growth of some potential pathogenic bacteria in the intestine should not be ignored under a long feeding period.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2017.12.014.

References

Anderson, J.M., Van Itallie, C.M., 2008. Tight junctions. Curr. Biol. 18, R941–R943.
Bai, N., Gu, M., Xu, X., Xu, B., Krogdahl, Å., 2017. Protective effects of mannan oligosaccharides on turbot *Scophthalmus maximus* suffering from soy enteropathy. Aquaculture 476, 141–151.
Barreau, F., Hugot, J., 2014. Intestinal barrier dysfunction triggered by invasive bacteria. Curr. Opin. Microbiol. 17, 91–98.
Behr, M., Riedel, D., Schuh, R., 2003. The claudin-like megatrachea is essential in septate junctions for the epithelial barrier function in Drosophila. Dev. Cell 5, 611–620.
Biddle, A., Stewart, L., Blanchard, J., Leschine, S., 2013. Untangling the genetic basis of fibrolytic specialization by Lachnospiraceae and Ruminococcaceae in diverse gut communities. Diversity 5, 627.
Bokulich, N.A., Subramanian, S., Faith, J.J., Gevers, D., Gordon, J.I., Knight, R., Mills, D.A., Caporaso, J.G., 2013. Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. Nat. Methods 10, 57–59.
Caballero-Franco, C., Keller, K., De Simone, C., Chadee, K., 2007. The VSL# 3 probiotic formula induces mucin gene expression and secretion in colonic epithelial cells. Am. J. Physiol. Gastrointest. Liver Physiol. 292, G315–G322.
Cai, Y.H., 2006. Effects of Antinutritional Factors in Soybean on the Growth Performance and Digestive Physiology of Japanese Flounder, *Paralichthys olivaceus*. Ocean University of China, Qingdao.
Cai, C.F., Wang, W.J., Ye, Y.T., Krogdahl, A., Wang, Y.L., Xia, Y.M., Yang, C.G., 2012. Effect of soybean meal, raffinose and stachyose on the growth, body composition, intestinal morphology and intestinal microflora of juvenile allogynogenetic silver crucian carp (*Carassius auratus gibelio*♀ × *Cyprinus carpio*♂). Aquac. Res. 43, 128–138.
Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Peña, A.G., Goodrich, J.K., Gordon, J.I., 2010. QIIME allows analysis of high-throughput community sequencing data. Nat. Methods 7, 335–336.
Chasiotis, H., Kolosov, D., Kelly, S.P., 2012. Permeability properties of the teleost gill epithelium under ion-poor conditions. Am. J. Phys. Regul. Integr. Comp. Phys. 302, R727–R739.
Chen, C., Hu, C., Chen, X., Zhang, L., 2002. Identification and characterization of *Shewanella algae* as a novel pathogen of ulcer disease of fish *Scinenops ocellata*. Oceanologia et Limnol. Sin. 34, 1–8.
Clemente, M.G., De Virgiliis, S., Kang, J.S., Macatagney, R., Musu, M.P., Di Pierro, M.R., Drago, S., Congia, M., Fasano, A., 2003. Early effects of gliadin on enterocyte intracellular signalling involved in intestinal barrier function. Gut 52, 218.
Clements, K.D., Angert, E.R., Montgomery, W.L., Choat, J.H., 2014. Intestinal microbiota in fishes: what's known and what's not. Mol. Ecol. 23, 1891–1898.
Dalié, D., Deschamps, A., Richard-Forget, F., 2010. Lactic acid bacteria–potential for control of mould growth and mycotoxins: a review. Food Control 21, 370–380.
De Filippis, F., Pellegrini, N., Vannini, L., Jeffery, I.B., La Storia, A., Laghi, L., Serrazanetti, D.I., Di Cagno, R., Ferrocino, I., Lazzi, C., 2015. High-level Adherence to a Mediterranean Diet Beneficially Impacts the Gut Microbiota and Associated Metabolome. (Gut, gutjnl-2015-309957).
de Medina, F.S., Romero-Calvo, I., Mascaraque, C., Martínez-Augustin, O., 2014. Intestinal inflammation and mucosal barrier function. Inflamm. Bowel Dis. 20, 2394–2404.
Denizot, J., Sivignon, A., Barreau, F., Darcha, C., Chan, H., Stanners, C.P., Hofman, P., Darfeuille-Michaud, A., Barnich, N., 2012. Adherent-invasive *Escherichia coli* induce

claudin-2 expression and barrier defect in CEABAC10 mice and Crohn's disease patients. Inflamm. Bowel Dis. 18, 294–304.
DeSantis, T.Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E.L., Keller, K., Huber, T., Dalevi, D., Hu, P., Andersen, G.L., 2006. Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. Appl. Environ. Microbiol. 72, 5069–5072.
Drago, S., El Asmar, R., Di Pierro, M., Grazia Clemente, M., Sapone, A.T.A., Thakar, M., Iacono, G., Carroccio, A., D'Agate, C., Not, T., Zampini, L., Catassi, C., Fasano, A., 2006. Gliadin, zonulin and gut permeability: effects on celiac and non-celiac intestinal mucosa and intestinal cell lines. Scand. J. Gastroenterol. 41, 408–419.
Duffy, N.M., Bui, P., Bagherie-Lachidan, M., Kelly, S.P., 2011. Epithelial remodeling and claudin mRNA abundance in the gill and kidney of puffer fish (*Tetraodon biocellatus*) acclimated to altered environmental ion levels. J. Comp. Physiol. B. 181, 219–238.
Edgar, R.C., 2013. UPARSE: highly accurate OTU sequences from microbial amplicon reads. Nat. Methods 10, 996–998.
Edgar, R.C., Haas, B.J., Clemente, J.C., Quince, C., Knight, R., 2011. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics 27, 2194–2200.
Engevik, M.A., Luk, B., Hall, A., Ganesh, B.P., Versalovic, J., 2016. *Lactobacillus reuteri* decreases adherent tumor mucins and enhances chemotherapeutic susceptibilities via ERK/JNK pathways. FASEB J. 30, 1107.1109.
Flint, H.J., Scott, K.P., Louis, P., Duncan, S.H., 2012. The role of the gut microbiota in nutrition and health. Nat. Rev. Gastroenterol. Hepatol. 9, 577–589.
Forberg, T., Sjulstad, E.B., Bakke, I., Olsen, Y., Hagiwara, A., Sakakura, Y., Vadstein, O., 2016. Correlation between microbiota and growth in Mangrove Killifish (*Kryptolebias marmoratus*) and Atlantic cod (*Gadus morhua*). Sci. Rep. 6, 21192.
Gajardo, K., Rodiles, A., Kortner, T.M., Krogdahl, Å., Bakke, A.M., Merrifield, D.L., Sørum, H., 2016. A high-resolution map of the gut microbiota in Atlantic salmon (*Salmo salar*): a basis for comparative gut microbial research. Sci. Rep. 6, 30893.
Givens, C.E., Ransom, B., Bano, N., Hollibaugh, J.T., 2015. Comparison of the gut microbiomes of 12 bony fish and 3 shark species. Mar. Ecol. Prog. Ser. 518, 209–223.
Hamer, H.M., Jonkers, D., Venema, K., Vanhoutvin, S., Troost, F., Brummer, R.J., 2008. The role of butyrate on colonic function. Aliment. Pharmacol. Ther. 27, 104–119.
Hau, H.H., Gralnick, J.A., 2007. Ecology and biotechnology of the genus Shewanella. Annu. Rev. Microbiol. 61, 237–258.
Hu, H., Zhang, Y., Mai, K., Ai, Q., Xu, W., Zhang, W., Li, Y., Liu, J., 2015. Effects of dietary stachyose on growth performance, digestive enzyme activities and intestinal morphology of juvenile turbot (*Scophthalmus maximus* L). J. Ocean Univ. China 14, 905–912.
Huddam, B., Koçak, G., Azak, A., Duranay, M., 2013. *Acinetobacter lwoffii* peritonitis in a patient receiving continuous ambulatory peritoneal dialysis. Ther. Apher. Dial. 17, 117–119.
Huyben, D., Nyman, A., Vidaković, A., Passoth, V., Moccia, R., Kiessling, A., Dicksved, J., Lundh, T., 2017. Effects of dietary inclusion of the yeasts *Saccharomyces cerevisiae* and *Wickerhamomyces anomalus* on gut microbiota of rainbow trout. Aquaculture 473, 528–537.
Izvekova, G., Izvekov, E., Plotnikov, A., 2007. Symbiotic microflora in fishes of different ecological groups. Biol. Bull. 34, 610–618.
Jacobs, J., Stine, C., Baya, A., Kent, M., 2009. A review of mycobacteriosis in marine fish. J. Fish Dis. 32, 119–130.
Jutfelt, F., 2011. Barrier function of the gut. In: Encyclopedia of Fish Physiology: From Genome to Environment, pp. 1322–1331.
König, J., Wells, J., Cani, P.D., García-Ródenas, C.L., MacDonald, T., Mercenier, A., Whyte, J., Troost, F., Brummer, R.-J., 2016. Human intestinal barrier function in health and disease. Clin. Translat. Gastroenterol. 7, e196.
Kosińska, A., Andlauer, W., 2013. Modulation of tight junction integrity by food components. Food Res. Int. 54, 951–960.
Krogdahl, Å., Hemre, G.I., Mommsen, T.P., 2005. Carbohydrates in fish nutrition: digestion and absorption in postlarval stages. Aquac. Nutr. 11, 103–122.
Leforestier, G., Blais, A., Blachier, F., Marsset-Baglieri, A., Davila-Gay, A.-M., Perrin, E., Tomé, D., 2009. Effects of galacto-oligosaccharide ingestion on the mucosa-associated mucins and sucrase activity in the small intestine of mice. Eur. J. Nutr. 48, 457–464.
Li, T., Lu, X., Yang, X., 2013. Stachyose-enriched α-Galacto-oligosaccharides regulate gut microbiota and relieve constipation in mice. J. Agric. Food Chem. 61, 11825–11831.
Li, Y., Hu, H., Liu, J., Yang, P., Zhang, Y., Ai, Q., Xu, W., Zhang, W., Mai, K., 2017. Dietary soya allergen β-conglycinin induces intestinal inflammatory reactions, serum-specific antibody response and growth reduction in a carnivorous fish species, turbot *Scophthalmus maximus* L. Aquac. Res. 48, 4022–4037.
Li, T., Lu, X.S., Yang, X.B., 2017. Evaluation of clinical safety and beneficial effects of stachyose-enriched alpha-galacto-oligosaccharides on gut microbiota and bowel function in humans. Food Funct. 8, 262–269.
Li, Y., Yang, P., Zhang, Y., Ai, Q., Xu, W., Zhang, W., Zhang, Y., Hu, H., Liu, J., Mai, K., 2017. Effects of dietary glycinin on the growth performance, digestion, intestinal morphology and bacterial community of juvenile turbot, *Scophthalmus maximus* L. Aquaculture 479, 125–133.
Liu, H., Guo, X., Gooneratne, R., Lai, R., Zeng, C., Zhan, F., Wang, W., 2016. The gut microbiome and degradation enzyme activity of wild freshwater fishes influenced by their trophic levels. Sci. Rep. 6, 24340.
Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-Delta Delta C) method. Methods 25, 402–408.
Luo, J.B., Feng, L., Jiang, W.D., Liu, Y., Wu, P., Jiang, J., Kuang, S.-Y., Tang, L., Zhang, Y.-A., Zhou, X.-Q., 2014. The impaired intestinal mucosal immune system by valine deficiency for young grass carp (*Ctenopharyngodon idella*) is associated with decreasing immune status and regulating tight junction proteins transcript abundance in the intestine. Fish Shellfish Immunol. 40, 197–207.
Magoč, T., Salzberg, S.L., 2011. FLASH: fast length adjustment of short reads to improve genome assemblies. Bioinformatics 27, 2957–2963.
Maloy, K.J., Powrie, F., 2011. Intestinal homeostasis and its breakdown in inflammatory bowel disease. Nature 474, 298–306.
Maranduba, C.M., De Castro, S.B.R., de Souza, G.T., Rossato, C., da Guia, F.C., Valente, M.A.S., Rettore, J.V.P., Maranduba, C.P., de Souza, C.M., do Carmo, A.M.R., 2015. Intestinal microbiota as modulators of the immune system and neuroimmune system: impact on the host health and homeostasis. J Immunol Res 2015, 931574.
Mattar, A., Teitelbaum, D.H., Drongowski, R., Yongyi, F., Harmon, C., Coran, A., 2002. Probiotics up-regulate MUC-2 mucin gene expression in a Caco-2 cell-culture model. Pediatr. Surg. Int. 18, 586–590.
McGuckin, M.A., Eri, R., Simms, L.A., Florin, T.H., Radford-Smith, G., 2009. Intestinal barrier dysfunction in inflammatory bowel diseases. Inflamm. Bowel Dis. 15, 100–113.
Merrifield, D.L., Ringo, E., 2014. Aquaculture Nutrition: Gut Health, Probiotics and Prebiotics. John Wiley & Sons.
Meyburgh, C., Bragg, R., Boucher, C., 2017. *Lactococcus garvieae*: an emerging bacterial pathogen of fish. Dis. Aquat. Org. 123, 67–79.
Mi, H., Mai, K., Zhang, W., Wu, C., Cai, Y., 2011. Effects of dietary soybean stachyose and phytic acid on gene expressions of serine proteases in Japanese flounder (*Paralichthys olivaceus*). J. Ocean Univ. China 10, 234.
Mukendi, C., Dean, N., Lala, R., Smith, J.J., Bronner, M.E., Nikitina, N.V., 2016. Evolution of the vertebrate claudin gene family: insights from a basal vertebrate, the sea lamprey. Int. J. Dev. Biol. 60, 39–51.
Mussatto, S.I., Mancilha, I.M., 2007. Non-digestible oligosaccharides: a review. Carbohydr. Polym. 68, 587–597.
Pacifici, S., Song, J., Zhang, C., Wang, Q., Glahn, R.P., Kolba, N., Tako, E., 2017. Intra amniotic administration of raffinose and stachyose affects the intestinal brush border functionality and alters gut microflora populations. Nutrients 9.
Pan, Q., Zeng, X., Pan, D., Peng, L., Wu, Z., Sun, Y., Wei, Y., 2017. The proliferation mechanism of *Lactobacillus plantarum* RB1 stimulated by Stachyose. Curr. Microbiol. 74, 732–738.
Panasevich, M.R., Kerr, K.R., Dilger, R.N., Fahey, G.C., Guérin-Deremaux, L., Lynch, G.L., Wils, D., Suchodolski, J.S., Steer, J.M., Dowd, S.E., 2015. Modulation of the faecal microbiome of healthy adult dogs by inclusion of potato fibre in the diet. Brit. J. Nutr. 113, 125–133.
Qiang, X., YongLie, C., QianBing, W., 2009. Health benefit application of functional oligosaccharides. Carbohydr. Polym. 77, 435–441.
Rastall, R.A., Maitin, V., 2002. Prebiotics and synbiotics: towards the next generation. Curr. Opin. Biotechnol. 13, 490–496.
Ray, A., Ghosh, K., Ringø, E., 2012. Enzyme-producing bacteria isolated from fish gut: a review. Aquac. Nutr. 18, 465–492.
Ringø, E., Gatesoupe, F.J., 1998. Lactic acid bacteria in fish: a review. Aquaculture 160, 177–203.
Ringø, E., Zhou, Z., Vecino, J.G., Wadsworth, S., Romero, J., Krogdahl, Å., Olsen, R.E., Dimitroglou, A., Foey, A., Davies, S., Owen, M., Lauzon, H.L., Martinsen, L.L., De Schryver, P., Bossier, P., Sperstad, S., Merrifield, D.L., 2016. Effect of dietary components on the gut microbiota of aquatic animals. A never-ending story? Aquac. Nutr. 22 (2), 219–282.
Saha, A.K., Ray, A.K., 1998. Cellulase activity in rohu fingerlings. Aquac. Int. 6, 281–291.
Sahlmann, C., Sutherland, B.J.G., Kortner, T.M., Koop, B.F., Krogdahl, Å., Bakke, A.M., 2013. Early response of gene expression in the distal intestine of Atlantic salmon (*Salmo salar* L.) during the development of soybean meal induced enteritis. Fish Shellfish Immunol. 34, 599–609.
Saitoh, Y., Suzuki, H., Tani, K., Nishikawa, K., Irie, K., Ogura, Y., Tamura, A., Tsukita, S., Fujiyoshi, Y., 2015. Structural insight into tight junction disassembly by *Clostridium perfringens* enterotoxin. Science 347, 775–778.
Scher, J.U., Sczesnak, A., Longman, R.S., Segata, N., Ubeda, C., Bielski, C., Rostron, T., Cerundolo, V., Pamer, E.G., Abramson, S.B., 2013. Expansion of intestinal *Prevotella copri* correlates with enhanced susceptibility to arthritis. elife 2, e01202.
Schmidt, V., Amaral-Zettler, L., Davidson, J., Summerfelt, S., Good, C., 2016. Influence of fishmeal-free diets on microbial communities in Atlantic Salmon (*Salmo salar*) recirculation aquaculture systems. Appl. Environ. Microbiol. 82, 4470–4481.
Segata, N., Izard, J., Waldron, L., Gevers, D., Miropolsky, L., Garrett, W.S., Huttenhower, C., 2011. Metagenomic biomarker discovery and explanation. Genome Biol. 12, R60.
Sekirov, I., Russell, S.L., Antunes, L.C.M., Finlay, B.B., 2010. Gut microbiota in health and disease. Physiol. Rev. 90, 859–904.
Silva, C.R.d., Delatorre, A.B., Martins, M.L.L., 2007. Effect of the culture conditions on the production of an extracellular protease by thermophilic Bacillus sp and some properties of the enzymatic activity. Braz. J. Microbiol. 38, 253–258.
Sørensen, M., Penn, M., El-Mowafi, A., Storebakken, T., Chunfang, C., Øverland, M., Krogdahl, Å., 2011. Effect of stachyose, raffinose and soya-saponins supplementation on nutrient digestibility, digestive enzymes, gut morphology and growth performance in Atlantic salmon (*Salmo salar*, L). Aquaculture 314, 145–152.
Sullam, K.E., Rubin, B.E., Dalton, C.M., Kilham, S.S., Flecker, A.S., Russell, J.A., 2015. Divergence across diet, time and populations rules out parallel evolution in the gut microbiomes of Trinidadian guppies. ISME J. 9, 1508.
Sun, X., Yang, H., Nose, K., Nose, S., Haxhija, E.Q., Koga, H., Feng, Y., Teitelbaum, D.H., 2008. Decline in intestinal mucosal IL-10 expression and decreased intestinal barrier function in a mouse model of total parenteral nutrition. Am. J. Physiol. Gastrointest. Liver Physiol. 294, G139–G147.
Sundell, K.S., Sundh, H., 2012. Intestinal fluid absorption in anadromous salmonids: importance of tight junctions and aquaporins. Front. Physiol. 3.
Suzer, C., Çoban, D., Kamaci, H.O., Saka, Ş., Firat, K., Otgucuoğlu, Ö., Küçüksari, H., 2008. Lactobacillus spp. bacteria as probiotics in gilthead sea bream (*Sparus aurata*, L.) larvae: effects on growth performance and digestive enzyme activities. Aquaculture 280, 140–145.

Tipsmark, C.K., Sørensen, K.J., Hulgard, K., Madsen, S.S., 2010. Claudin-15 and-25b expression in the intestinal tract of Atlantic salmon in response to seawater acclimation, smoltification and hormone treatment. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 155, 361–370.

Tsuchiya, C., Sakata, T., Sugita, H., 2008. Novel ecological niche of *Cetobacterium somerae*, an anaerobic bacterium in the intestinal tracts of freshwater fish. Lett. Appl. Microbiol. 46, 43–48.

Tsukahara, T., Iwasaki, Y., Nakayama, K., Ushida, K., 2003. Stimulation of butyrate production in the large intestine of weaning piglets by dietary fructooligosaccharides and its influence on the histological variables of the large intestinal mucosa. J. Nutr. Sci. Vitaminol. 49, 414–421.

Turner, J.R., 2009. Intestinal mucosal barrier function in health and disease. Nat. Rev. Immunol. 9, 799–809.

Ulluwishewa, D., Anderson, R.C., McNabb, W.C., Moughan, P.J., Wells, J.M., Roy, N.C., 2011. Regulation of tight junction permeability by intestinal bacteria and dietary components. J. Nutr. 141, 769–776.

van Vliet, M.J., Harmsen, H.J., de Bont, E.S., Tissing, W.J., 2010. The role of intestinal microbiota in the development and severity of chemotherapy-induced mucositis. PLoS Pathog. 6, e1000879.

Wen, H., Feng, L., Jiang, W., Liu, Y., Jiang, J., Li, S., Tang, L., Zhang, Y., Kuang, S., Zhou, X., 2014. Dietary tryptophan modulates intestinal immune response, barrier function, antioxidant status and gene expression of TOR and Nrf2 in young grass carp (*Ctenopharyngodon idella*). Fish Shellfish Immunol. 40, 275–287.

White, J.R., Nagarajan, N., Pop, M., 2009. Statistical methods for detecting differentially abundant features in clinical metagenomic samples. PLoS Comput. Biol. 5, e1000352.

Xing, M., Hou, Z., Yuan, J., Liu, Y., Qu, Y., Liu, B., 2013. Taxonomic and functional metagenomic profiling of gastrointestinal tract microbiome of the farmed adult turbot (*Scophthalmus maximus*). FEMS Microbiol. Ecol. 86, 432–443.

Xu, H., Ai, Q., Mai, K., Xu, W., Wang, J., Ma, H., Zhang, W., Wang, X., Liufu, Z., 2010. Effects of dietary arachidonic acid on growth performance, survival, immune response and tissue fatty acid composition of juvenile Japanese seabass, *Lateolabrax japonicus*. Aquaculture 307, 75–82.

Zhang, J., Xiong, F., Wang, G.T., Li, W.X., Li, M., Zou, H., Wu, S.G., 2017. The influence of diet on the grass carp intestinal microbiota and bile acids. Aquac. Res. 48, 4934–4944.

Zhou, X.L., Kong, X.F., Lian, G.Q., Blachier, F., Geng, M.M., Yin, Y.L., 2014. Dietary supplementation with soybean oligosaccharides increases short-chain fatty acids but decreases protein-derived catabolites in the intestinal luminal content of weaned Huanjiang mini-piglets. Nutr. Res. 34, 780–788.

Zhou, Z., Ringø, E., Olsen, R.E., Song, S.K., 2017. Dietary effects of soybean products on gut microbiota and immunity of aquatic animals: a review. Aquac. Nutr. 1–22.