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Sodium butyrate supplementation in high-soybean meal diets for turbot (*Scophthalmus maximus* L.): Effects on inflammatory status, mucosal barriers and microbiota in the intestine

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A R T I C L E I N F O

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A B S T R A C T

A 12-week feeding trial was conducted to evaluate the effects of dietary sodium butyrate (NaBT) on the intestinal health of juvenile turbot (*Scophthalmus maximus* L.), in terms of inflammatory status, mucosal barriers and microbiota. Three isonitrogenous and isolipidic practical diets were used: (1) fish meal based group (FM); (2) soybean meal group (SBM), soy protein replacing 40% fish meal protein in FM; (3) NaBT group, 0.2% NaBT supplemented in SBM. Each diet was fed to triplicate tanks (30 fish in each tank). The current results showed that 0.2% dietary NaBT improved the growth performance of fish and alleviated the enteropathy, increasing the absorptive surface and mitigating the infiltration of mixed leukocytes in lamina propria. Fish fed the NaBT diet presented increased activities of intestinal brush border enzyme and similar nutrient digestibility with the FM group. Compared to SBM, the inclusion of 0.2% NaBT in diet significantly up-regulated the intestinal gene expression of tight junction proteins and down-regulated the gene expression of TNF-α and NF-κB. The gut microbial communities of the NaBT group were closer to the FM group than to the SBM group, in terms of PCoA, UPGMA and Heatmap analyses based on weighted Unifrac distance. The relative abundance of several dominant bacteria at the phylum (Proteobacteria, Bacteroidetes, Deinococcus-Thermus and Actinobacteria) and genus level (*Thermus*, *Acinetobacter*, *Bacteroides* and *Silanimonas*) were altered by dietary NaBT. In conclusion, dietary NaBT had positive roles in protecting the intestinal health of turbot from the impairment of soybean meal.

1. Introduction

The gut serves as a complex and dynamic ecosystem comprising interactions among the epithelial barrier, immune cells and gut microflora. Gut health is crucial for animal body health [1–3], especially for marine carnivorous fish which have inferior intestinal length index. Plant protein sources have been widely used in fish feed due to rapidly growing aquaculture but relatively stable fish meal production. However, application of plant protein in feeds for marine fish easily causes intestinal impairment. In turbot, for example, Liu et al. reported that high dose of soybean meal (SBM) in diet led to obvious enteropathy, as well as impaired growth performance [4]. Therefore, it was worthwhile to investigate the nutritional strategies for mitigating the intestinal impairment and stress caused by plant protein sources in the fish diets. Many relevant efforts have been made, including those in our laboratory. In our previous studies, positive roles of some functional ingredients such as glutamine and arginine in protecting intestinal health of turbot have been observed when plant protein sources were used in the diets [4,5].

Butyrate is known to be a primary energy source for intestinal epithelial cells and exerts important roles in the maintenance of gut homeostasis [6,7]. Besides, butyrate has been reported to promote wound healing in intestinal surgery and to alleviate inflammatory symptoms in the intestine of mammals [8]. In medical applications, sodium butyrate (NaBT) enema has been used in the treatment of bowel inflammation and positive effects have been observed *in vivo* and *in vitro* [9–11]. In piglet, several studies have shown that supplementation of NaBT in diets improved the growth performance and exerted beneficial

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Abbreviations

ADC	Apparent digestibility coefficient
AKP	Alkaline phosphatase
AMPK	Adenosine 5′-monophosphate-activated protein kinase
FER	Feed efficiency ratio
FI	Feed intake
FM	Fish meal
FMW	Final mean weight
GC	Goblet cell
HSI	Hepatosomatic index
IL-1β	Interleukin-1β
ILI	Intestinal length index
ISI	Intestinal somatic index
LP	Lamina propria
NaBT	Sodium butyrate
NF-κB	Nuclear factor-kappa B
OTU	Operational taxonomic unit
PCoA	Principle coordinate analysis
PR	Perimeter ratio
SBM	Soybean meal
SGR	Specific growth rate
SR	Survival rate
TGF-β	Transforming growth factor-β
TNF-α	Tumor necrosis factor-α
UPGMA	Unweighted pair group method with arithmetic
WGR	Weight gain rate
ZO-1	Zonula occluden-1

effects on health status of digestive tract in weanling piglets [12,13]. In fish, it had been showed that dietary NaBT could improve the growth, antioxidant ability and intestinal absorption capacity of the juvenile grass carp [14], as well as microencapsulated NaBT can be used as a dietary supplement to repair or prevent intestinal damage in carp fed oxidized soybean oil [15]. However, Owen et al. (2006) pointed out that supplementation of 0.2 g/kg NaBT in diets for African catfish was not effective [16], as observed in some studies with weanling piglets [17–19]. Up to date, very few studies are available regarding the effects of NaBT on intestinal health of fish despite the widely reported studies with mammals. The present study was aimed at investigating effects of dietary NaBT on the intestinal health status of turbot (*Scophthalmus maximus* L.), which is a typical marine carnivorous fish species and thus the intestinal health of which is sensitive to dietary supplementation of plant protein sources.

In intestinal homeostasis, the intestinal epithelium provides a barrier which prevents the passage of toxic and inflammatory molecules from the external milieu into the submucosa [6]. The impaired epithelial barrier function induced by high dose of SBM led to increased permeability, reduced gene expression of tight junction proteins, and increased gene expression of pro-inflammatory cytokines and NF-κB [4]. Previous studies on NaBT, *in vivo* and *in vitro*, have proved that NaBT could decrease the expression and release of pro-inflammatory cytokine via inhibition of NF-κB, affecting the innate immune response [17,20–25]. Interestingly, previous studies also pointed out that butyrate at lower concentrations (up to 2 mM) could induce decreased permeability in a Caco-2 and HT-29 cell lines [26,27]. However, at higher concentrations (8 or 10 mM) butyrate increased the permeability in a Caco-2 cell line and adult rat distal colon mucosa [26,28]. The present study evaluated the effects of dietary NaBT supplementation on the intestinal homeostasis in terms of inflammatory status, mucosal barriers and related gene expressions.

Besides, intestinal microbiome also plays important roles in the development of epithelial mucosa and intestinal immune systems [29,30]. One important role of gut microbiota studies in aquaculture research is to provide the scientific basis for enhancing effective strategies to manipulate gut microbiota via the diet and improving productivity [31]. However, the effects of NaBT on gut microbiota have been rarely studied in carnivorous fish. Piazzon et al. (2017) pointed out that the addition of butyrate could slightly reduce cumulative mortality after bacterial challenge and increase gut microbial diversity of gilthead sea bream [32]. On the contrary, a study on common carp showed that no significant effects of sodium butyrate on gut microbial communities were observed. The divergent results remains to be elucidated [33]. To investigate the effects of NaBT on intestinal microbiome in fish, the intestinal microbiota in response to dietary NaBT was also studied, with Illumina HiSeq sequencing analysis of bacterial hyper-variable V4-region of the 16 S rRNA.

2. Materials and methods

2.1. Ethical considerations

The protocols for animal care and handling used in this study were approved by the Animal Care Committee of Ocean University of China. Facilities for turbot husbandry were optimally equipped to ensure refinement of breeding and accommodation to minimize fish suffering. No pathological symptoms were observed during the experimental periods.

2.2. Experimental diets

Three isonitrogenous and isolipidic diets (52% crude protein and 10% crude lipid) included a fish meal based diet (named as FM group), a diet that FM with 40% fish meal protein replaced by soybean protein (named as SBM group) and a diet supplementing 0.2% sodium butyrate into the SBM diet (named as NaBT group) (Table 1). Yttrium oxide (Y_2O_3, 1 g/kg) was used in each diet for determining apparent digestibility coefficient of dry matter.

All the ingredients that were grounded into fine powder through a 320-μm mesh were thoroughly mixed with fish oil and soy lecithin, and an appropriate volume of water was added to produce stiff dough. Then the dough was pelleted with an experimental feed mill (F [II]-26, South China University of Technology, Guangzhou, China), dried for about 12 h in a ventilated oven at 50 °C, and stored at −20 °C until further use.

2.3. Fish husbandry

Juvenile turbot (*Scophthalmus maximus* L.) in this feeding trial were yearlings obtained from a commercial farm in Weihai, Shandong Province, China. After arrival, fish were fed the control diet and acclimatized to the experimental system for 2 weeks. During the acclimation period, fish with extreme sizes, malformation, surface damage, incomplete gill cover and low vitality were excluded. At the beginning of the experiment, fish were fasted for 24 h and weighted. Fish of homogenous size, having a mean initial body weight of appropriate 9.60 g, were randomly distributed into 9 tanks in the flow-through system. Triplicate groups of fish were assigned to each dietary treatment (30 fish in each tank). Fish were fed by hand to visual satiation twice daily at 8:00 and 18:00 for 12 weeks. The uneaten feed was collected in 30 min after feeding, dried to a constant weight, and weighed for the calculation of feed intake. Water conditions and fish behavior were observed and recorded every day during the feeding period. The monitored water quality parameters were: water temperature 19–25 °C, DO > 7.0 mg/L, salinity 23–26, NH_4^+-N < 0.3 mg/L, and pH 7–8.

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

Ingredients	FM	SBM	0.2% NaBT
Fish meal[a]	68	40.8	40.8
Soybean meal[b]	0	37.9	37.9
α-Starch	16	11.55	11.35
Fish oil	4.8	6.7	6.7
Soy lecithin	0.5	0.5	0.5
Vitamin premix[c]	1	1	1
Mineral premix[d]	0.5	0.5	0.5
Choline chloride	0.3	0.3	0.3
Monocalcium phosphate	0.5	0.5	0.5
Ethoxyquin	0.05	0.05	0.05
Yttrium oxide	0.1	0.1	0.1
Calcium propionate	0.1	0.1	0.1
Cellulose	8.15	0	0
Sodium butyrate[e] (NaBT)	0	0	0.2
Proximate composition			
Dry matter content	97.07	97.11	97.41
Crude protein	50.41	50.15	51.10
Crude lipid	9.52	10.29	10.15

Abbreviations: FM, fish meal diet; SBM, soybean meal diet; NaBT, sodium butyrate.

[a] Purchased from Qingdao Seven Great Bio-tech Company Limited (Qingdao, China), crude protein: 74.04%, crude lipid: 9.97%.

[b] Purchased from Qingdao Seven Great Bio-tech Company Limited (Qingdao, China), crude protein: 53.12%, crude lipid: 2.12%.

[c] Vitamin mixture: providing for per kg diet: VA 32 mg, VB_1 25 mg, VB_2 45 mg, VB_6 20 mg, VB_{12} 10 mg, Niacinaminde 200 mg, Inositol 800 mg, Calcium pantothenate 60 mg, VH 60 mg, Folate 20 mg, VE 240 mg, VK 10 mg, VC phosphate 2000 mg, VD 5 mg, Antioxidant 3 mg, Microcrystalline cellulose 6470 mg.

[d] Mineral mixture: providing for per kg diet: Mg 313 mg, Fe 79.10 mg, Zn 62.60 mg, Mn 46.60 mg, I 2 mg, Se 0.90 mg, Cu 6.40 mg, Zeolite powder 3485 mg.

[e] Purchased from *Sigma-Aldrich Co*. (USA). The batch number was 303,410–500G and the purity was more than 98%.

2.4. Sampling

All the fish were fully anesthetized with eugenol (1:10,000) (purity 99%, Shanghai Reagent Corp, Shanghai, China) before handling. The total number and body weight of fish in each tank were measured respectively. Three distal intestine samples each tank were collected for morphological measurements, fixed with Bouin's fixative solution. Five distal intestine samples each tank were frozen in liquid nitrogen immediately and stored at −80 °C for gene expression analysis. Another five distal intestine samples each tank were collected for enzyme activity analysis. All the above samples were taken only from the fish with digesta throughout the intestinal tract, to ensure intestines exposure to the diets until sampling. Feces was collected quantitatively after sampling, and then frozen in liquid nitrogen and stored at −80 °C. Three fish each tank were sterilized with 70% alcohol, and the whole mucosa layer of distal intestines were removed with sterilized instruments around an alcohol lamp and then transferred to 2 ml sterile tubes (Axygen, America) stored at −80 °C for intestinal microbiota analysis.

2.5. Intestinal enzyme activities

The activities of intestinal AKP and Na^+-K^+-ATP were determined spectrophotometrically according to the detailed users' manual of commercial kits (Jiancheng Bioengineering Institute, Nanjing, China).

2.6. Intestinal morphology

Preserved tissue segments were routinely dehydrated in ethanol, equilibrated in xylene and embedded in paraffin according to the standard histological procedures [34,35]. The segments were cut in 7 μm longitudinal sections with a rotary microtome (Lecia Jung RM 2016, Germany) and stained with hematoxylin-eosin method (HE). Observation was performed by Nikon eclipse Ti-S microscope (Japan). The thickness of the LP, the PR and the number of goblet cells were determined by analyzing the micrographs from light microscopy with the image analysis software, Image Pro Plus® 6.0 (Media Cybernetics, Silver Spring, MD, USA) [34,36–38].

2.7. Real-time quantitative PCR

Distal intestines were ground to powder in liquid nitrogen and then processed with RNAiso Plus (9109; Takara Biotech, Dalian, China) for extraction and purification of RNA. The quality and concentration of RNA were detected by electrophoresis on 1.2% denatured agarose gels and assessed by Nano Drop®2000 spectrophotometer (Thermo Fisher Scientific, USA), respectively. The cDNA was generated by reversely transcribing 1 μg total RNA using PrimeScript RT reagent Kit with gDNA Eraser (RR047A; Takara Biotech, Dalian). Specific primers for the genes were generated by Sangon (Shanghai, China) and the application efficiency was assessed (Table 2). RPSD and β-actin were used as the reference gene to normalize cDNA loading. The detailed methods was described in our previous work [4].

2.8. Bacterial DNA extraction, amplification, sequencing and bioinformatic analysis

Bacterial DNA of the distal intestinal samples was extracted with QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) with slight modifications as follows: (i) the samples were transferred from −80 °C to ice for a short while; (ii) the samples were opened longitudinally on sterile petri dishes on ice before thawing; (iii) the mucosal layers and contents were collected and transferred immediately to a 5 ml sterile tubes with sufficient InhibitEX buffer (proportional to the tissue weight); (iv) the tubes were subjected to vortex at maximum speed for 1 min, and 1 ml of the homogenate was taken for the downstream DNA extraction [36]. The integrity and quality of each DNA sample were determined on a 1% agarose gel electrophoresis. DNA purity and concentration were monitored with NanoDrop ND-1000 spectrophotometer (Peqlab, Erlangen, Germany). DNA was diluted to 1 ng/μl using sterile water. The detailed methods was described in our previous work [4].

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

Target gene	Primer sequence (5′-3′)	Amplicon size (bp)	AE	Genbank accession number
Claudin-4	F:ATGTGGAGTGTGTCGGCTT R:AGACCTTGCACTGCATCTG	237	1.06	MF370857
Occludin	F: ACTGGCATTCTTCATCGC R: GGTACAGATTCTGGCACATC	583	1.01	KU238181
ZO-1	F: AGAGAACCTGTCACTGATAGATGC R:CTGTCGGAATTGTTGCCTGATG	1697	0.95	KU238184
TNF-α	F: GGACAGGGCTGGTACAACAC R: TTCAATTAGTGCCACGACAAAGAG	186	0.91	AJ276709.1
TGF-β	F: CTGCAGGACTGGCTCAAAGG R: CATGGTCAGGATGTATGGTGGT	749	0.97	KU238187
β-actin	F: GCTGTCTTCCCTTCTATCGTCG R: TCCATGTCATCCCAGTTGGTC	543	0.97	AY008305.1
RPSD	F: CCTCATGTCGCGGATGCT R: CCTCGGAAAGTTCCTGCTC	545	1.10	DQ848899

F, forward primer; R, reverse primer; AE, the primer amplification efficiencies.

An OTU table was generated and the rarefaction curve was produced based on the metric of observed species for the estimation of alpha diversity. PCoA, UPGMA clustering and Heatmap based on weighted UniFrac phylogenetic distance were constructed to visualize the distance matrix from complex and multidimensional data. To assess the changes in microbial community structure, differentially abundant taxa among treatments were identified by Metastats analysis [39]. Tukey's test and wilcox's test were used to test statistical difference of α-diversity and β-diversity between groups.

2.9. Calculations and statistical analysis

Survival (%) = 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

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)

Intestinal length index, ILI (%) = intestinal length (cm) / body length (cm)

Intestinal somatic index, ISI (%) = intestinal weight (g) / body weight (g)

Absorptive surface (Perimeter ratio, PR) = IP/EP, arbitrary units. IP, the internal perimeter of the gut lumen (villi and mucosal folding length). EP, the external of the gut lumen (perimeter of internal muscularis mucosa) [38].

All data were subjected to one-way analysis of variance (ANOVA) with SPSS 17.0. Homogeneity of variance test was conducted to ensure that variance was homogeneous. Tukey's multiple-range test was applied to treatment means which showed a statistically significant variation in the samples. Differences were considered significant at $P < 0.05$. The results were presented as means ± standard error.

3. Results

3.1. Growth performance

Fish fed all diets had high survival rate (ranging from 97.78% to 100%) and no significant difference was observed among treatments ($P > 0.05$). Compared to the FM group, significant ($P < 0.05$) decreases in FMW, WGR and SGR were observed in the SBM group, while compared to the SBM group, dietary supplementation of 0.2% NaBT resulted in significantly ($P < 0.05$) higher FMW, WGR and SGR. The SBM group showed significantly ($P < 0.05$) increased FI and reduced FER compared to the FM group, whereas the NaBT group showed similar levels with the FM group ($P > 0.05$). The inclusion of 0.2% NaBT in diet caused the lowest ILI, ISI and HSI of fish among treatments ($P < 0.05$), and there was no significant difference observed between the NaBT group and the FM group ($P > 0.05$) (Table 3).

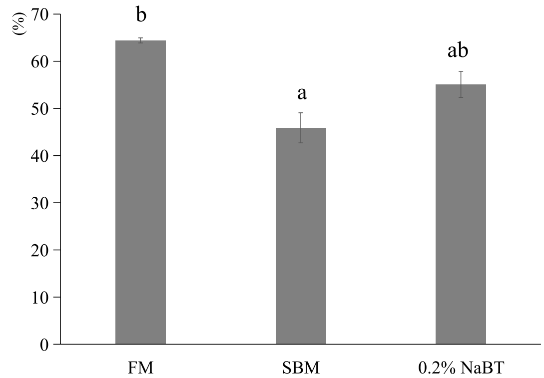

Fig. 1. Effects of NaBT on apparent digestibility coefficient of juvenile turbot fed high dose of soybean meal in diet. [Ab] Different superscript letters indicate significant difference ($P < 0.05$).

3.2. Gut digestive and absorptive ability

Compared to the FM group, the SBM group showed significantly ($P < 0.05$) decreased digestibility and intestinal AKP activity, while the NaBT group showed similar levels with the FM group (Fig. 1 and Fig. 2). Fish fed the NaBT diet showed significantly ($P < 0.05$) increased intestinal Na^+-K^+-ATP activity compared to the SBM group (Fig. 3).

3.3. Gut morphology

Fish fed the SBM diet exhibited distinct inflammatory symptoms, with wizened enterocytes, disordered nucleus position of enterocytes, and obvious infiltration of mixed leukocytes in the lamina propria, as well as significantly ($P < 0.05$) decreased PR and increased LP in gut morphometry. No inflammatory symptom was observed in gut morphology of fish from the NaBT group, and no difference ($P > 0.05$) in gut PR and LP were observed between the FM group and the NaBT group (Fig. 4 and Table 4).

Details of distal intestine section from fish in FM (A, D and G), SBM (B, E and H) and 0.2% NaBT (C, F and I) groups. MF: mucosal fold; MM: muscularis mucosa; SML: submucous layer; LP: lamina propria; GC: goblet cell (arrows); MV: microvilli (arrowheads); N: nucleus; EGC: eosinophilic granular cell (red arrows). Scale bar of A, B, C, 200 μm; scale bar of D, E, F, 50 μm; scale bar of G, H, I, 20 μm.

Table 3
Effects of NaBT on growth performance of juvenile turbot fed high dose of soybean meal in diet.

Parameter	FM	SBM	0.2% NaBT
Survival (%)	100 ± 0.00	97.78 ± 2.22	98.89 ± 1.11
Initial mean weight (g)	9.64 ± 0.01	9.55 ± 0.03	9.64 ± 0.02
Final mean weight (g)	62.21 ± 1.66^b	45.86 ± 1.83^a	57.45 ± 3.98^b
Weight gain (%)	545.33 ± 16.46^b	380.42 ± 20.50^a	496.31 ± 41.07^b
Specific growth rate (%/d)	2.22 ± 0.03^b	1.87 ± 0.05^a	2.12 ± 0.08^b
Feed intake (%)	1.48 ± 0.02^a	1.63 ± 0.02^b	1.61 ± 0.02^{ab}
Feed efficiency ratio	1.18 ± 0.01^b	0.94 ± 0.02^a	1.05 ± 0.03^{ab}
Intestinal length index (%)	53.42 ± 0.72^b	47.55 ± 0.71^a	53.44 ± 1.75^b
Intestinal somatic index (%)	1.15 ± 0.02^b	0.98 ± 0.01^a	1.22 ± 0.01^b
Hepatosomatic index (%)	1.24 ± 0.06^b	0.84 ± 0.03^a	1.11 ± 0.03^b

Values are means ± S. E. and values within the same row with different letters are significantly different ($P < 0.05$).

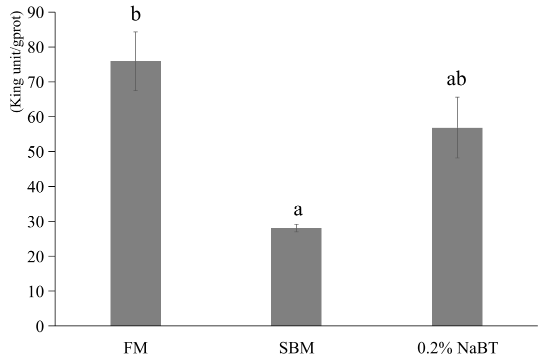

Fig. 2. Effects of NaBT on intestinal alkaline phosphatase activity of juvenile turbot fed high dose of soybean meal in diet. [Ab] Different superscript letters indicate significant difference ($P < 0.05$).

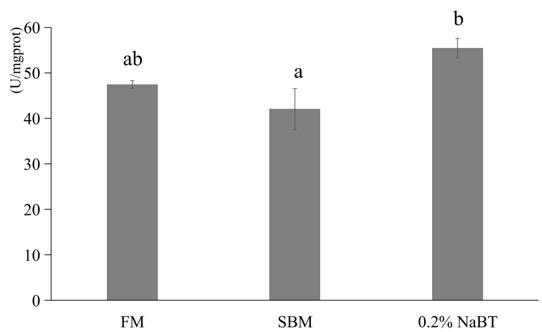

Fig. 3. Effects of NaBT on Na^+-K^+-ATP activity of juvenile turbot fed high dose of soybean meal in diet. [Ab] Different superscript letters indicate significant difference ($P < 0.05$).

3.4. Gut mucosal barrier

Fish in the SBM group showed significantly ($P < 0.05$) reduced gene expression levels of tight junction proteins (claudin-4, occludin and ZO-1) in the intestine, while the NaBT diet resulted in similar gene expression levels of these proteins with the FM group (Fig. 5A). The SBM diet led to significant increases in gut gene expression levels of TNF-α and NF-κB and significant decrease in TGF-β gene expression ($P < 0.05$) compared to the FM group, however, fish from NaBT group showed similar gene expression levels with those from the FM group (Fig. 5B).

3.5. Gut bacterial community

To characterize and compare the gut microbial community of fish from different groups, Illumina HiSeq sequencing analysis of bacterial hyper-variable V4-region of the 16 S rRNA was conducted with mucosa of distal intestine. Triplicate samples per treatment were used for this analysis. After trimming the low-quality sequences and adaptors, a total of 541,751 effective tags for the experimental samples were obtained, clustering a total of 4842 OTUs with over 97% sequence similarity. The OTUs were assigned to 502 genera, 238 families, 138 orders, 73 class, and 37 phyla. Based on the rarefaction curves shown in Supplementary Fig. S1, similar trends were observed in the microbial diversity in all nine samples, approaching the saturation plateau. The Good's coverage of all the samples reached 0.997, indicating adequate sequencing depth (Supplementary Table S1).

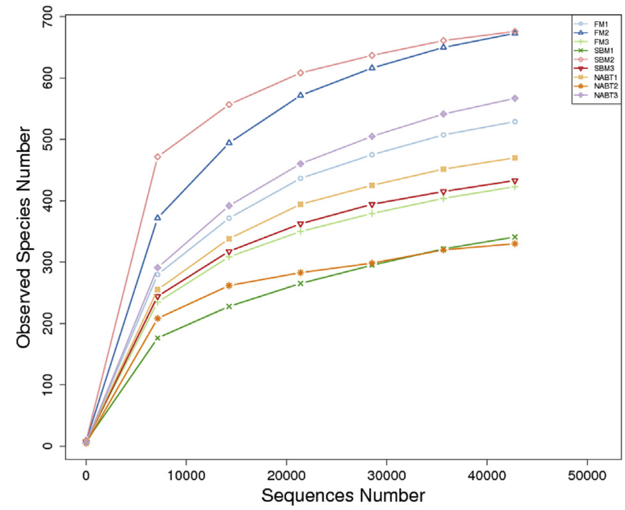

Fig. S1. Rarefaction curve of gut microbiota in juvenile turbot

The rarefied curves for observed species number tended to approach the saturation plateau.

Abbreviation: FM1, FM2 and FM3 are the three replicates of FM group; SBM1, SBM2 and SBM3 are the three replicates of SBM group; NABT1, NABT2 and NABT3 are the three replicates of NaBT group.

Table S1
Alpha diversity index of gut microbiota of experimental turbot

	FM	SBM	0.2% NaBT
OTUs	601.67 ± 75.32	534.67 ± 86.44	477.67 ± 61.41
Good's coverage	0.997 ± 0.000	0.998 ± 0.000	0.998 ± 0.001
Observed species	541.67 ± 72.45	483.33 ± 99.93	455.67 ± 68.79
Chao1	602.55 ± 63.92	565.18 ± 88.45	518.49 ± 82.59
ACE	623.14 ± 65.56	576.88 ± 77.57	530.19 ± 83.20
Shannon	4.45 ± 0.76	5.82 ± 0.70	4.37 ± 0.29
Simpson	0.81 ± 0.12	0.95 ± 0.02	0.87 ± 0.03

Values are means ± S. E. and values within the same row with different letters are significantly different ($P < 0.05$).

A Venn diagram was used to show the unique and shared OTUs (Fig. 6). The numbers of unique OTUs in the gut of FM, SBM and NaBT groups were 342, 529 and 279, respectively. Interestingly, the FM and SBM samples shared 277 OTUs, while the FM and NaBT samples shared 522 OTUs. Beta diversity heatmap, PCoA and UPGMA based on weighted unifrac distances were used to compare the similarity in the microbial community composition of 9 samples (Fig. 7). According to the beta diversity heatmap, the NaBT group has smaller coefficient (ranging from 0.145 to 0.518) than the SBM group (ranging from 0.516 to 0.756) when compared with the FM group (Fig. 7A). As shown in Fig. 7B, the clusters of FM and NaBT samples scattered on the left of the coordinate axis, which distinctly separated from the cluster of SBM samples. Similar as the UPGMA analysis (Fig. 7C) was performed.

Every circle with different colour represented 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.

At the phylum level, the gut microbial community structure of the three groups was analyzed (Fig. 8A). Proteobacteria, Bacteroidetes, Firmicutes and Deinococcus-Thermus constituted four common dominant phyla, which accounted for 94.68%, 98.59% and 94.29% of the total sequencing number in 9 samples of three groups, respectively. MetaStat analysis on the dominant phyla was also conducted (Table 5). Significant decreases in relative abundance of Proteobacteria and Actinobacteria as well as marked increases in relative abundance of Bacteroidetes and Deferribacteria from SBM samples were observed when

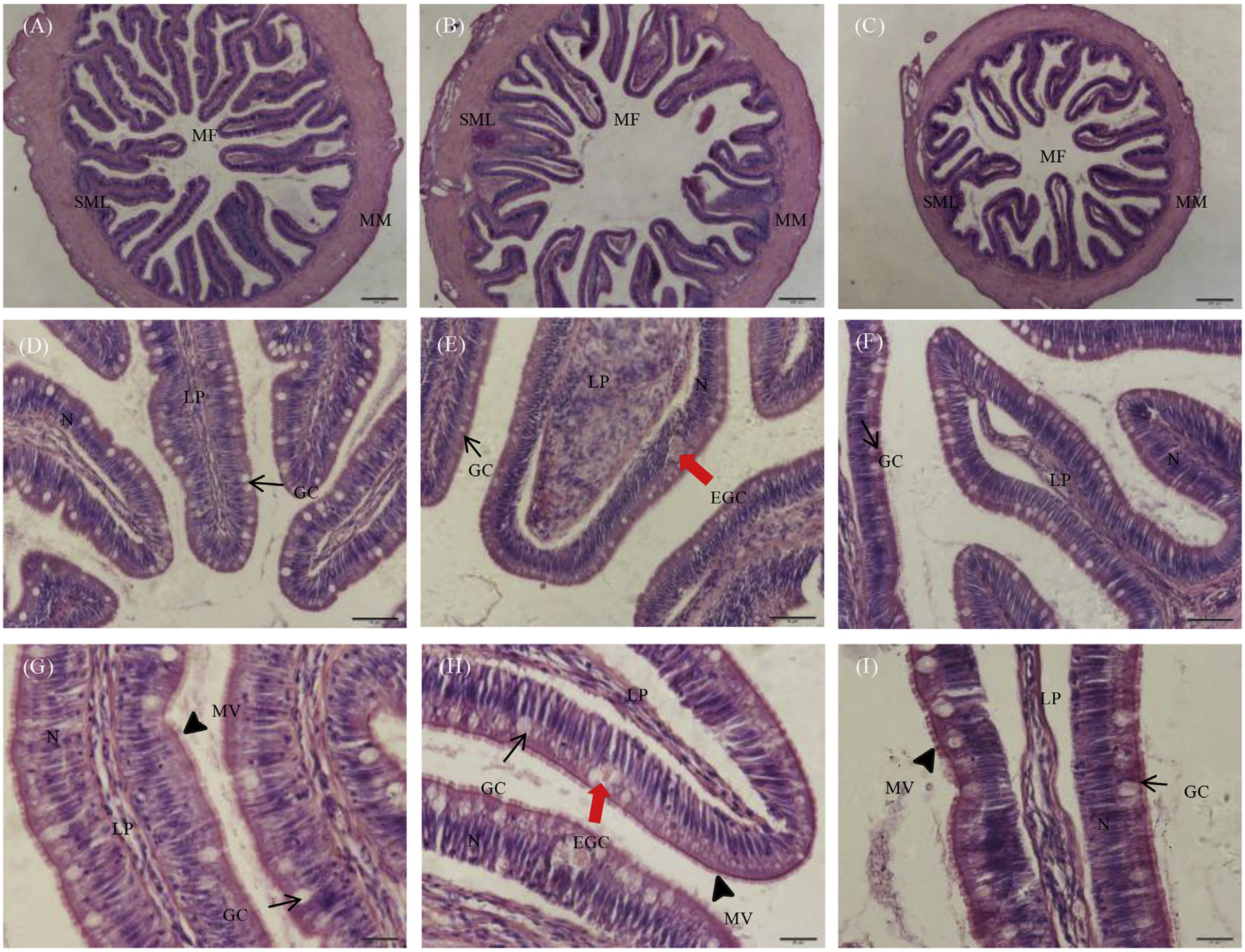

Fig. 4. Effects of NaBT ondistal intestine morphology of juvenile turbot fed high dose of soybean meal in diet for twelve weeks.

Table 4
Effects of NaBT on the distal intestine morphometry of juvenile turbot fed high dose of soybean meal in diet (n = 3).

Parameters	FM	SBM	0.2% NaBT
Perimeter ratio	4.93 ± 0.13^{b}	3.50 ± 0.23^{a}	4.22 ± 0.18^{ab}
Lamina propria (μm)	23.08 ± 0.81^{a}	31.96 ± 2.81^{b}	27.10 ± 2.00^{ab}
Goblet cell	46.58 ± 4.12	36.45 ± 2.04	35.58 ± 2.39

Values are means ± S. E. and values within the same row with different letters are significantly different ($P < 0.05$).

compared with FM group ($P < 0.05$), whereas supplementing 0.2% NaBT in SBM-based diet significantly increased the relative abundances of Proteobacteria, Deinococcus-Thermus and Actinobacteria, and reduced the relative abundances of Bacteroidetes and Deferribacteria compared to the SBM treatment ($P < 0.05$). At the genus level, the top 10 dominant genera of turbot gut microbial communities were *Limnobacter*, *Thermus*, *Methyloversatilis*, *Vibrio*, *Acinetobacter*, *Bacteroides*, *Allobaculum*, *Silanimonas*, *Lachnospiraceae_NK4A136_group* and *Ferrovibrio*. The rest were categorized as 'others'. When representative bacteria were classified into genera, strong distinction emerged between the SBM group and the other two groups. Compared with SBM group, the samples from NaBT group showed significantly increased abundances of *Thermus*, *Acinetobacter*, *Silanimonas*, *Escherichia-Shigella*, *Schlegelella*, *Oxalophagus*, *Halomonas*, *Pseudomonas*, *Azospirillum* and *Paracoccus* as well as reduced abundances of *Bacteroides*, *Lachnospiraceae_NK4A136_group*, *Alistipes* and *Prevotellaceae_UCG-001* ($P < 0.05$).

Only top 10 most abundant (based on relative abundance) bacteria phyla and genera were shown in the figures, other phyla and genera were all classified as 'others'.

4. Discussion

Results of the present study showed that fish suffered from high dose of soybean meal in diet, showing remarkably suppressed growth performance and obvious enteropathy, whereas supplementation of 0.2% NaBT in diet alleviated the phenomena, in terms of significantly increased growth performance, nutrient digestibility, and intestinal brush border enzyme activities, as well as alleviated inflammatory symptoms. The present results were consistent with the previous report on common carp which showed that microencapsulated NaBT at a dose of 300 mg/kg diet increased weight gain and intestinal microvillus density of fish [15]. To date, most relevant results were reported with terrestrial animals. Information on the effects of NaBT in fish remains limited. It has been found that feeding NaBT at 8 g/kg diet to piglets in the first week after weanling improved the average daily weight gain and the average daily feed intake [12]. Furthermore, similar reports have shown that feeding weanling piglets with 1 g/kg diet of NaBT increased villus height and villus height to crypt depth ratio at the small intestinal mucosa [20,40]. That might be due to the fact that NaBT could be quickly absorbed and utilized directly as a major energy source for epithelial cells to promote sodium and water absorption as well as mucosa proliferation, especially when the gut was injured [6,18,41–43]. However, different from above studies, some

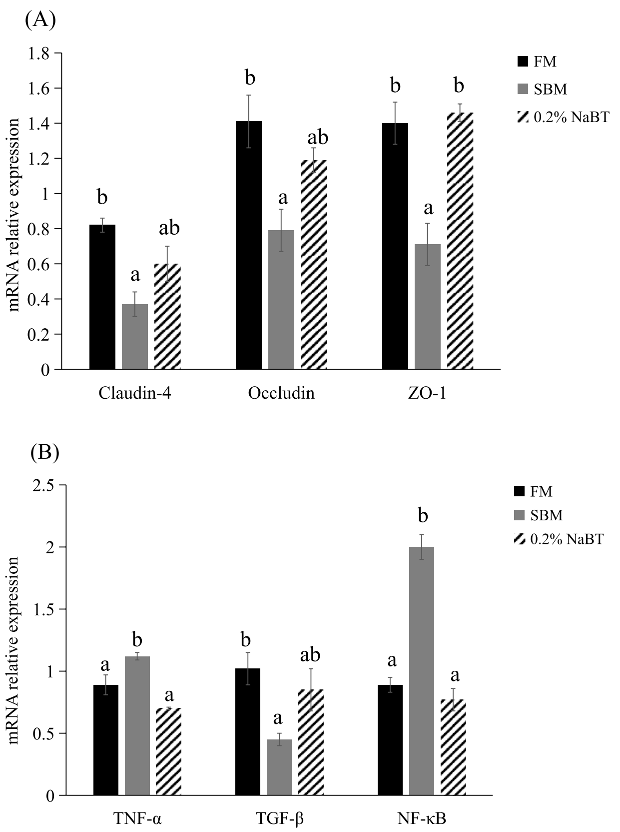

Fig. 5. Effects of NaBT on the expression of key genes in intestinal mucosal barrier of juvenile turbot fed high dose of soybean meal in diet for 12 weeks. (A) The expression of distal intestine tight junction proteins and key genes in mechanical barrier; (B) the expression of distal intestine cytokines and key genes in immune barrier. [Ab] Different superscript letters indicate significant difference ($P < 0.05$).

controversial results were also reported. In the studies with fish, Owen et al. found that when 0.2 g/kg NaBT was used as feed additive for African catfish, there was non-significant difference among dietary treatments [16]. In rainbow trout, dietary supplementation with 10 g acid moiety/kg of a sodium formate and butyrate blend (ratio 2:1 on the acid-moiety weight basis) did not improve the growth rate and feed utilization of fish [44], which was consistent with the results in some studies with piglets [17,18,45]. The different models, dose and form of NaBT, feed composition and rearing environment may partly explain this paradox [6,15,18,45–47]. Regarding the present study, one possible reason explaining this paradox could be the gut maturation status. Turbot, as a marine carnivorous fish, has inferior intestinal length index to other species and thus NaBT was rapidly absorbed to affect the epithelial cells of distal intestine [18], while in other species, NaBT may be absorbed in the upper gastrointestinal tract and therefore hardly reach the lower digestive tract [17,18,48].

The intestinal epithelium provides a physical barrier and innate immune defense, preventing a variety of gut pathogens entering host tissues. The impaired epithelial barrier function may lead to increased permeability and uncontrolled antigen and toxic factors into the host immune system [8,49,50]. The present study showed high dose of dietary soybean meal significantly elevated the intestinal gene expression levels of pro-inflammatory cytokine TNF-α and NF-κB, and suppressed the gene expression of anti-inflammatory cytokine TGF-β. Mounting evidence proved that TNF-α and TGF-β are the primary cellular messengers triggering the cascade of metabolic alterations following the innate immune response [51,52], and NF-κB, as a crucial transcription factor, involves in the regulation of the inflammatory response and controls the expression of genes encoding pro-inflammatory cytokines [6,20,53]. In this study, fish fed 0.2% NaBT alleviated enteropathy via inhibiting NF-κB signaling pathways and decreasing TNF-α gene expression. This was consistent with other previous *in vivo* and *in vitro* studies [17,21,23,24,54]. Additionally, one major component of the intestinal barrier is the formation of tight junctions between epithelial cells [55–57]. The present study showed that dietary supplementation of 0.2% NaBT increased the intestinal gene expression of claudin-4, occludin and ZO-1 of juvenile turbot compared to the SBM

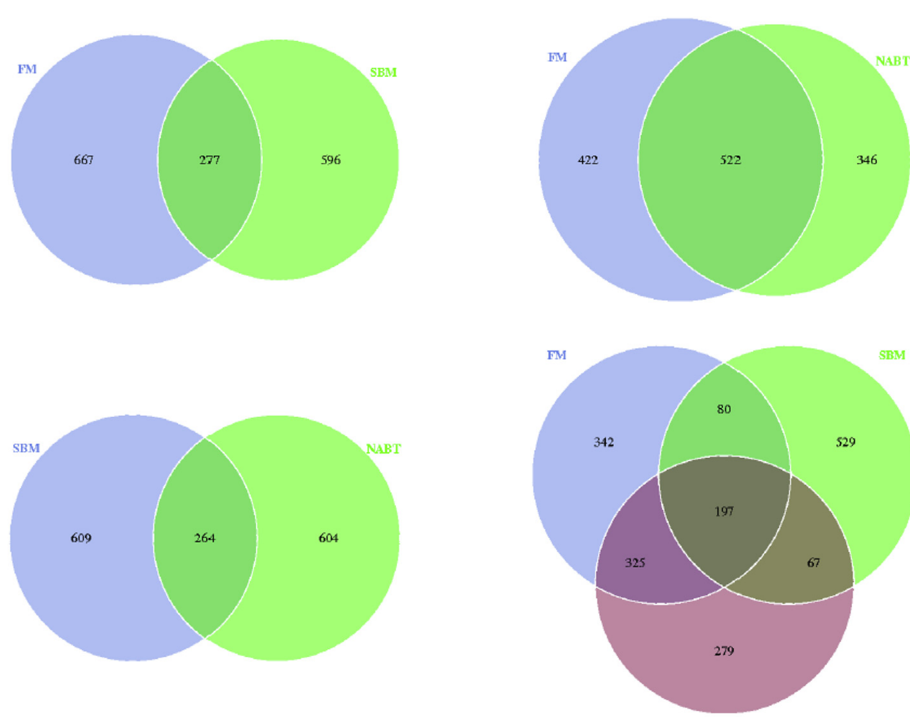

Fig. 6. Venn diagram of unique and shared OTUs.

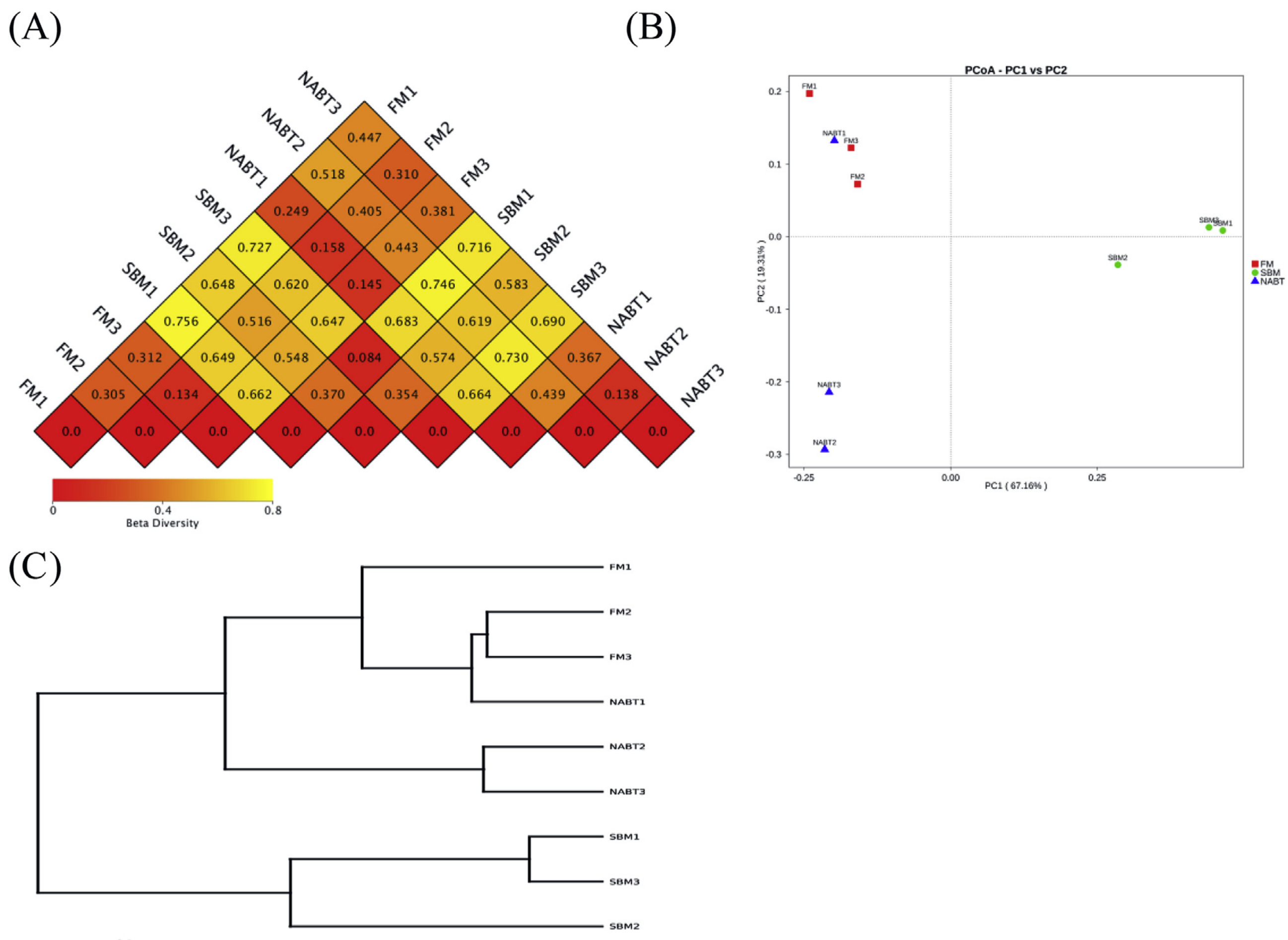

Fig. 7. Beta diversity of gut microbiota of juvenile turbot. (A): Beta diversity heatmap. The value of one square was based on weighted unifrac distance. The smaller coefficient means top-left sample was more similar with top-right sample. (B) and (C): Principal Coordinate Analysis (PCoA) against PC1versus PC2 axes and UPGMA-clustering trees were both based on weighted unifrac distance.

group, resulting in comparable values to the FM group. Similarly, a study with IPEC-J2 cell model by Ma et al. suggested that treatment with 4 mM NaBT promoted the process of wound healing by increasing ZO-1 gene expression [1]. That might be due to the fact that NaBT could exert a role of activating AMPK to facilitate the assembly of tight junctions [55]. To our knowledge, information regarding the effects of NaBT on the regulation of intestinal mechanical barrier *in vivo* is rarely reported. The mechanisms of NaBT effects on the intestinal barrier function *in vivo* warrant further investigation.

The intestinal barrier function, which is in close association with a myriad of microbes and their products [6], plays important roles in metabolic, nutritional, physiological and immunological processes in the host [58]. Results of the present study showed that the intestinal microbial compositions of fish were highly sensitive to diets, consistent with the previous studies with fish or terrestrial animals [59–62]. From α-diversity, both the indices of diversity and species richness in NaBT group showed partially decreased but not statistically in the present study. The studies on gilthead sea bream and common carp have been

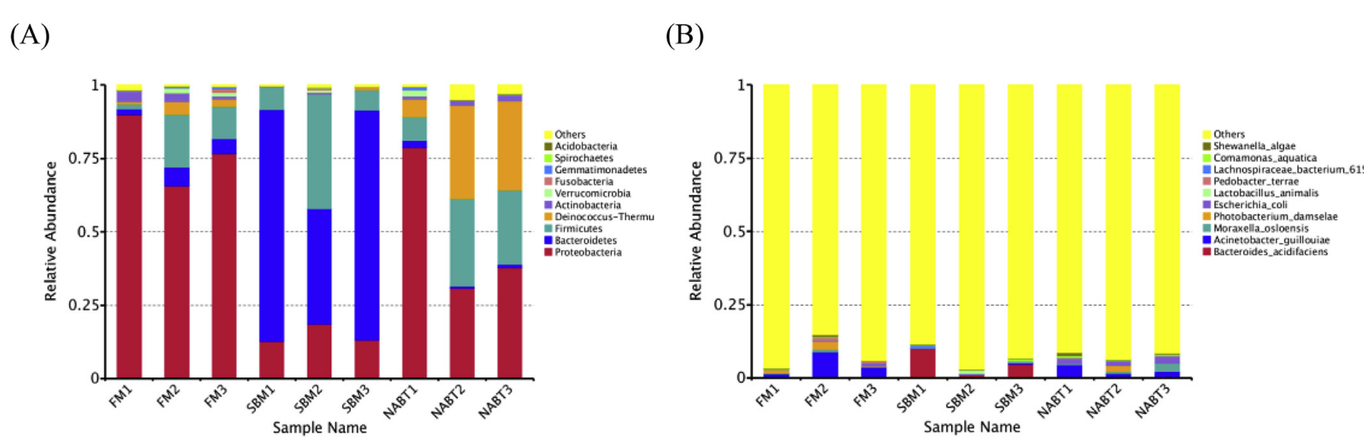

Fig. 8. Taxonomy classification of reads at the phylum (A) and genus (B) taxonomic levels in gut microbiota of juvenile turbot.

Table 5
The MetaStat analysis of intestinal microbiota of fish among groups (× 10^-4).

Phylum	FM		SBM		NABT	
	Mean	S. E.	Mean	S. E.	Mean	S. E.
Proteobacteria	7738.77^{c}	701.40	1479.46^{a}	189.08	4915.63^{b}	1495.32
Bacteroidetes	448.18^{a}	126.12	6550.43^{b}	1309.44	134.46^{a}	52.66
Deinococcus-Thermus	263.00^{a}	107.50	32.02^{a}	28.52	2278.68^{b}	836.42
Actinobacteria	253.65^{b}	80.63	26.49^{a}	18.03	167.49^{b}	32.04
Deferribacteres	0^{a}	0	23.14^{b}	1.59	0.39^{a}	0.21

Genera	FM		SBM		NABT	
	Mean	S. E.	Mean	S. E.	Mean	S. E.
Thermus	233.94^{a}	114.77	31.16^{a}	27.78	2268.94^{b}	835.11
Bacteroides	1.17^{a}	0.93	687.65^{b}	203.68	2.42^{a}	0.08
Acinetobacter	606.56^{b}	243.77	102.83^{a}	64.83	536.68^{b}	93.33
Silanimonas	534.18^{b}	236.04	2.73^{a}	1.52	447.40^{b}	119.44
Lachnospiraceae_NK4A136_group	0.62^{a}	0.31	395.83^{b}	127.22	0.86^{a}	0.34
Alistipes	0.31^{a}	0.21	212.13^{b}	71.16	0^{a}	0
Escherichia-Shigella	49.47^{a}	22.93	16.67^{a}	14.34	200.13^{b}	24.85
Schlegelella	113.27^{b}	44.96	8.34^{a}	7.40	161.81^{b}	71.97
Oxalophagus	26.02^{b}	12.17	0.62^{a}	0.41	153.00^{b}	73.57
Halomonas	99.09^{ab}	53.33	29.37^{a}	18.17	122.15^{b}	32.32
Pseudomonas	98.55^{b}	28.40	11.06^{a}	5.14	73.62^{b}	9.61
Azospirillum	67.31^{b}	35.12	0^{a}	0	27.81^{b}	13.31
Prevotellaceae_UCG-001	0^{a}	0	35.13^{b}	14.96	0^{a}	0
Paracocccus	21.42^{b}	10.39	0.16^{a}	0.08	7.87^{b}	0.74

Values are means and S. E. and values within the same row with different letters are significantly different ($P < 0.05$).

proved the similar results [31,33]. However, supplementing 0.4% sodium butyrate could avoid growth retardation in parasitized fish and increase intestinal microbiota diversity with a higher representation of butyrate-producing bacteria [32]. To date, the underlying mechanisms of sodium butyrate on the gut microbiota of fish are still considerably understudied compared to that of humans and mammals. One possible reason of the present result might be the capacity of acidifier [63]. In the digestive tract of fish, sodium butyrate, as one kind of organic acid, caused a pH reduction and inhibited overgrowth of pH-sensitive pathogenic bacteria [64]. These differences indicate that not all the datasets are comparable due to the nature of the samples, the protocols, experimental environment, feeding time, diet intervention and so on [65,66]. Furthermore, according to the results of Venn diagram and beta diversity of gut microbiota, supplementation of 0.2% NaBT in diet presented closer microbial composition and structure to the FM group, indicating that NaBT contributed positively to the delicate balance in gut microbial environment. Previous studies have proved that gut bacteria have beneficial effects on the digestive processes of fish via providing a variety of enzymes [67–70], and the interaction between the indigenous microbiota and the host may have important implications [71]. For instance, some members of Proteobacteria could utilize outside environmental reservoirs to proliferate, reaching the relatively high prevalence in the gut tract of fish [72–74]. Bacteria of Actinobacteria could serve to produce extracellular enzymes and form kinds of secondary metabolites [67,75]. In this study, the inclusion of NaBT in diet significantly elevated the relative abundances of Proteobacteria and Actinobacteria compared to the SBM group, which was consistent with our previous study [4]. In the genus level, *Bacteroides* belonging to the phylum Bacteroidetes could secret proteins to break down polysaccharides and metabolize sugars [59,76]. Some members of Deinococcus-Thermus (such as *Thermus*) are known for their resistance to extreme stresses including oxidation and high temperature [77]. Our results showed dietary NaBT could increase the relative abundance of *Thermus* and decrease the relative abundance of *Bacteroides* by comparation to the SBM group, which could reveal that NaBT played a beneficial effect on gut microbiota by regulating digestive enzyme levels produced by bacteria. Additionally, Williams et al. (2018) reported that *Escherichia-Shigella* as the dominant genus of feces in red pandas furing weaning exerted an important role in food digestion and absorption, however, the study is not comparable with our results [78]. As to other shifted bacteria in our present results, little information on the function of the bacteria *in vivo* was reported. Therefore, we could not clearly elucidate the alteration of microbiota at genus level. Moreover, no literature regarding the influence of NaBT on gut microbiota and bacteria functions in fish has been available. Further detailed work is needed in this research area.

5. Conclusions

The present study suggested that supplementation of 0.2% NaBT in the soybean meal-based diet improved the growth performance of juvenile turbot and alleviated the soybean-induced enteropathy by enhancing digestive and absorptive ability and regulating the intestinal morphology. Dietary NaBT increased the gene expression of intestinal tight junction proteins (claudin-4, occludin and ZO-1) and decreased the gene expression of pro-inflammatory cytokine (TNF-α) and NF-κB, which were probably involved in the reinforcement of intestinal mucosal barrier function. Additionally, the inclusion of NaBT in diet led to closer intestinal microbial composition to the fish meal based group than to the soybean meal based group.

Declaration of interest

None of the authors has any conflicts of interest to declare.

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