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Effects of fish meal replacement by soybean meal with supplementation of functional compound additives on intestinal morphology and microbiome of Japanese seabass (*Lateolabrax japonicus*)

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Abstract

A 15-week trial was carried out to estimate the effects of functional compound additives (FCA) on intestinal morphology and microbiome in Japanese seabass, *Lateolabrax japonicus*, fed diets with soybean meal (SBM) partially replacing fish meal (FM). The formulation of FCA was the mixture of antioxidant, immunopotentiator and Mintrex® trace elements. Four isonitrogenous (45%) and isolipidic (11%) diets, including FM42 (the control group without FCA), FM35 (15% FM protein substitution level with FCA), FM21 (50% FM protein substitution level with FCA) and FM21-N (50% FM protein substitution level without FCA) were formulated. Quadruplicate groups of seabass (initial average weight 125.65 ± 0.60 g) were randomly handfed each diet twice daily in seawater floating cage. No significant differences were found in microvillus height (HMV) and muscular thickness (MT) of distal intestine among fish fed FM42, FM35 and FM21, whereas the HMV, MT and fold height (HF) in seabass fed FM42 were significantly higher than those of seabass fed FM21-N ($P < 0.05$). Seabass fed FM21-N had more obvious enteritis parameters such as tissue disruption, wider lamina propria and less mucosal fold than those in seabass fed FM21. High-throughput sequencing technology of gut flora showed that *Proteobacteria* was the most dominant bacteria at phylum level among groups. There was no significant difference in relative abundance and composition of gut microflora among groups. In conclusion, supplementation of FCA might partially eliminate SBM-induced enteritis, but the composition and relative abundance of intestinal microflora were not affected by dietary treatments.

Keywords: *Lateolabrax japonicus*, functional compound additives, soybean meal, intestinal morphology, intestinal microbiome

Introduction

Soybean meal (SBM) has been widely researched as a cheap and alternative resource for fish meal (FM) in aquafeeds (Laporte & Trushenski 2012). However, some antinutritional factors in SBM such as lectin and isoflavones are well known to have negative effects on palatability and intestinal morphology of fish (Baeverfjord & Krogdahl 1996). The reduction in mucosal folding, loss of mucosal integrity, abnormal vacuolization and inflammatory cell infiltration are the most common morphological changes, which may negatively affect the growth and health of aquatic animals (Burrells, Williams, Southgate & Crampton 1999; Refstie, Landsverk, Bakke-McKellep, Ringø, Sundby, Shearer & Krogdahl 2006).

Meanwhile, SBM-induced negative changes in the balance of gut flora were found in Atlantic cod, *Gadus morhua* (Refstie *et al.* 2006), and rainbow trout, *Oncorhynchus mykiss* (Heikkinen, Vielma, Kemiläinen, Tiirola, Eskelinen, Kiuru &

Wright 2006). Intestinal flora plays an important role in defencing against pathogens and keeping normal intestinal function in fish (Ringø, Olsen, Mayhew & Myklebust 2003; Sekirov, Russell, Antunes & Finlay 2010). Some studies also observed that several lactic bacteria may improve immunity and prevent intestinal inflammation in rainbow trout, *O. mykiss* (Kim & Austin 2006). Therefore, studies about the importance of gut microflora to the gut morphology and the health of animals were still need to be further researched, especially for fish (Wu, Gao, Zheng, Wang, Cheng & Wang 2010).

Previous study showed that compound rather than single substance was more effective to improve physiological function of fish (Kasumyan & DÖving 2003). The functional compound additives (FCA) were the mixture of antioxidant (Agrado®), immunopotentiator (NE-150®) and Mintrex® trace elements (Cu, Mn and Zn). Although no studies were found to evaluate the effects of FCA on intestinal morphology or bacterial community in fish and livestock, the single substance in FCA has been widely studied in fish and livestock. The primary chemical of Agrado® is ethoxyquin, which has been used for many years as a feed preservative in the feed of fish, livestock and pets (Yamashita, Katagiri, Pirarat, Futami, Endo & Maita 2009). Agrado® was also reported to benefit oxidative balance and growth performance in livestock and fish by improving metabolism balance (Kegley, Hellwig, Gill & Owens 2000; Vázquez-Añón & Jenkins 2007). NE-150® is a commercial essential oil product, which is characterized by a 1:1 standardized combination of carvacrol and thymol. Carvacrol and thymol are essential oils from oregano and thyme extracts, which were found to enhance populations of beneficial gut bacteria and improve gut barrier morphology and function in rainbow trout, *O. mykiss* (Ahmadifar, Falahatkar & Akrami 2011; Giannenas, Triantafillou, Stavrakakis, Margaroni, Mavridis, Steiner & Karagoun 2012), channel catfish, *Ictalurus punctatus* (Zheng, Tan, Liu, Zhou, Xiang & Wang 2009) and carp, *Cyprinus carpio* (Mahmoud, Yamazaki, Miyashita, Il-Shik, Dong-Suk & Suzuki 2004). Mintrex® is a combination of Alimet® and metal basing on the ratio of 2:1, and is having high stability in feed and water. Previous studies found that Mintrex® Cu, Mn and Zn not only keep balance of the trace elements but also could keep normal growth, antioxidant ability and balance of amino acid in tutor, *Scophthalmus maximus* (Ma, Hou, Mai, Bharadwaj, Ji & Zhang 2014a,b). Recent study also found that Mintrex® trace minerals in broiler diets could increase intestinal integrity and improve immune response to *coccidiosis vaccination* (Richards, Dibner, Hampton, Wuelling & Wehmeyer 2006). Some substances in FCA have been used to improve the intestinal function in livestock (Richards *et al.* 2006; Michiels, Missotten, Fremaut, De Smet & Dierick 2007) and fish, but the effects of FCA on intestinal morphology and microbiota of Japanese seabass have not been investigated.

Japanese seabass, *Lateolabrax japonicus*, is a euryhaline fish that has been largely cultured in East Asia, especially in China (Islam, Ueno & Yamashita 2015). Studies of FM replacement have been widely conducted in seabass and have observed that low replacement level of FM protein (below 30%) did not significantly affect the growth performance and gut integrity (Laporte & Trushenski 2012; Li, Ai, Mai, Xu & Cheng 2012; Hu, Yun, Xue, Wang, Wu, Zheng & Han 2013). However, reports relating to the effects of dietary SBM and compound additives on intestinal morphology and microbiota of Japanese seabass have not been investigated. On the basis of those studies, this study was designed to explore whether fish fed the diet with low replacement level of FM protein and FCA supplementation might have better intestinal function compared to that in commercial feed. Also, the study was mainly purposed to evaluate whether FCA could partially suppress negative effects on intestinal morphology and bacterial community in Japanese seabass when fed diets with high FM protein substitution level.

Materials and methods

Experimental procedure

The major protein sources were FM and SBM, and the major lipid sources were fish oil and lecithin. The formulation of FCA was the mixture of antioxidant (0.02% Agrado®), immunopotentiator (0.006% NE-150®) and trace elements (0.0055% Mintrex® Cu, 0.01% Mintrex® Mn and 0.025% Mintrex® Zn) (Novus International, St Louis, MO, USA). Four isonitrogenous (45% crude protein) and isolipidic (11% crude lipid) diets including FM42 (the control group based on commercial feed without FCA), FM35 (15% FM protein

substitution level with FCA), FM21 (50% FM protein substitution level with FCA) and FM21-N (50% FM protein substitution level without FCA) were formulated (Table 1). Procedures and storage of experimental diets were similar with those described by Li *et al.* (2012).

The Japanese seabass were gained from a commercial farm in Qinzhou, China. Before the feeding trial, all seabass were raised in floating seawater cages (4 × 5.0 × 5.0 m), and were acclimated to adapt the experimental conditions through feeding commercial feed for 14 days. During the feeding trial, fish with homogenous sizes of 40 fish per cage (initial average weight 125.65 ± 0.60 g) were divided into four replicate floating cages (1.5 × 3.0 × 3.0 m) in seawater. All Japanese seabass from 16 cages were handfed to apparent satiation twice daily at 06:00 and 18:00 for 15 weeks respectively. The water temperature ranged from 15 to 26.5°C. The salinity was about 20‰ and dissolved oxygen was above 7 mg L^{-1}.

Table 1 Ingredient and proximate compositions of the experimental diets (% dry matter)

Ingredients	FM42	FM35	FM21-N	FM21
Fish meal*	42	35.5	21	21
Soybean meal*	12	21.8	30	30
Wheat*	25.8	21.9	22.1	22.1
Peanut meal*	10	10	10	10
Squid meal*	2.5	2.5	3.5	3.5
Spray blood meal*	0.0	0.0	2.8	2.8
Soy protein concentrate*	0.0	0.0	2	2
Lecithin	1	1	1	1
Fish oil	4	4.4	5.5	5.5
Vitamin and mineral premix†	1	1	1	1
Vitamin C	0.1	0.1	0.1	0.1
Calcium propionic acid	0.1	0.1	0.1	0.1
Choline	0.5	0.5	0.5	0.5
Monocalcium phosphate	1	1	1	1
MeraMet‡	0.0	0.08	0.26	0.26
Threonine‡	0.0	0.01	0.1	0.1
Ethoxyquin	0.02	0.02	0.02	0.02
Functional compound additives§	0.0	0.0665	0.0	0.0665
Proximate analysis				
Crude protein	45.22	44.7	45.06	44.95
Crude lipid	11.60	11.42	11.35	11.15
Lysine	2.74	2.63	2.66	2.68
Methionine	0.96	0.97	0.99	1.02

*Wheat (dry mater %): protein 16.50, lipid 1.76; fish meal (dry mater %): protein 63.90, lipid 10.15; squid meal (dry mater %): protein 56.67, lipid 28.85; peanut meal (dry mater %): protein 55.99, lipid 6.46; spray blood meal (dry mater %): protein 98.92, lipid 0.99; soybean meal (dry mater %): protein 54.51, lipid 1.48; soy protein concentrate (dry mater %): protein 73.32, lipid 1.04. Spray blood meal and soy protein concentrate were used for balancing protein content in diets. These ingredients were obtained from Great seven Bio-Tech (Qingdao, China)

†These vitamin and mineral premix were obtained from Great seven Bio-Tech.

‡MeraMet is methionine hydroxy calcium. Methionine and threonine were satisfied in all the treatments.

§The formulation of functional compound additives were 0.02% Agrado®, 0.0055% Mintrex® Cu, 0.01% Mintrex® Mn, 0.025% Mintrex® Zn and 0.006% NE-150® (combination of thymol and carvacrol).

Sampling

After the feeding experiment, all seabass were fasted for 24 h and anaesthetized before sampling. The samples for intestinal morphology analysis were collected (three fish from each cage). The sterile scalpel was used to cut about 1 cm of distal intestine. After cutting, physiological saline was used to rinse the guts in order to remove intestinal contents. Then, distal guts were fixed in phosphate-buffered 4% formalin solution. After 24 h, all distal guts were transferred into 70% ethanol until further analysis. The whole intestines of three seabass per cage were aseptically dissected; then, all intestinal contents were squeezed out and collected in sterile tubes under a sterile environment; and finally tubes were stored at −80°C for further analysis.

Distal intestinal morphology analysis

The distal intestinal micromorphology was determined based on the method described by Peng, Xu, Ai, Mai, Liufu and Zhang (2013). Briefly, the fixed distal guts were washed and dehydrated with gradient alcohol; then, the guts were paraffin-embedded, sectioned and stained with haematoxylin and eosin. Four morphological parameters including microvillus height (HMV), muscular thickness (MT), fold height (HF) and enterocyte height (HE) were measured by light microscopy (Olympus, DP72, Tokyo, Japan). Microvillus height was determined from bottom to tip of microvillus (10 random measured values of a seabass, 12 seabass per group). Fold height was determined from bottom to tip of fold (10 random measured values of a seabass, 12 seabass per group). Muscular thickness was determined from the bottom of serosa to the top of submucosa (10 random measured values of a seabass, 12 seabass per group).

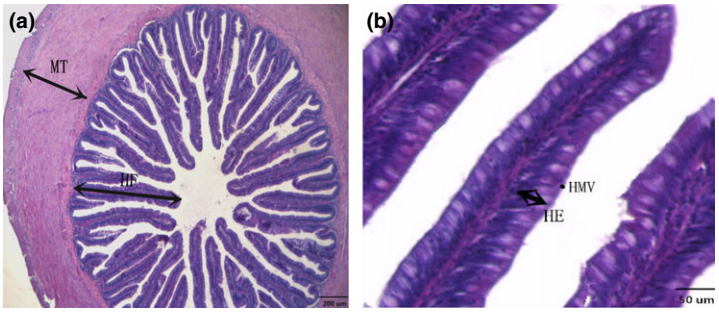

Figure 1 (a and b) Transversal section photomicrographs of Japanese seabass in distal gut. Intestinal section from fish fed FM42. (a) Fold height and muscular thickness were analyzed using a light microscope (magnification ×40). (b) Enterocytes height and microvilli height were analyzed in a higher objective lens of light microscope (magnification ×200). HE, enterocyte height; HF, fold height; HMV, microvillus height; MT, muscular thickness (hematoxylin and eosin). [Colour figure can be viewed at wileyonlinelibrary.com].

Enterocyte height was determined from bottom to tip of enterocyte (10 random measured values of a seabass, 12 seabass per group). The light microscope was used to analyze the HF, HMV, HE and MT (Fig. 1a and b). Three parameters about intestinal enteritis were evaluated semi-quantitatively according to the method described by Laporte and Trushenski (2012). Three enteritis parameters included: (i) size of the submucosa; (ii) width of lamina propria; (iii) the appearance of the mucosal folds. Each of the parameters was marked by a gradient from i to iv, where iv indicated the most obvious signs of intestinal enteritis (Table 2).

Table 2 Description of the histological scoring system used to evaluate the morphological status of gut in Japanese seabass

Parameter	Score	Appearance
Size of the submucosa	i	Thin layer of connective tissue between muscularity and fold
	ii	Slightly increasing number of connective tissue under mucosal folds
	iii	Clear increase in connective tissue under most mucosal folds
	v	Thick layer of connective tissue under many folds
	iv	Largest submucosa under many folds
Width of lamina propria	i	Thin and delicate core of connective tissue in most folds
	ii	The lamina propria slightly shows in many folds
	iii	Thick lamina propria largely appear and increase in many folds
	v	Lmina propria appears large and thick in most of the mucosal folds
	iv	Larger lamina propria with thick areas in all folds
The appearance of the mucosal folds	i	Long and thin folds
	ii	Some folds bloating and shrinkage
	iii	Diffused shrinkage and start of tissue disruption
	v	Large amount of tissue disruption
	iv	Total tissue disruption

The method was according to Laporte and Trushenski (2012) with slight modification.

Intestinal microflora analysis

DNA extraction and purification

DNA was extracted according to a method from a QIAamp® DNA Stool Mini Kit (Qiagen, Hilden, Germany). Then, E-Spect ES-2 (Malcom, Tokyo, Japan) was used to analyze the DNA purity and concentration. The extracted DNA was stored at −20°C for further analysis.

PCR amplification and pyrosequencing

The PCR from intestinal samples was conducted to generate small fragments of the 16S-rRNA genes (420 bp, covering the V4 hypervariable regions). The common microbacillary primers 515F (5′-GTGCCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′) were adjusted by adding ligation adapters. Then, the PCR was operated in duplicate with a 20 μL reaction volume which included 2 μL 10× Ex Taq buffer, 1.25 U DNA polymerase (Takara Ex Taq), 1 μL BSA (10 mg mL^{-1}) and DNA template, forward and reverse primers, 1.6 μL dNTP mixture (TransGen Biotech., Beijing, China) and sterile water. The reaction conditions of PCR included initial denaturing step at 95°C for 5 min, followed by 30 cycles, where each cycle for 30 s at 94°C, 30 s at 56°C (annealing) and 30 s at 72°C (extension) and final extension of

10 min at 72°C. The PCR product was removed on agarose gels and purified with a gel extraction Kit (Qiagen). The pyrosequencing was operated at BGI Tech in Wuhan, China. The sequencing platform was Illumina Miseq.

Statistical and bioinformatics analysis

The intestinal morphological data were analyzed by one-way analysis of variance (ANOVA) where using SPSS 17.0. Differences between the means were tested by Tukey's multiple range tests. Differences were regarded as significant when $P < 0.05$. The intestinal morphological results were presented as means ± SD (Standard deviation).

The software Mothur (v1.31.2) was used to perform the operational taxonomical unit (OTU) and phylotype analysis. The corresponding rarefaction curve was drawn by software *R* (v3.0.3). The principal component analysis (PCA) of the fast UniFrac metric matrix was used to compare the microflora according to phylogenetic information (Lozupone, Hamady & Knight 2006). Heatmap figures were produced with the *R* project for statistical computing. Alpha diversity was applied for analyzing the complexity of species diversity among samples using OTUs, ACE, Chao, Shannon and Simpson indices. Kruskal–Wallis test was used for comparison among groups. Differences were regarded as significant when $P < 0.05$ and the results were presented as means ± SD (Standard deviation).

Result

Distal intestinal morphology analysis

One-way ANOVA analysis showed that FM substitution level significantly affects the morphological changes of distal intestine. The HF, HMV and MT of distal intestine showed a decreasing trend with the increase in FM substitution level. There was no significant difference in HF of distal intestinal between seabass fed the control group (FM42) and FM35. However, fish fed the control group and FM35 had significantly higher HF than that in seabass fed FM21 and FM21-N ($P < 0.05$). Although no significant differences in distal intestinal HMV and MT were found between fish fed FM21 and FM21-N, the data in fish fed FCA supplemented diet were slightly higher than those in fish fed diet without FCA. No significant difference was found in HE of distal intestinal among dietary treatments ($P > 0.05$) (Table 3). Moreover, when 50% FM protein was replaced, fish fed diet FM21-N had more pathological changes such as tissue disruption, the increase in lamina propria width and reduction in mucosal HF and number than those in seabass fed diet FM21. Fish fed the control group and FM35 appeared unobvious symptoms of enteritis (Table 4; Fig. 2).

Intestinal microbiological analysis

After filtering the low-quality reads, 1 820 377 effective tags were collected from 16 samples. All the matching tags were delineated into operational taxonomic units (OTUs). The different tagging similarity values were about 97%. All samples produced 3050 OTUs including 669 singletons OTUs and 2381 no-singletons OTUs. After removing the singletons, each sample contained 175–883 OTUs; and the statistical result of OTUs is shown in Table 6. All OTUs were classified from genus to phylum using default setting of the program Mothur. The rarefaction curves trended to close to the saturation plateau. Good's coverage estimations indicated that up to 97% of microorganism were gained from 16 samples (Fig. 3). In this study, relative reading abundance of bacterial phyla and genus within the different samples

Table 3 Effects of dietary fish meal protein partially replaced by soybean meal on micromorphology of the intestine in Japanese sea bass with supplementation of functional compound additives

	Group			
Index	**FM42**	**FM35**	**FM21-N**	**FM21**
Enterocyte height (μm)	26.06 ± 4.06	26.62 ± 3.74	26.40 ± 4.18	24.68 ± 5.96
Fold height (μm)	732.88 ± 72.46^{a}	724.42 ± 60.57^{a}	487.17 ± 31.96^{b}	554.76 ± 70.60^{b}
Microvillus height (μm)	2.09 ± 0.20^{a}	1.90 ± 0.28^{ab}	1.38 ± 0.16^{b}	1.80 ± 0.38^{ab}
Muscular thickness (μm)	325.36 ± 35.91^{a}	268.72 ± 25.04^{ab}	202.88 ± 51.08^{b}	261.37 ± 50.00^{ab}

Values are represented as means ± SD ($n = 4$); values in the same row with the same superscripts are not significantly different ($P > 0.05$).

Table 4 Histological measurements and scores for the different parameters used to assess the degree of enteritis developed by Japanese seabass fed four feeds

	FM42					FM35					FM21-N					FM21				
Quantitative measurements	**Frequency of scores (%)**					**Frequency of scores (%)**					**Frequency of scores (%)**					**Frequency of scores (%)**				
Semiquantitative evaluation*	i	ii	iii	v	iv	i	ii	iii	v	iv	i	ii	iii	v	iv	i	ii	iii	v	iv
Size of the submucosa	91.7	8.3	–	–	–	100	–	–	–	–	–	–	–	25	75	–	–	58.3	25	16.7
Width of lamina propria	83.4	8.3	8.3	–	–	91.7	8.3	–	–	–	–	–	8.3	25	66.7	–	–	50	33.3	16.7
The appearance of the mucosal folds	91.7	8.3	–	–	–	75.1	16.6	8.3	–	–	–	–	8.3	33.3	58.3	–	8.3	33.3	33.3	25

*Frequencies are based on composite scores (based on multiple images and views) for 12 fish per group.

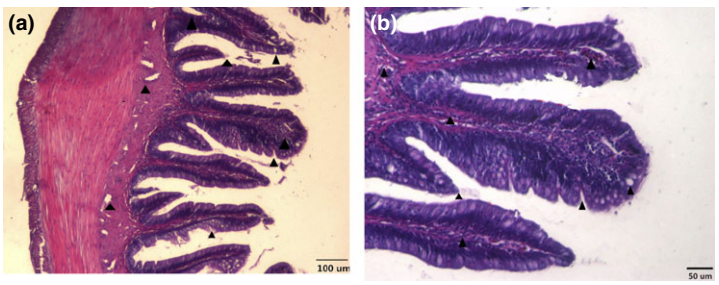

Figure 2 (a and b) Transversal section photomicrographs of Japanese seabass in distal gut. Intestinal section from fish fed FM21-N. (a) Small arrow point shows that broken fold and abnormal submucosa were observed in a light microscope (magnification ×100). (b) Small arrow point indicates severe enlargement of enteritis parameters in fish fed FM21-N (magnification ×200). [Colour figure can be viewed at wileyonlinelibrary.com].

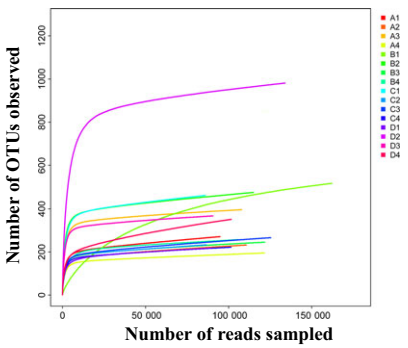

Figure 3 Rarefaction analysis of the different samples. Rarefaction curves of OUTs clustered at 97% sequence identity among samples. A = FM42, B = FM35, C = FM21-N, D = FM21. [Colour figure can be viewed at wileyonlinelibrary.com].

included 21 different phyla and 39 different genera (ratio >0.5%).

The intestinal content samples were dominated by six major phyla, representing above 90% of the sequences, including *Proteobacteria* (FM42, 82.87%; FM35, 75.76%; FM21-N, 81.46%; FM21, 69.2%), *Bacteroidetes* (FM42, 6.44%; FM35, 6.89.%; FM21-N, 6.91%; FM21, 10.76%), *Firmicutes* (FM42, 2.74%; FM35, 2.43%; FM21-N, 4.31%; FM21, 6.88%), *Spirochaetes* (FM42, 2.25%; FM35, 0.03%; FM21-N, 0.15%; FM21, 0.06%), *Acidobacteria* (FM42, 1.24%; FM35, 2.15%; FM21-N, 1.07%; FM21, 3.2%), *Actinobacteria* (FM42, 0.36%; FM35, 0.39%; FM21-N, 2.1%; FM21, 1.75%). There were no significant differences in the relative abundance of major bacterial phyla among dietary treatments ($P > 0.05$; Kruskal–Wallis) (Table 5).

Community richness and Alpha indices (OTUs, ACE, Chao, Shannon and Simpson) were showed in Table 6. There were no significant differences in the diversity and the richness of microflora in the intestinal content among dietary treatments, as evaluated with the Alpha indices ($P > 0.05$). At the phylum and genus levels except group B1, the 16 samples showed highly similarity of relative abundance. The group B1 (the first cage of FM35) showed relatively simple species compared with the other three groups in FM35 causing the abnormal elevate in *Spirochaetes* belonging to the

Table 5 Main gut bacterial composition at phylum level per group (%)

	Group			
Phylum	**FM42**	**FM35**	**FM21-N**	**FM21**
Proteobacteria	82.87 ± 9.28	75.76 ± 6.61	81.46 ± 7.42	69.20 ± 12.12
Bacteroidetes	6.44 ± 1.51	6.89 ± 2.36	6.91 ± 0.67	10.76 ± 9.01
Firmicutes	2.74 ± 1.03	2.43 ± 0.20	4.31 ± 1.04	6.88 ± 1.17
Spirochaetes	2.25 ± 3.79	0.03 ± 0.03	0.15 ± 0.23	0.06 ± 0.05
Acidobacteria	1.24 ± 2.45	2.15 ± 1.99	1.07 ± 1.82	3.20 ± 3.90
Actinobacteria	0.36 ± 0.23	0.39 ± 0.31	2.10 ± 2.02	1.75 ± 1.54
Others	4.10 ± 2.57	12.34 ± 3.54	4.01 ± 2.56	8.14 ± 4.21

Values are represented as means ± SD ($n = 4$); values in the same row with the same superscripts are not significantly different ($P > 0.05$).

Table 6 Number of sequences analyzed, observed diversity richness (OTUs), estimated OTU richness (ACE and Chao) and diversity index (Shannon and Simpson) for 16S rRNA libraries of four groups*

Alpha	**FM42**	**FM35**	**FM21-N**	**FM21**	***P*-value**
OTUs†	273 ± 87	374 ± 143	295 ± 112	479 ± 341	0.5801
Chao‡	534 ± 109	692 ± 269	759 ± 408	701 ± 378	0.8770
Ace‡	894 ± 432	722 ± 361	1110 ± 447	843 ± 392	0.5439
Shannon‡	2.43 ± 0.48	2.15 ± 1.26	2.51 ± 0.50	3.02 ± 1.22	0.8137
Simpson‡	0.28 ± 0.07	0.39 ± 0.34	0.28 ± 0.08	0.24 ± 0.14	0.9363

*Values are presented as means ± SD ($n = 4$). Values in the same row with the same superscripts are not significantly different ($P > 0.05$).
†The operational taxonomic units (OTU) were defined at the 97% similarity level.
‡The richness estimators (ACE and Chao) and diversity indices (Shannon and Simpson) were generated with Mothur program.

normal deviation, which was ignored in statistical process (Fig. 4a). Hence, the analysis of relative read abundance showed high similarity of intestinal microflora in different groups (Fig. 4b).

The score plot of PCA indicated that most of the samples harboured characteristic bacterial communities and grouped to the left of the plot close to PC1, which made up 34.55% of the total variations. The two PCA axes showed 50.68% of the variation between the two different communities (Fig. 5). At genus level, hierarchically clustered heatmap analysis according to the profiles indicated that gut bacterial community of the same species of fish harboured the same features even if fed different diets. The composition of gut microflora of 16 samples shared high similarity except group B1 (the first cage of FM35) (Fig. 6).

Discussion

The HF, HMV and MT of distal intestine in Japanese seabass showed a decreasing trend with the increase in FM protein substitution level, which was consistent with previous studies in Atlantic salmon, *Salmo salar*, and Japanese flounder, *Paralichthys olivaceus* (Baeverfjord & Krogdahl 1996; Chen, Ai, Mai, Xu, Liufu, Zhang & Cai 2011). These results of morphological parameters indicated that high SBM level might have negative effects on distal intestinal structure due to antinutritional factors such as saponin (Chen *et al.* 2011) or lectin (Peng *et al.* 2013) in SBM. It was reported that lectin combining with polysaccharides on intestinal epithelial cell surface could damage gut microvillus, which might result in poor absorption and nutrient digestion in Atlantic salmon, *S. salar*, and Rainbow trout, *O. mykiss* (Buttle, Burrells, Good, Williams, Southgate & Burrells 2001). Moreover, when 50% FM protein was replaced, the data in fish fed FCA supplemented diet showed slightly higher HMV and MT than those in fish fed diet without FCA, which indicated supplementation of FCA might partially eliminate the negative effects on intestinal morphology of Japanese seabass. This might be deduced that some substances in FCA might improve gut barrier structure and function of seabass (Mahmoud *et al.* 2004; Zheng *et al.* 2009). However, no significant differences were observed in HE among groups, which was not consistent with the result in turbot, *S. maximus* (Peng *et al.* 2013). This could possibly be attributed to that different morphological parameters might respond differently to different feed composition and fish species.

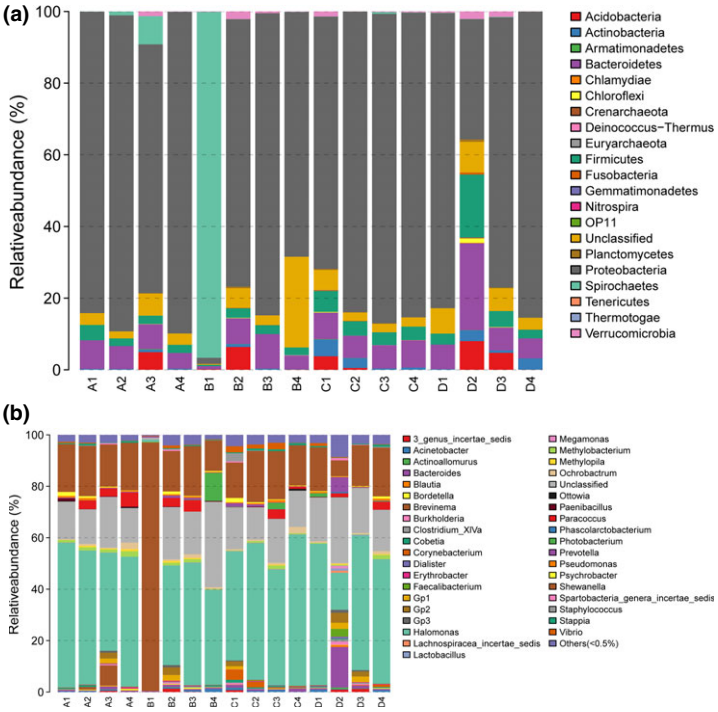

Figure 4 (a and b) Relative abundance of gut bacterial composition at phylum and genus level per group (%). (a) Twenty-one different phyla were found in 16 samples. (b) There were 39 different kinds of genera in gut bacterial of Japanese seabass. A = FM42, B = FM35, C = FM21-N, D = FM21. [Colour figure can be viewed at wileyonlinelibrary.com].

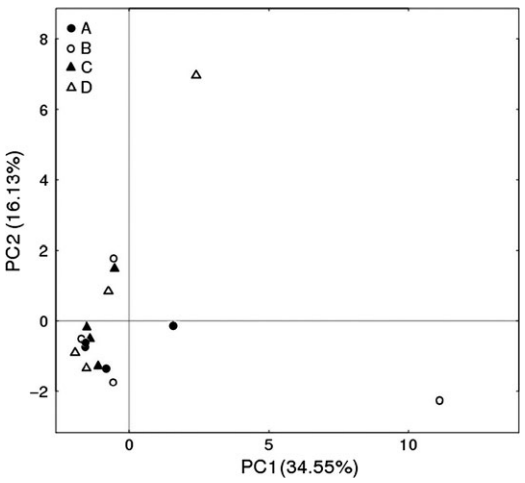

Figure 5 Principal coordinates analysis (PCA): scatter plot of PCA indicating variance of fingerprints derived from different microflora. Main components 1 and 2 were 34.55% and 16.13% of the variance. A = FM42, B = FM35, C = FM21-N, D = FM21.

Similarly, when 50% FM protein was replaced, fish fed the diet without FCA had more intestinal pathological changes than those in seabass fed the diet with FCA, whereas other two groups appeared unobvious symptoms of enteritis, which further indicated that fish fed high SBM level had negative effects on intestine and FCA might partially suppress the negative effects. These results of enteritis in high SBM level were parallel with previous studies in rainbow trout, *O. mykiss* (Romarheim, Skrede, Penn, Mydland, Krogdahl & Storebakken 2008), and salmonids, *S. salar* (Baeverfjord & Krogdahl 1996). NE-150® was widely reported to enhance populations of beneficial gut bacteria, improve gut barrier and reduce enteritis (Ahmadifar *et al.* 2011; Giannenas *et al.* 2012). Similarly, Mintrex® trace elements had great effects on improving fish physiological functions (Ma *et al.* 2014a,b). Therefore, it might be deduced that when fish fed the diets with FCA supplementation, these additives might be one reason of alleviating SBM-induced negative effects on intestinal pathological changes. However, Laporte and Trushenski (2012) found that sunshine bass could tolerate high SBM content with respect to the balance of intestinal function and integrity. This discrepancy might be due to different fish species, feed composition and living conditions.

Recent studies had showed that gut flora plays an important role in growth performance, defencing

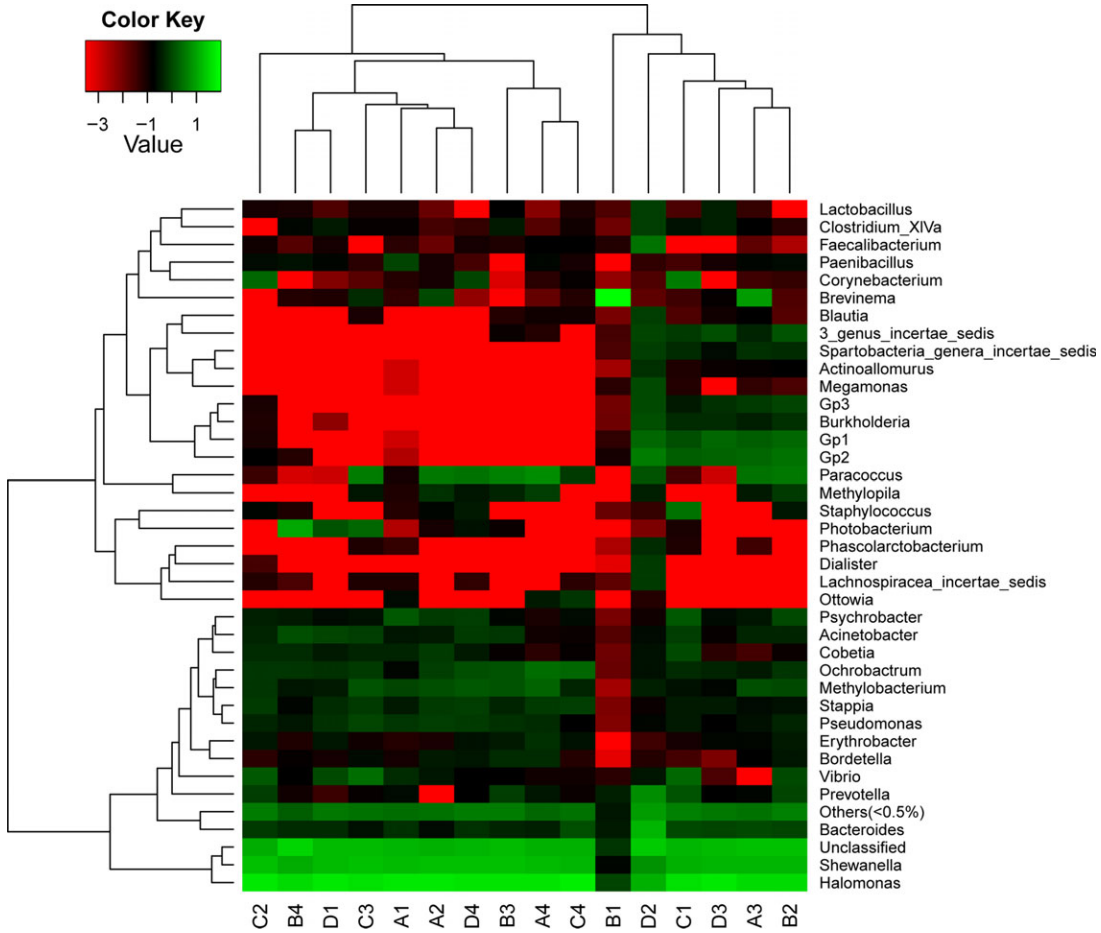

Figure 6 Bacterial distribution of genus level among 16 samples. Rows depict the 39 main bacteria at genus level, columns depict 16 samples. At the top right corner of picture, the colour intensity with the legend indicates square-root-transformed values of bacteria at genus level. The bacteric phylogenetic trees were indicated using neighbour-joining method and the relation among different samples was shown by Bray distance. A = FM42, B = FM35, C = FM21-N, D = FM21. [Colour figure can be viewed at wileyonlinelibrary.com].

against pathogens and keeping normal intestinal function of animals (Sekirov *et al.* 2010). However, studies about the importance of gut microflora to the gut morphology and the health of aquatic animals were still poorly reported (Li, Long, Gatesoupe, Zhang, Li & Gong 2015). The research about the effects of feeds and additives on the composition of intestinal bacterial community in Japanese seabass was also limited.

In this study, samples from the intestinal contents were analyzed using pyrosequencing of V4 region of the 16S rRNA gene. The intestinal microflora analysis indicated that no significant differences were found in the relative abundance of major bacterial phyla among dietary treatments. Moreover, the most abundant bacteria in the gut of Japanese seabass was *Proteobacter* at phylum level, which agreed with previous studies based on both culture-dependent and culture-independent methods (Kim, Brunt & Austin 2007; Desai, Links, Collins, Mansfield, Drew, Van Kessel & Hill 2012). Many animals including fish took *Proteobacteria* as the most dominant member of intestinal bacteria (Li *et al.* 2015). However, the results of dominant bacteria were not consistent with the previous studies which showed that *Frimicutes* and *Actinobacteria* were the most dominant phyla in the intestinal contents in grass carp, *Ctenopharyngodon idellus* (Zhou, Chen, Zhang & Chen 1998), yellow catfish, *Pelteobagrus fulvidraco* (Wu *et al.* 2010) and zebra-fish, *Brachydanio rerio* (Roeselers,Mittge, Stephens, Parichy, Cavanaugh, Guillemin & Rawls

2011). Previous studies found that feed had a great effect on the composition of gut microbiota and the same feed could result in similar intestinal bacteria in grass carp, *Ctenopharyngodon idellus* (Wu, Wang, Angert, Wang, Li & Zou 2012), and rainbow trout, *O. mykiss* (Desai *et al.* 2012). However, the composition and dominant bacteria of intestinal microflora were not affected by SBM level or FCA in this study. The original members of gut microflora harboured in the gut and co-evolved with the hosts significantly responding the living conditions, the feed and fish species (Navarrete, Magne, Araneda, Fuentes, Barros, Opazo & Romero 2012; Li *et al.* 2015). Previous study in rainbow trout, *O. mykiss*, also revealed that the microflora from intestinal contents was similar with those from living conditions (Merrifield, Dimitroglou, Bradley, Baker & Davies 2009). Therefore, the incongruence of the dominant phyla in the intestinal bacteria might be due to different fish species and habitat.

Although the significant differences were found in intestinal morphological changes, no significant differences were observed in the diversity and richness indices of microbial community among groups in this study, which was consistent with the previous study in juvenile allogynogenetic silver crucian carp, *Carassius auratus gibelio* (Cai, Wang, Ye, Krogdahl, Wang, Xia & Yang 2012), but was inconsistent with the report in rainbow trout, *O. mykiss* (Merrifield *et al.* 2009). The reasons for discrepancy might attributed to be that the diversity and richness of intestinal microflora were influenced by exogenous and endogenous factors of aquatic animals such as feed composition, intestinal morphology, fish stage, phylogenetic position and living conditions (e.g. water temperature and salinity) (Sullam, Essinger, Lozupone, O'Connor, Rosen, Knight & Russell 2012). In this study, the living environment and the host itself might be the major influential factors of richness and diversity of intestinal microflora in Japanese seabass.

Similarly, the PCA score plot and hierarchically clustered heatmap analysis according to the microflora profiles at genus level supported the hypothesis that the intestinal microflora of the same species of fish harboured the same features even if they were fed different feeds. The composition of intestinal microbiota among dietary treatments shared high similarity, which might reveal that the local environment and host itself might play more important role in the composition of microbial communities in this study. However, as showed in the clustering heatmap analysis, even if seabass fed the same feed and lived in same habitat, the differences in the seabass intestinal microflora of different samples were still existed, showing the impossibility to make generalizations of the microbial diversity from different intestinal samples (Heikkinen *et al.* 2006). These results might prove that the gut microflora composition of fish might be not only the reflection of the microbiome in fish species or local living conditions, but also result from selective pressures within the intestine or individual variances, depending on species-specific behaviour or metabolism (Larsen, Mohammed & Arias 2014).

In conclusion, results of this study showed that: (i) FCA supplementation might partially eliminate SBM-induced enteritis; and (ii) the composition and relative abundance of intestine microbiota in Japanese seabass were not influenced by SBM level or FCA

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