



Original Research Article

Soybean β -conglycinin and glycinin reduced growth performance and the intestinal immune defense and altered microbiome in juvenile pearl gentian groupers *Epinephelus fuscoguttatus*♀ \times *Epinephelus lanceolatus*♂

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ABSTRACT

The utilization efficiency of soy protein is affected by its 2 anti-nutritional substances—the antigens β -conglycinin and glycinin. This study investigated their effects on the growth performance, intestinal immune defense, and microbiome in juvenile pearl gentian groupers (*Epinephelus fuscoguttatus*♀ \times *Epinephelus lanceolatus*♂). Three isonitrogenous and isolipidic diets were formulated containing fishmeal supplemented with 70 g/kg β -conglycinin or 100 g/kg glycinin, or no supplementation (control). Each experimental diet was fed to quadruplicate groups with 30 fish in each tank for 8 weeks. Dietary inclusion of either β -conglycinin or glycinin significantly reduced weight gain and specific growth rates, and cell proliferation of the distal intestine. Histological evaluation of the intestine tract revealed the inflammation signs, characterized by reducing of plica height and width as well as the number of the goblet cells, and widening of the lamina propria. The group fed the β -conglycinin diet had reduced lysozyme activity, contents of immunoglobulin M and complements 3 and 4. Increased activities of caspase-3 and -9 were observed in the group fed the β -conglycinin diet compared to the other 2 groups. In the intestinal microbiota, the relative abundances of the potentially pathogenic genera *Photobacterium* and *Vibrio* were significantly higher in the glycinin group than those in others. Therefore, the existence of soybean antigens (β -conglycinin or glycinin) could damage the structural integrity of the intestine, reduce immune defense, reshape the intestinal microbiome and, ultimately, impair growth in fish.

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1. Introduction

Soybean meal is a rich protein source for use in fish feeding (Lilleeng et al., 2007). However, more soybean meal in feed decreased growth in juvenile carnivorous fish such as cobia (*Rachycentron*

canadum; Chou et al., 2004), silvery-black porgy (*Sparidentex hasta*; Yaghoubi et al., 2016), and turbot (*Scophthalmus maximus* L.; Liu et al., 2018). These adverse effects are related to the presence of anti-nutritional factors in soybean meal, especially β -conglycinin (7S) and glycinin (11S) (Buttle et al., 2001). These 2 antigenic proteins accounting for 70% to 80% of the total protein in soybean are considered the major cause of reduced growth and increased intestinal disease in animals. Fish fed 4% to 8% of dietary 7S and/or 11S could damage the structural integrity of the intestine, reduce the immune function and lead to a decline in growth performance in different fish species, such as Jian carp (*Cyprinus carpio* var. Jian; Zhang et al., 2013; Jiang et al., 2015b), juvenile golden crucian carp (*Carassius auratus*; Li et al., 2019a; 2019b), *Rhynchocypris lagowski* (Li et al., 2020; Zhu et al.,

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2021), turbot (Li et al., 2017), and grass carp (*Ctenopharyngodon idella*; Duan et al., 2019a; Zhang et al., 2019).

Microbiota located in the intestine takes on an important role in regulating immune responses and forming a defensive barrier against invading pathogens in fish (Nayak, 2010; Ingerslev et al., 2014); therefore, fish health and well-being require a healthy gut microbiome. Many factors, including species, environmental conditions, growth phase, and diet, can regulate the intestinal microbiome (Nayak, 2010). High-throughput sequencing technique has been used to prove that inflammatory bowel disease might be closely related to dysbiosis of the intestinal microbiota (Ridaura et al., 2013). Although diet is an essential influence on the intestinal microbiome (Kim and Kim, 2013), the effects of feed on intestinal health remain unclear. The effects of soybean meal on fish gut microbiome have been studied previously (Ringø et al., 2006; Miao et al., 2018); however, few studies have reported the influences of soy antigen protein on gut microbiome, and the correlation between gut microbes and immune functions has not been well documented.

The pearl gentian grouper (*Epinephelus fuscoguttatus*♀ × *Epinephelus lanceolatus*♂), mainly farmed in China, is a carnivorous fish species in aquaculture with experienced increased market demand in recent years (Jiang et al., 2015a; He et al., 2020a). Our previous study reported that replacing 50% of the fishmeal in pearl gentian grouper feed with high-soybean meal reduced growth performance and caused enteritis (He et al., 2020b). This study aims to examine the adverse influences of both 7S and 11S on growth, intestinal immune function, and microbiome in this species. The results of this study help to offer insights into the potential to use high soybean meal in carnivorous fish feeds.

2. Materials and methods

2.1. Ethical statement for animal experiments

All hybrid groupers and their caretaking procedures conformed to the NIH guidelines (NIH Pub. No. 85 to 23, revised 1996), and were approved by the Institutional Animal Care and Use Committee of Guangdong Ocean University (Zhanjiang, China).

2.2. Diets

The protein profile of the 7S (90.08%) and 11S (79.97%) (China Agricultural University, patent no. 200410029589.4, China) fractions was presented by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis (Fig. 1). It is well-known that 7S and 11S account for approximately 30% and 40% of the total soybean proteins, respectively (Utsumi et al., 1997). Our previous study reported that 37.1% soybean meal (crude protein 50%, approximately 6.2% 7S or 9.3% 11S) in the diet significantly reduced growth and impaired the intestinal integrity of pearl gentian grouper juveniles (He et al., 2020b). Seven percent of 7S and 10% of 11S were added to the basal diet (48% crude protein, 11% crude lipid), respectively (Table 1). The 3 isonitrogenous and isolipidic test feeds are named FM (control), 7S, and 11S, respectively. Procedures for experimental feed preparation and storage method were the same as those described previously (He et al., 2020a).

2.3. Feeding trial

Juvenile pearl gentian groupers were purchased from a local hatchery (Zhanjiang, China). Groupers (mean initial body weight = 8.50 ± 0.01 g) were randomly distributed into 3 groups in quadruplicate with 30 fish per tank. Fish were fed until apparent

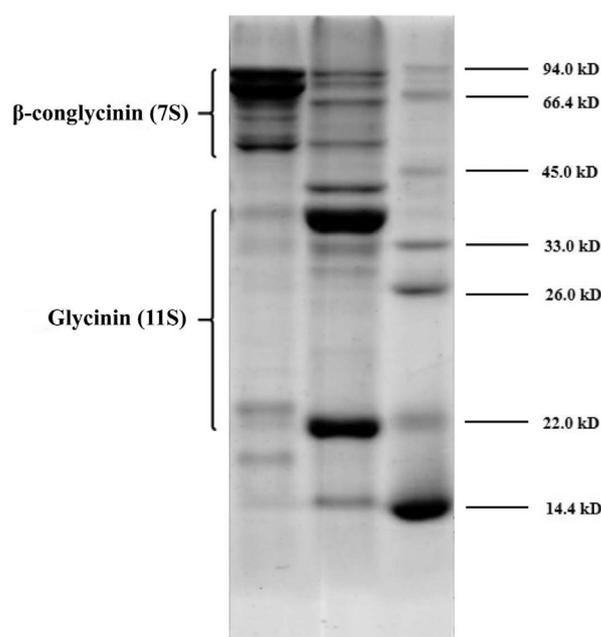


Fig. 1. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis patterns of the isolated β -conglycinin (7S) and glycinin (11S) fractions. Lane 1: 7S fraction; lane 2: 11S fraction; lane 3: protein marker.

Table 1

Formulation and nutrient composition of the experimental diets (DM basis, %).

Item	FM (control)	β -Conglycinin (7S)	Glycinin (11S)
Ingredients			
Brown fishmeal	40.00	40.00	40.00
Casein	11.20	6.00	4.00
Gelatin	2.80	1.50	1.00
β -Conglycinin	0.00	7.00	0.00
Glycinin	0.00	0.00	10.00
Wheat flour	20.00	20.00	20.00
Corn starch	13.13	13.26	12.69
Fish oil	5.00	5.00	5.00
Vitamin premix ¹	0.20	0.20	0.20
Mineral premix ¹	0.50	0.50	0.50
L-Lysine	0.06	0.00	0.11
L-Methionine	0.32	0.43	0.39
L-Arginine	1.69	1.24	1.22
L-Leucine	0.42	0.19	0.21
Others ²	4.68	4.68	4.68
Total	100.00	100.00	100.00
Nutrient levels³			
Lysine	3.30	3.21	3.21
Methionine	1.34	1.28	1.31
Arginine	3.68	3.46	3.79
Leucine	3.75	3.53	3.68
Crude protein	48.78	47.66	48.21
Crude lipid	11.36	11.00	11.03
Ash	8.00	8.13	8.38
Moisture	7.51	7.35	7.55

¹ The vitamin and mineral premixes were purchased from Qingdao Master Biotech Co., Ltd (Qingdao, China).

² Others: soybean lecithin, 1.50%; soybean oil, 1.50%; calcium monophosphate, 1.00%; ethoxyquin, 0.03%; vitamin C (35%), 0.05%; attractant, 0.10%; choline chloride, 0.50%.

³ All the nutrient levels were measured values.

satiation twice daily at 08:00 and 17:00 for 8 weeks. The water quality of all tanks was controlled within the following ranges: temperature from 28 to 30 °C, ammonia nitrogen < 0.03 mg/L and dissolved oxygen > 5 mg/L.

2.4. Sample collection

After 8 weeks of feeding experiment, all pearl gentian groupers were fasted for 24 h, then all fish per tank were counted and weighed for growth performance analysis. The livers and intestines of 3 fish per tank were collected quickly, then weighed and measured the intestinal length. The proximal intestine (PI), mid intestine (MI), and distal intestine (DI) of another 2 fish per tank were stripped and immediately transferred to 4% paraformaldehyde solution for histological study with hematoxylin-eosin (H&E) staining. The intestinal tracts of 2 fish from each tank were collected, homogenized, and then centrifuged (3,000 × g, 10 min) at 4 °C for enzyme assay. The DI of 2 fish per tank were merged into one sample and then taken for microbiotic analysis.

2.5. Intestinal morphology

The fixed PI, MI, and DI samples from each tank were dehydrated. After being embedded in paraffin, thin sections (3 μm) of intestine were cut, stained with H&E, and then observed using an optical microscope (ECLIPSE 90i, Nikon, Japan). Ten measurements of muscle layer thickness (MLT), plica width (PW), and plica height (PH) in each slice were performed using image acquisition software (NIS Elements version 4.60, Nikon, Japan).

Table 2
Growth performance of fish fed the experimental diets (n = 4).

Item	FM (control)	7S	11S	P-value
FBW	50.83 ± 0.55 ^b	46.47 ± 0.63 ^a	45.31 ± 1.66 ^a	0.019
WGR ¹ , %	497.63 ± 6.49 ^b	446.86 ± 7.21 ^a	432.69 ± 19.47 ^a	0.019
SGR ² , %/d	3.19 ± 0.02 ^b	3.03 ± 0.02 ^a	2.98 ± 0.06 ^a	0.023
PER ³	2.44 ± 0.11	2.05 ± 0.14	2.12 ± 0.08	0.100
FCR ⁴	0.84 ± 0.04 ^a	1.03 ± 0.07 ^b	0.98 ± 0.04 ^{ab}	0.049
FI ⁵ , % BW/d	2.11 ± 0.08	2.35 ± 0.02	2.33 ± 0.07	0.070
SR ⁶ , %	97.50 ± 1.60	97.50 ± 0.83	98.34 ± 0.96	0.849

FM = fishmeal; 7S = β-conglycinin; 11S = glycinin; FBW = final body weight.
^{a, b}Different superscript letters in each row indicate significant difference (P < 0.05).
¹ Weight gain rate (WGR) = 100 × (Final body weight – Initial body weight)/Initial body weight.
² Specific growth rate (SGR) = 100 × [ln (Final body weight) – ln (Initial body weight)]/Days.
³ Protein efficiency ratio (PER) = Wet weight gain/Total protein intake.
⁴ Survival rate (FCR) = Total diet intake/Total wet weight gain.
⁵ Feed intake (FI) = 100 × Total diet intake/[(Initial body weight/Final body weight)/2 × Days].
⁶ Survival rate (SR) = 100 × The final fish number/The initial fish number.

Table 3
Morphologic indexes of fish fed the experimental diets (n = 4).

Item	FM (control)	7S	11S	P-value
CF ¹ , g/cm ³	3.23 ± 0.07	3.41 ± 0.10	3.55 ± 0.17	0.189
VSI ² , %	8.85 ± 0.26	9.52 ± 0.30	9.13 ± 0.35	0.309
ISI ³ , %	2.96 ± 0.17	3.03 ± 0.13	2.77 ± 0.17	0.487
HIS ⁴ , %	0.72 ± 0.04	0.80 ± 0.05	0.77 ± 0.05	0.433
ILI ⁵ , %	116.63 ± 2.70 ^a	126.63 ± 3.29 ^b	117.01 ± 3.66 ^a	0.048

FM = fishmeal; 7S = β-conglycinin; 11S = glycinin.
^{a, b}Different superscript letters in each row indicate significant difference (P < 0.05).
¹ Condition factor (CF) = 100 × Body wet weight (g)/Body length (cm)³.
² Viscerosomatic index (VSI) = 100 × Viscerosomatic wet weight (g)/Body wet weight (g).
³ Intestinal somatic index (ISI) = 100 × Intestinal somatic wet weight (g)/Body wet weight (g).
⁴ Hepatosomatic index (HSI) = 100 × Liver wet weight (g)/Body wet weight (g).
⁵ Intestinal length index (ILI) = 100 × Intestine length (cm)/Total body length (cm).

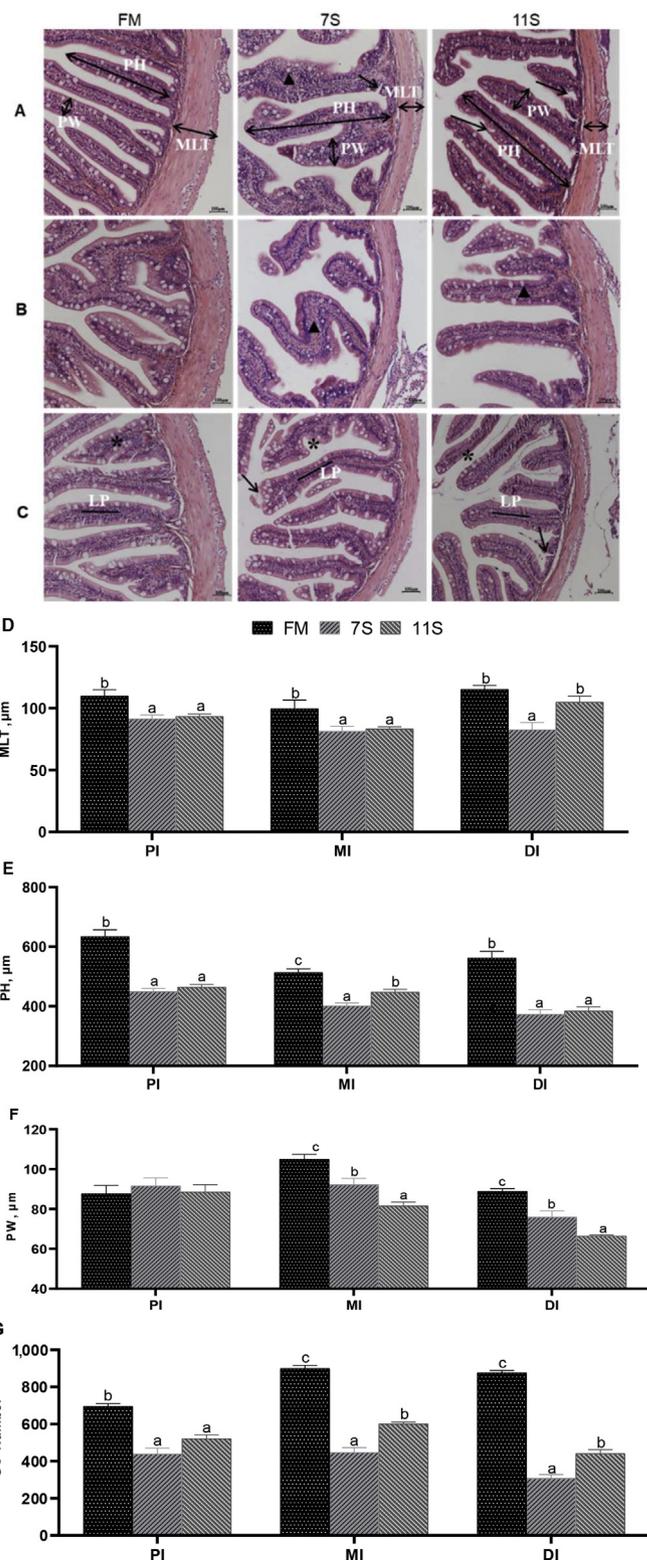


Fig. 2. Histological examinations of the proximal intestine (PI, A), mid intestine (MI, B), and distal intestine (DI, C) in fish fed differential experiment diets for 8 weeks (200 ×, scale bar = 100 μm). (D), (E) and (F) represent the thickness of the intestinal muscular layer (MLT), plica height (PH), plica width (PW), and goblet cell (GC) number, respectively. Results are represented as means ± SEM (n = 4) and analyzed using ANOVA followed by Tukey's test. Different letters indicate significant difference (P < 0.05). FM = fishmeal (control); 7S = β-conglycinin; 11S = glycinin. LP = lamina propria.

2.6. 5-Ethynyl-2'-deoxyuridine (EdU) labeling of the distal intestine

After the 8-week sampling period, 10 mg of EdU (RiboBio, Guangzhou, China) was dissolved with phosphate buffered saline (PBS) solution to make a 1 mg/mL stock solution. This dose is based on a study in mice (100 µg/g body weight; Guo et al., 2009). The DI of 10 fish from each group were harvested at 12 h after intraperitoneal injection and fixed with 4% paraformaldehyde for 24 h (Guo et al., 2009). Then, Apollo buffer (RiboBio) was used to incubate all fixed DI samples for 30 min away from light. These samples were washed with 0.5% TritonX-100 solution for 10 min, and then stained with Hoechst 33342 (RiboBio) for 30 min away from light. Images of these DI samples were captured using fluorescent microscopy (Nikon Eclipse Ci, Japan).

2.7. Enzyme activity analysis

According to manufacturer's protocol, lysozyme (LYS), acid phosphatase (ACP), caspase-3, and caspase-9 activities and complements (C3, C4), immunoglobulin M (IgM), and cathelicidin contents in the intestinal tract were measured using commercial kits (Catalog#ml036413, ml626531, ml662603, ml661711,

ml003460, ml003461, ml306423, and ml622805; Shanghai Enzyme-link Biotech Co., Ltd., Shanghai, China).

2.8. Analysis of distal intestine microbiota

DNA from each sample was obtained with DNA isolation kits (Qiagen, Germany) polymerase chain reaction amplification was carried out to produce small fragments of the bacterial 16S-rRNA gene using the universal primers 3338F/806R (F: 5'-ACTCC-TACGGGAGGCAGCA-3'; R: 5'-GGACTACHVGGGTWCTAAT-3'). Amplification products were then purified and recovered using agarose gel electrophoresis (1.0%). Finally, library construction and sequencing steps were conducted by Beijing Biomarker Technologies Co. Ltd. (Beijing, China).

Bioinformatics analysis was performed on the Biomarker Bio-cloud platform (www.biocloud.org). Raw paired-end reads were merged by FLASH (Magoč and Salzberg, 2011). Then, the raw reads and chimeric sequences were filtered and removed, respectively. Operational taxonomic units (OTU) were obtained by clustering clean reads using USEARCH at a 97% similarity level. The unique and common OTU were identified by Venn analysis. The α -diversity indexes including ACE, Chao1, Shannon, and Simpson were

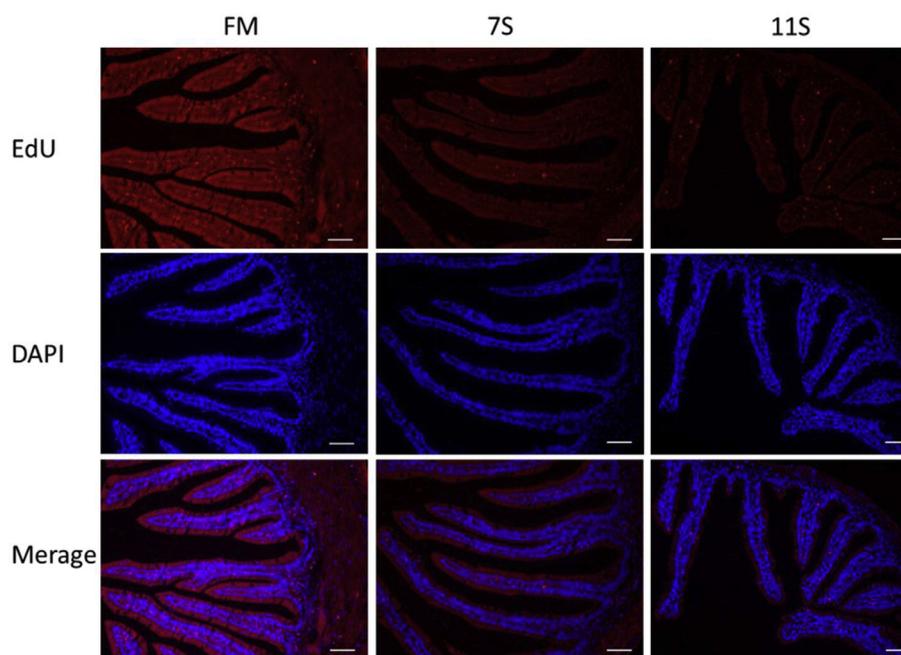


Fig. 3. Detection of cell proliferation of distal intestine in fish after 12 h of intraperitoneal injection using EdU (200 ×, scale bar = 100 µm). FM = fishmeal (control); 7S = β-conglycinin; 11S = glycinin.

Table 4

The intestinal immune indexes of fish fed the experimental diets (n = 4).

Item	FM (control)	7S	11S	P-value
LYS, U/g tissue	0.06 ± 0.00 ^c	0.04 ± 0.00 ^a	0.05 ± 0.00 ^b	0.001
ACP, U/g tissue	0.07 ± 0.00 ^a	0.06 ± 0.00 ^a	0.08 ± 0.00 ^b	0.001
C3, µg/g tissue	471.65 ± 32.01 ^b	322.53 ± 17.13 ^a	633.42 ± 37.63 ^c	0.001
C4, µg/g tissue	1,452.00 ± 39.43 ^b	947.77 ± 26.38 ^a	1,653.72 ± 73.23 ^c	<0.001
IgM, µg/g tissue	213.23 ± 6.67 ^b	150.21 ± 14.86 ^a	208.73 ± 9.41 ^b	0.004
Cathelicidin, µg/g tissue	42.11 ± 2.04	44.96 ± 0.60	43.62 ± 0.83	0.354
Caspase-3, U/g tissue	392.71 ± 20.25 ^a	475.23 ± 21.04 ^b	394.34 ± 21.68 ^a	<0.001
Caspase-9, U/g tissue	0.61 ± 0.02 ^a	0.82 ± 0.03 ^b	0.76 ± 0.02 ^b	<0.001

FM = fishmeal; 7S = β-conglycinin; 11S = glycinin; LYS = lysozyme; ACP = acid phosphatase; C3 = complement 3; C4 = complement 4; IgM = immunoglobulin M.

^{a, b}Different superscript letters in each row indicate significant difference (P < 0.05).

Table 5
Alpha diversity analysis of the distal intestine of fish fed the experimental diets ($n = 3$).

Item	FM (control)	7S	11S	P-value
ACE	651.76 ± 77.81	778.34 ± 127.37	469.25 ± 15.19	0.112
Chao1	685.60 ± 84.45	800.35 ± 131.65	501.56 ± 20.69	0.143
Simpson	0.03 ± 0.01	0.03 ± 0.00	0.08 ± 0.04	0.208
Shannon	4.79 ± 0.31	4.95 ± 0.24	4.20 ± 0.50	0.231

FM = fishmeal; 7S = β -conglycinin; 11S = glycinin.

examined to get each sample's community richness and diversity. According to taxonomic information, a statistical analysis of community abundance was carried out at each classification level. The raw reads were uploaded to the NCBI's SRA database (PRJNA739276).

2.9. Statistical analysis

SPSS 24.0 was used for statistical analysis. P -values < 0.05 were considered statistically significant. Raw data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's tests and presented as means ± standard error of mean (SEM).

3. Results

3.1. Growth performance

The survival rate (SR) and protein efficiency ratio (PER) were not influenced by the type of experimental diet ($P > 0.05$, Table 2). The final body weight (FBW), weight gain rate (WGR), and specific growth rate (SGR) in groups 7S and 11S were significantly lower than those in group FM ($P < 0.05$). Compared to group FM, group 7S fish had significantly higher feed conversion ratio (FCR). However,

no significant differences in FCR were found between groups FM and 11S ($P > 0.05$).

3.2. Morphologic indexes

No significant differences were found in condition factor (CF), viscerosomatic index (VSI), intestinal somatic index (ISI), and hepatosomatic index (HSI) among the 3 groups ($P > 0.05$, Table 3). Intestinal length index (ILI) in group 7S was significantly higher than that in groups FM and 11S ($P < 0.05$).

3.3. Histological examination and intestinal morphometry of the 3 intestinal segments

In the PI, the MLT and PH were significantly lower in groups 7S and 11S than in the FM group ($P < 0.05$, Fig. 2). No significant difference was found in PW among the 3 groups ($P > 0.05$). In the MI, groups 7S and 11S had significantly lower MLT, PH, and PW than controls ($P < 0.05$). In the DI, the MLT of group 7S was significantly lower than that in controls ($P < 0.05$). Groups 7S and 11S had significantly lower PH and PW than controls ($P < 0.05$). The goblet cell (GC) numbers of PI, MI, and DI in both treatment groups were significantly lower than those in the FM group (Fig. 2A to C, $P < 0.05$). Compared to the FM group, the lamina propria (LP) of the treated group became wider.

3.4. Use of EdU to assay DNA synthesis in distal intestines

As shown in Fig. 3, EdU staining from the distal intestine was present at the muscular layer and plica. Cell proliferation in control DI showed extremely obvious positive signaling compared to those of groups 7S and 11S.

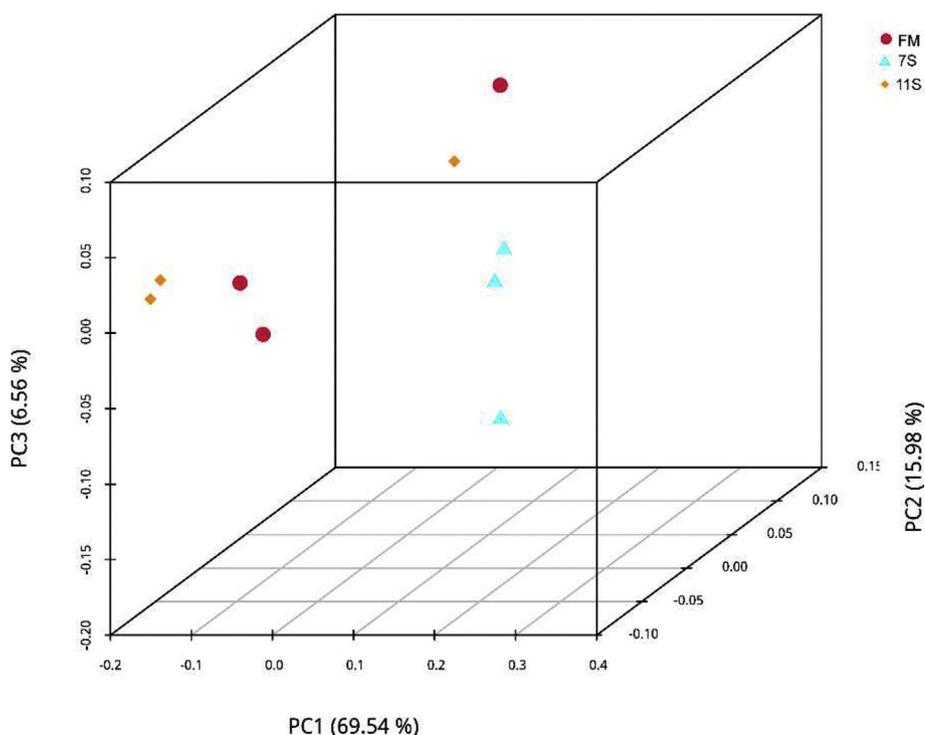


Fig. 4. Principal co-ordinates analysis of intestinal flora. FM = fishmeal (control); 7S = β -conglycinin; 11S = glycinin.

3.5. Intestinal immune-related enzyme activities

Lysozyme activities were significantly higher in FM than those in the other 2 groups ($P < 0.05$, Table 4). Acid phosphatase activities showed no significant differences between groups FM and 7S ($P > 0.05$), which were both significantly lower than those of group 11S ($P < 0.05$). The C3, C4, and IgM contents of the 7S group were significantly higher than those of the FM group ($P < 0.05$). There were no significant differences in cathelicidin content among the 3 groups ($P > 0.05$). Caspase-3 activities in the 7S group were significantly higher than those in the other 2 groups ($P < 0.05$). Caspase-9 activities were significantly lower in controls than in treatment groups ($P < 0.05$).

3.6. Distal intestine microbiota

An average of 65,176 effective sequencing lengths and 609 OTU were found in each group (Appendix Fig. 1). A total of 525 OTU were

commonly observed in FM, 7S, and 11S (Appendix Fig. 2). A total of 89, 321, and 28 OTU were uniquely found in FM, 7S, and 11S, respectively. No significant differences in the Chao 1, ACE, and the Simpson and Shannon index were shown among the experimental groups ($P > 0.05$, Table 5). The results of weighted UniFrac of PCoA showed that the 3 PCoA axes account for the variation among the 3 groups 92.08%, and group 7S was separately clustered from the groups FM and 11S (Fig. 4). The average intestinal microbiotic community in pearl gentian groupers was dominated by Proteobacteria (relative abundance 42.60%) and Firmicutes (40.02%). Bacteroidetes (5.84%) and Actinobacteria (5.66%) were subdominants (Fig. 5A and Appendix Table 1). However, the abundance of these 4 phyla levels among the 3 groups did not show significant differences (Fig. 5B, $P > 0.05$). At the class level, the average intestinal microbiotic community in pearl gentian groupers was dominated by Clostridia (relative abundance 36.68%), Gammaproteobacteria (28.25%), and Alphaproteobacteria (13.64%). Bacteroidia (5.83%) and Actinobacteria (4.77%) were subdominants

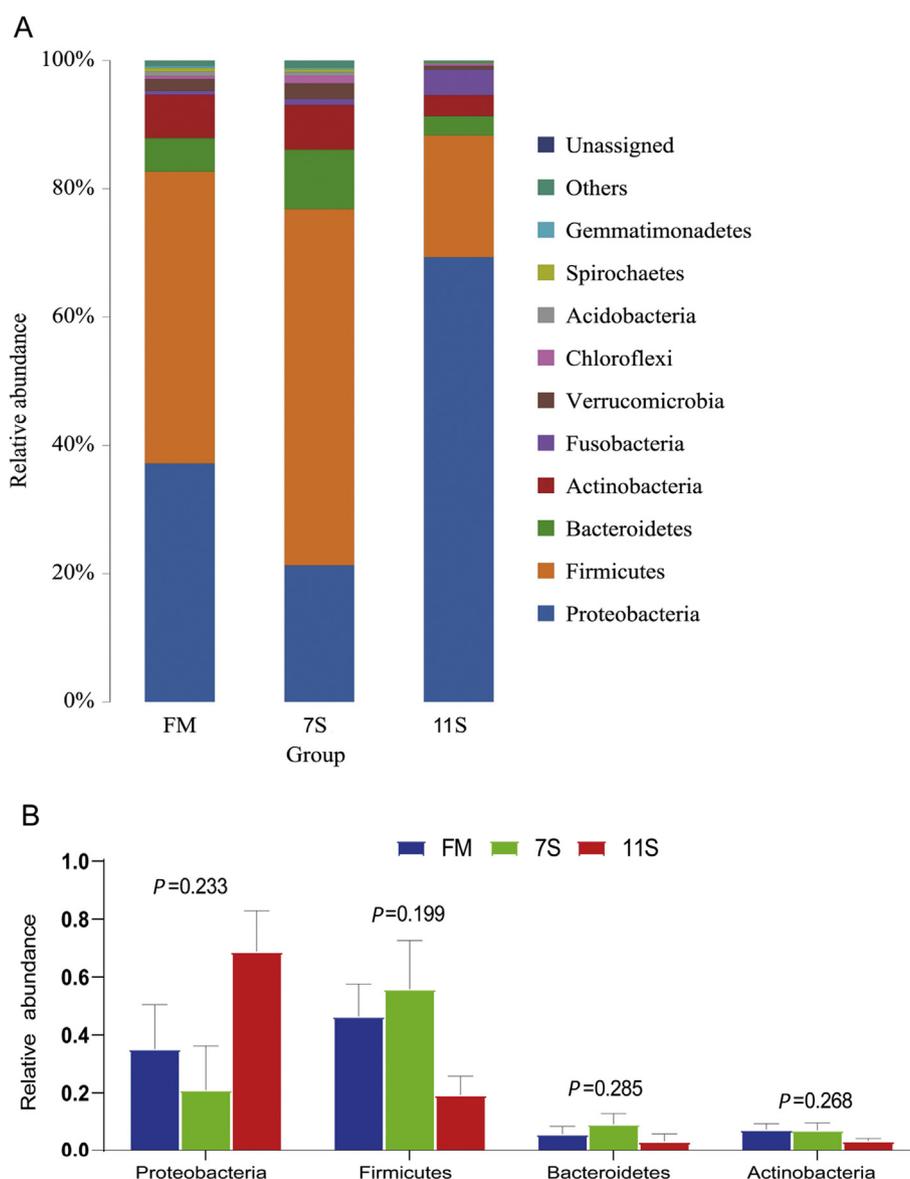


Fig. 5. The distal intestine microbiota at phylum levels in the 3 groups of fish fed the differential experimental diets. (A) Relative abundances of the distal intestine microbiota. (B) Results for the top-four phylum level are represented as means \pm SEM ($n = 3$) and analyzed using ANOVA followed by Tukey's test. Same or no letters indicate no significant difference ($P > 0.05$). FM = fishmeal (control); 7S = β -conglycinin; 11S = glycinin.

(Fig. 6A and Appendix Table 2). The relative abundance of Clostridia was significantly lower in group 7S than in group 11S (Fig. 6B, $P < 0.05$). The relative abundance of Gammaproteobacteria was significantly higher in group 11S than those in others ($P < 0.05$). The most highly-represented genera were *Photobacterium* (12.90%), *Christensenellaceae_R-7_group* (8.96%), *Vibrio* (7.11%), and *Marvinbryantia* (6.11%) (Fig. 7A and Appendix Table 3). Among the subdominant genera, no significant differences were found in the abundances of *Marvinbryantia*, *Nautella*, *Rhodococcus*, and *Acinetobacter* among the 3 groups ($P > 0.05$). The most highly-represented were *Nautella* (4.95%) and *Blautia* (3.72%). The relative abundances of *Photobacterium* and *Vibrio* were significantly higher in group 11S than those in the other 2 groups. In contrast, the relative abundances of *Christensenellaceae_R-7_group*, *Blautia*, *Romboutsia*, and *Ruminococcaceae_UCG-002* were considerably lower in group 11S than those in others ($P < 0.05$, Fig. 7B).

Paired comparisons of the abundances of the potentially beneficial or pathogenic bacterial genera in each group are shown in Fig. 8. The relative abundances of the potentially beneficial genera *Akkermansia*, *Parabacteroides*, *Brevibacterium*, *Bacillus*, and *Ruminiclostridium 9* were significantly lower in Group 7S than in Group FM ($P < 0.05$). The relative abundances of the potentially pathogenic genera *Sarcina*, *Muribaculum*, and *Photobacterium* were significantly higher in Group 7S than in Group FM ($P < 0.05$). The relative abundances of the potentially beneficial genera *Brevibacterium*, *Rhodanobacter*, *Arthrobacter*, and *Bdellovibrio* were significantly lower in Group 11S than in Group FM ($P < 0.05$). The microbial function between groups was predicted from the amplicon data using PICRUSt2. According to Kyoto Encyclopedia of Genes and Genomes (KEGG) level 2 analysis, the presumptive functions were mainly enriched in carbohydrate metabolism, amino acid metabolism, membrane transport, energy metabolism, etc (Appendix Fig. 3). It was further found that no significant difference in microbial function between the group 7S or 11S compared to group FM ($P > 0.05$, Appendix Fig. 3).

4. Discussion

In this study, dietary 7S or 11S decreased fish WGR and SGR but did not affect SR compared to the control group, suggesting that adding 7% of 7S or 10% of 11S to the feed may depress grouper growth but is not lethal, which is consistent with studies of Jian carp (Zhang et al., 2013) and grass carp (Duan et al., 2019b). Furthermore, in the present study, feed intake was not influenced by diet, which is in accordance with a survey of juvenile Chinese mitten crabs (*Eriocheir sinensis*; Han et al., 2019). However, inconsistent results have been reported in other aquatic animals regarding the influences of dietary 7S and 11S on feed intake (Zhang et al., 2013; Jiang et al., 2015b). Notably, in this study, dietary 7S and 11S increased the FCR, implying that the growth inhibition can be partly attributed to low feed efficiency by adding soybean antigenic protein.

In addition, fish growth highly depends on the healthy development of the intestine, which can be reflected by the MLT and PH. In this study, dietary 7S or 11S significantly reduced the PH, PW, and GC of the whole intestine, and the MLT in the MI and DI. Widening of the lamina propria of the PI, MI, and DI in groups 7S and 11S indicates varying degrees of damage and inflammation. Similar results were also observed in turbot (Li et al., 2017) and grass carp (Duan et al., 2019b). Stimulation of antigen-active macromolecules can prompt an immune response that causes atrophy of the intestinal villi, thus damaging the intestinal morphology (Li et al., 2019a; 2019b). Furthermore, cell proliferation in the DI of groups 7S and 11S showed less positive signaling compared to that of controls, suggesting that soybean antigenic protein reduced DNA

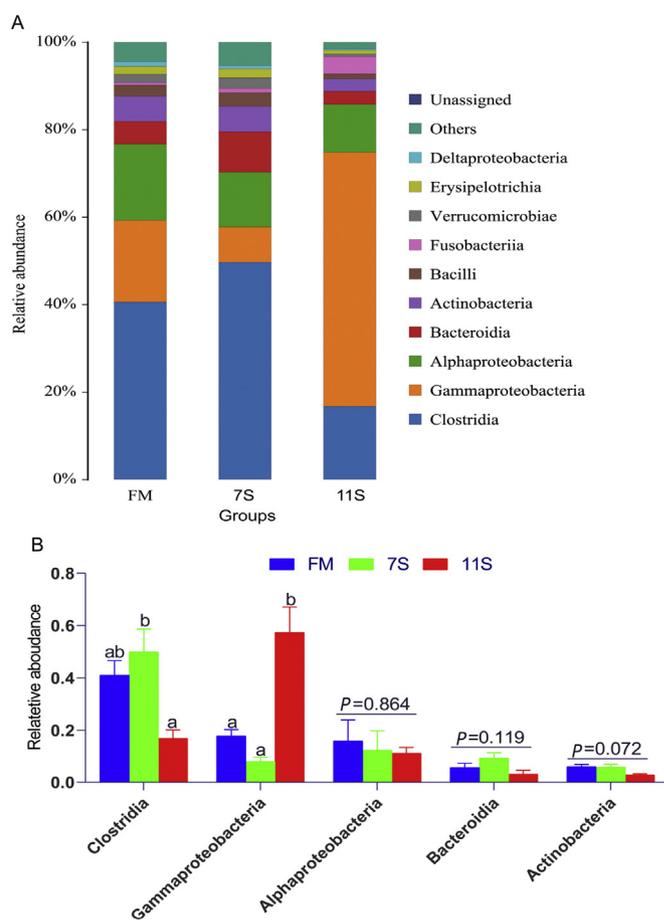


Fig. 6. The distal intestine microbiota at class levels in the 3 groups of fish fed the differential experimental diets. (A) Relative abundances of the distal intestine microbiota. (B) Results for the top-five class level are represented as means \pm SEM ($n = 3$) and analyzed using ANOVA followed by Tukey's test. Same or no letters indicate no significant difference ($P > 0.05$). FM = fishmeal (control); 7S = β -conglycinin; 11S = glycinin.

replication activity and cell proliferation. An in vitro study reported that > 0.25 mg/mL of 7S in the medium reduced the cellular activity of enterocytes in Jian carp (Zhang et al., 2013).

The innate immune system of fish, including LYS, C3, C4, ACP, and antimicrobial components, provides the first line of immune defense and includes some humoral components (Ye et al., 2020). In the present study, fish fed 7S diet showed notably higher LYS activity and C3, C4 and IgM contents compared to fish fed FM diet. Similar results have been reported in juvenile golden crucian carp (Li et al., 2019a; 2019b), grass carp (Duan et al., 2019a), and *R. lagowski* (Li et al., 2020). The above results imply that the DI's immune function was impaired by dietary 7S. Interestingly, the ACP activity and C3 and C4 contents were notably higher in group 11S than in controls. A noticeable difference in the response of these 3 parameters to 7S and 11S emerged. The different influences of 7S and 11S on intestinal immune function in pearl gentian groupers may be caused by the various digestive functions of these 2 proteins. The 11S acidic subunits degrade rapidly (within 0.5 h), which means that free amino acids produced by 11S hydrolysis could be preferentially absorbed in the PI. Meanwhile, even the most digestible α' -subunit of 7S requires more than 0.5 h for hydrolysis (Liu et al., 2007). Some amino acids in feed, such as isoleucine (Zhao et al., 2013), leucine (Ren et al., 2015), and tryptophan (Zehra and Khan, 2015), can increase ACP activity and C3 and C4 contents in fish. Caspase plays a key role in the extrinsic and intrinsic pathways

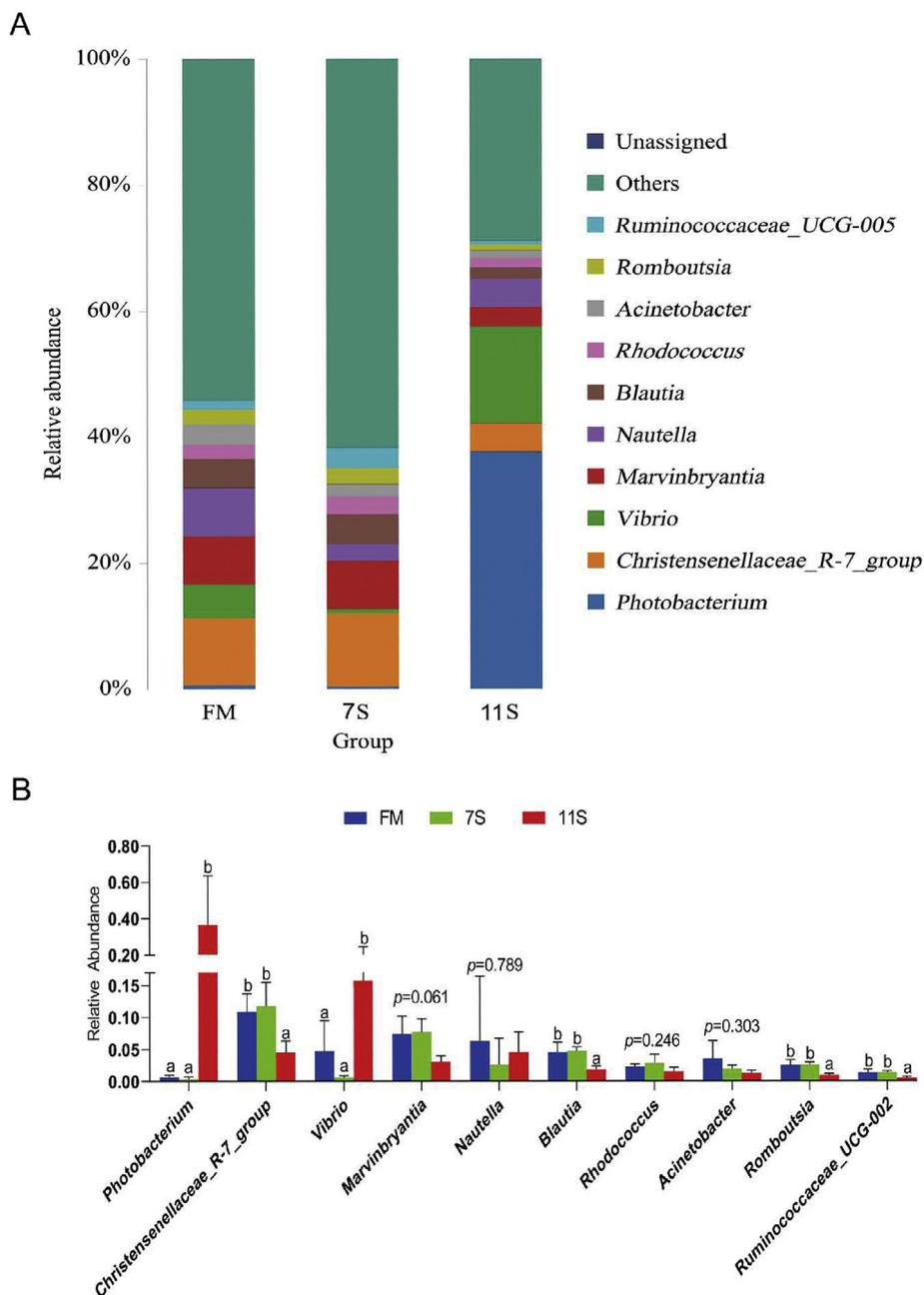


Fig. 7. The distal intestine microbiota at genus levels in the 3 groups of fish fed the differential experimental diets. (A) Relative abundances of the distal intestine microbiota. (B) Results for the top-ten genus level are represented as means ± SEM (n = 3) and analyzed using ANOVA followed by Tukey's test. Different letters indicate significant difference (P < 0.05). FM = fishmeal (control); 7S = β-conglycinin; 11S = glycinin.

involved in apoptosis (Budihardjo et al., 1999). Dietary 7S and 11S increase caspase-3, caspase-8, and caspase-9 at the enzymatic or transcriptional levels in fish intestines (Duan et al., 2019a). In this study, the intestinal caspase-3 and -9 activities in group 7S, and the caspase-9 activity in group 11S, were notably higher than in controls, suggesting that dietary 7S and 11S may induce apoptosis by affecting caspase activities.

The different compositions in diet can cause changes in fish by changing the population size and metabolism of key symbiotic species of the intestinal microbiome (Ringø et al., 2006). The flora located in fish intestine are critical to the regulation of nutrient digestion, immune response, colonization by potential pathogens, and disease resistance (Nayak, 2010). In the current study, no

notable differences were found in α-diversity among treatments. Similar results were observed in pearl gentian groupers fed peptides from swine blood (He et al., 2020a) and cottonseed protein concentrate (Ye et al., 2020). The results of PCoA showed that group 7S was separately clustered from groups FM and 11S, and the sum of the percentages for the X, Y, and Z axes of this analysis is 92.08%, implying that this analysis is a true representation of the variation between samples. In addition, the intestinal flora of the pearl gentian groupers was mainly composed of Firmicutes, Proteobacteria, and Bacteroidetes, which constitute the normal core intestinal flora of juvenile groupers (He et al., 2020b; Ye et al., 2020). These phyla account for more than 90% of the intestinal flora in various freshwater and marine fish species (Apper et al., 2016; Liu

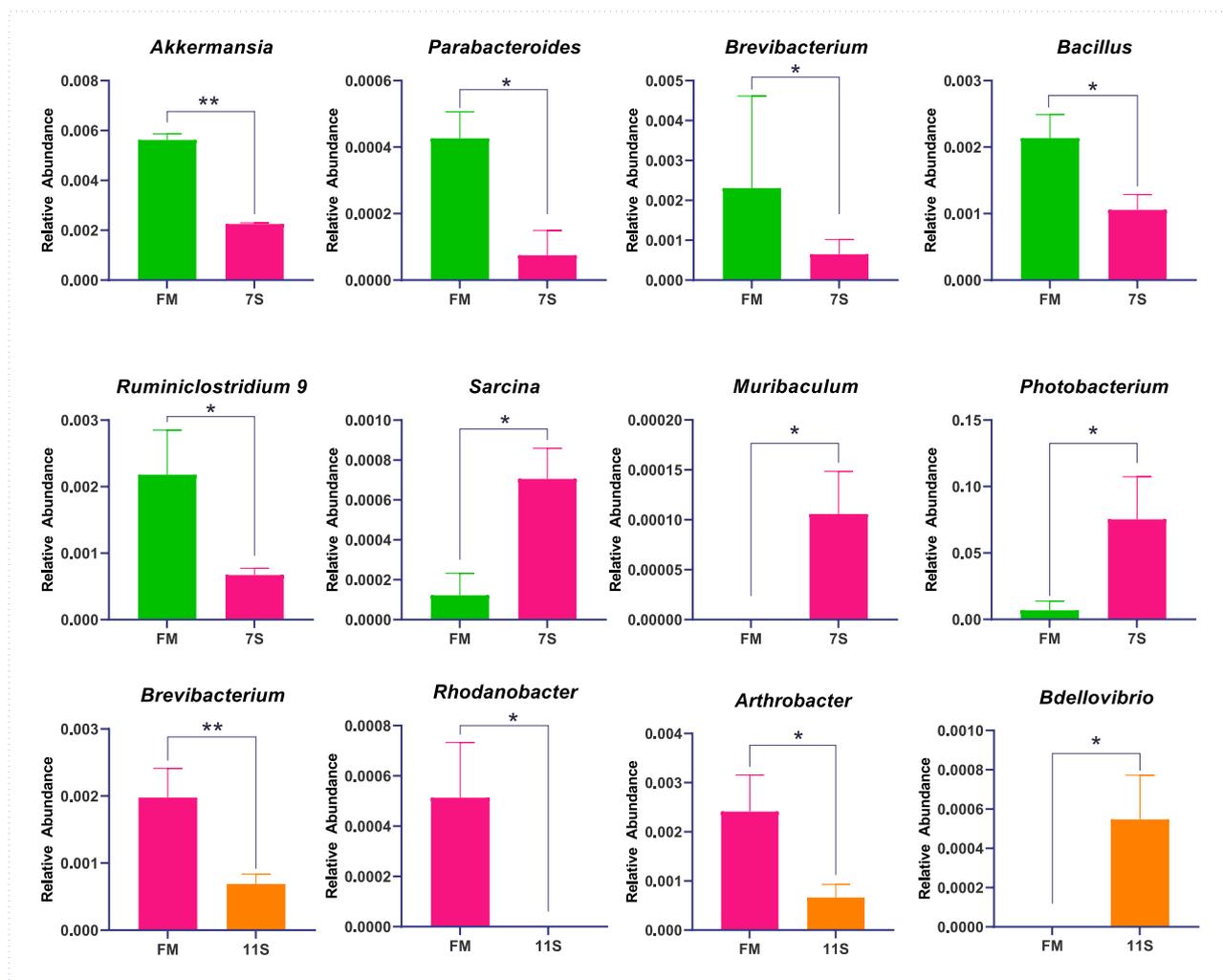


Fig. 8. Comparisons of the abundance of potentially beneficial or pathogenic microbe genera in group FM with that in groups 7S and 11S. Values are means \pm SEM of the 3 replicates. Asterisk (*) represents a significant difference of $P < 0.05$ between groups. Double asterisks (**) denote a highly significant difference of $P < 0.01$ between groups. FM = fishmeal; 7S = β -conglycinin; 11S = glycinin.

et al., 2018; Rimoldi et al., 2018), indicating that these bacteria are essential for nutrient digestion, absorption and immune response (Ghanbari et al., 2015). At the class levels, the intestinal microbiotic community in pearl gentian groupers was dominated by Clostridia and Gammaproteobacteria. Class 'Clostridia' is currently the only class in the phylum Firmicutes grouping both gram-positive and Gram-negative taxa (Marchandin et al., 2010). Gammaproteobacteria are an important class of the Proteobacteria phylum, although Gammaproteobacteria has only the taxonomic rank of class within the phylum Proteobacteria, and it is richer in genera (approximately 250) than all bacterial phyla except Firmicutes (Raina et al., 2019). The relative abundance of Clostridia was significantly lower in group 7S than in group 11S, while the Gammaproteobacteria abundance was significantly higher in group 11S than in others, which implies a difference between dietary 7S and 11S on the intestinal flora of grouper at the class level. The relative abundances of the genera *Photobacterium* and *Vibrio* were notably higher in group 11S than in FM. *Vibrio* is a diverse genus of Proteobacteria, of which *V. vulnificus*, *V. salmonicida*, and *V. anguillarum* are major bacterial pathogens of invertebrate species and marine fish (Austin and Austin, 1999). *Vibrio*'s relative abundance was higher when golden pompano (*Trachinotus ovatus*) were fed a cottonseed protein concentrate diet instead of a fishmeal diet (Shen et al., 2020).

Photobacterium, a member of the family Vibrionaceae, was usually observed on the surface of healthy fish and was initially related to luminescent organs (Cahill, 1990). However, some *Photobacterium* can generate harmful neuraminidases (Sugita et al., 2000). For example, *P. damsela* is a neuraminidase producer and is related to skin ulcers, and is also an infectious source for fish pasteurellosis (Urbanczyk et al., 2011). The possible cause for poor growth in group 11S could be related to the presence of potentially pathogenic *Vibrios* and *Photobacterium*. These 2 potential pathogens may increase ACP activity in response to intestinal inflammation in pearl gentian groupers. Previous study suggests that ACP activity is significantly increased after infection with 3 pathogenic *Vibrio* species: *V. alginolyticus*, *V. harveyi*, and *V. parahaemolyticus* (Ge et al., 2012). Acid phosphatase activity plays an important role in cell-mediated immunity and appears to be a marker for macrophage infiltration in the lamina propria and submucosa of the DI, as presented by the influences of soybean meal exposure in Atlantic salmon (*Salmo salar* L.; Bakke-McKellep et al., 2008). In addition, the relative abundances of *Brevibacterium* were significantly lower in both group 7S and 11S than in group FM. *Brevibacterium* is of interest industrially because it produces various products such as amino acids (especially glutamic acid and lysine) and enzymes important to cheese ripening (Weimer 1999). The genus of bacteria

has been described as beneficial bacteria for hosts, such as *Brevibacterium* spp., which contributes to nutritional processes in Arctic charr and (Ringø et al., 1995) and yellowtail kingfish (*Seriola lalandi*; Ramírez and Romero, 2017). Notably, top-ten relative abundances of the genus-level and α -diversity showed no significant differences between groups 7S and FM, suggesting that fish fed the 7S diet did not alter the abundance and diversity of their intestinal flora. In addition, differences in the intestinal flora of groups 7S and 11S may be related to the different digestive functions of these 2 antigenic proteins in groupers. Some digestive functions of the digestive tract are achieved through a process of bacterial metabolism (Hooper and Macpherson, 2010). Analyzing the functional diversity of the bacterial community may reflect the effects of the gut microbiota on host physiology (Wang et al., 2019). In this study, they are mainly enriched in carbohydrate metabolism, amino acid metabolism, membrane transport, energy metabolism between the 11S-vs-FM and 7S-vs-FM comparison groups. However, none of these pathways above showed significant differences between groups. The possible reason is that these different genera cannot concentrate on a pathway, resulting in no significant difference in functional classification between them. As discussed above, metabolites of some differentially varying bacterial genera are able to participate in intestinal immune function, which further leads to the inhibition of fish growth. However, the predicted function and the actual metabolic profile may exhibit some degree of discrepancy, and further studies are needed to show the close relationship between the differential genera and intestinal dysfunction in response to soybean antigenic protein.

5. Conclusion

In conclusion, the findings of this study show that the existence of soybean antigens (7% of β -conglycinin or 10% of glycinin) could damage the structural integrity of the intestine, reduce immune defense, reshape the intestinal microbiome and, ultimately, impair growth in fish. Fish fed β -conglycinin and glycinin diets exhibit some degree of discrepancy in gut microbial patterns, not α -diversity. This may be related to the strength of the antigenicity of these 2 antigenic proteins.

Author contributions

Yuanfa He: Conceptualization, Methodology, Investigation, Writing—Original Draft Preparation; **Jinfang Liang:** Investigation, Feeding Experiment; **Xiaohui Dong:** Formal Analysis; **Qihui Yang:** Formal Analysis; **Hongyu Liu:** Formal Analysis; **Shuang Zhang:** Formal Analysis; **Shuyan Chi:** Conceptualization; Writing—Review and Editing, Project Administration; **Beiping Tan:** Resources, funding Acquisition. All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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Appendix

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.aninu.2021.11.001>.

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